Respiration of mitochondria in living organisms is controlled by the ATP/ADP ratio and phosphorylation pattern of cytochrome c oxidase

Dissertation

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Rabia Ramzan

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2. Kadenbach, B., Ramzan, R., Vogt, S. Degenerative diseases, oxidative stress and cytochrome c oxidase function, Trends Mol. Med. 15 (2009) 139-147.

3. Kadenbach, B., Ramzan, R., Wen, L., Vogt, S. New extension of the Mitchell Theory for oxidative phosphorylation in mitochondria of living organisms, Biochim. Biophys. Acta 1800 (2010) 205–212.

4. Ramzan, R., Staniek, K., Kadenbach, B., Vogt, S. Mitochondrial respiration and membrane potential are regulated by the allosteric ATP-inhibition of cytochrome c oxidase. Biochim. Biophys. Acta 1797 (2010) 1672-1680.

To my lovely Mahveen

Abstract

The 'second mechanism of respiratory control' (allosteric ATP-inhibition of cytochrome c oxidase (CcO)) is demonstrated for the first time in intact isolated rat liver and heart mitochondria. The problems of measuring the kinetics of allosteric ATP-inhibition in isolated mitochondria were investigated. And it was found that only at very high ATP/ADP ratios, this inhibition is obtained and requires an ATP-regenerating system consisting of phosphoenolpyruvate (PEP) and pyruvate kinase (PK). The allosteric ATP-inhibition can be switched off probably by dephosphorylation of a serine at CcO subunit-I. The phosphorylation of CcO at serine, threonine and tyrosine was studied in isolated mitochondria by extracting complex IV of the respiratory chain (CcO) by BN-PAGE (blue-native-polyacrylamide-gel-electrophoresis), SDS-PAGE and Western blotting with the corresponding antibodies against the phosphorylated amino acids. The extent of allosteric ATP-inhibition of CcO varied in different preparations of mitochondria, which was suggested to be based on the psychological situation of the animal at the time of killing. Incubation of bovine heart tissue slices with insulin decreased the allosteric ATP-inhibition and phosphorylation of CcO subunit-I at serine. By using a TPP^+ (tetraphenylphosphonium)-electrode, and in collaboration with Katrin Staniek, Veterinary University, Vienna, a reversible decrease of the mitochondrial membrane potential ($\Delta\Psi_m$) by PEP and PK through the $\Delta\Psi_m$-independent allosteric ATP-inhibition of CcO at high ATP/ADP ratios was demonstrated in isolated rat liver mitochondria for the first time. It is proposed that respiration in living eukaryotic organisms is normally regulated by the $\Delta\Psi_m$-independent 'allosteric ATP-inhibition of CcO', and only when the allosteric ATP-inhibition is switched off under stress, respiration is regulated by 'respiratory control', based on $\Delta\Psi_m$ according to the Mitchell Theory.

Zusammenfassung

Der “zweite Mechanismus der Atmungskontrolle” (allosterische ATP-Hemmung der Cytochrom c Oxidase (CcO)) wird zum erstenmal an intakten Mitochondrien aus Rattenleber und Rattenherz gezeigt. Die Probleme bei der Messung der Kinetik der allosterischen ATP-Hemmung an isolierten Mitochondrien wurden untersucht. Nur bei hohen ATP/ADP Verhältnissen wird diese Hemmung erhalten und erfordert ein ATP-regenerierendes System bestehend aus Phosphoenolpyruvat (PEP) und Pyruvat Kinase (PK). Die allosterische ATP-Hemmung kann abgeschaltet werden, wahrscheinlich durch Dephosphorylierung an einem Serin der CcO Untereinheit-I. Die Phosphorylierung der CcO an Serin, Threonin und Tyrosin wurde an isolierten Mitochondrien durch Extraktion von Complex IV (CcO) mittels BN-PAGE (Blau-Native Polyacrylamid Gelelektrophorese), SDS-PAGE und Western Blots mit den entsprechenden Antikörpern gegen die phosphorylierten Aminosäuren untersucht. Das Ausmass der allosterischen ATP-Hemmung der CcO variierte in verschiedenen Mitochondrienpräparationen, was auf die psychologische Situation des Tieres beim Töten zurückgeführt wurde. Inkubation von Rinderherzschnitten mit Insulin verminderte die allosterische ATP-Hemmung und die Phosphorylierung der CcO Untereinheit-I am Serin. Mittels einer TPP^+ (Tetraphenylphosphoniuim)-Elektrode, und in Zusammenarbeit mit Katrin Staniek, Veterinärmedizinische Universität Wien, wurde erstmals eine reversible Verminderung des mitochondrialen Membranpotentials ($\Delta\Psi_m$) durch PEP und PK über die $\Delta\Psi_m$-unabhängige allosterische ATP-Hemmung der CcO bei hohen ATP/ADP Verhältnissen an isolierten Rattenlebermitochondrien gezeigt. Es wird postuliert, das die Atmung in lebenden eukaryotischen Organismen normalerweise durch die $\Delta\Psi_m$-unabhängige ‚allosterische ATP-Hemmung der CcO’ kontrolliert wird. Nur wenn unter Stress die allosterische ATP-Hemmung abgeschaltet ist, wird die Atmung durch die ‚Atmungskontrolle’, die entsprechend der Mitchell Therie auf dem $\Delta\Psi_m$ beruht, reguliert.

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1. Introduction

Fundamentally, life is a process of energy-handling because living and non-living things can be differentiated on the basis of a series of characteristics e.g. metabolism, growth, responsiveness to stimuli, homoeostasis (self-maintenance), movement, reproduction etc. All these processes require energy in the form of ATP and in order to fulfil this high cellular energy requirement, most aerobic bacteria and all eukaryotic cells have developed a specific mechanism of 'oxidative phosphorylation' which generates about 15 times more ATP by respiration compared to 'glycolysis'. In eukaryotes, glycolysis takes place in the cytosol and oxidative phosphorylation in mitochondria.

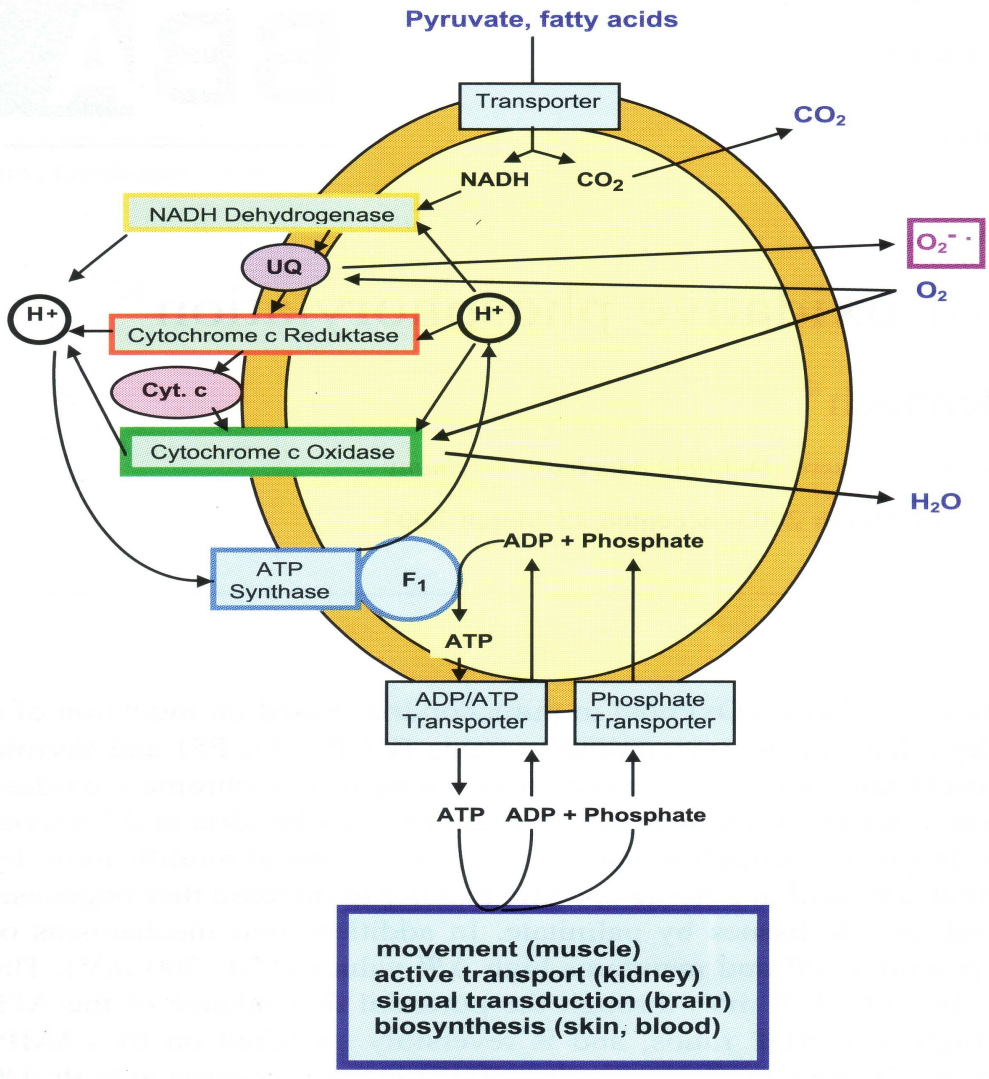


Fig. 1. Oxidative phosphorylation in mitochondria
The figure represents the inner mitochondrial membrane with the proton pumps of respiratory chain i.e. complex I (NADH dehydrogenase), complex III (cytochrome c reductase), complex IV (cytochrome c oxidase) and of complex V (F_0F_1-ATP synthase). In order to simplify the figure, complex II is not shown here.

Mitochondria are the cellular organelles containing maternally inherited genome (e.g. human mtDNA = 16569 bp) that encodes only 13 polypeptides which all in turn represent subunits of different enzyme complexes of oxidative phosphorylation. Three enzyme complexes of the mitochondrial respiratory chain (complex I: NADH dehydrogenase; complex III: cytochrome c reductase and complex IV: cytochrome c oxidase) are electron transport-driven proton pumps which transfer reducing equivalents from nutrition to dioxygen accompanied by the transfer of protons across the inner mitochondrial membrane, and thus generate a proton motive force (see Fig. 1). This proton motive force in turn consists of an electrochemical gradient and a pH gradient as follows:

$$\underline{\Delta p = \Delta\Psi - Z \bullet \Delta pH}, \qquad Z = 2.303 \bullet RT / F = 59 \text{ (mV, at 25°C)}, \qquad \Delta p \bullet F = \Delta\mu H^{+}$$

This proton motive force is used by the F_0F_1-ATP synthase (complex V) in mitochondria to produce ATP from ADP and inorganic phosphate [Abrahams et al., 1994; Junge et al., 1997].

Complex IV i.e. Cytochrome c oxidase (CcO) is the terminal enzyme of the mitochondrial respiratory chain which transfers electrons from cytochrome c to molecular oxygen, coupled with the uptake of protons from the matrix forming water, and with the translocation of protons across the inner mitochondrial membrane.

The mammalian CcO is composed of 13 subunits encoded both by mitochondrial DNA (subunits I, II, and III) and nuclear DNA (subunits IV, Va, Vb, VIa, VIb, VIc, VIIa, VIIb, VIIc, VIII). The enzyme from bovine heart crystallizes as a dimer [Tsukihara et al., 1996], as shown in Fig. 2. In contrast, the bacterial enzyme is monomeric and consists of only 2-4 subunits [Iwata et al., 1995].

Subunit I contains heme a (the redox center) and heme a_3-Cu_B (the oxygen binding binuclear center), while subunit II contains Cu_A and the cytochrome c binding site. Although, heme a and heme a_3 are chemically identical but, due to the difference in environment of subunit I, both differ on the basis of their spectral properties. From bacterial to mammalian CcO, the properties of both, subunit I and II are nearly identical and have been shown to resemble each other in the crystal structures of the bovine heart enzyme [Tsukihara et al., 1996] and *Paracoccus denitrificans* enzyme [Iwata et al., 1995]. Additionally, one of each Zn, Mg, and Na (or Ca) are also observed in the mammalian enzyme [Yoshikawa et al., 1998], but their functions are still unknown.

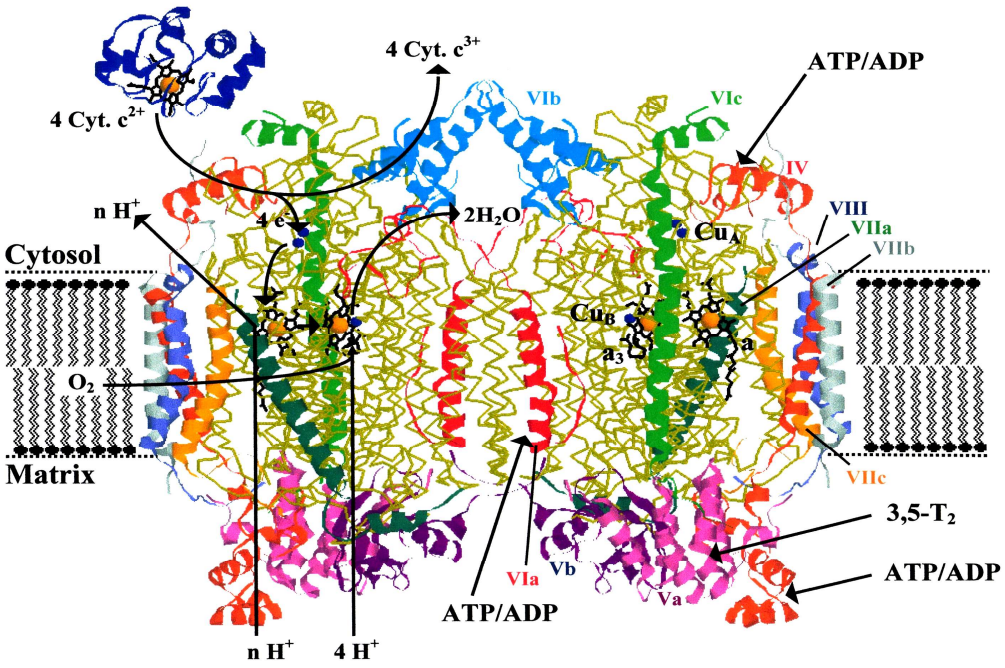


Fig. 2. Crystal structures of cytochrome c oxidase and cytochrome c from bovine heart (taken from Kadenbach et al., 2000).
Each monomer of the dimeric enzyme contains 3 mitochondrial-encoded subunits (I, II, and III, in light green) containing the cytochrome c binding site and two copper atoms (Cu_A) at subunit II, and heme a and the dioxygen binding site at the binuclear center heme a_3/Cu_B in subunit I. The 10 nuclear-encoded regulatory subunits (IV, Va, Vb, VIa, VIb, VIc, VIIa, VIIb, VIIc, and VIII) are indicated as coloured ribbons. Left monomer indicates the catalytic reaction. Right monomer shows the binding sites for ATP or ADP and 3, 5- diiodothyronine. Data source for CcO: protein data bank Brookhaven, Tsukihara et al. [1996]. This modeling was prepared with the program "RasMol 2.6".

The reaction of CcO with oxygen is coupled with the translocation of protons from the matrix to the intermembrane space of the mitochondria and has the following stoichiometry:

$$4\ \text{cytochrome}\ c^{2+} + 4\ H^+ + n\ H^+_{matrix} + O_2 \longrightarrow 4\ \text{cytochrome}\ c^{3+} + n\ H^+_{cytosol} + 2\ H_2O$$

The pathway of electrons from cytochrome c via Cu_A (two electronically coupled copper atoms), heme a, and the binuclear center (composed of heme a_3 and Cu_B) is mostly established [Ludwig et al., 2001]. CcO represents the only enzyme which reacts with oxygen without formation of reactive oxygen species (ROS) [Muramoto et al., 2010]. This is based on the unique structure of the oxygen binding binuclear center which simultaneously transfers four electrons to the bound dioxygen molecule (see Fig. 3). Oxygen, bound to the reduced binuclear center (Fe^{2+}/Cu_B^{1+}) is immediately reduced to the redox state of water by the uptake of 4 electrons: two from iron (forming the ferri state Fe^{4+}), one from copper (forming Cu^{2+}), and the fourth from a tyrosine side chain, forming a radical. The formation of a

tyrosine radical is possible by a covalent bridge between Tyr^{280} and His^{276} (nomenclature of the *P. denitrificans* enzyme) which stabilizes the tyrosine radical.

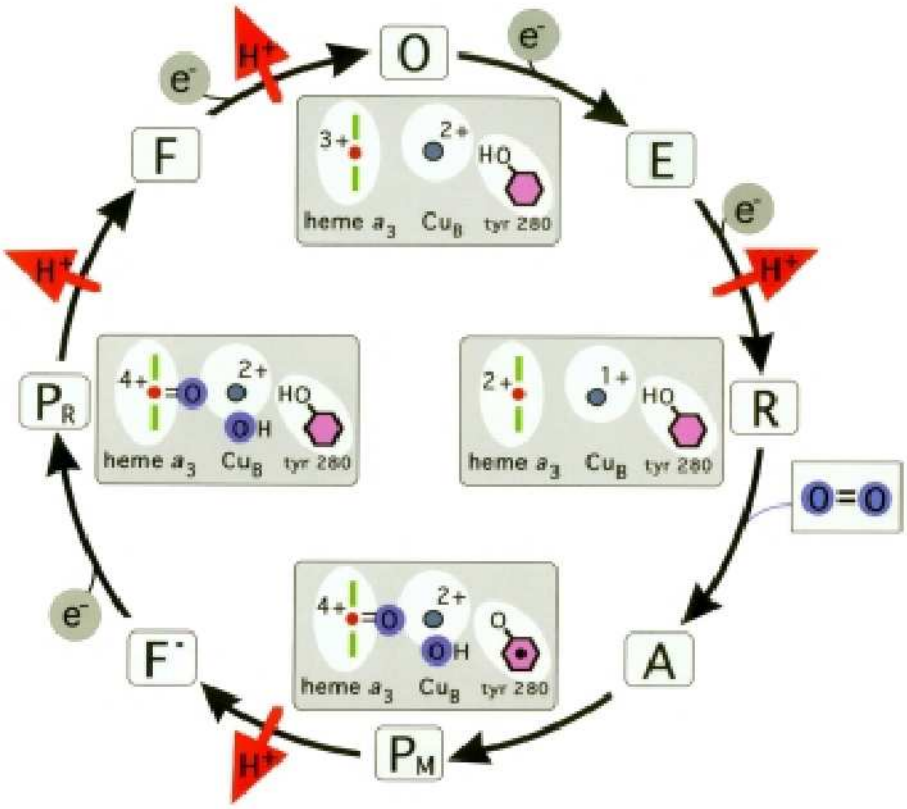


Fig. 3. Simplified scheme of the O_2 reduction cycle catalyzed by cytochrome c oxidase (taken from Ludwig et al., 2001).
Key intermediates (white boxes labeled "O", "E", "R" etc.) are listed clockwise along with the input steps for the four electrons (gray circles), the binding of dioxygen (blue), and the presumed transmembrane proton translocation steps (red arrows; according to Michel [1999]). The four gray shaded boxes within the reaction circle indicate various states of cytochrome c oxidase, "O", "R", "R_M", "P_R". Proton uptake steps, water release, and the assignment of formal charges to the oxygen atoms have been omitted for clarity.

In contrast to the known mechanism of electron transfer in CcO, the mechanism of its coupling to the translocation of protons across the membrane is mostly unknown [Michel, 1999; Brzezinski et al., 2008; Qin et al., 2009], but models have been suggested [Sharpe and Ferguson-Miller, 2008; Siegbahn and Blomberg, 2008]. Two proton pathways, consisting of hydrophilic amino acids and water molecules, have been identified in the bacterial enzyme [Iwata et al., 1995] and were corroborated in the bovine heart enzyme [Tsukihara et al., 1996]. But a third proton pathway, including Asp^{51} as the possible outlet amino acid at the

cytosolic side, was identified in the bovine heart enzyme [Yoshikawa et al., 1998; 2006], but could not be found in the bacterial enzyme [Pfitzner et al., 1998; Salje et al., 2005].

Seven of the nuclear-encoded subunits of bovine heart CcO are located as transmembraneous polypeptides and the other three are located on the outside of the inner membrane: subunit VIb at the cytosolic side and subunits Va and Vb at the matrix side (see Fig. 2). Similar to the enzyme from mammals, CcO from birds (turkey) [Hüttemann et al., 2000] and fish (tuna) [Arnold et al., 1997] also contain thirteen subunits. On the other hand, 11 subunits were found in the enzyme from yeast [Geier et al., 1995], seven in *Dictyostelium discoideum* [Bisson et al., 1986], but only four subunits occur in CcO from the bacteria, *P. denitrificans* [Iwata et al., 1995].

For a long time, the regulatory function of the 10 nuclear-encoded subunits in mammalian CcO [Kadenbach and Merle, 1981; Kadenbach et al., 1983; Tsukihara et al., 1996], which do not occur in the bacterial enzyme [Iwata et al., 1995], was questioned [Saraste, 1983]. The identification of tissue-, species-, and developmental-specific isoforms of nuclear-encoded subunits, however, suggested specific regulatory functions. The heart type subunits of VIa, VIIa, and VIII (VIaH, VIIaH, and VIIIH) are expressed in heart and skeletal muscle, whereas the liver type subunits (VIaL, VIIaL, and VIIIL) are ubiquitously expressed [Schlerf et al., 1988; Lightowlers et al., 1990; Kennaway et al., 1990; Seelan and Grossman, 1991; Linder et al., 1995]. Moreover, the cDNA of an isoform of subunit IV (IV-2) was found and its transcript was observed in lungs of adult and fetal human and adult rat, as well as in the muscle of fetal human by Northern Blot analysis [Hüttemann et al., 2001].

Only one isoform was found for subunit VIII in human, for subunit VIIa in rat and for subunit VIa in fish [Grossman and Lomax, 1997; Linder et al., 1995; Hüttemann et al., 1997]. In the adult rat heart, the CcO subunit VIa consists of two thirds of the heart type isoform (VIaH) and one third of the liver type isoform (VIaL) [Kadenbach et al., 1990], whereas in the skeletal muscle almost 100 % of subunit VIaH is expressed [Anthony et al., 1990]. In fetal heart and skeletal muscle, mostly the liver type of subunits VIa and VIIa (VIaL and VIIaL) are expressed, but switch to the heart type isoforms after birth [Taanman et al., 1992; Bonne et al., 1993; Grossman et al., 1995; Parsons et al., 1996]. Interestingly, a different subunit composition of CcO from wheat germ and wheat seedling suggests the existence of developmental- or tissue-specific isoforms also in the plant enzyme [Peiffer et al., 1990]. In yeast, the expression of subunit V isoforms (corresponding to subunit IV in mammals), Va and Vb, are dependent on the oxygen concentration in the growth medium [Burke and Poyton, 1998].

For most nuclear-encoded subunits of CcO the function is still unknown. Nevertheless, all 10 subunits appear essential for the function of CcO in higher organisms, since mutations in nuclear-encoded CcO structural subunits were searched for but never found. In contrast, many mitochondrial diseases based on mutations of mitochondria-encoded subunits I-III of CcO have been described [Barrientos et al., 2002]. Only recently, in a patient with leukodystrophic encephalopathy a mutation in the nuclear-encoded CcO subunit VIb was identified that is a cause for the disease [Massa et al., 2008]. In drosophila the mutation in CcO subunit VIa was found to cause neurodegeneration [Liu et al., 2007].

The nuclear-encoded subunits of CcO, which do not occur in bacteria, are suggested to have a regulatory function. But also in subunit I of bacterial CcO, as well as in mammalian subunit I a conserved steroid binding site was identified [Qin et al., 2008].

In bovine heart CcO, seven high-affinity binding sites for ATP or ADP and three additional sites only for ADP were identified by equilibrium dialysis with radioactive ATP and ADP [Napiwotzki et al., 1997; Napiwotzki and Kadenbach, 1998]. The ATP/ADP ratio was found to regulate the efficiency of proton translocation in CcO of heart and skeletal muscle. By increasing the intraliposomal ATP/ADP-ratio, a decrease of the H^+/e- ratio from 1.0 to 0.5 was measured with the reconstituted enzyme from bovine heart [Frank and Kadenbach, 1996; Hüttemann et al., 1999]. The decrease was half-maximal at an ATP/ADP-ratio of 100, and occurred by the exchange of bound ADP by ATP at the matrix domain of subunit VIaH, since preincubation of the enzyme with a monoclonal antibody against subunit VIaH prevented the decrease. The decrease of H^+/e- ratio of CcO from heart and skeletal muscle (heart-type of subunit VIaH) at high ATP/ADP-ratios was suggested to participate in thermogenesis at rest, e.g. during sleep when the ATP/ADP ratio is high. In CcO from liver and kidney containing subunit VIaL (liver-type of subunit VIa) palmitate but not high ATP/ADP ratios were found to decrease the H^+/e- ratio from 1.0 to 0.5 (half-maximal at 0.5 µM) [Lee and Kadenbach, 2001].

The control of mitochondrial respiration is generally understood as ‘respiratory control’, first described by Lardy and Wellman [1952], where the rate of respiration is strictly controlled by the availability of ADP. The energetic intermediate between oxygen reduction and ATP synthesis was identified by Peter Mitchell [1966] as the electrochemical proton gradient across the inner mitochondrial membrane (proton motive force), composed mainly of the membrane potential $\Delta\Psi_m$ [Nicholls and Ferguson, 2002]. ‘Respiratory control’ is thus explained by the Mitchell theory as inhibition of the mitochondrial proton pumps (respiratory chain complexes I, III, and IV) at high $\Delta\Psi_m$.

Later on, in the last decade, an additional and $\Delta\Psi_m$-independent regulation of respiration was described based on "allosteric ATP-inhibition of CcO" [Arnold and Kadenbach, 1997]. This 'second mechanism of respiratory control' [Kadenbach and Arnold, 1999] is characterized by the sigmoidal inhibition curves of CcO in the presence of high ATP/ADP ratios, whereas hyperbolic curves are obtained at low ATP/ADP ratios. Both hyperbolic and allosteric kinetics are measured polarographically using ascorbate as a substrate and at increasing concentrations of cytochrome c. The allosteric ATP-inhibition was correlated with the exchange of bound ADP by ATP at the matrix domain of subunit IV, since a monoclonal antibody against subunit IV prevented the allosteric ATP inhibition. Half-maximal inhibition of activity was obtained at an intramitochondrial ATP/ADP ratio of 28, and the inhibition was independent of the proton motive force [Arnold and Kadenbach, 1999]. The maximal Hill-coefficient of 2 [Arnold and Kadenbach, 1997] has suggested the cooperativity of two cytochrome c binding sites which are assumed to be located at each monomer of the dimeric enzyme. Interestingly, the allosteric ATP-inhibition of CcO is abolished by the thyroid hormone 3,5-diiodothyronine through binding to subunit Va [Arnold and Kadenbach, 1998]. Subunit Va was found to be post-translationally modified in hearts of transgenic mice which are resistant to the ROS-inducing reagent doxorubicin [Merten et al., 2005].

An abolition of the allosteric ATP-inhibition of CcO was also obtained in cultivated astrocytes and cerebellar granule cells under hypoxic conditions which resulted in an elevated transcription level of the CcO subunit IV-2 isoform [Horvath et al., 2006]. According to the crystal structure of the bovine heart enzyme [Tsukihara et al., 1996], a binding pocket for ATP has been postulated which is formed by amino acids of subunits I, II, and IV while two cysteines occur in the subunit IV-2 isoform but not in subunit IV-1 [Hüttemann et al, 2001]. These two cysteine residues may form a disulfide bridge (regulated by the oxygen concentration) which inturn may prevent the binding of ATP and thus the allosteric ATP-inhibition.

An allosteric ATP-inhibition was also found with the yeast enzyme, but not with the enzyme from *Rhodobacter sphaeroides* [Follmann et al., 1998] which lacks a subunit similar to subunit IV of the eukaryotic enzyme. Regulation of enzymatic activity by the addition of ATP was also measured with CcO from cyanobacteria. This oxidase contains a fourth subunit which is homologous to the eukaryotic subunit IV [Alge et al., 1999].

The allosteric ATP-inhibition of CcO has been proposed to keep $\Delta\Psi_m$ in vivo at low values (100-140 mV) via feedback inhibition of CcO by ATP [Lee et al., 2001; Kadenbach et

al., 2004, 2009; 2010]. Since the production of reactive oxygen species (ROS) in mitochondria increases exponentially at $\Delta\Psi_m$ > 140 mV [Liu, 1997; Korshunov et al., 1997; Starkov and Fiskum, 2003; Rottenberg et al., 2009], the allosteric ATP-inhibition of CcO was suggested to prevent the formation of ROS in mitochondria. ROS are known to stimulate aging and the formation of degenerative diseases [for review see Dalle-Donne et al., 2006; Valko et al., 2007; Trachootham et al., 2008]. However, for the synthesis of ATP in mitochondria by the F_0F_1-ATP synthase, only 130 mV are required for the maximal rates of ATP synthesis [Kaim and Dimroth, 1999]. In fact, such low $\Delta\Psi_m$ values (100-140 mV) have been measured in living cells and tissues [Wan et al., 1993; Mollica et al., 1998; Zhang et al., 2001; Berkich et al., 2003]. In contrast, with isolated mitochondria, high $\Delta\Psi_m$ values of 180-230 mV were measured [Fox et al., 1993; Nicholls and Ferguson, 2002; O'Brien et al., 2008]. Therefore, it was assumed that isolated mitochondria do not represent the actual bioenergetic situation of mitochondria within living cells. The allosteric ATP-inhibition of CcO was proposed to extend the Mitchell Theory for eukaryotic organisms in vivo [Kadenbach et al., 2010], because low $\Delta\Psi_m$ values not only prevent the formation of ROS but also the backflow of protons across the inner mitochondrial membrane at high $\Delta\Psi_m$ values [O'Shea et al., 1984] which decreases the efficiency of energy transduction.

Objectives of the present dissertation

Up till now, the 'allosteric ATP-inhibition of CcO: (second mechanism of respiratory control) has not been accepted generally as a regulatory principle of respiration because of the difficulties to obtain reproducible results with isolated mitochondria. The intention of this dissertation was to demonstrate the allosteric ATP-inhibition of CcO in intact isolated mitochondria, and to characterize the inherent problems of measuring the kinetics in mitochondria from rat heart and liver and from bovine tissues. In addition, to investigate the relationship between reversible phosphorylation of CcO and the extent of allosteric ATP-inhibition. Finally, to demonstrate the postulated reversible decrease of $\Delta\Psi_m$ in mitochondria at high ATP/ADP ratios, in collaboration with Prof. Katrin Staniek (Veterinary University of Vienna).

2. Materials

2.1 Animals

Wistar rats	Animal house, BMFZ, Marburg
SD rats	Animal house, BMFZ, Marburg

2.2 Tissues

Bovine heart, liver and kidney	Slaughterhouse Marburg
Rat heart and liver	BMFZ, Marburg

2.3 Chemicals

All substances used were of analytical grade and purchased from Calbiochem (Darmstadt), Fluka (Steinheim ans Seelze), Merck (Darmstadt), Roth (Karlsruhe), Serva (Heidelberg), Sigma (Steinheim and USA) and VWR (Darmstadt). Distilled water was obtained from "Millipack 40" with a macromolecule filter (pore size 0.22µm), Millipore, Schwalbach.

2 Mercaptoethanol	Fluka, Steinheim
2-Propanol	Fluka, Seelze
6-Aminohexanoic acid	Sigma, Steinheim
Acetic Acid 100%	VWR, Darmstadt
Acetonitril	Merck, Darmstadt
Acrylamide (2X)	Serva, Heidelberg
N,N'-Methyl-bis acrylamide	Roth, Karlsruhe
Albumin, Bovine Serum Fraction V, Fatty Acid Free	Calbiochem, Darmstadt
APS (ammonium persulfat)	Roth, Karlsruhe
Bis-TRIS 99%	Fluka, Steinheim
DMSO	Sigma, Seelze
Glycerol anhydrous min 99.5%	KMF, Lohmar
Glycin	Roth, Karlsruhe
Hepes	Roth, Karlsruhe
Joklik medium	PAN Biotech, Aidenbach
KCl, 3 mol/L electrolyte (for oxygen electrode)	Mettler Toledo, Switzerland
Roti-Block	Roth, Karlsruhe

Sodium hydrosulfite, ca 85%	Aldrich, Steinheim
Tricine 99%	Sigma, Steinheim
TRIS 99.9%	Roth, Karlsruhe
Tris HCl	Fluka, Steinheim
Urea ≥ 99%	Fluka, Steinheim
TEMED	Roth, Karlsruhe

2.3.1 Antibodies

anti-Cox subunit I, mouse IgG_{2a}	Mo Bi Tec, Göttingen
anti-Cox subunit IV, mouse IgG_{2a}	Mo Bi Tec, Göttingen
anti-phosphoserine kit, 6 monoclonal Ab from mouse	nanotools, München
anti-phosphothronine kit, 3 monoclonal Ab from mouse	nanotools, München
anti-phosphotyrosine, clone 4G10	Millipore, Schwalbach
Goat Anti-Mouse IgG, HRP-conjugated	Biomol, Hmaburg

2.3.2 Enzymes

PK (pyruvate kinase) from rabbit muscle Type II, ammonium sulfate suspension	Sigma, Steinheim
PKA (protein kinase A) from bovine heart	Sigma, Steinheim

2.3.3 Detergents

Dodecylmaltoside (laurylmaltoside)	Sigma, Steinheim
Digitonin	Fluka, Steinheim
SDS (sodium dodecylsulfate)	Fluka, Steinheim
Tween 20	Fluka, Steinheim
Triton X-100	Sigma, Steinheim
Triton X-114	Sigma, Steinheim

2.3.4 Dyes

Bromophenol Blue	Merck, Darmstadt
Coomassie Brilliant Blue G-250	Fluka, Steinheim

2.3.5 Nucleotides

ADP monopotassium salt dihydrate	Sigma, Steinheim

ATP disodium salt, Grade II, min 99%	Sigma, Steinheim

2.3.6 Protein inhibitors

H89, Dihydrochloride in sloution	Calbiochem, Darmstadt
Okadaic acid, potassium salt	Calbiochem, Darmstadt
Oligomycin	Calbiochem, Darmstadt
Rotenone	Sigma, Steinheim
Wortmanin	Calbiochem, Darmstadt
Forskolin min 98%	Sigma, Steinheim
IBMX (3-isobutyl-1-methylxanthine)	Sigma, Steinheim

2.3.7 Substrates

Cytochrome c from bovine heart	Sigma, Steinheim
L (+)- Ascorbate	DM, Karlsruhe
L. Glutamic acid monosodium salt hydrate	Sigma, Steinheim
L (-) Malic acid disodium salt 98% purity	Sigma, Steinheim
Succinic acid 99%	Aldrich, Steinheim
PEP (phosphoenolpyruvate)	Sigma, Steinheim

2.3.8 Uncoupler

CCCP	Sigma, Steinheim

2.4 Apparatuses

Biofuge fresco, Mikroliter rotor 24 x 2ml	Heraeus, Langenselbold
Clark-oxygen electrode	Hansatech Instrument, England
Digital pH meter	Mettler Toledo, Switzerland
Fastblot B43	Whatman Biometra, Göttingen
Gel electrophoresis apparatus	Biometra, Göttingen
Gel gradient mixer	Workshop, Fachbereich Chemie, Marburg
In Lab pH combination, liquid filled electrodes	Mettler Toledo, Switzerland
Multifuge 1S	Heraeus, Langenselbold
Mikroliter rotor 48 x 2ml	Sorvall, Heraeus, Langenselbold
Festwinkel rotor FA 12.94 High conic	Sorvall, Heraeus, Langenselbold
Multiskan photometric microplate reader	Thermo, Finland

Loose and tightly fitting glass Potters	Kobe, Marburg
Nicolet Evolution 100 spectrophotometer	Thermo Electron Corporation, Cambridge UK
Power Pack P25	Biometra, Göttingen
Speed-vac concentrator	Bachhofer, Reutlingen
Table top microcentrifuge	Eppendorf, Hamburg
Ultracentrifuge L5-65, 60 Ti Rotor	Beckmann, Frankfurt
Varioperpex 12000 peristatic pump	LKB, Sweden
Vortex	Heidolph, Schwabach
Gel blotting paper	Whatman, Dassel

2.5 Kits

BCA™ Protein Assay Kit	Thermo, USA
ECL (Enhanced Chemiluminescence) substrate	Amersham, Sweden

2.6 Chromatographic materials

DEAE Cellulose	Serva, Heidelberg
DEAE-Sephacel	Pharmacia, Freiburg
Sephadex G-25	Pharmacia, Freiburg

2.7 Membranes

Immobilion PVDF Transfer membrane 0.45µM	Kobe, Marburg
Membrane (PTFE 0.125mm x 25mm) 30m reel for Clark-oxygen electrode	H. Saur, Reutlingen

3. Methods

3.1 Animals

Bovine heart, liver and kidney were obtained from the slaughterhouse in Marburg and transported to the laboratory on ice. They were either used directly for the isolation of mitochondria or kept at -80 °C after the removal of connective tissue and fat. Wistar or SD rats were obtained from the animal house in the Biomedical Research Center (BMFZ), Marburg.

3.2 Incubation of the bovine heart tissue

5 g of frozen bovine heart tissue was first thawed up on ice for half an hour. By the use of scalpel, the heart was cut into slices with the thickness of less than 1 mm. The minced tissue was washed with 50 ml Joklik medium two times, filtered using cheese cloth and divided into equal parts each of 0.5 g. Using 15 ml Erlenmeyer flasks, the minced tissue was incubated with or without additions under shaking at RT (room temperature) for the indicated times as mentioned in the legends. After incubation, each tissue sample was filtered and homogenized with 5 ml of the isolation medium-A in a glass/Teflon potter and mitochondria were isolated as described below.

3.3 Isolation of mitochondria

All the steps involved in isolation of mitochondria were performed at 4°C and the glassware used was also precooled in an ice-bath before starting the procedures in order to minimize the activity of phospholipases and proteases.

3.3.1 Isolation of mitochondria from bovine heart, liver and kidney

Isolation buffer-A (pH 7.4)	250 mM Sucrose 20 mM Hepes 1 mM EGTA 0.2% BSA (freshly added)

The tissues were washed 2-3 times with ice-cold isolation buffer, cut into cubes of about 5 mm^3 using scissors, washed again with ice-cold isolation buffer, decanted and homogenized in 5 volumes of isolation buffer using a loose-fitting glass/Teflon potter (1000 rpm; 5-10 strokes).

The tissue homogenates were transferred into 50 ml Falcon tubes and centrifuged at 800g for 10 min at 4°C. The supernatant containing mitochondria was filtered through a

cheese cloth to remove connective tissue and centrifuged at 11000 g for 15 min at 4°C. After centrifugation, the supernatant was discarded and mitochondrial pellet was washed with 5 ml of ice-cold isolation buffer and centrifuged again at 11000 g for 15 min. After discarding the supernatant, mitochondrial pellet was resuspended in small amount of isolation buffer and transferred into the Eppendorf cups and either kept on ice for immediate use or shock freezed in liquid nitrogen and stored at -80°C for further use.

3.3.2 Isolation of intact mitochondria from rat heart and liver

Isolation buffer-B (pH 7.4)	250 mM Sucrose 10 mM Hepes 1 mM EDTA 0.2% BSA (freshly added)

After killing the rats by decapitation mainly, the heart and liver were taken out rapidly, immersed in 30 ml of ice-cold isolation medium in small beakers separately and rinsed to remove the blood completely. While keeping the beaker on ice, tissues were minced by scissors into small pieces, filtered, decanted and homogenized in 5 volumes of isolation buffer using the loose-fitting glass/Teflon potter (operated at 1000 rpm; 5-10 strokes) for heart and tightly-fitting glass/Teflon potter for the liver (operated at 1600 rpm; 5-10 strokes). Centrifugation was performed as described above for bovine tissues.

3.3.3 Prepration of mitoplasts from rat liver mitochondria

Rat liver mitoplasts were prepared with digitonin as described previously [Schnaitman and Greenawalt, 1968; Carabez and Sandoval, 1974]. Equal aliquots of rat liver mitochondrial suspension (100 mg mitochondrial protein/ml) in isolation buffer and ice-cold digitonin solution were mixed with continuous stirring for 15 min at 0°C. Digitonin solution was prepared as 12 mg digitonin/ml in the same isolation buffer-B just prior to use. After 15 min, the resulting suspension was diluted with three volumes of isolation buffer-B and centrifuged at 11000g for 10 min. The supernatant was carefully drawn off and the pellet was gently suspended in the same volume of isolation buffer-B and left on ice for polarographic measurements.

3.3.4 Incubation of rat heart mitochondria

After isolation, equal aliquots of rat heart mitochondria were incubated with additions after 1:15 dilution in the isolation buffer-B. The time, temperature and the additions are

mentioned in the legends to the figures accordingly. After incubation, samples were cooled on ice, centrifuged at 11000g and suspended in the minimal volume of isolation buffer-B. Finally 10 µl of each were used for kinetics measurement and the rest were stored at -80°C or proceeded directly for the Western blots.

3.4 Determination of protein concentration by BCA method

Mitochondrial protein concentration was determined using Pierce BCA protein assay kit which is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein.

Kit contents

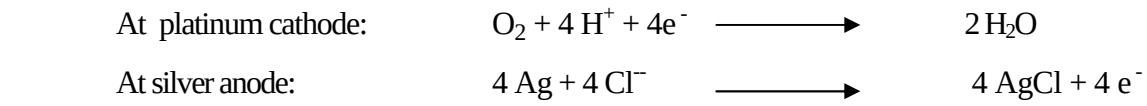
BCA Reagent A contains:	Sodium carbonate Sodium bicarbonate Bicinchoninic acid Sodium tartarate in 0.1M sodium hydroxide
BCA Reagent B contains:	4% cupric sulphate
Albumin Standard Ampules:	bovine serum albumin (BSA) at 2.0 mg /ml in 0.9% saline and 0.05% sodium azide

The contents of one Albumin Standard (BSA) ampule was diluted into several Eppendorf cups using distilled water as a diluent as described in the kit procedure. The working reagent was prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (in a ratio of 50:1, Reagent A: B).

Protein sample was diluted with distilled water (D.W.) in 3 different dilutions of 1:20, 1:50 and 1:100 and 10 µl of each of standard and unknown sample replicates were pipetted into a microplate well. And 200 µl of the working reagent was added to each well and then the plate was covered and incubated at 37°C for 30 min. After this, the plate was cooled to room temperature and the absorbance was measured at 562 nm on a Multiskan photometric microplate reader using Multiskan Ex software. Finally, after subtracting 562 nm absorbance of the Blank from the absorbance of all other individual standard and unknown samples, a standard curve was plotted for BSA standards vs. their concentrations in µg / ml using Microsoft Excel program and the protein concentration of each unknown sample was determined.

3.5 Polarographic measurement of oxygen consumption

Polarographic measurements of oxygen consumption were performed with a Clark type electrode [Ferguson-Miller, 1998] from Hansatech (UK). When a potential difference of 600 mV is applied, the following chemical reactions occur at the electrodes:



Electrons generated at the anode are used to reduce oxygen at the cathode. The oxygen tension at the cathode then drops and this acts as a sink so that more oxygen diffuses towards it to make up the deficit. Since the rate of diffusion of oxygen through the membrane is the limiting step, the current produced by the electrode is proportional to the oxygen tension in the sample. For electrode calibration, the measuring cell was filled with air saturated corresponding buffer either for respiration or for kinetics.

3.5.1 Measurement of mitochondrial respiration

Sucrose-buffer (pH 7.2):
250 mM sucrose
10 mM Hepes
5 mM $MgSO_4$
0.2 mM EDTA
5 mM KH_2PO_4
0.5% fatty acid-free BSA

KCl-buffer (pH 7.4):
130 mM KCl
3 mM Hepes
0.5 mM EDTA
2 mM KH_2PO_4
0.5% fatty acid-free BSA

Respiration of intact rat liver mitochondria was measured polarographically at 25°C in 0.5 ml of sucrose-buffer while that of heart mitochondria from both rat and bovine was measured in KCl-buffer under the same conditions unless otherwise stated in the legends. Finally, the Respiratory Control Ratio (RCR) was calculated as:
RCR = respiration rate at state 3 / respiration rate at state 4 [Chance and Williams, 1955a].

3.5.2 Measurement of enzyme kinetics

Kinetics-buffer (pH 7.4):	250 mM Sucrose 20 mM Hepes 1 mM EDTA 2 mM EGTA 25 mM NaF 10 nM okadaic acid 5 mM $MgSO_4$ 1% Tween-20

The kinetics of CcO activity was also measured polarographically at 25°C in a volume of 0.5 ml in the kinetics-buffer and oxygen consumption was recorded at increasing concentrations of cytochrome c (0.2 – 60 µM) in the presence of 17 mM ascorbate and either 5 mM ADP, or 5 mM ATP and a regenerating system consisting of 10 mM phosphoenolpyruvate (PEP) and 20 or 160 U/ml pyruvate kinase (PK). The rates of oxygen consumption (nmol O_2 min^{-1} ml^{-1}) were calculated at each concentration of cytochrome c and presented graphically using Microsoft Excel program.

3.6 Isolation of cytochrome c oxidase (CcO)

Cytochrome c oxidase was isolated from mitochondria either by the Blue Native polyacrylamide gel electrophoresis (BN-PAGE) [Schäger et al., 1991; Wittig et al., 2006] or by Triton X-100 method [Kadenbach et al., 1986]. Both procedures were performed in the cold room. Also the equipments and buffers used were placed on ice (0-4 °C) during the whole isolation procedures.

3.6.1 Isolation of CcO by BN-PAGE

CcO was isolated from mitochondria in a one step procedure by BN-PAGE (first dimension, 1D).

Solutions

3xGB - 3xGel buffer (pH 7.0):	1.5 M Aminocaproic acid 150 mM Bis-tris
AB - Acylamide/bisacrylamide mix (99.5 T, C):	48% Acylamide, 1.5% Bisacrylamide
10% Amonium persulfate (APS):	

Anode buffer (pH 7.0):	50 mM Bis-tris
Blue cathode buffer (pH 7.0):	15 mM Bis-tris, 50 mM Tricine 0.02% Serva Blue G (SBG)
Colourless cathode buffer (pH 7.0):	15 mM Bis-tris, 50mM Tricine
SDS, sodium dodecylmaltoside (20%):	
Sample solubilization buffer: (pH 7.0 at 4°C)	1 mM EDTA 2 mM 6-Aminocaproic acid 50 mM Bistris 50 mM NaCl
5% stock of Coomassie blue G-250:	suspended in 500 mM of 6-Aminocaproic acid
Destining solution of 1D blots:	10% Acetic acid , 25% Methanol
Solution I (adjusted to pH 7.0 with HCL):	4% SDS 10% Glycerol 2% Mercaptoethanol 0.03% Serva blue G 50 mM Tris

Preparation of linear 6-13% acrylamide gradient gels

For separation of CcO by BN-PAGE, linear gradient gels of 6-13% acrylamide range were used because these are suitable for separation of protein complexes in the molecular mass range from 10^6 Da to 10^5 Da. The gel mixtures were prepared as described below:

	Stacking 4% gel (5ml)	Separating 6% gel (10ml)	Separating 13% gel (10ml)
3xGB	1.64ml	3.3ml	3.3ml
AB	0.4ml	1.19ml	2.6ml
Water	2.87ml	5.43ml	2.35ml
Glycerol	0	0	1.67ml
APS	60µl	42.9µl	33.3µl
TEMED	6µl	4.3µl	3.3µl

For preparation of gradient gels, Biometra minigel apparatus was used with 1 mm spacers and combs and the casting of gradient separation gels were performed in the cold room at 4°C using a gradient mixer.

After mounting the mini gel glass plates together, the separating gel mixture solutions each of 10ml were prepared as mentioned in the table. The front reservoir of gradient mixer was filled with 3 ml of 6% gel mixture and the rear reservoir with 2.5 ml of 13% gel mixture. The conical insert was put into the rear reservoir. And then the gel cassette was filled in by gel mixture using peristaltic pump at speed 9. Use of more volume of 6% acrylamide solution than of 13% acrylamide solution assures that the two solutions initially do not mix when the connecting tube is opened and a linear gradient is obtained. Then the gels were overlaid with isopropanol and transferred to room temperature for polymerization which took almost 2 hours. After polymerization, isopropanol was removed from the top by draining and afterwards the gels were washed with water and sample gels were cast at room temperature and a comb was inserted and allowed to polymerize for 15 mins. After polymerization, the comb was removed and the gels were overlaid with 1x gel buffer and stored at 4°C until used.

Preparation of samples

400 µg Mitochondrial protein was pelleted by centrifugation at 13,000g for 10 min. This centrifugation was performed only in the case when the protein concentrations were less than 40 mg/ml. After the addition of 40 µl of sample solubilization buffer, mitochondria were homogenized by twirling with a tiny spatula. And 5 µl (2.5 g/g) of the detergent SDS (20% sodium dodecylmaltoside) was added and the samples were left to solubilize for 5-10 min. After centrifugation for 20 min at 20,000g, the supernatant was retained and 5 µl of 50% glycerol was added. Finally, 2.5 µl of Coomassie blue dye from 5% stock suspension was added to give the detergent/dye ratio of 8 (g/g) and 11 µl of each sample was loaded into the gels.

Electrophoretic conditions

Gel cassettes were placed into the apparatus and first the spaces in wells were filled with blue cathode buffer and then the samples were underlaid into the wells. After filling the upper and lower reservoir with blue cathode and anode buffer, respectively, electrophoresis was started at 80 V and when the dye front reached to the stacking gel, the voltage was increased to 200 V. Blue cathode buffer was replaced with colourless cathode buffer when the

dye front reached to 2/3 of the gel and the electrophoresis was continued until the dye front reached the end.

After removing the gel of BN-PAGE, it was used for one of the following procedures:

a) directly for blotting
b) the complex IV bands (CcO) were excised and either directly used for second dimension (SDS-PAGE), Western Blotting and staining, or freezed and stored at -80°C for further use
c) the complex IV bands (CcO) were freeze dried immediately by speed vacuum for mass spectrometric analysis.

a) Electroblotting of native proteins from BN-PAGE gels

Electroblotting of BN-PAGE gels was performed under the conditions as described in the procedure of Western Blot. The difference include only the destaining of the background on the 1D blot after blotting on PVDF membrane with '1D-blot destaining solution'. The blot was rinsed with water and dipped in methanol for 3 min with shaking for complete destaining of the protein bands which were immediately visible after transfer. Again, the blot was rinsed with water, dried and used for Western blot analysis as described later.

b) Denaturing SDS-PAGE for second dimension (2D)

After first-dimensional BN-PAGE, the second dimension i.e. SDS-PAGE was performed for the resolution of subunits of CcO. For this, the same procedure and conditions were followed as described in the procedure of SDS-PAGE except these modifications:

After mounting the glass gel plates together, first 7.5 ml of the separating gel mixture containing 18% acrylamide and high amounts of urea (6M) was added. Then the stacking gel mixture was added leaving 2.3 cm space from the top (for the gel bands to be placed) and without inserting the comb allowed to polymerize at room temperature. At the end, gels were overlaid with running buffer in order to avoid dryness.

Then the bands of complex IV which either have been stored at -80°C (left at room temperature for few minutes) or have been directly excised from ID gels were soaked for 15 min in 1% SDS, rinsed briefly with water and placed at the top of the already polymerized SDS gels. After mounting these gels in the electrophoresis chamber, 'Solution I' was added as a 1-2 mm layer which covered the bands of the first dimension. Then electrophoresis was performed under the same conditions as discussed in procedure for SDS-PAGE. After the

second dimension, the same procedures were used for Western blotting and staining as described later.

c) For analysis by mass spectrometry

CcO bands after excision from ID BN-PAGE were freeze dried for 2-3 hrs using a speed vacuum and stored at 4°C for further analysis by mass spectrometry.

3.6.2 Isolation of CcO by Triton X-100 method

CcO was also isolated from mitochondria using the non-ionic detergents Triton X-114 and Triton X-100, followed by DEAE-Sephacel chromatography, exchange of the nonionic detergent by cholate, and fractionated precipitation with ammonium sulfate ($AmSO_4$) [Kadenbach et al., 1986].

Solutions

1 M KPi, pH 7.2

20% Triton X-114 (w/v)

Washing buffer: 200 mM KPi, pH 7.2

Extraction buffer: 200 mM KPi, pH 7.2, 5% Triton X-100

Equilibration buffer: 50 mM KPi, pH 7.2, 0.1% Triton X-100

Elution buffer: 200 mM KPi, pH 7.2, 0.1% Triton X-100

Saturated $AmSO_4$ (at 4°C): adjusted to pH 7.2 with concentrated NH_4OH

Regeneration of DEAE-Sephadex columns

First the columns were washed with at least 20 volumes of 0.1 M NaOH (the contact of NaOH with the ion exchanger should be at least one hour but not more than two hours) and then with > 10 volumes of water. Then the columns were washed (overnight) with a large volume of equilibration buffer (40 mM KPi, pH 7.2; 0.1% Triton X-100). This was continuously performed until the pH of the effluent from the column became equal to the pH of the buffer and confirmed by pH paper.

Extraction by Triton X-114 and Triton X-100

The thawed mitochondrial suspensions from bovine heart were adjusted to 20 mg protein/ml with isolation medium and then to 4 parts of mitochondrial suspension, 1 part of 1 M KPi, pH 7.2 was added under continuous mixing to give a final concentration of 200 mM KPi. Mitochondria were solubilized by adding 2 ml of 20 % Triton X-114 under continuous

vortexing and the suspension was incubated for 5 min under stirring. Then these solubilized mitochondria were ultracentrifuged at 45,000 rpm for 60 min (using 60 Ti rotor). The heme aa3 content of the supernatant was determined with a spectrophotometer (difference spectrum). Normally no heme aa3 was detected in the supernatant after Triton X-114 treatment. The CcO pellet was washed with washing buffer using a potter homogenizer and centrifuged for 10 min at 13,000 rpm. The pellet containing CcO was solubilized in Triton X-100 extraction buffer and after centrifugation at 45,000 rpm for 30 min, the green supernatants (dark brown at high enzyme concentration) were collected. This extraction step was repeated until all heme aa3 was extracted from the sediment (or until all greenish colour was disappeared from green brown pellets). The heme aa3 content of the supernatants was determined spectrophotometrically.

DEAE-Sephadex anion exchange chromatography

The combined CcO supernatants were diluted with 4 volumes of water and loaded on 6 pre-equilibrated DEAE sephadex mini columns. The columns were kept in the cold room. Unbound proteins were washed out from each mini column with two column volumes of equilibration buffer. CcO was eluted with elution buffer (5 ml) and fractionated into portions of ca. 2 ml. The green fractions, containing enzyme were then combined.

Purification of CcO by fractionated ammonium sulfate precipitation

The green fractions were supplemented with solid Na-cholate to give a final concentration of 1% (w/v) and the pH was adjusted to 7.4 with 1 N NaOH. Ammonium sulphate ($AmSO_4$) precipitation was performed in an ice bath by the drop by drop addition of saturated $AmSO_4$ solution under continuous stirring until a saturation of 28 % was reached and the pH was readjusted. Readjustment of pH was required whenever $AmSO_4$ was added. The solution was stirred overnight (at least longer than 14 h) and centrifuged for 10 min at 27,000 g (15,000 rpm, JA-20 rotor) to precipitate impure CcO. Then $AmSO_4$ was added to the supernatant to a final concentration of 37 %, stirred for 10 min and centrifuged again. The precipitated CcO was dissolved in a minimal volume of mitochondrial isolation buffer-B (without BSA) and kept at -80 °C.

Spectrophotometric determination of CcO concentration

The CcO concentration (µ moles heme aa_3/ml) was determined spectrophotometrically by measuring from a diluted aliquot the oxidized spectrum from 380-500 nm, using the following extinction coefficient:

Cytochrome aa_3: $\varepsilon_{(\text{oxidized, 421-490 nm})} = 140\ mM^{-1}\ cm^{-1}$

3.7 SDS polyacryamide gel electrophoresis (SDS-PAGE)

The subunit pattern of CcO was determined by SDS-PAGE according to Kadenbach et al. [1983]. The applied system uses 18 % acrylamide and high amounts of urea (6 M) in the separation gel.

Solutions

Acrylamide solution:	50%	Acrylamide (w/v)
	1.3%	N, N'-methylenbisacrylamide (w/v)
Separating gel buffer: (pH 8.3)	0.75 M	Tris-HCl
	7.2 M	Urea
	0.2%	SDS
Stacking gel buffer: (pH 6.8)	0.125 M	Tris-HCl
	0.125%	SDS
Electrophoresis buffer:	25 mM	Tris base
	192 mM	Glycin
	0.1%	SDS (w/v)

Gel mixtures

Separating gel with 18% acrylamide (w/v):	2.9 g	Urea
	7.5 ml	Acrylamide solution
	10 ml	Separating gel buffer
	50 µl	8.3% ammonium peroxidisulfate solution
	10 µl	TEMED
Stacking gel with 10% acrylamide (w/v):	2 ml	Acrylamide solution
	4 ml	Stacking gel buffer
	25 µl	8.3% ammonium peroxidisulfate solution
	5 µl	TEMED
	62.5 mM	Tris-Cl
Sample buffer for CcO: (pH 6.8)	20%	Glycerol (w/v)
	8%	SDS

0.01%	Bromophenol blue (w/v)
5%	β-mercaptoethanol (v/v)

For SDS-PAGE, mini gels with the gel size of 10 x 10 x 0.1 cm were used. The readjustment of the pH of separation gel buffer was necessary for each gel preparation to get a good resolution Polymerization of the acrylamide gel was started by the addition of ammonium peroxidisulfate and TEMED. After polymerization of separating gel, the stacking gel was molded on the top and a comb was inserted. After polymerization (for ca. 45 min) the comb was removed from the gel and wells were rinsed with electrophoresis buffer. 3 µg of the denatured enzyme mixed with sample buffer in a total volume of about 6 µl were loaded in each well and the electrophoresis was started. For better separation, electrophoresis was first run at 11 mA for stacking gel and when the dye front was about to enter into separation gel, the current was increased to 25 mA.

Staining of protein bands with Coomassie Blue

Staining solution:	0.25%	Coomassie Brilliant blue G-250
	10%	Acetic acid (v/v)
	30%	Isopropanol (v/v)
Destaining solution:	5%	Ethanol
	4.5%	Acetic acid

Staining and destaining of gels were performed at room temperature with shaking. Gels were taken from the apparatus and gently rinsed with D.W. to remove remaining urea. They were soaked in staining buffer and stained overnight. Then gels were carefully washed with D.W. and destained overnight again in destaining buffer under slow shaking until the protein bands were clearly distinguished from the background.

3.8 Western Blotting

Blotting buffer (pH 8.0):	25 mM	Tris-base
	150 mM	Glycin
	10%	Methanol

Western blotting was performed under standard conditions. For Western blot analysis, proteins separated by SDS-PAGE were transferred onto PVDF (Polyvinylidene fluoride) membranes by "semi-dry-blotting" at room temperature [Eckerskorn et al., 1988]. PVDF

membranes (Immobilion PVDF Transfer Membrane, Kobe, Marburg) were used since its hydrophobicity prevented blotting-through of proteins and because more protein is bound after prolonged transfer time, compared to nitrocellulose. For electroblotting, the apparatus "Fastblot B43" with plate electrodes from glass carbon (Whatman Biometra, Göttingen) was used [Kyhse-Andersen, 1984].

PVDF membranes were first washed in methanol for 5 min. The remaining methanol was removed by 15 min washing with blotting buffer including 3 times change of the buffer. The gel containing electrophoresd enzyme was cut to the size of the membrane and washed briefly with D.W. In addition, the plate electrodes of the blotting apparatus were also made wet before use. In order to make the so-called "blotting sandwich", first two blotting papers (already soaked in blotting buffer) were placed above the anode plate followed by the placement of PVDF membrane and then gel on it and finally 2 papers soaked in blotting buffer to complete the sandwich. Air bubbles in the "sandwich" were removed by rolling a glass rod on it. Finally, the blotiing apparatus was closed by putting the cathode plate on the top. A weight of 2 kg was placed on the top of the apparatus. The blotting was performed at 80 mA (ca. 1mA/cm^2) and the transfer time was 120 min [Eckerskorn et al., 1988].

3.8.1 Detection of immunoreactivity using Horseradish peroxidase (HRP)

Solutions

Blocking reagent:	1X	Roti-Block
PBS (pH 7.4):	2.7mM	KCl
	1.5mM	KH_2PO_4
	137mM	NaCl
	8mM	$Na_2HPO_4.2H_2O$
Washing buffer:	0.1%	Tween-20 in PBS
Stripping buffer (pH 6.8):	2%	SDS
	62.5mM	Tris-HCl
	100mM	ß-mercaptoethanol

The sequence of manipulations for immunochemical detection with Horseradish peroxidase (HRP) was as follows:

a. Separation of proteins by SDS-PAGE (or BN-PAGE)
b. Transfer of proteins on PVDF
c. Blocking of the membrane with (1X) Roti-Block
d. Incubation with primary antibody
e. Incubation with horse-raddish-peroxidase (HRP) conjugated secondary antibody
f. Colour detection by ECL (enhanced chemiluminescence) substrate

Western blot analysis is based on the antigen-antibody interaction. The primary antibody recognizes and binds to the corresponding binding site on the protein (under detection) followed by the recognition and binding of secondary antibody (which is enzyme-linked, in this case was HRP-conjugated) to primary antibody depending on the binding site on primary antibody. Finally, when the substrate of this secondary antibody linked enzyme is given, it reacts and gives the reaction which is then detected.

The PVDF membrane containing blotted proteins was incubated with blocking reagent at room temperature with shaking for one hour to reduce non-specific binding of proteins. Then blots were incubated for 1 hour at room temperature under shaking with primary antibodies prepared in blocking buffer against phosphoserine from mouse (1µg/ml each of 6 purified monoclonal Abs: clones IC8, 4A3, 4A9, 4H4 and 7F12, anti-phosphoserine kit from nanotools, München); against phosphothreonine from mouse, 3 monoclonal Abs (1µg/ml each of 3 clones IE11, 4D11 and 14B3, anti-phosphothronine kit from nanotools, München) and monoclonal antibody against phosphotyrosine from mouse (1:1000, clone 4G10, Millipore, Schwalbach). Non-specific binding of the proteins was removed by washing 5 times each for 5 min with the washing buffer. Then secondary antibody (Goat Anti-Mouse IgG, HRP-conjugated, from Biomol, Hamburg) in blocking reagent (1:10000) was added and incubated for one hour with shaking at room temperature. The blot was washed again 5 times each for 5 min. Finally, immunodetection was performed using the ECL substrate that contained horseradish peroxidase coupled antibodies against mouse secondary antibody (ECL Western Blotting detection reagents from Amersham, Sweden) and the blots were visualized on X-ray films.

After immunodetection, the antibodies at the blot were stripped off by incubation of the blot in stripping buffer for 30 min at 60°C followed by 3x washing with the washing buffer, each for 15 min with shaking. Then after on all the blots, subunits I and IV of CcO were detected using antibodies against these subunits (anti-Cox subunit I and subunit IV

mouse Abs from Mo Bi Tec, Göttingen) diluted as 1:10000 in blocking buffer and the rest was done as described above.

3.9 Membrane potential measurements

The membrane potential was measured in collaboration with Prof. Dr. Katrin Staniek, Veterinär-Medizinische Universität, Wien, Austria, using a tetraphenyl phosphonium (TPP^+) electrode in a thermostated chamber at 25°C, as previously described [Kamo et al., 1979]. Two Ag/AgCl REF200 electrodes (Radiometer, Copenhagen), one of which was covered with a TPP^+-permeable polyvinylchloride-based membrane, were connected to the pH-5 module of a Gilson 5/6 Oxygraph (Middleton, WI, USA). As a reference solution 10 mM TPP^+ was filled into the tube of the TPP^+-selective electrode. The potential difference between the TPP^+- and the reference electrode depends on the TPP^+ concentration in the incubation medium and was recorded on a linear y-t chart recorder. Before each measurement the system was calibrated by increasing concentrations of TPP^+ (0.5 - 5 µM). Assuming that the distribution of TPP^+ between the mitochondria and the medium follows the Nernst equation, and that the conservation of mass holds true, the $\Delta\Psi_m$ was calculated according to the following equation:

$$\Delta\Psi_m = -59.16mV \times \log \frac{V_e \times 10^{\frac{\Delta U - \Delta U_e}{59.16mV}} - V + V_m}{V_m}$$

(V_m, mitochondrial matrix volume, 1 µl/mg protein [Griffiths and Halestrap, 1993] as approximation; V, current volume of the medium; V_e, V after the addition of the uncoupler 2,4-dinitrophenol; ΔU, current deflection of the TPP^+ electrode potential from the baseline and ΔU_e, ΔU after the addition of the uncoupler). In this way, the $\Delta\Psi_m$ was corrected for dilution and non-specific binding of TPP^+ [Murastugu, 1979].

The membrane potential of rat liver mitochondria was measured in the sucrose-buffer, while that of rat heart mitochondria was measured in the KCl-buffer, as used for mitochondrial respiration measurements. Other additions are indicated in the legends to the figures.

3.10 Mass Spectrometry

The analysis of phosphorylated amino acids in CcO subunits by mass spectrometry was performed in collaboration with a group in Bochum (Dr. S. Helling, Prof. K, Marcus Medizinisches Proteom-Center, Funktionelle Proteomik, Ruhr-Universität Bochum).

3.10.1 Enzymatic digestion of purified CcO

This was performed in the laboratory of Dr. Helling and Prof. Katrin Marcus in Bochum as detailed in [Helling, Vogt, Rhiel, Ramzan, Wen, Marcus and Kadenbach, 2008].

3.10.2 Chemical cleavage of proteins in BN-PAGE gels by cyanogen bromide (CNBr)

The dried or dehydrated bands from BN-PAGE were rehydrated for 10 min in 200 µl of 50 mM NH_4HCO_3 solution. After removal of this solution, 200 µl of acetonitril solution (prepared as 1:1 in water) was added and left for 10 mins further at RT. This process of dye removal was repeated with NH_4HCO_3 solution and acetonitril solution one after the other several times unless all the Coomassie blue dye was removed from the gel bands. Finally, the gel bands were smashed with a pipette tip, dried for 15-20 min in a speed vacuum and after adding 20 µl of CNBr (dilution 10 % CNBr with 70% formic acid), left for 2 hours for peptide cleavage at RT in the dark in Eppendorf cups. The reaction was stopped by the addition of 50 µl H_2O for 10 min (quenching). Peptide extraction was performed once with with 0.2% TFA (in 50% ACN) and once with 50 µl of 100 % acetonitril solution (in water). The two supernatents were pooled and sonicated for 15 min in ultrasonic bath. After this, the supernatants were divided into two parts, one for proceeding directly and the other for performing trypsin digestion in addition. All samples were left for 15 min at -80°C for freezeing and then placed into lyophilizator overnight for the evaporation of the solvent.

3.10.3 Trypsin digestion of peptides from BN-PAGE gels

After lyophilization, samples for protein digest were solubilized in 5 µl of 5 M urea for 5 min, followed by the addition of 60 µl of 50 mM NH_4HCO_3 (in 10% acetonitril) and left for 15 min sonication in ultrasonic bath at RT. At this step, pH was checked to be around 7.6. 20 µg of trypsin (Serva) was activated for 3 min in 5 µl of 10 mM HCl, and subsequently diluted with 95 µl of 45 mM NH_4HCO_3 (in 10% ACN). To the lyophilized samples, 5µl of trypsin solutin was added, vortexed and incubated for 2 hours at 45°C on thermomixer.

3.10.4 TiO_2 enrichment of phosphorylated peptides

The samples without trypsin digestion was treated with 20 µl of 5 M urea, spin and vortexed as required. Finally, the identification of phosphorylated peptides required their enrichment with TiO_2 material in self-made columns. The column preparation and slightly differing sample treatments were described earlier (Thingholm et al., 2007; Mazarek et al., 2006). The TiO_2 enrichment of digested frgaments from purified CcO and BN-PAGE gels

extracted CcO was performed as described [Helling, Vogt, Rhiel, Ramzan, Wen, Marcus, Kadenbach, 2008].

3.10.5 NanoLC-ESI-MS/MS and data analysis

These procedures were performed in the laboratory of Dr. Helling and Prof. Katrin Marcus in Bochum as detailed in [Helling, Vogt, Rhiel, Ramzan, Wen, Marcus and Kadenbach, 2008].

4. Results

4.1 Mitochondrial respiration is regulated by two mechanisms

The allosteric ATP-inhibition of CcO was previously shown with the purified enzyme or with detergent-suspended mitochondria [Arnold and Kadenbach, 1997; 1999; Bender and Kadenbach, 2000; Lee et al., 2002] but not with intact isolated mitochondria. The $\Delta\Psi_m$-independent inhibition of respiration of intact mitochondria by ATP is shown here for the first time, as presented in Figures 4 and 5 [Ramzan et al., 2010]. Except the mitochondria prepared from frozen bovine heart (Fig. 4, lower graph), mitochondria from rat liver and rat heart exhibit the classical “respiratory control”, characterized by stimulation of respiration by ADP (state 3 respiration rate) followed by inhibition after conversion of ADP into ATP (state 4 respiration rate). If mitochondria are uncoupled by CCCP (uncoupler of oxidative phosphorylation) respiration increases to the rate of state 3. Subsequent addition of ATP results in partly inhibition of respiration. Further addition of ADP abolished the ATP-inhibition, since it decreases the ATP/ADP ratio (Fig. 4, upper graph). The ATP-inhibition is shown for rat liver and heart mitochondria respiring with succinate (Fig. 5). If the outer membrane of rat liver mitochondria is removed by digitonin treatment, the resulting mitoplasts also exhibit the inhibition of uncoupled glutamate + malate respiration by ATP (Fig. 6). The digitonin treatment partly “uncouples” the mitoplasts (= increased proton permeability), resulting in very low "respiratory control". It was found that the extent of ATP-inhibition of uncoupled respiration is variable depending on the individual mitochondrial preparation. In some cases no ATP-inhibition of uncoupled respiration was found, as shown for example in Fig. 7 for a rat liver preparation. The variable extent of ATP-inhibition in different mitochondrial preparations was expected from the postulated switching off of the allosteric ATP-inhibition of CcO under stress conditions [Kadenbach, Ramzan and Vogt, 2009; Kadenbach, Ramzan, Wen and Vogt, 2010].

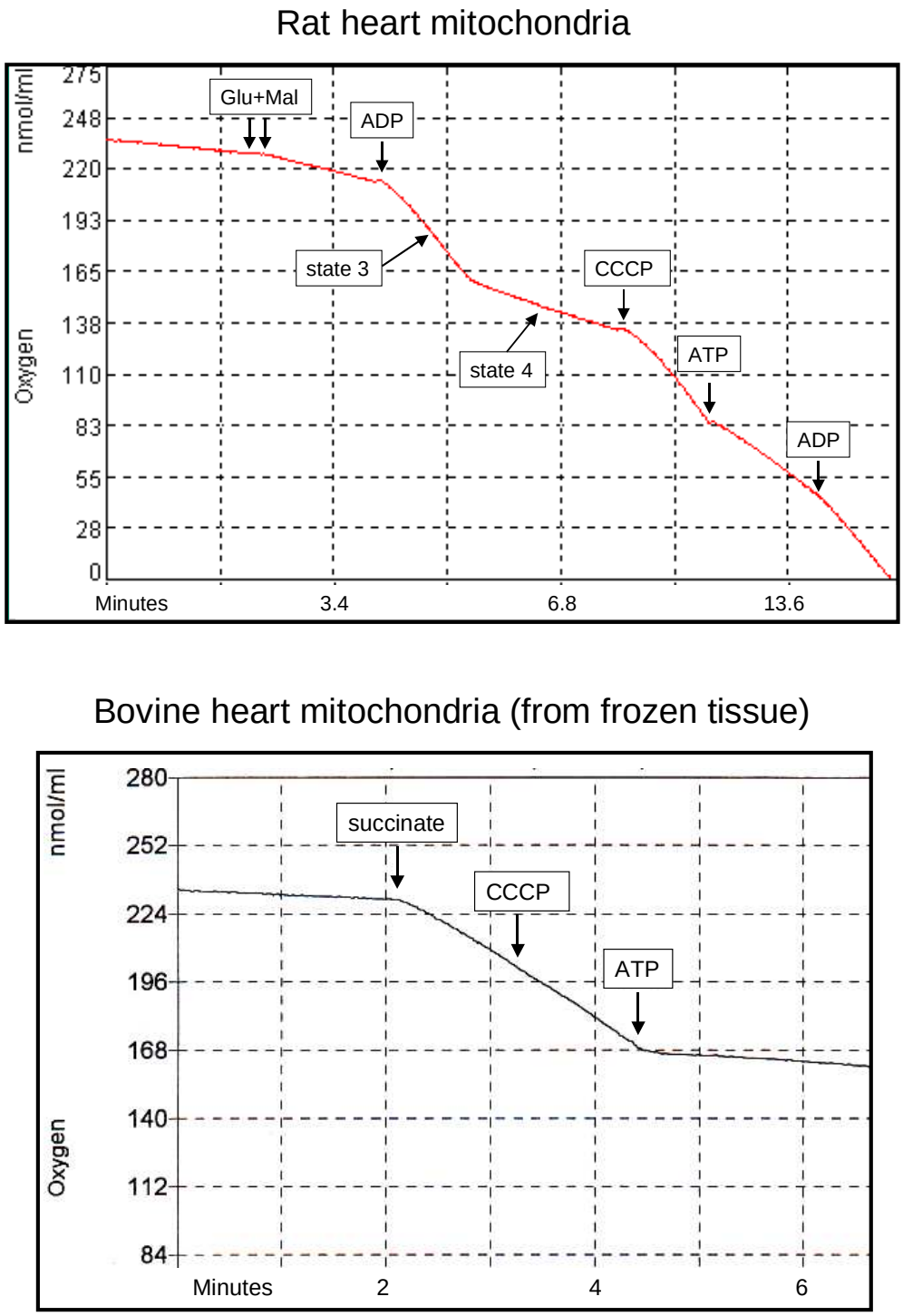


Fig. 4. Respiration of intact mitochondria is inhibited by high $\Delta\Psi_m$-values (classical respiratory control) and by high ATP/ADP ratios in a $\Delta\Psi_m$-independent way

Upper figure; Intact rat heart mitochondria were prepared in isolation buffer-B and the oxygen consumption was measured in KCl-buffer. The indicated additions were: 5 mM glutamate (Glu) + 5 mM malate (Mal) + 0.2 mM ADP + 1 µM CCCP + 10 mM ATP + 1 mM ADP. The final concentration of mitochondria in the measuring chamber was 0.86 mg protein/ml. Lower figure: Bovine heart mitochondria were prepared from heart tissue which had been frozen for two months at -80°C. The oxygen consumption of the mitochondria (0.7 mg protein/ml) was measured in KCl-buffer. The indicated additions include: 5 mM succinate + 1 µM CCCP + 10 mM ATP.

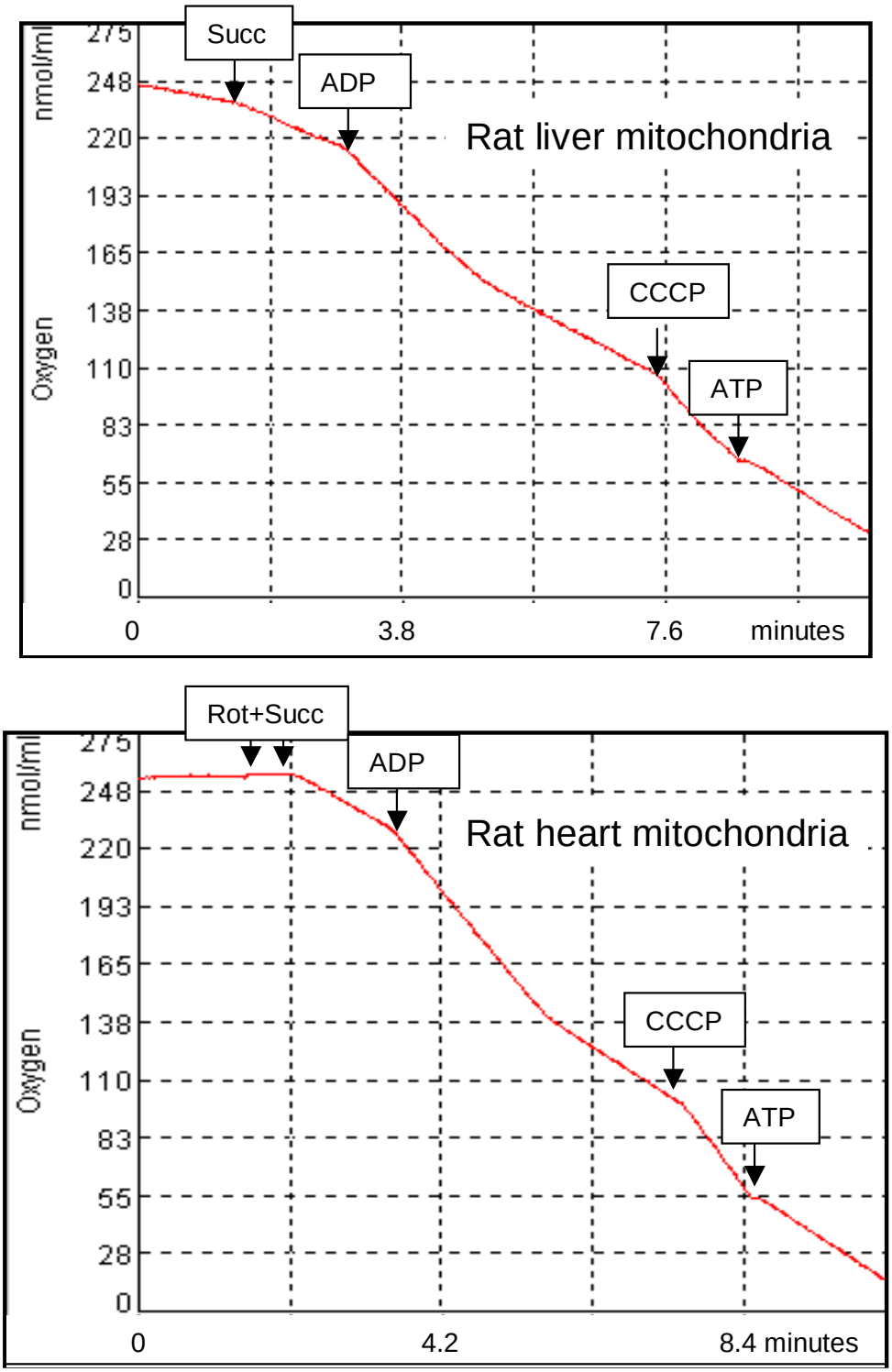


Fig. 5 Succinate-respiration of intact rat liver and heart mitochondria is inhibited by both, high $\Delta\Psi_m$-values and the $\Delta\Psi_m$-independent allosteric ATP-inhibition of CcO
Upper figure: Respiration of intact rat liver mitochondria was measured in KCl-buffer with the following additions: 5 mM succinate + 0.1 mM ADP + 1 µM CCCP + 10 mM ATP (0.52 mg protein/ml). Lower figure: Respiration of intact rat heart mitochondria was measured in KCl buffer with the following additions: 1µM rotenone + 5 mM succinate + 0.2 mM ADP + 0.2 µM CCCP + 10 mM ATP (0.68mg protein/ml).

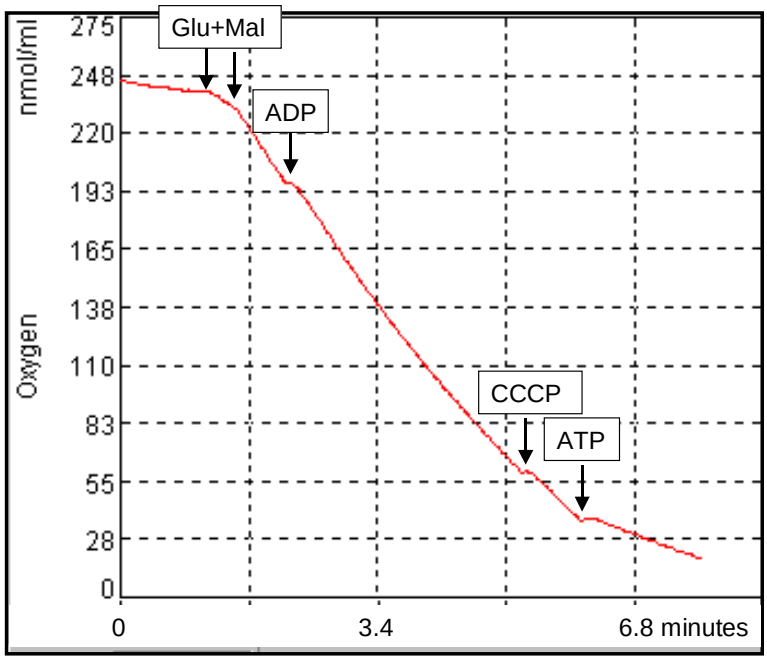


Fig. 6. Rat liver mitoplasts show the $\Delta\Psi_m$-independent inhibition of respiration by ATP
Rat liver mitoplasts were prepared from liver mitochondria using digitonin as described under Methods. The respiration was measured in sucrose buffer-B and additions were as follows: 5 mM glutamate + 5 mM malate + 0.2 mM ADP + 2 µM CCCP + 10 mM ATP.

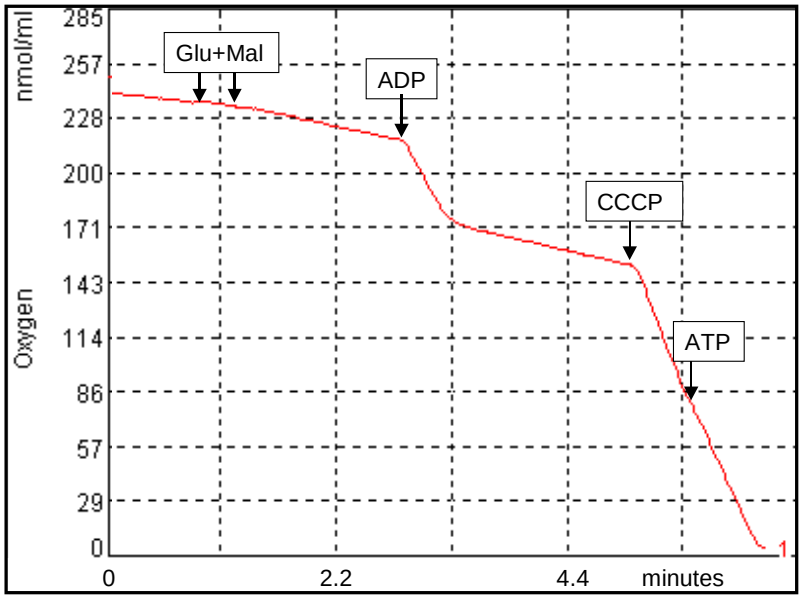


Fig. 7. No $\Delta\Psi_m$-independent inhibition of respiration by ATP in a preparation of intact rat liver mitochondria
Respiration of rat liver mitochondria was measured in sucrose-buffer-B with the following additions: 5 mM glutamate + 5 mM malate + 0.2 mM ADP + 1 µM CCCP + 10 mM ATP. (1.39mg protein/ml)

4.2 Measurement of the kinetics of allosteric ATP-inhibition of CcO in mitochondria

Since one aspect of this dissertation was to characterize the allosteric ATP-inhibition of CcO in intact mitochondria, I investigated the optimal conditions for kinetic measurements. The polarographic analysis of CcO kinetics requires increasing concentrations of reduced cytochrome c. This is achieved by addition of 15 mM ascorbate which reduces added and reoxidised cyctochrome c rapidly to ferrocytochrome c [Ferguson-Miller et al., 1978]. Ascorbate, however, is auto-oxidable, as shown in Fig. 8. In this figure the ascorbate oxidation is measured in the kinetics-buffer at increasing concentrations of cytochrome c,

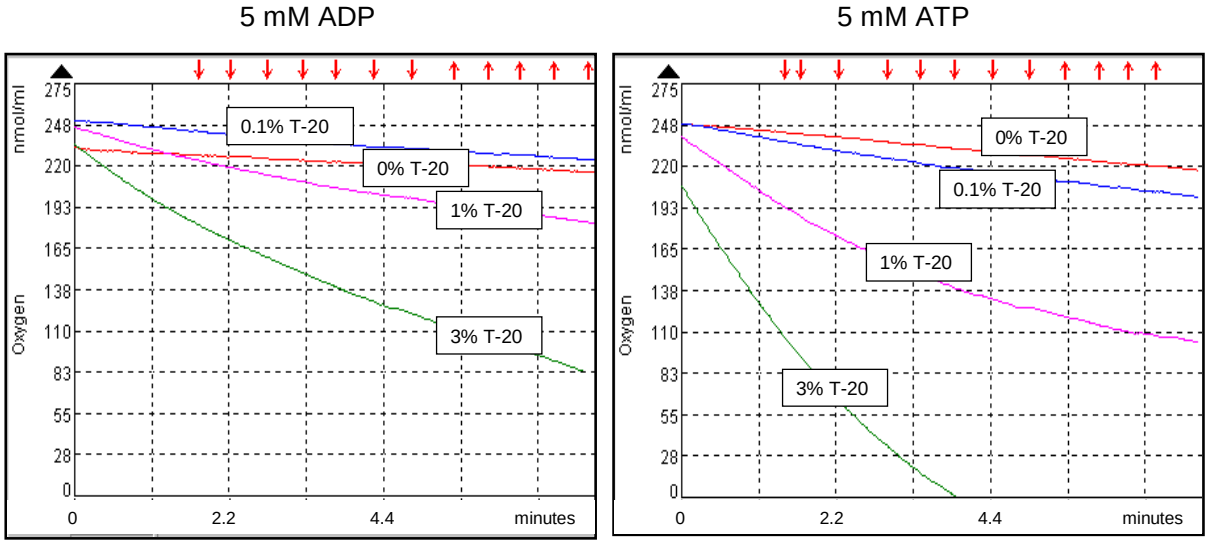


Fig. 8. Influence of Tween-20 and nucleotides on the auto-oxidation of 17 mM ascorbate in kinetics-buffer
Ascorbate auto-oxidation was measured polarographically in kinetics-buffer with 0% - 3% Tween-20 (T-20), without mitochondria, and titrated with cytochrome c concentrations of 0.4, 0.8, 1.2, 1.6, 2.0, 2.8, 5, 10, 20, 30, 40 and 60 µM in the presence of either 5 mM ADP (left figure) with no (red), 0.1% (blue), 1% (pink) and 3% (green) Tween-20 or 5 mM ATP (right figure) with no (red), 0.1% (blue), 1% (pink) and 3% (green) Tween-20. The red arrows indicate the additions of cytochrome c.

used for the kinetic measurements of CcO, which, however, had no effect on the oxygen consumption of ascorbate. Surprisingly, the ascorbate auto-oxidation is stimulated by Tween-20 (required for measurement of CcO activity in mitochondria) and furthermore by ATP. The polarographic recordings of the oxygen consumption of rat liver and rat heart mitochondria in the presence of 1% Tween-20 and at increasing concentrations of cytochrome c are presented

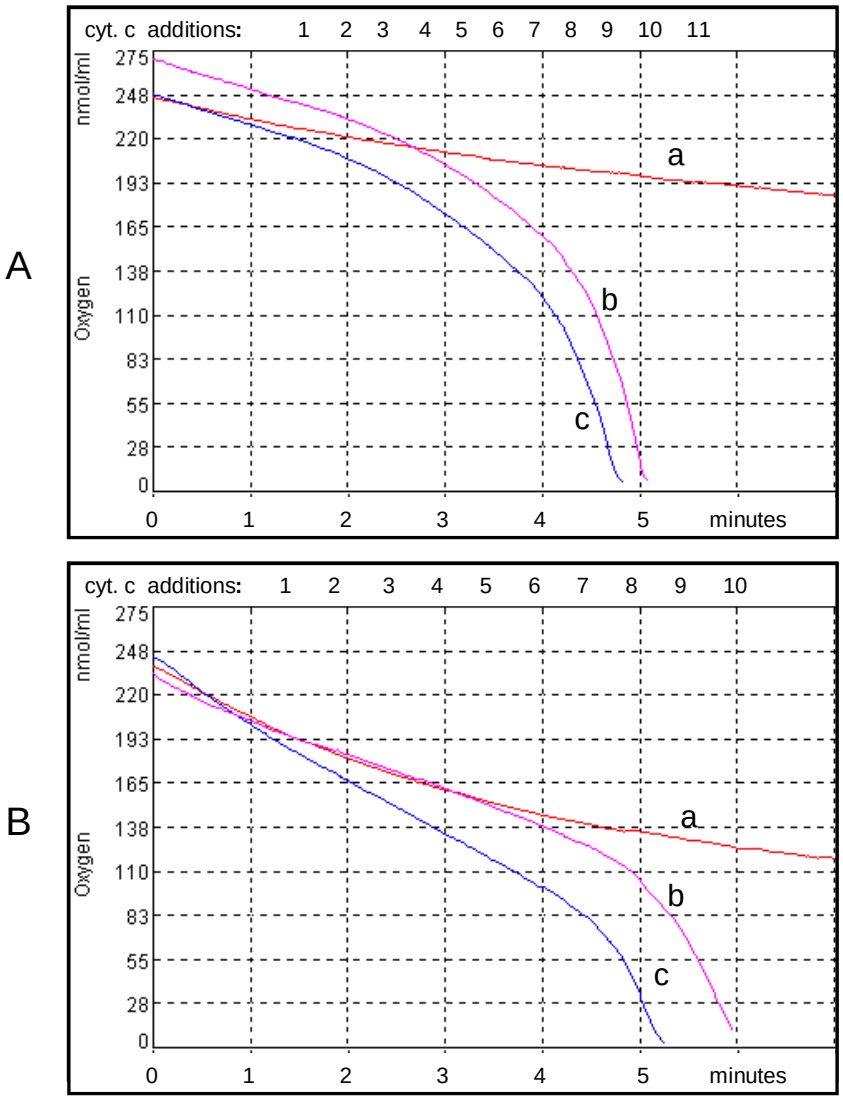


Fig. 9. Polarographic recordings of oxygen consumption of ascorbate in kinetics-buffer without (a) and with rat liver (b) and heart mitochondria (c) in the presence of ADP (A) and ATP (B)
The indicated additions of cytochrome c ranged from 0.4 µM (1) to 40 µM (11) for a. The trace marks for b and c are not indicated. The recordings were performed in kinetics-buffer in the presence of 17 mM ascorbate and 5 mM ADP (A) without mitochondria (a, red), with 10 µl rat liver mitochondria (b, pink) (0.93 mg protein/ml) or 10 µl rat heart mitochondria (c, blue) (0.31 mg protein/ml). Recordings in the presence of 17 mM ascorbate and 5 mM ATP + 10 mM PEP + 20 U/ml PK (B) were performed without mitochondria (a, red), with rat liver mitochondria (b, pink) or rat heart mitochondria (c, blue).

in Fig. 9. On the upper part (A) the recordings in the presence of 5 mM ADP, on the lower part (B) recordings in the presence of 5 mM ATP and an ATP-regenerating system (10 mM PEP and 20 U/ml PK) are presented. It is evident that the recordings with ATP + PEP + PK are different from those with ADP. From the rates of oxygen uptake at increasing cytochrome c concentrations graphs of CcO kinetics were calculated by the Microsoft Excel program as

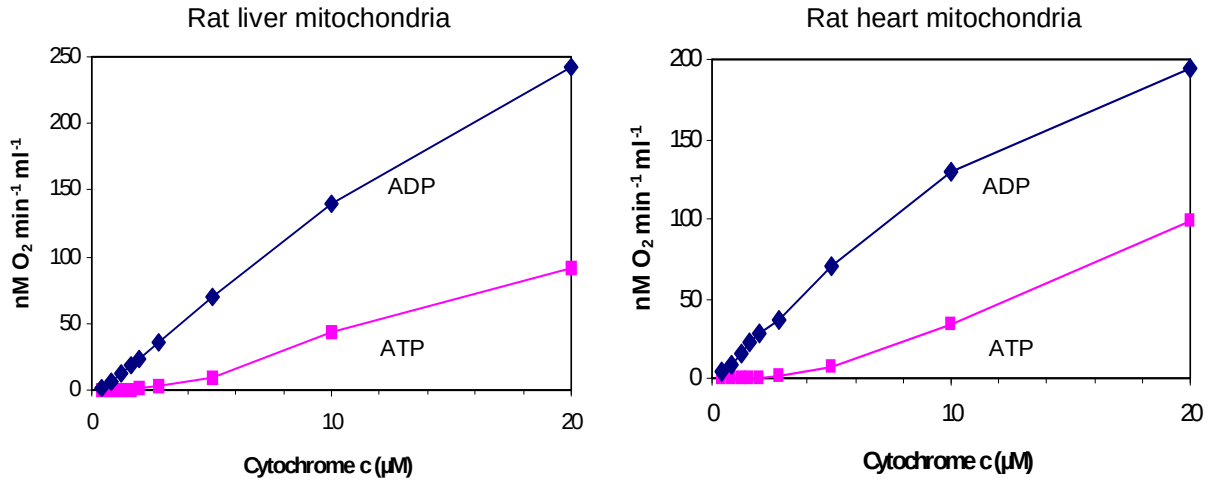


Fig. 10. Kinetics of CcO from rat liver and rat heart mitochondria
These are the kinetics measurements of the mitochondrial samples from Fig.9, prepared in isolation buffer-B and measured in the kinetics-buffer under standard conditions in the presence of ADP (blue) or ATP (pink) as indicated.

presented in Fig. 10. In the presence of ATP, the graphs show sigmoidal inhibition curves of oxygen consumption with increasing cytochrome c concentrations, as compared to hyperbolic curves in the presence of ADP. This 'allosteric ATP-inhibition of CcO' is obtained with rat liver and rat heart mitochondria, but the extent of ATP-inhibition was found to vary in different mitochondrial preparations.

In order to determine the optimal Tween-20 concentration for measuring the kinetics of CcO in mitochondria, the CcO kinetics were measured at increasing Tween-20 concentrations in the presence of ADP at 5, 10 and 20 µM cytochrome c, shown in Fig. 11. The CcO activity increases with increasing Tween-20 concentration, due to increasing accessibility of cytochrome c to CcO. With rat liver mitochondria almost no CcO activity was found in the absence of Tween-20 in contrast to heart mitochondria, where about 50% of maximal activity was measured. Isolated rat liver mitochondria have an intact outer membrane excluding the access of cytochrome c to CcO, whereas the outer membrane of isolated rat heart mitochondria is usually disrupted. Maximal rates were measured between 0.5 and 2% Tween-20. Therefore I used in the following polarographic measurements 1% Tween-20.

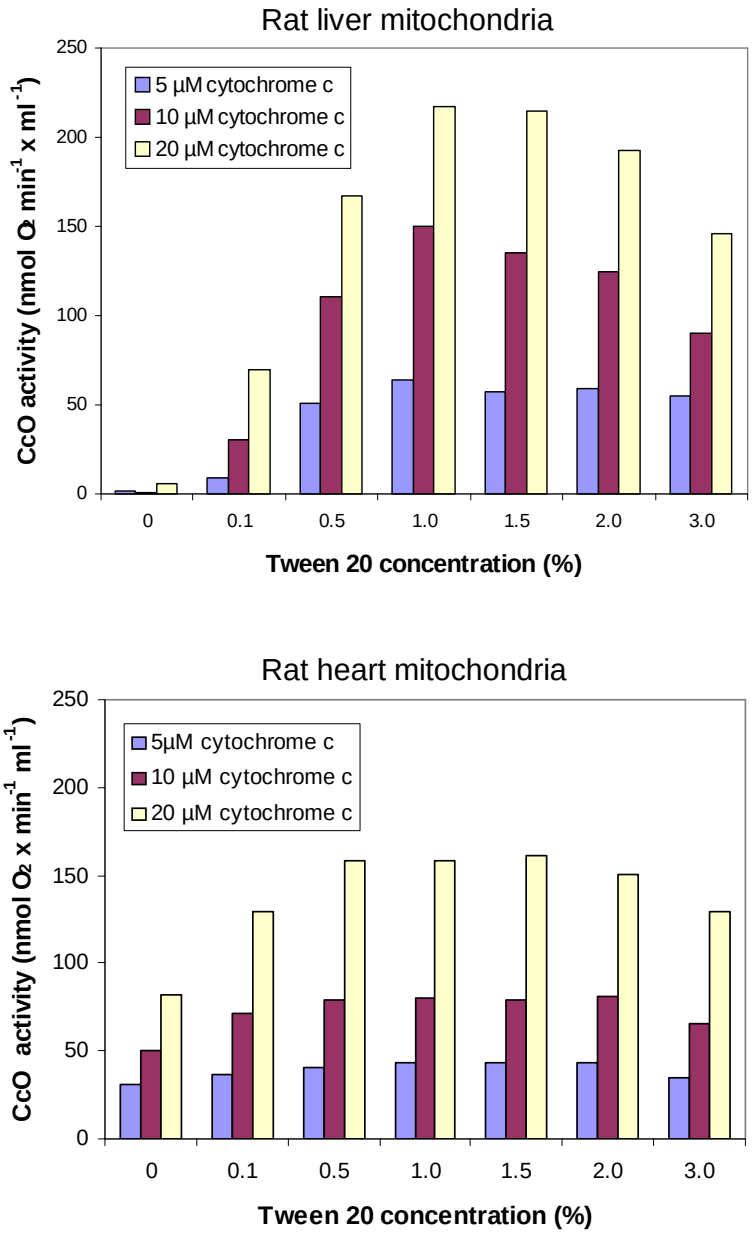


Fig. 11. Effect of Tween-20 on CcO activity of mitochondria from rat liver and rat heart in the presence of ADP
The CcO activity of rat liver (0.94 mg protein/ml, upper graph) and rat heart mitochondria (0.34 mg protein/ml, lower graph) was measured in the kinetics-buffer in the presence of 17 mM ascorbate, 5 mM ADP with cytochrome c concentrations of 5 µM (blue), 10 µM (brown) and 20 µM (yellow) at 0% - 3% Tween-20.

The effect of 1% Tween-20 on the structure of rat heart and liver mitochondria is presented in the electron microscopic pictures shown in Fig. 12. Incubation of rat heart and rat liver mitochondria for 10 min at RT with 1% Tween-20 results in swelling and fragmentation of mitochondria, but the membrane structures are still preserved.

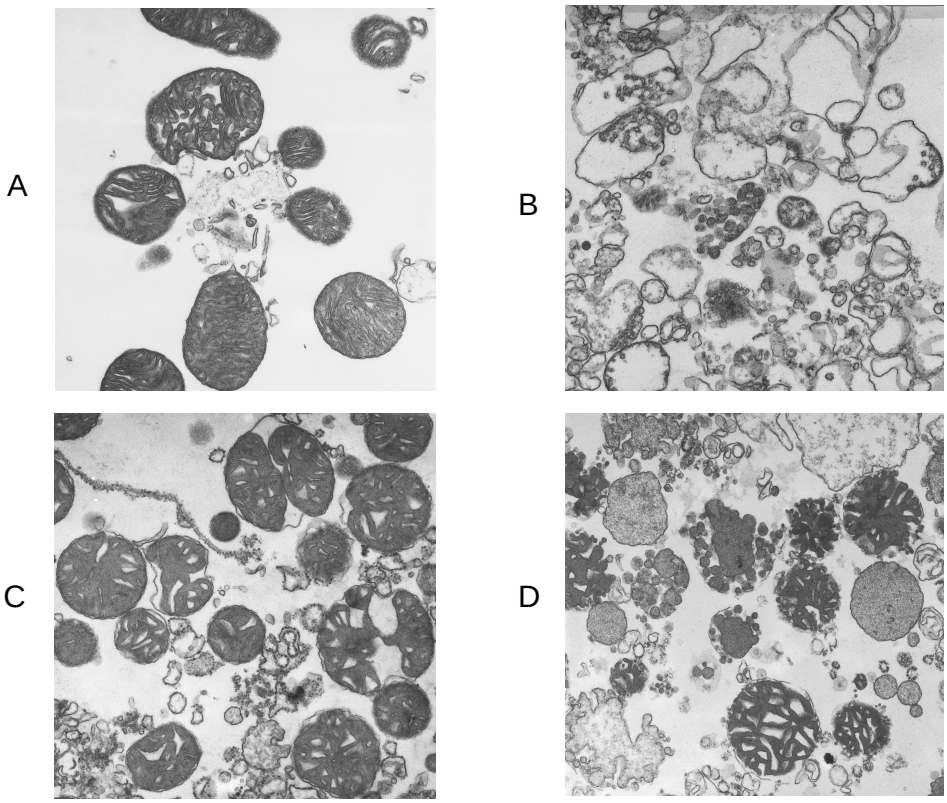


Fig. 12. Electron microscopic pictures of isolated rat liver and rat heart mitochondria after incubation in the absence and presence of 1% Tween-20
Mitochondria were incubated for 10 min at RT in kinetics-buffer without (A and C) and with 1% Tween-20 (B and D) isolated from rat heart (A, B) and rat liver mitochondria (C, D).

The sucrose buffer of the polarographic experiments contained 5 mM $MgSO_4$ because protein kinase (PK) requires Mg^{2+} ions as a cofactor. Mg^{2+} ions, however, stimulate the mitochondrial F_0F_1-ATPase resulting in decreased ATP/ADP ratios, as apparent from Fig. 13, where addition of $MgCl_2$ has strongly stimulated the rate of state 4 respiration, which is inhibitited by oligomycin, a specific inhibitor of mitochondrial ATPase.

As complete allosteric ATP-inhibition of CcO is only measured when the amount of ADP in the polarographic measuring cell is less than 2% of total [ADP + ATP] (half-maximal inhibition at ATP/ADP = 28 [Arnold and Kadenbach, 1999]), the hydrolysis of ATP by ATPases in the mitochondrial preparation must be compensated by an ATP-regenerating

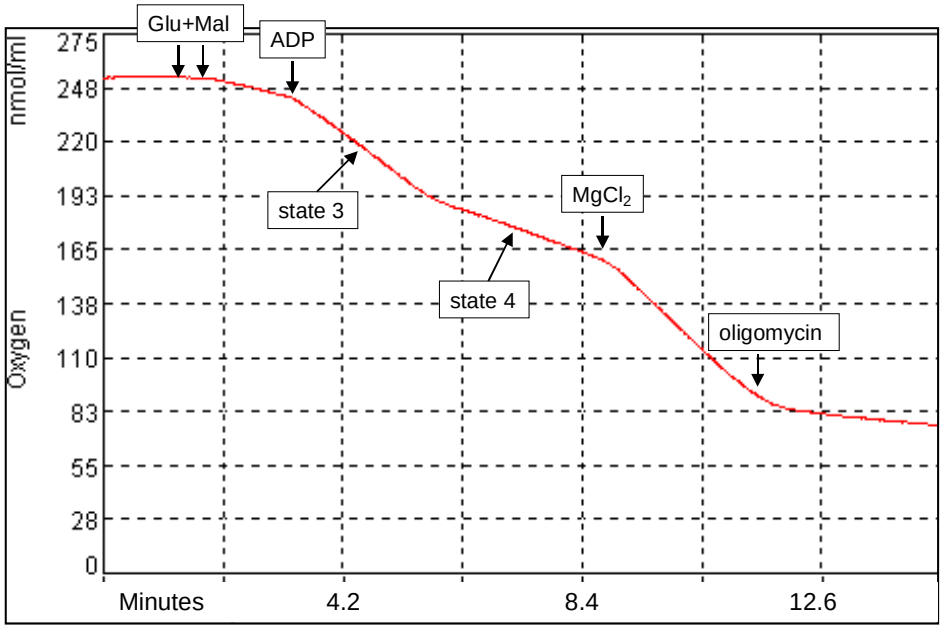


Fig. 13. Magnesium ions strongly stimulate F_0F_1-ATPase of rat heart mitochondria, indicated by the stimulation of state 4 respiration which is inhibited by oligomycin
The oxygen consumption of rat heart mitochondria (0.45 mg protein/ml) was measured in KCl-buffer. The indicated additions include: 5 mM glutamate (Glu) + 5 mM malate (Mal) + 0.2 mM ADP + 5.3 mM $MgCl_2$ and 1 µg/ml oligomycin.

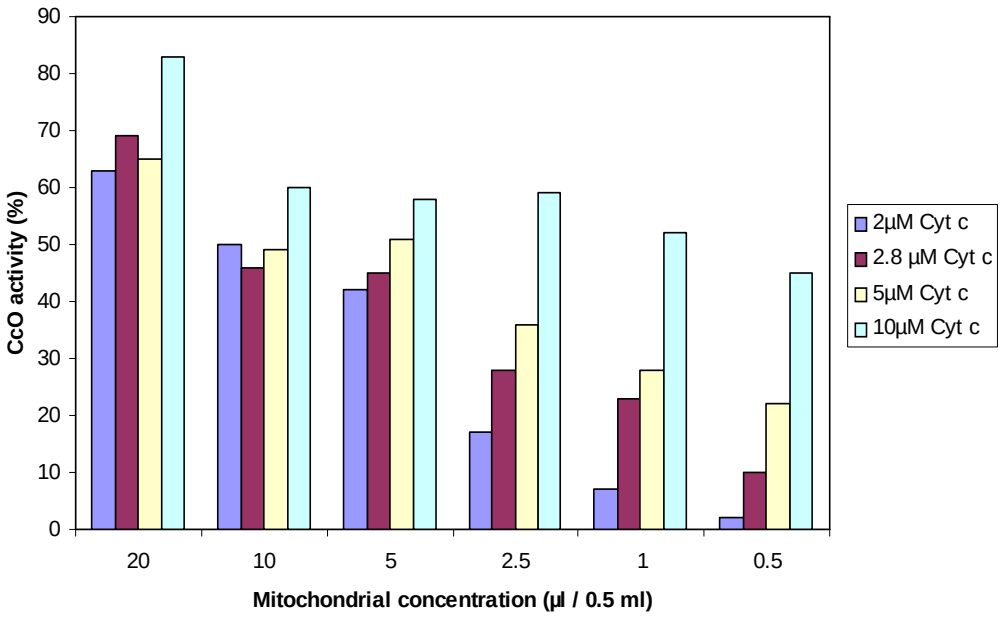


Fig. 14. The concentration of rat heart mitochondria influences the extent of allosteric ATP-inhibition of CcO
The activity of CcO at decreasing concentrations of mitochondria was measured in the kinetics-buffer under standard conditions at indicated concentrations of cytochrome c, i.e. 2 µM (blue), 2.8 µM (brown), 5 µM (yellow) and 10 µM(light green) either with ADP or ATP, and the percentage CcO activity with ATP at each concentration of mitochondrial protein and cytochrome c was related to the activity with ADP taken as 100 %.

system consisting of PEP and PK. This conclusion is supported by the experiment shown in Fig. 14, where the CcO activity was measured at increasing dilution of mitochondria in the measuring cell at 4 different cytochrome c concentrations. With increasing dilution of mitochondria the percentage of CcO activity in the presence of ATP + PEP + PK, related to the activity with ADP = 100%, decreased and reached to almost 0% in the presence of 2 µM cytochrome c. This result clearly indicates a shift of the steady state ATP/ADP ratio to higher values by dilution of mitochondria, due to the reduction of the amount of ATPases, while the activity of the ATP-regenerating system (PEP+ PK) remains constant.

The ATP-regenerating system is essential for the measurement of allosteric ATP-inhibition of CcO in isolated mitochondria, as apparent from Fig. 15. The hyperbolic curve of oxygen consumption of rat heart mitochondria at increasing cytochrome c concentrations is increased by 5 mM ADP. Addition of 5 mM ATP induces a sigmoidal inhibition curve which is further lowered by the addition of the ATP-regenerating system (PEP + PK) which compensates the activities of ATPases and keeps the ATP/ADP ratio at high levels. In some cases up to 160 U/ml of PK were required to obtain full allosteric ATP-inhibition of CcO with rat heart mitochondria, depending on the amount of ATPases in the mitochondrial preparation (data not shown).

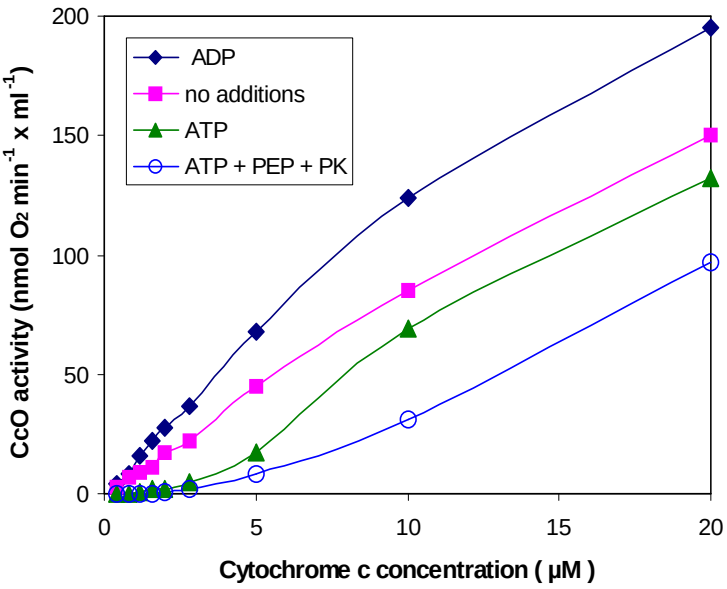


Fig. 15. Effect of the ATP-regenerating system on CcO activity of rat heart mitochondria.
The CcO activity of mitochondria (0.45 mg protein/ml) was measured in the kinetics-buffer in the presence of 17 mM ascorbate. Titrations with increasing cytochrome c concentrations were done either without any addition (pink), with 5 mM ADP (dark blue), 5 mM ATP (green), or 5 mM ATP + 10 mM PEP + 160 U/ml PK (light blue), as indicated. The curves have been drawn by using Microsoft excel program.

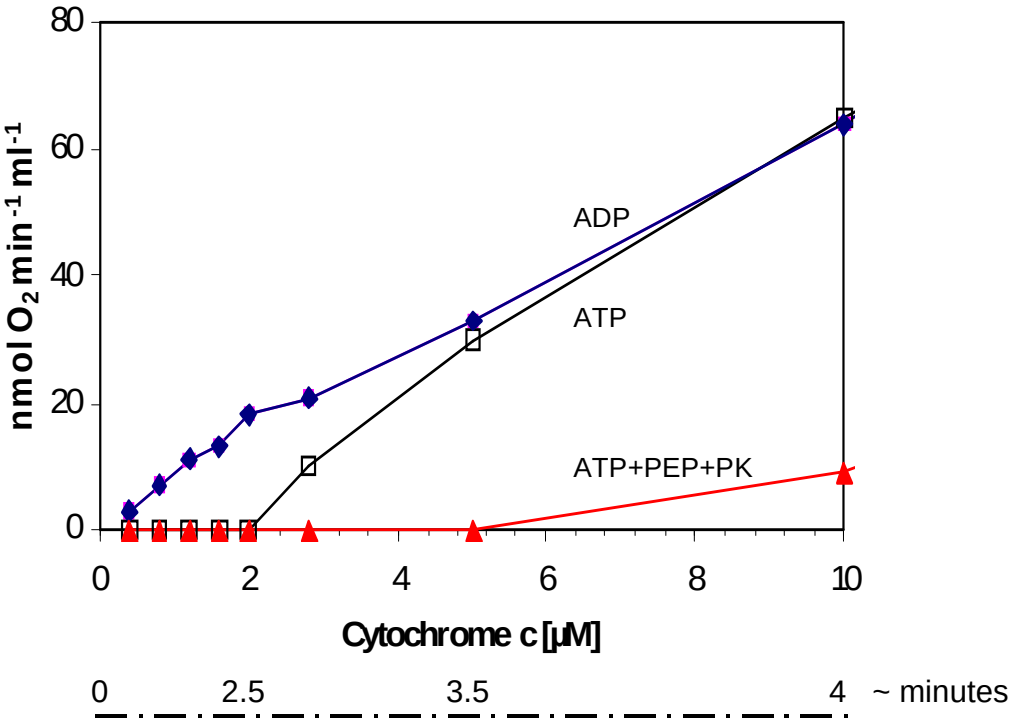


Fig. 16. The allosteric ATP-inhibition of CcO in rat heart mitochondria is abolished with time unless PEP and PK are present
Oxygen consumption of rat heart mitochondria (0.28 mg protein/ml) was measured in kinetics-buffer in the presence of 5 mM ADP (blue), 5 mM ATP only (black), or 5 mM ATP + 10 mM PEP + 160 U/ml PK (red) and titrated with increasing concentrations of cytochrome c in the presence of ascorbate. The time scale has been drawn in order to show the time course of ATP hydrolysis by ATPases, which is compensated by the ATP-regenerating system.

Furthermore, the influence of ATPases on the polarographic measurement of CcO kinetics in the mitochondrial preparation is proven by the experiment of Fig. 16 with rat heart mitochondria where a continuous increase of oxygen consumption is found with ADP. Addition of 5 mM ATP alone results in full inhibition of activity for about 2 minutes (up to the fifth addition of cytochrome c = 2 µM), but then the activity increases to the value obtained with 5 mM ADP (at 10 µM cytochrome c). The presence of PEP and PK in addition to ATP, however, keeps the activity at very low values. This result is a confirmation of previous results with isolated CcO reconstituted in liposomes where the allosteric ATP-inhibition was only measured at very high intraliposomal ATP/ADP ratios (> 50) but was completely lost at slightly lower ATP/ADP ratios (< 16) [Arnold and Kadenbach, 1999].

4.3 The allosteric ATP-inhibition is reversibly switched on by phosphorylation of CcO

CcO was purified from bovine heart by standard procedures [Kadenbach et al., 1986], and the kinetics and Western blots were performed with antibodies against phosphoserine, phosphothreonine and phosphotyrosine and against a mixture of CcO subunit I and IV, as

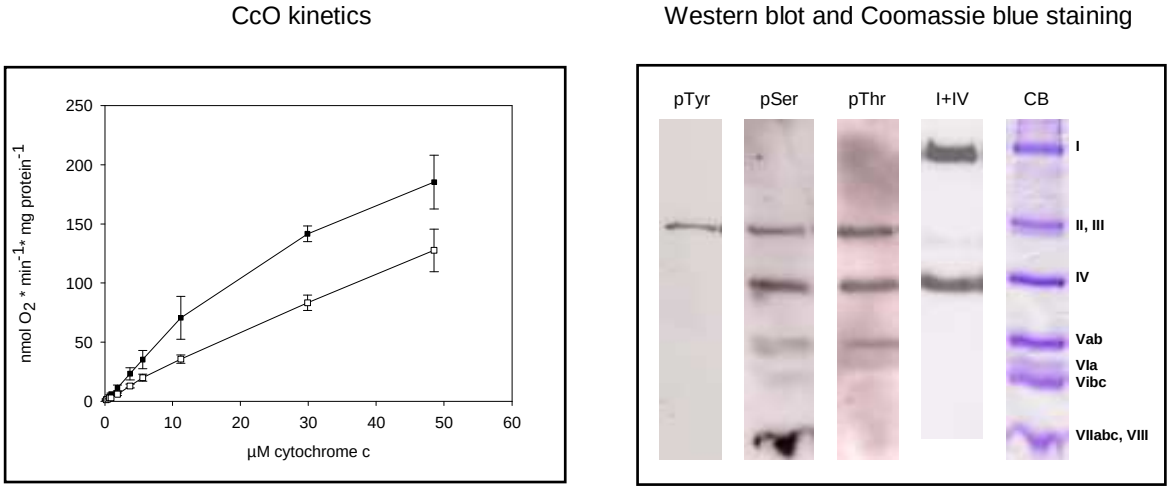


Fig. 17. Purified CcO from bovine heart shows no allosteric ATP-inhibition and no phosphorylation of subunit I
CcO was isolated by the Triton X-100 method and kinetics (left figure) was measured in kinetics-buffer in the presence of ADP (closed circle) and ATP + PEP + PK (open circle). The average of three determinations is shown. Subunits of purified CcO were separated by SDS-PAGE, stained with Commassie blue and blotted with antibodies against phosphoserine (pSer), phosphothreonine (pThr), phosphotyrosine (pTyr) and CcO subunits I+IV (right figure) (taken from [Helling et al., 2008]).

shown in Fig. 17. This purified CcO preparation had no allosteric ATP-inhibition and reacted with antibodies against phosphoserine, -threonine and -tyrosine at various subunits but not with subunit I. Treatment of the enzyme with ATP, protein kinase A (PKA) and cAMP induces an allosteric ATP-inhibition accompanied by phosphorylation of CcO subunit I at serine and threonine.

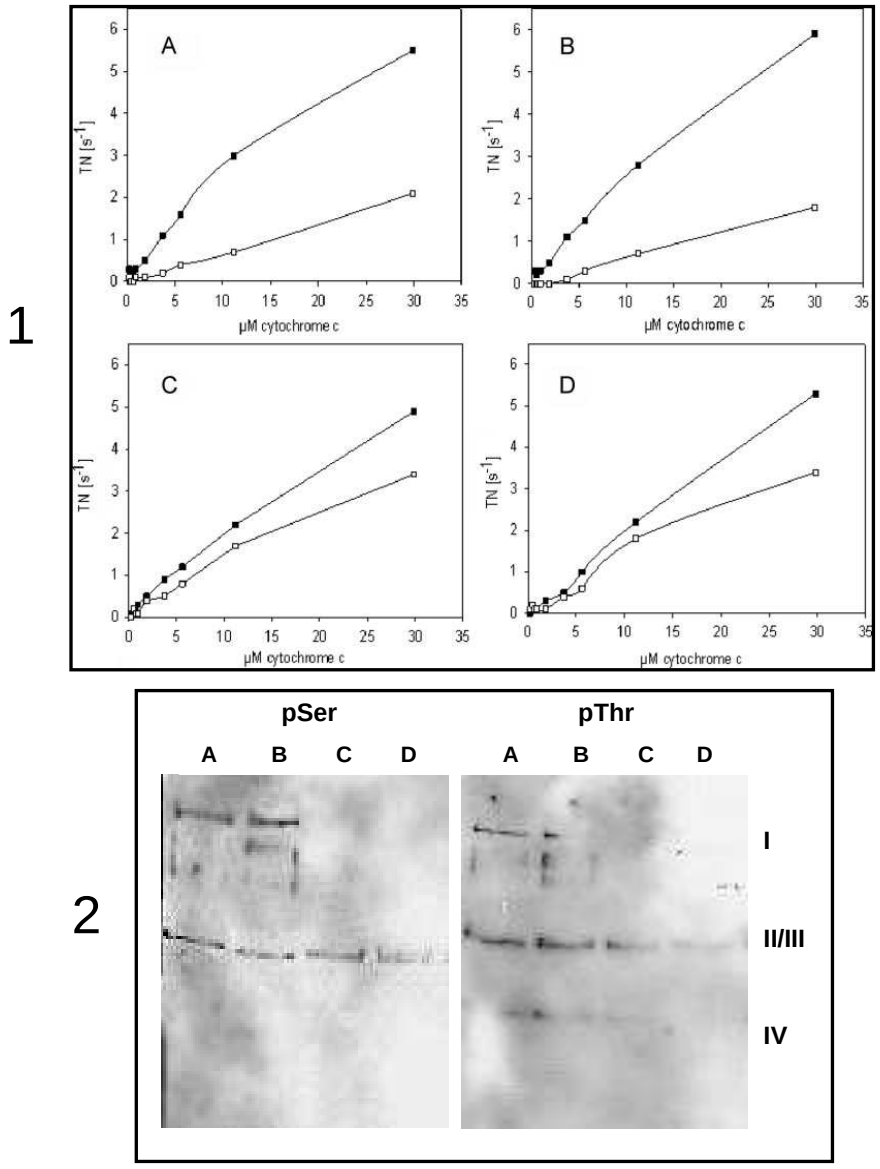


Fig. 18. PKA induces the allosteric ATP-inhibition of CcO and phosphorylates subunit I of purified CcO from bovine heart at serine and threonine
CcO was isolated from bovine heart with the standard Triton X-100 procedure. The kinetics (1) were measured using ascorbate as substrate in the presence of ADP (closed circle) and ATP + PEP + PK (open circle), after incubation of CcO for 40 min at 30°C with (A and B) or without PKA (C and D) under identical conditions. The turnover number (TN) is presented as µmol cytchrome c x µmol heme aa_3^{-1} x s^{-1}. (2) Western blots (2) were performed with antibodies against pSer and pThr (taken from [Helling et al., 2008]).

In order to study the phosphorylation of CcO subunits in small mitochondrial samples, and to isolate CcO in a one-step procedure (faster than isolation by column chromatography), I established in the laboratory the procedure of blue native polyacrylamide gel electrophoresis (BN-PAGE), as described by Schägger and coworkers [Schägger, 1991; Wittig, 2006]. In Fig. 19 is presented the Coomassie blue staining of a BN-PAGE gel with rat heart mitochondria,

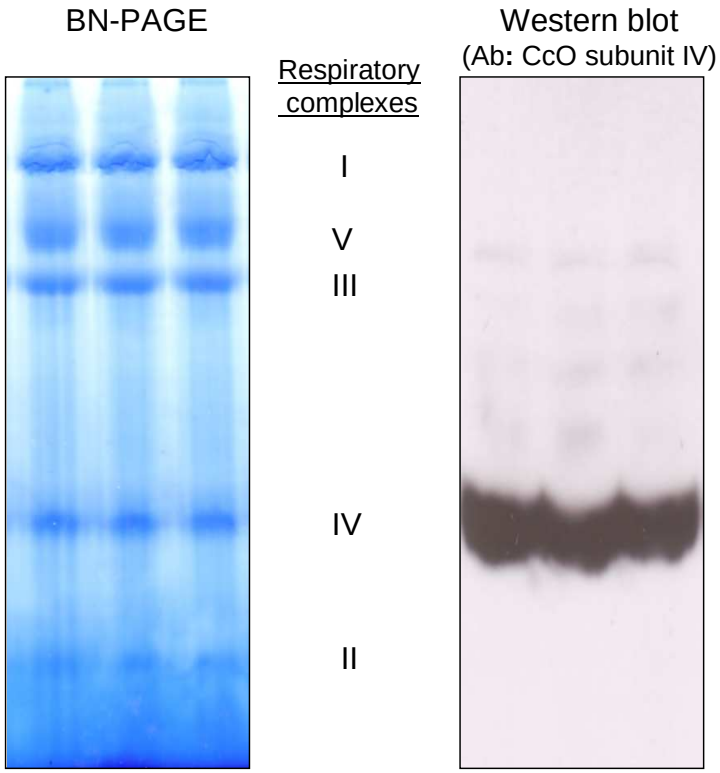


Fig. 19. The antibody against CcO subunit IV reacts with the 4th band (complex IV) of BN-PAGE gels.
BN-PAGE (left) was performed with the mitochondria isolated from rat heart. The Western blot was done after blotting the BN-PAGE gel, and the CcO subunit IV antibody reaction was performed in order to find out the position of CcO (right).

and the Western blot of the gel with an antibody against CcO subunit IV. In the Western blot of the BN-PAGE gel, the antibody reacted only with the fourth band of the Coomassie blue-stained gel, known to represent complex IV (CcO) of the mitochondrial respiratory chain. Following this, SDS gels were run with the fourth band of BN-PAGE gels, blotted on PVDF membranes, and separate Western blots were performed with antibodies against phosphoserine, phosphothreonine and phosphotyrosine.

Moreover, some allosteric ATP-inhibition of CcO was also found with freshly isolated rat mitochondria (Fig. 20). The Western blot exhibited phosphorylation of CcO subunit I at serine, threonine and tyrosine. From these results it was concluded that the allosteric ATP-inhibition can be reversibly switched on and off by serine/threonine phosphorylation/dephodphorylation of CcO at subunit I [Kadenbach, Ramzan, Vogt, 2009; Kadenbach, Ramzan, Wen, Vogt, 2010].

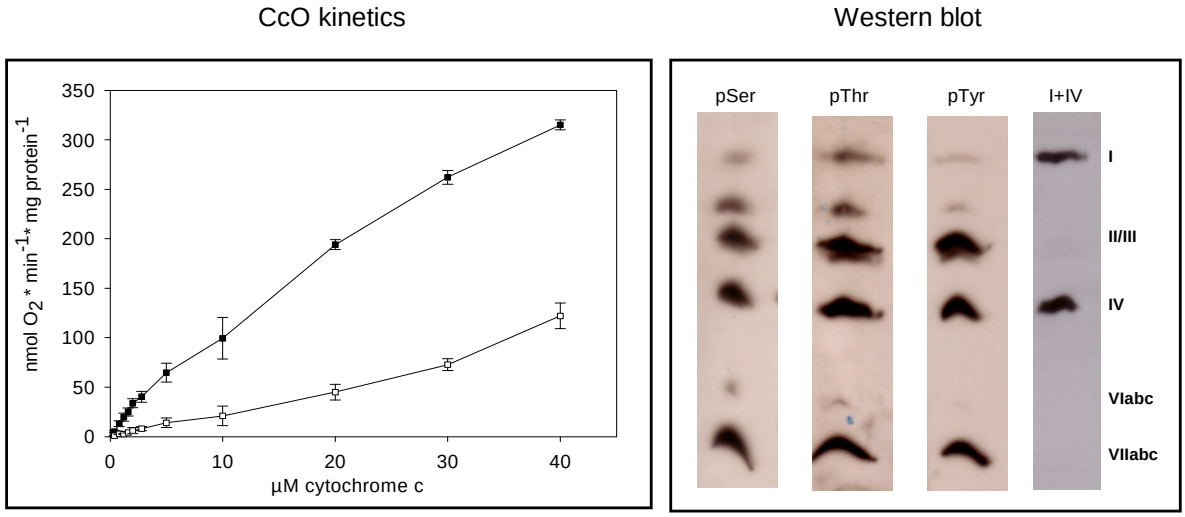


Fig. 20. Isolated rat heart mitochondria show allosteric ATP-inhibition and phosphorylation of CcO subunit I
Rat heart mitochondrial kinetics was measured in kinetics-buffer in the presence of ADP (closed circle) and ATP (open circle). The average of three determinations is shown. In order to see the phosphorylation pattern of CcO in mitochondria, complex IV was isolated by BN-PAGE and the subunits of CcO were separated by SDS-PAGE and Western blot was performed with antibodies against phosphoserine (pSer), phosphothreonine (pThr), phosphotyrosine (pTy), and CcO subunits I+IV (I+IV) (taken from Helling et al., 2008).

4.4 Determination of phosphorylation sites in CcO subunits

After isolation of mitochondria from frozen bovine heart tissue, the CcO complex was purified by BN-PAGE. Either the whole complex IV from BN-PAGE or the subunits after SDS-PAGE followed by staining with Coomassie blue were excised, dried and further investigated by mass spectrometry in collaboration with Dr. Stefan Helling in the laboratory of Prof. Katrin Marcus in Bochum. In parallel, Western blots were continued from other SDS-gels, with antibodies against phosphoserine, -threonine, and -tyrosine. This collaboration resulted in the identification of 6 new phosphorylation sites in CcO. On the other hand, the correlation between these phosphorylation sites and the allosteric ATP-inhibition of CcO has not been found so far.

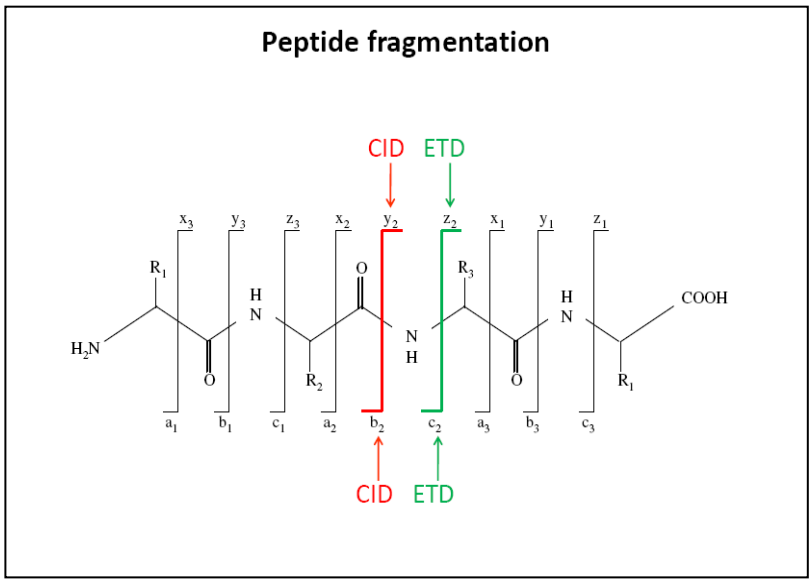


Fig. 21 Schematic representation of the cleavage sites of proteins by collision induced dissociation (CID) and electron transfer dissociation ETD).
The figure was prepared by S. Helling, Bochum.

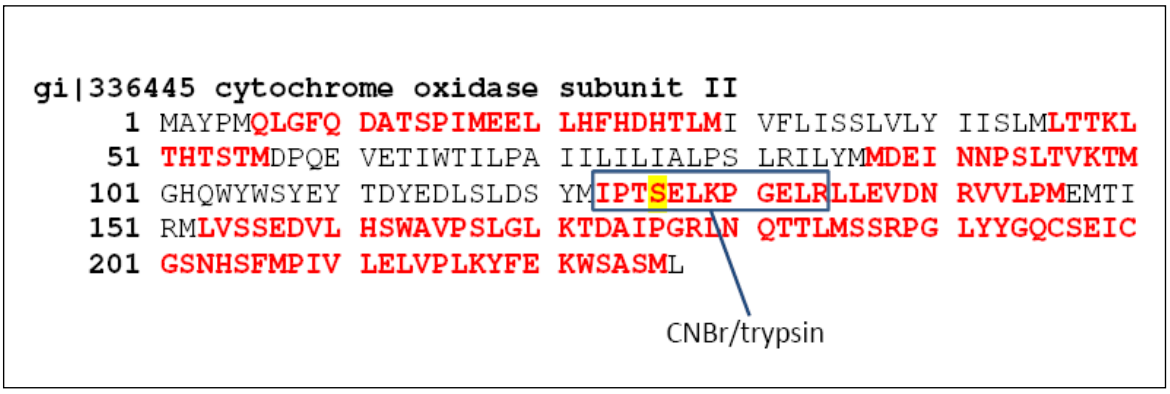


Fig. 22. Serine-126 phosphorylation site in a fragment of CcO subunit II from bovine heart isolated by CNBr/trypsin cleavage
CcO was isolated from bovine heart mitochondria with and without allosteric ATP-inhibition by BN-PAGE. Fragments indicated in red have been identified by mass spectrometry. The figure was prepared by S. Helling, Bochum.

In Fig. 21 are presented two procedures, CID (collision induced dissociation) and ETD (electron transfer dissociation) to obtain fragments for mass spectrometric analysis of phosphorylated amino acids in CcO subunits. In Fig. 22 is shown a fragment (boxed) of CcO subunit II obtained by cyanbromide/trypsin cleavage of complex IV from a BN-PAGE gel of bovine heart mitochondria which exhibited allosteric ATP-inhibition. The mass spectra of the

fragments obtained by CID and ETD are presented in Figures 23 and 24, respectively. The two mass spectra clearly identify Ser^{126} of bovine heart subunit II as phosphorylated

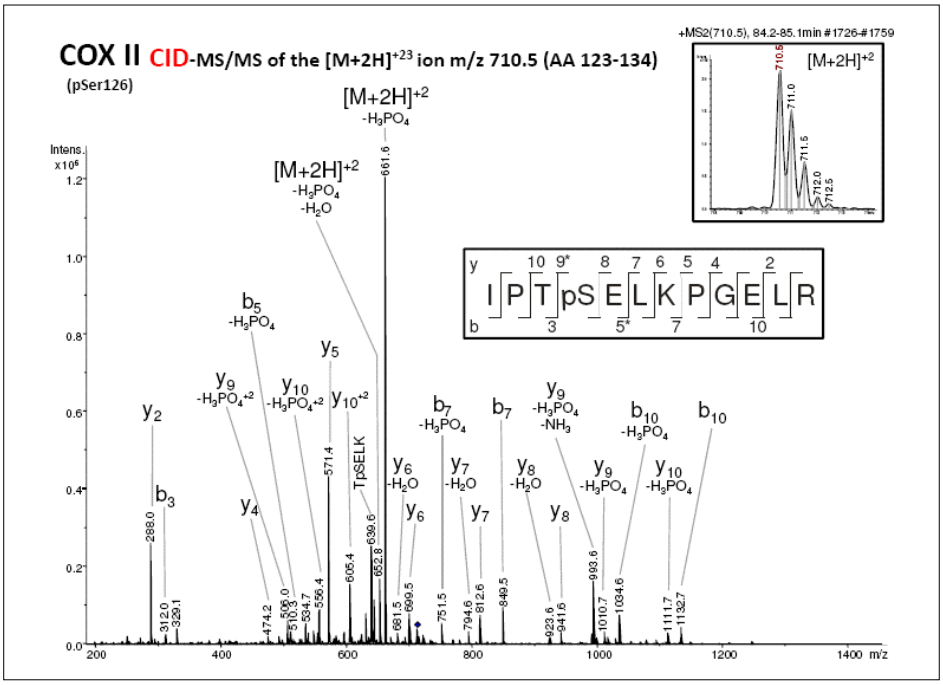


Fig.23. Mass spectrum of a fragment of CcO subunit II obtained by CID

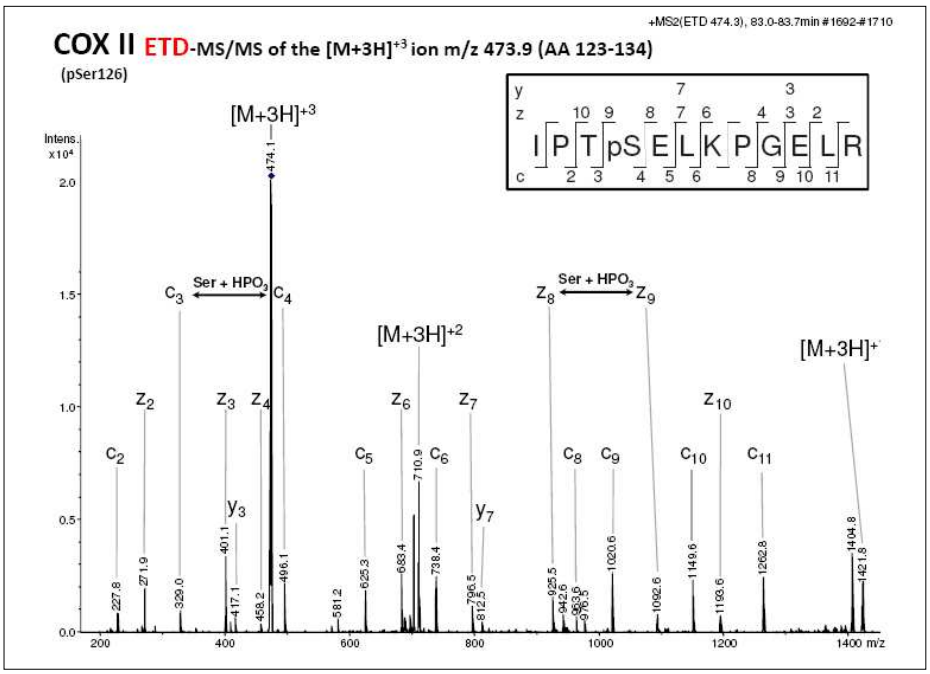


Fig. 24. Mass spectrum of a fragment of CcO subunit II obtained by ETD

amino acid. Three other phosphorylated amino acids have been identified in CcO by the collaboration with the Bochum group as shown in Fig. 25. In this figure, the newly identified phosphorylated amino acids in CcO subunits [Helling et al., 2008] are indicated in the crystal structure of bovine heart CcO [Tsukihara et al., 1996] by red balls. Another identified phosphorylated amino acid at Ser^{1} of CcO subunit-VIIc is not included in the Fig. 25. However, in no case a clear correlation between one of the phosphorylated amino acid and the allosteric ATP-inhibition of CcO was found.

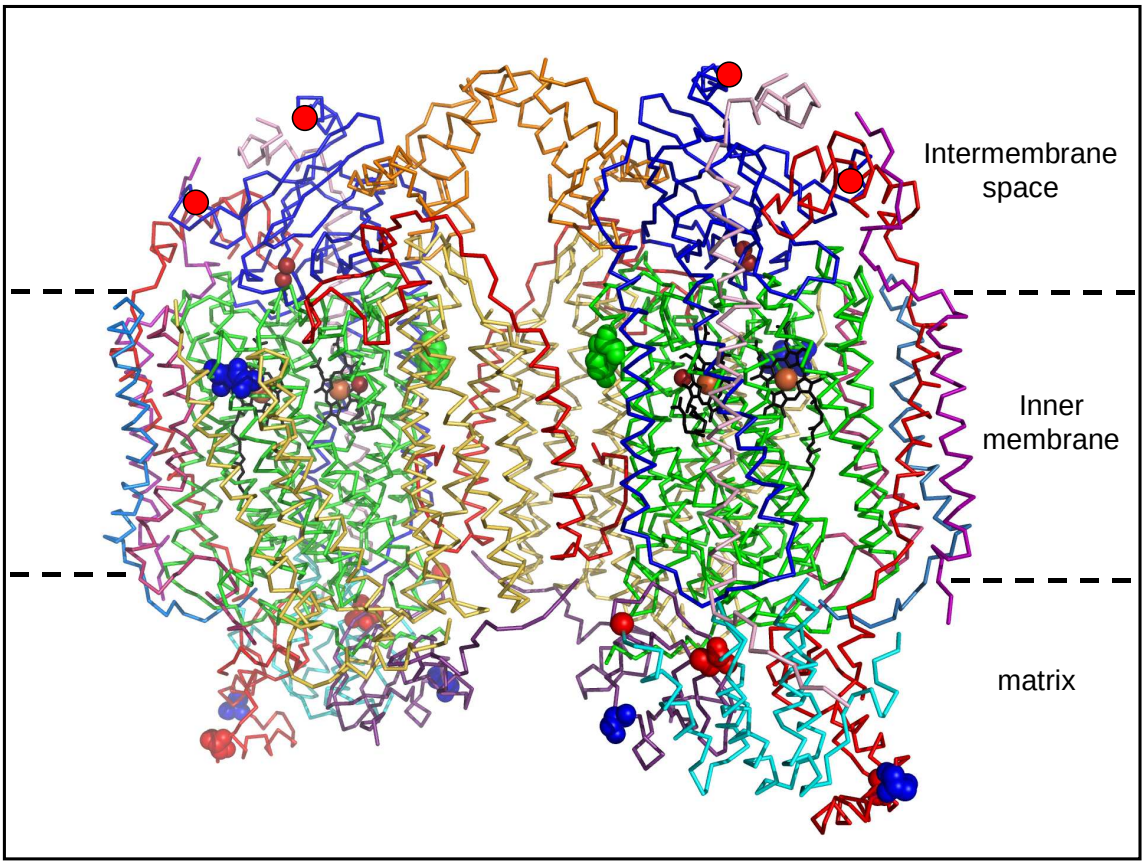


Fig. 25. Crystal structure and known phosphorylated amino acids in bovine heart CcO subunits
In the crystal structure of CcO [Tsukihara et al., 1996] is indicated in green: Tyr^{304} in subunit-I [Lee et al. 2005]; in blue: Ser^{115} and Ser^{116} in subunit-I, Ser^{52} in subunit-IV, Ser^{40} in subunit-Vb [Fang et al. 2007]; in red: Ser^{34} in subunit-IV, Ser^{4} and Thr^{35} in Va [Helling, Vogt, Rhiel, Ramzan, Wen, Marcus and Kadenbach, 2008], Ser^{126} and Tyr^{118} in subunit-II [Helling, Ramzan, Vogt, Kadenbach, in preparation].

4.5 Influence of various effectors on the allosteric ATP-inhibition of isolated heart mitochondria

In order to exclude the possibility that CcO is dephosphorylated during isolation of mitochondria in sucrose medium, mitochondria were isolated in the presence and absence of okadaic acid (a specific inhibitor of the protein phosphatases PP1 and PP2a), NaF (an unspecific inhibitor of protein phosphatases) and EGTA (an essential activator of the Ca^{2+}-dependent protein phosphatase PP2B, also known as calcineurin [Klumpp and Krieglstein, 2002]). From Fig. 26,]). From Fig. 26, it is clear that neither the kinetics nor the phosphorylation pattern of CcO is influenced by the presence of protein phosphatase inhibitors during isolation of mitochondria. Also, rat heart mitochondria standing on ice for 5

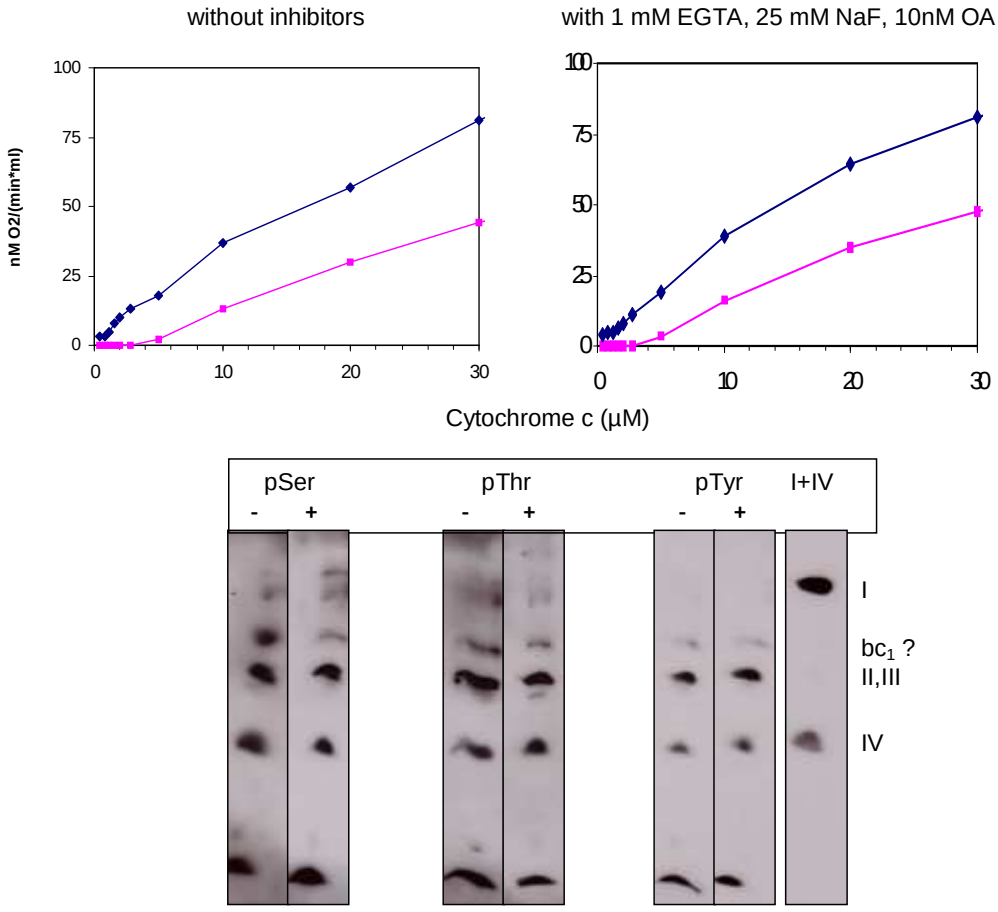


Fig. 26. The presence of NaF, okadaic acid and EGTA during isolation of rat heart mitochondria did not change the kinetics and phosphorylation pattern of CcO
A rat heart was divided into two parts and the mitochondria were isolated with isolation buffer-B in the absence (left side kinetics) and presence of inhibitors (1 mM EGTA, 25 mM NaF and 10 nM okadaic acid (OA)) (right side kinetics) and kinetics were performed in kinetics-buffer in the presence of ADP (blue) and ATP (pink). CcO was isolated by BN-PAGE and Western blots were performed after SDS-PAGE with antibodies against phosphoserine (P-serine), phosphothreonine (P-threonine), phosphotyrosine (P-tyrosine), and CcO subunits 1+IV (I+IV).

hours in the standard isolation medium did not change the kinetics of CcO (not shown). Therefore, in the followings, mitochondria were isolated in sucrose medium without inhibitors of protein phosphatases. Also the pH of the isolation buffer had little effect on the kinetics of CcO in the isolated bovine heart mitochondria, as indicated in Fig. 27.

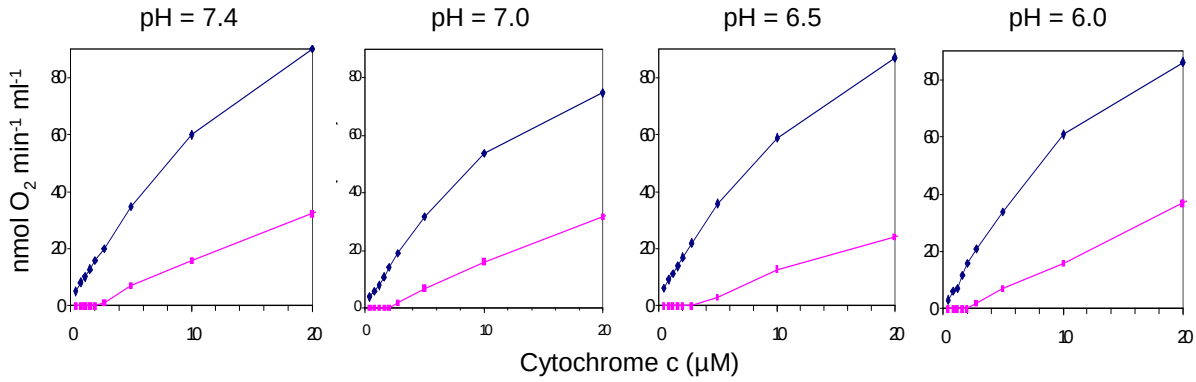


Fig. 27. Effect of pH in the isolation medium on the allosteric ATP-inhibition of CcO of bovine heart mitochondria
Bovine heart mitochondria were isolated from the same frozen heart tissue in isolation buffer-A with different pH (7.4, 7.0, 6.5 and 6.0). CcO kinetics were measured with kinetics-buffer under the standard conditions in the presence of ADP (blue) and ATP (pink).

The incubation of rat heart mitochondria for 5 min at room temperature with metal ions (Mg^{2+}, Mn^{2+}, Ca^{2+}) decreased the activity with ADP and to some extent the allosteric ATP inhibition (Fig. 28). The reason for this effect is not clear yet.

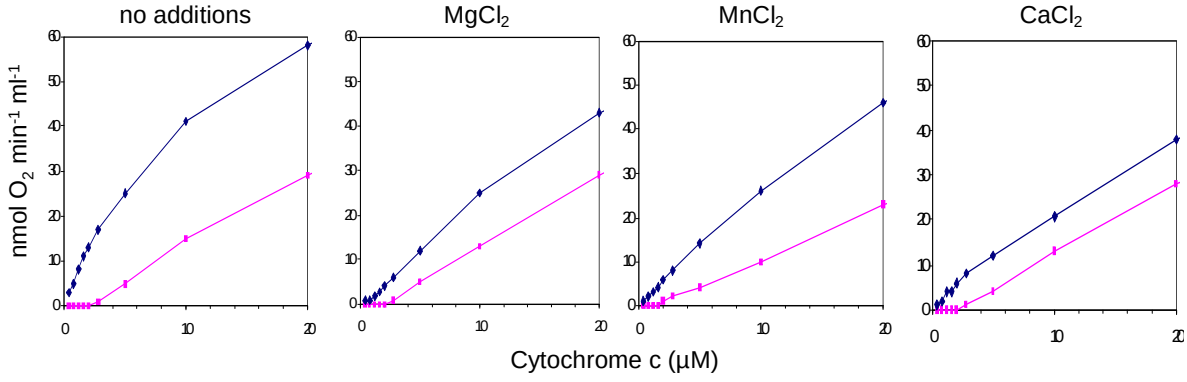


Fig. 28. Metal ions decrease the respiration with ADP of rat heart mitochondria during incubation for 5 minutes at room temperature
Rat heart mitochondria were isolated with isolation buffer-B, incubated in the same buffer for 5 min at room temperature without and with 2.1 mM each of $MgCl_2$, $MnCl_2$ and $CaCl_2$ as shown. Measurement of CcO kinetics were performed with 10 µl of each incubated sample in the kinetics-buffer in the presence of ADP (blue) and ATP+PEP+PK (pink).

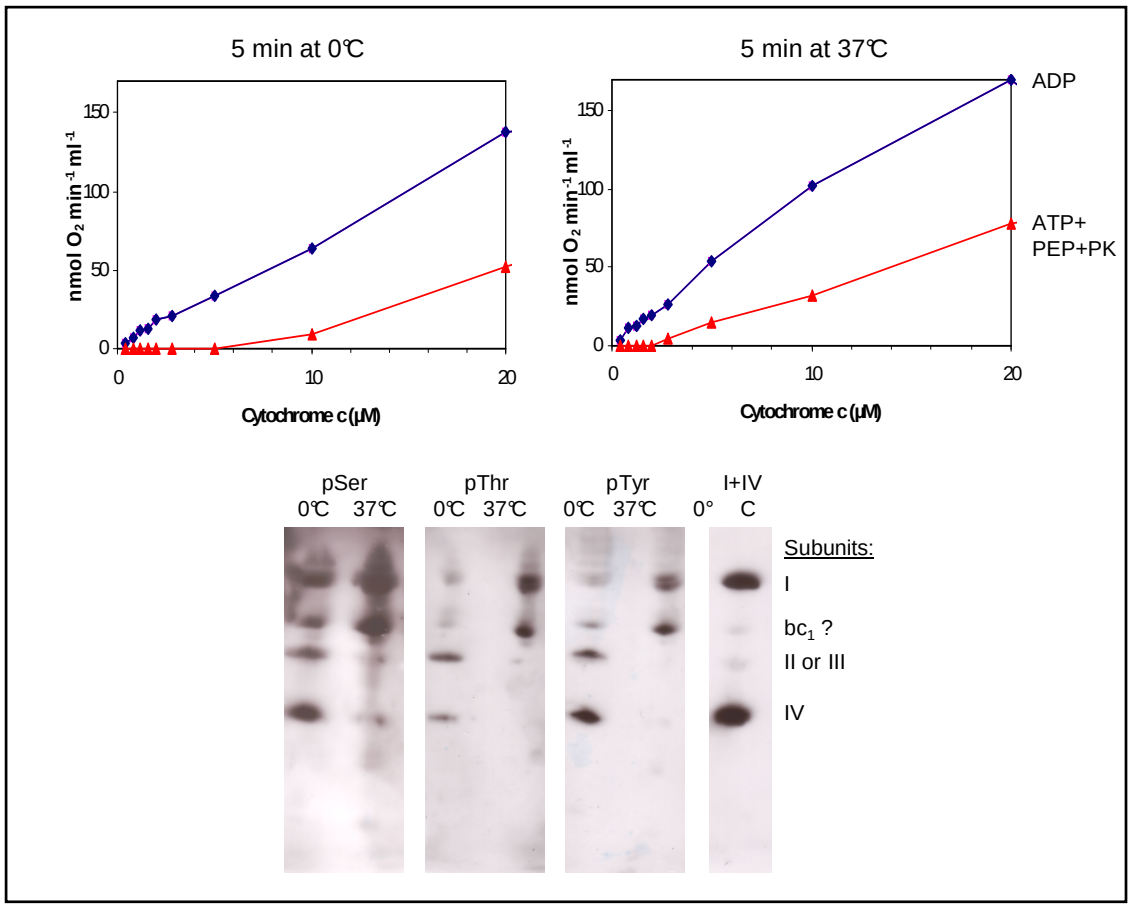


Fig. 29. Anaerobic incubation of rat heart mitochondria for 5 min at 37 °C decreased the allosteric ATP-inhibition and phosphorylation pattern of CcO
Isolated rat heart mitochondria were divided into two parts and centrifuged. One pellet was incubated for 5 min at 37 °C, the other pellet was kept at 0°C. CcO kinetics were measured with ADP (blue) and with ATP+PEP+PK (pink). Aliquots were separated by BN-PAGE and the CcO complexes by SDS-PAGE. Western blots were performed with antibodies against phosphoserine (pSer), phosphothreonine (pThr), phosphotyrosine (pTyr) and CcO subunit I+IV (I+IV).

Incubation of rat heart mitochondrial pellet for 5 min at 37°C, i.e. under anaerobic conditions, decreased the allosteric ATP-inhibition to some extent but had profound effects on the phosphorylation pattern of CcO (Fig. 29). The phosphorylation of subunits II or III and of IV at serine, threonine and tyrosine disappeared almost completely, whereas the phosphorylation of subunit I increased markedly (together with phosphorylation of a postulated subunit of the bc_1-complex III). This is in accordance with the results of Fang et al. [2007] who observed during ischemia/reperfusion of rabbit hearts, phosphorylation of CcO subunit-I at Ser^{115} and Ser^{116}.

In previous studies, it was shown that the allosteric ATP-inhibition of CcO in isolated mitochondria from frozen bovine liver was increased by incubation with cAMP [Bender and

Kadenbach, 2000]. I have investigated the effect of cAMP up to 500 µM concentrations on the allosteric ATP-inhibition of mitochondria from rat and bovine heart, but in more than 10 experiments, no stimulation was found (data not shown). This could be explained, if so, by a different signaling pathway in liver and heart to switch on and off the allosteric ATP-inhibition.

In another experiment, incubation of bovine heart mitochondria for 10 min at 37°C increased the allosteric ATP-inhibition together with the strong phosphorylation of CcO subunit I at serine. The increase of allosteric ATP-inhibition and serine phosphorylation of CcO subunit I was prevented if the incubation was performed in the presence of 10 nM okadaic acid (Fig. 30). I have tried to reproduce this experiment several times under identical conditions, but in no case neither the allosteric ATP-inhibition nor the serine phosphorylation of CcO subunit I could be reproduced (see discussion for a possible interpretation).

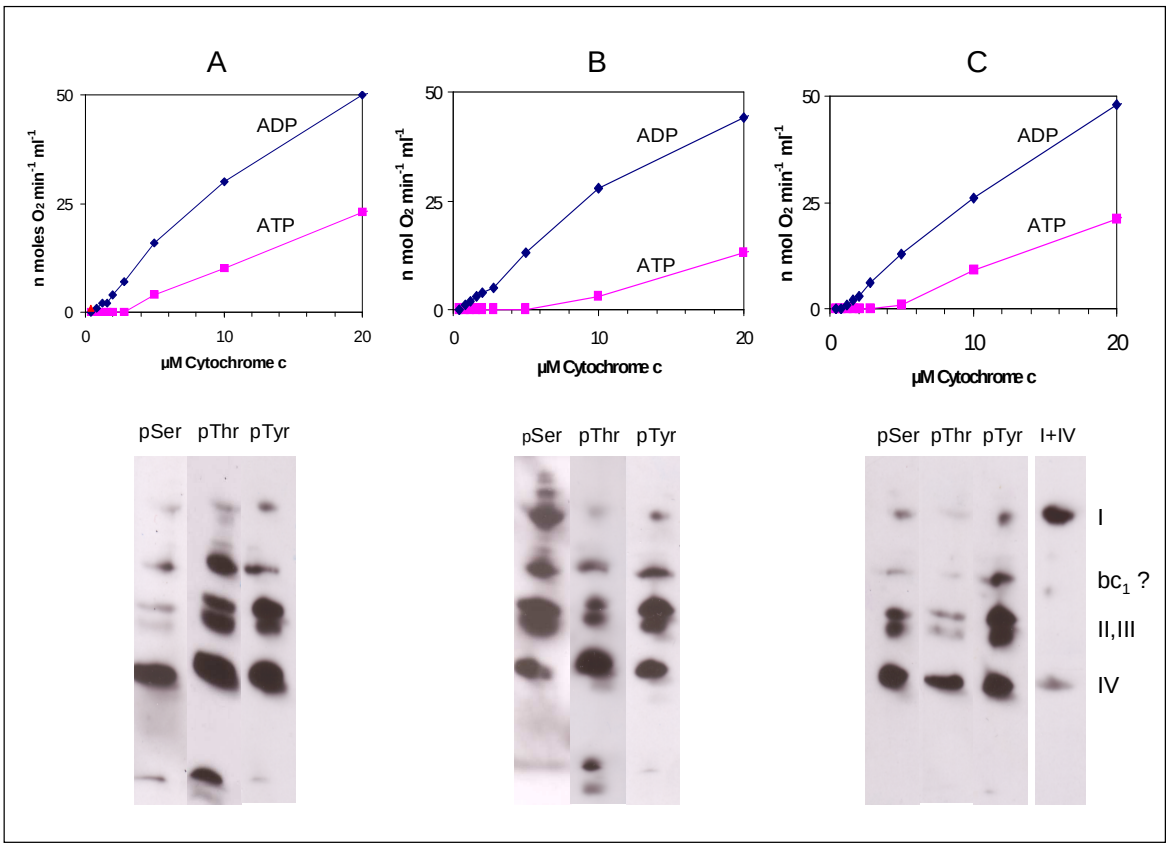


Fig. 30. Incubation of bovine heart mitochondria increases allosteric ATP-inhibition and serine phosphorylation of CcO subunit I

A: No incubation of mitochondria; B: Incubation of mitochondria for 10 min at 37°C under shaking; C: Incubation of mitochondria for 10 min at 37°C under shaking in the presence of 10 nM okadaic acid. The Western blots after SDS-PAGE of complex IV from BN-PAGE gels were performed with antibodies against phosphserine (pSer), phosphothreonine (pThr), phosphotyrosine (pTyr) and CcO subunits I and IV (I+IV).

4.6 The allosteric ATP-inhibition of CcO in mitochondria is variable and proposed to be related to the psychological situation of the animal at the time of killing

In Fig. 31 are presenrted the individual values of allosteric ATP-inhibition of CcO of isolated rat heart mitochondria from 42 animals. Each spot corresponds to a separate preparation of rat heart mitochondria isolated within two years. The data are presented for each rat as percentage of CcO activity at 1.6, 2.8, 5, and 10 µM cytochrome c in the presence of 5 mM ATP + 10 mM PEP + 20 U/ml PK related to the activity with 5 mM ADP taken as 100%.

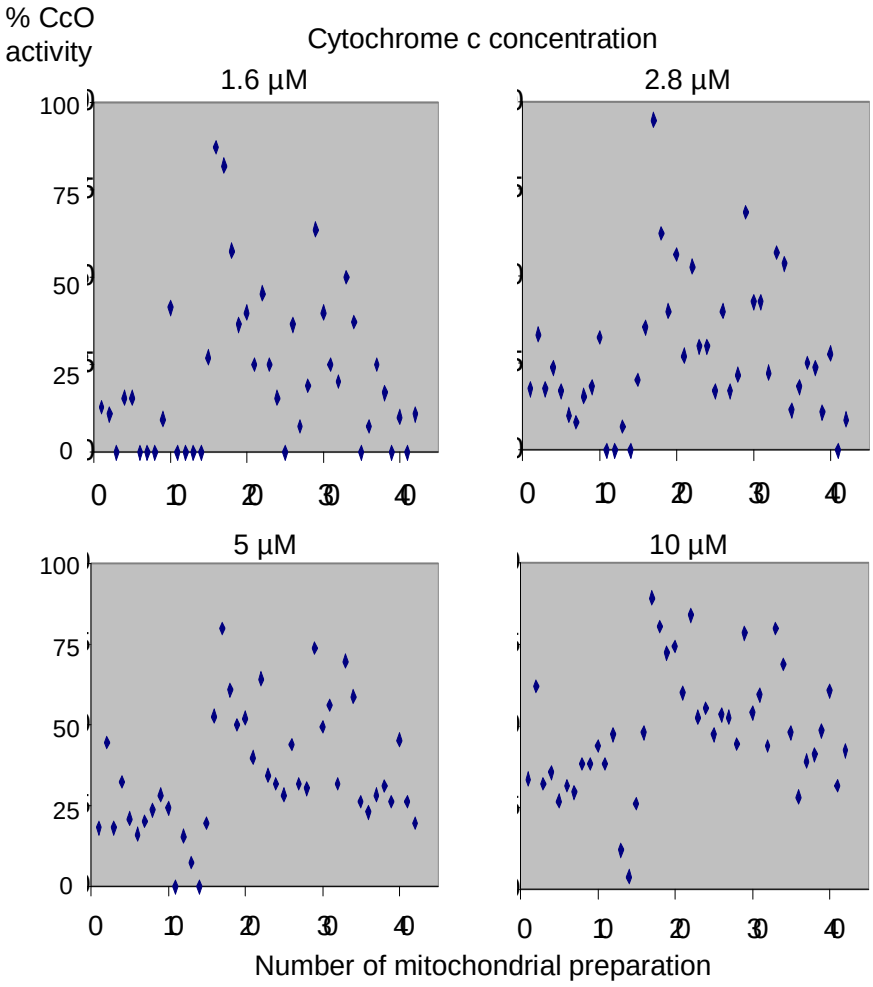


Fig. 31. Inhibition of CcO activity by ATP related to the activity in the presence of ADP = 100% of heart mitochondrial preparations from 42 different rats
Kinetics of isolated mitochondria were measured in the presence of ADP and of ATP+PEP+PK, and the percentage inhibition by ATP related to ADP = 100% was calculated at 4 different cytochrome c concentrations (1.6, 2.8, 5 and 10 µM).

Clearly, the extent of allosteric ATP-inhibition varies markedly from almost none to 100% inhibition; despite the fact that the preparation of mitochondria and the activity measurements

were performed under identical conditions i.e. isolation with isolation buffer-B and polarographic measurement in the kinetics-buffer containing the protein phosphatase inhibitors NaF and okadaic acid. I also investigated the effect of the killing method (decapitation, anesthetizing by strike on head or with CO_2, isofluran, or ether) on the extent of allosteric ATP-inhibition of CcO in the isolated rat heart mitochondria and did not find any relationship (data not shown).

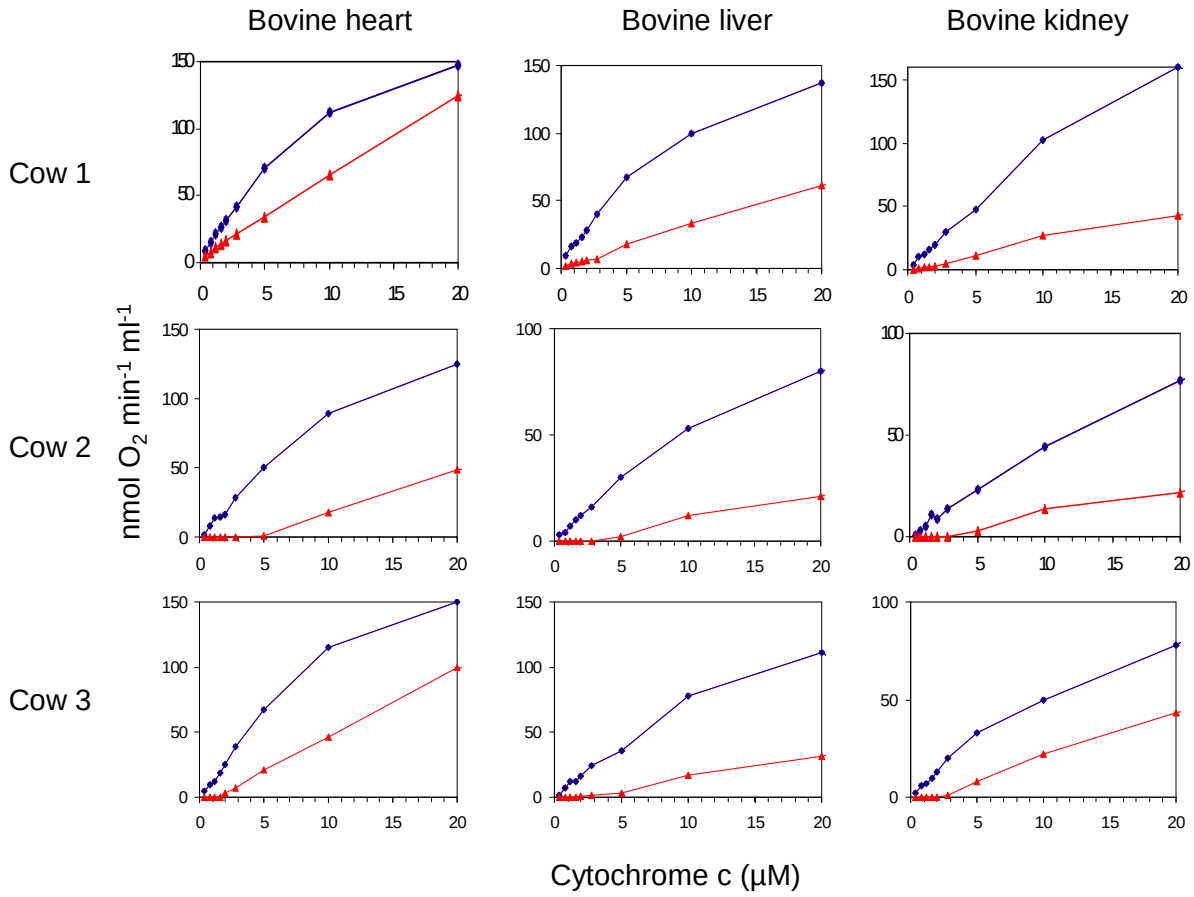


Fig. 32. The extent of allosteric ATP-inhibition of CcO in mitochondria from fresh bovine heart, liver and kidney varies among individual cows
The kinetics of CcO activity was measured in the kinetics-buffer immediately after the isolation of mitochondria from bovine heart, liver and kidney each from 3 different cows, with the isolation buffer-A. The three different tissues from each cow were taken at an interval of one week. In the 9 graphs, the upper curves represent the activity measured in the presence of ADP (blue), the lower curves show the activity measured in the presence of ATP (red). The final mitochondrial protein content used for kinetic measurements varied in the different preparations between 0.56-0.68 mg protein/ml for heart, 1.0-1.5 mg protein/ml for liver and 0.94-1.18 mg protein/ml for kidney.

These results with rat heart mitochondria were confirmed when compared with mitochondria from heart, liver and kidney of cows prepared immediately after transportation of the tissues from the slaughterhouse to the laboratory on ice, as shown in Fig. 32. The tissues were obtained from 3 different animals within 3 weeks in February. Whereas no allosteric ATP-inhibition was found in mitochondria from the first cow, a strong effect was obtained from the second, and an average extent of allosteric ATP-inhibition in mitochondria from the third cow. Surprisingly, the different kinetics in mitochondria from heart correlated to some extent with those from liver and kidney of the same animal, suggesting a central control of allosteric ATP-inhibition in most tissues. From all mitochondria Western blots were performed, but no differences could be detected between the corresponding mitochondria of the three animals (data not shown, see below). From kinetics data, I concluded that the extent of allosteric ATP-inhibition of CcO in the mitochondria must depend on the psychological situation of the animal at the time of killing, as proposed in previous review articles (Kadenbach, Ramzan, Vogt, 2009; Kadenbach, Ramzan, Wen, Vogt, 2010).

4.7 Phosphorylation and kinetics of CcO after incubation of minced heart tissue from bovine and rat

In order to investigate the signal pathways which turn on and off the allosteric ATP-inhibition of CcO I studied the effect of various parameters during incubation of minced bovine heart tissue under shaking at room temperature. After incubation, mitochondria were isolated, the kinetics were measured and Western blots were performed under standard conditions. The time of incubation (up to 40 min) of minced tissue in Joklik medium (cell culture medium without calcium) at room temperature had little effect on the allosteric ATP-inhibition of the isolated mitochondria, as shown in Fig. 33. Also the Western blot of complex IV from BN-PAGE gels did not show any clear change with the time of incubation. It should be mentioned that the two lower bands in the Western blots of the samples after 10 and 20 minutes of incubation run different from those of the other incubation times, because the samples were run on a different SDS-PAGE. It appears that the signal pathways which switch on and off the allosteric ATP-inhibition of CcO are blocked after killing the animal.

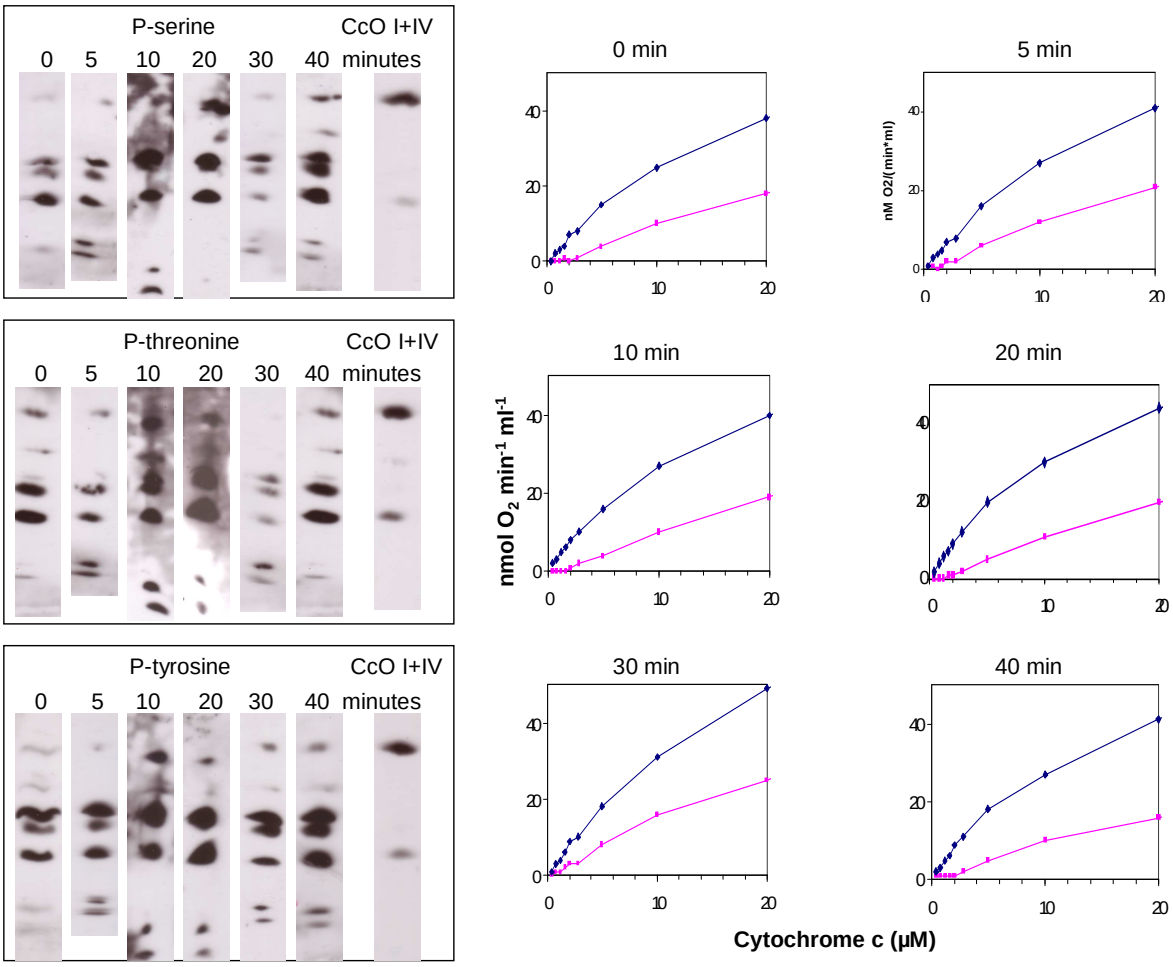


Fig. 33. The time of incubation of bovine heart tissue in Joklick medium at room RT does not influence the allosteric ATP-inhibition and phosphorylation of CcO
Bovine heart tissue was incubated in Joklick medium for 0, 5, 10, 20, 30 and 40 min at RT with shaking. After isolation of mitochondria with isolation buffer-A, the CcO kinetics measurement were performed in the presence of ADP (blue) and ATP (pink) as shown. After isolation of complex IV by BN-PAGE from incubated mitochondria, SDS-PAGE and Western blot analysis were performed with antibodies as shown in the figures on the left.

Insulin (1U/ml) decreased the allosteric ATP-inhibition of CcO in isolated bovine heart mitochondria during incubation of the heart tissue for 20 min at 25°C in Joklik medium (without Ca^{2+} ions) but found to have little effect on the phosphorylation pattern of CcO, except for a decrease of subunit-I serine phosphorylation (Fig. 34). The same result was obtained with the experiment shown in Fig. 35, where bovine heart tissue was incubated for 20 min at 25°C with 200 mU/ml insulin. The phosphorylation pattern, however, differed from that of Fig. 34 since very little phosphorylation of CcO subunit-I on serine and tyrosine was

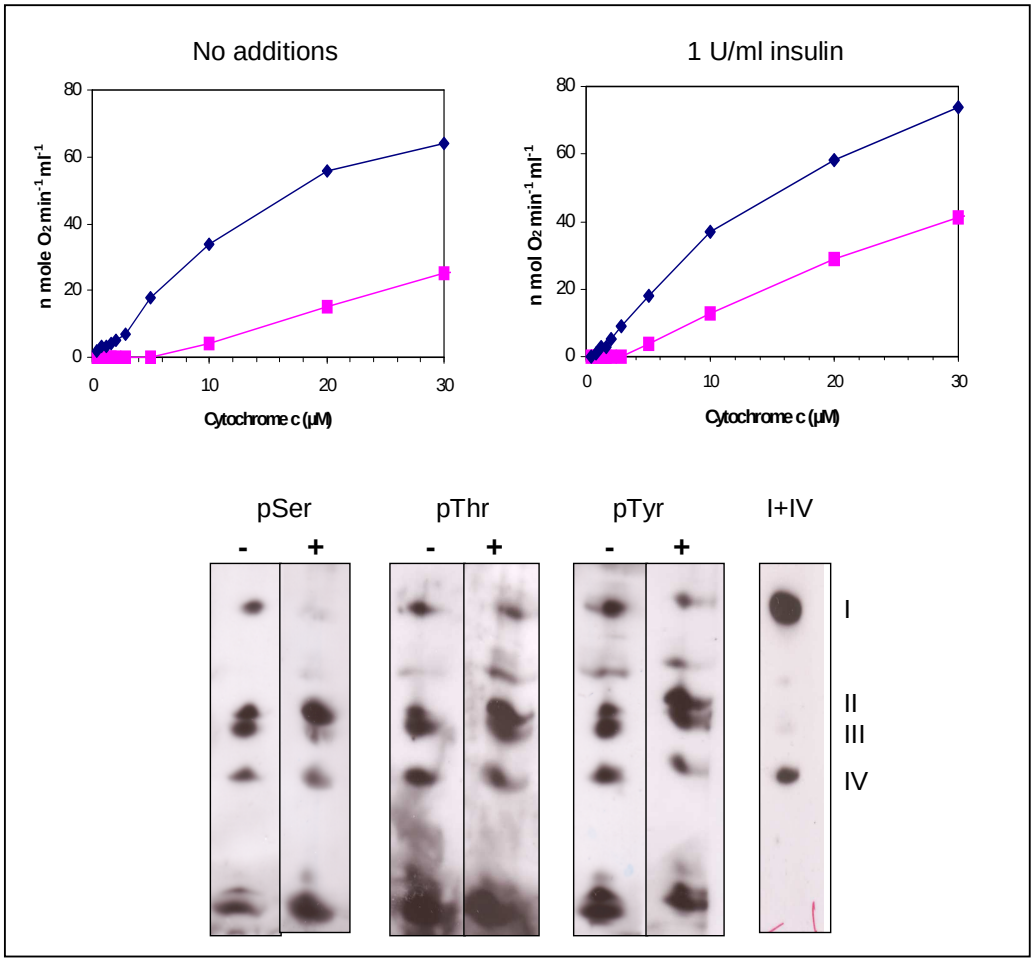


Fig. 34. Insulin (1 U/ml) decreases the allosteric ATP-inhibition of CcO and decreases the serine phosphorylation of CcO subunit-I during incubation of minced bovine heart tissue

Minced bovine heart tissue was incubated without and with 1U/ml insulin (- and +) for 20 min at 25°C in Joklik medium with shaking. CcO kinetics of isolated mitochondria were measured with ADP (blue) and ATP+PEP+PK (pink). Western blot analysis was performed after isolation of CcO by BN-PAGE and CcO subunit separation by SDS-PAGE with the antibodies against phosphserine (pSer), phosphothreonine (pThr), phosphotyrosine (pTyr) and CcO subunits I and IV (I+IV).

found. The different extent of phosphorylation of serine at CcO subunit-I may be explained by the activity of a serine/threonine-specific phosphatase during BN-PAGE (see Fig. 36).

In other experiments, bovine heart tissue slices were incubated for 40 min at 25°C under shaking with 1.2 mM $CaCl_2$, 5 µM forskolin (a diterpene and specific stimulator of adenylyl cyclase), 0.3 mM IBMX (3-isobutyl-1-methylxanthine, inhibitor of phosphodiesterases), 5 µM acetylcholine (neurotransmitter), 10 µM PP2 (inhibitor of

nonreceptor tyrosine kinase cSrc) or 40 nM Wortmanin (inhibitor of phosphatidylinositol-3-phosphate kinase), but in no case a marked change of the allosteric ATP-inhibition of CcO in the isolated mitochondria and of the phosphorylation pattern of CcO was found (data not shown).

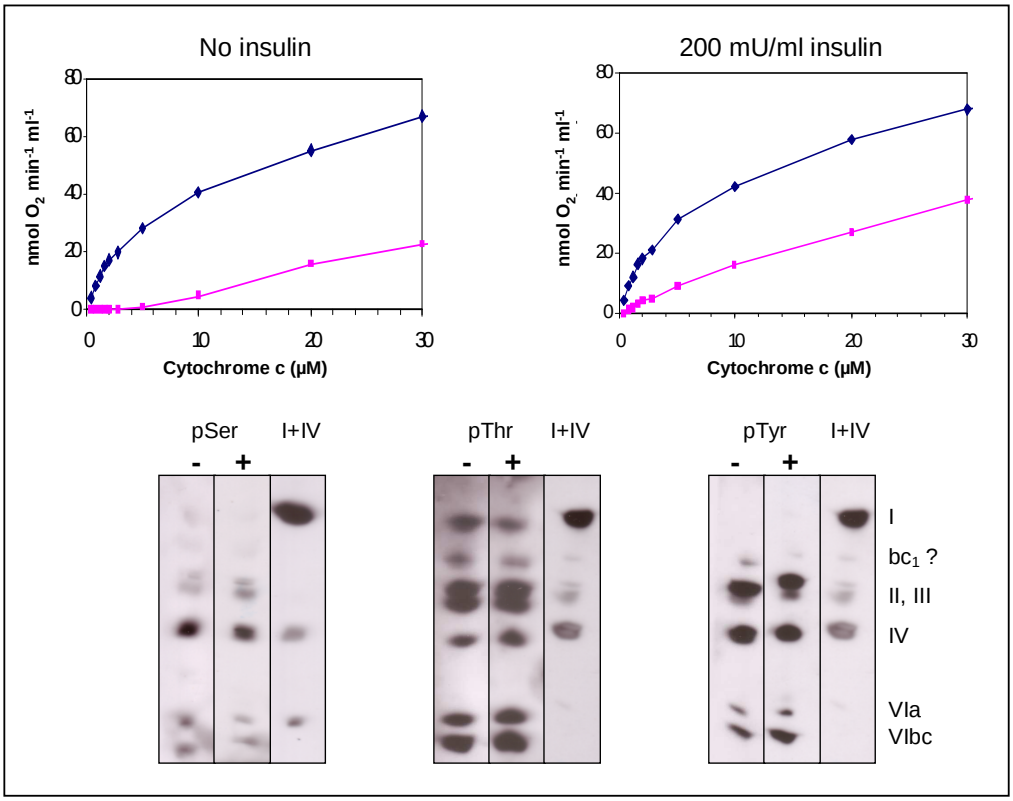


Fig. 35. Insulin (0.2 U/ml) decreases the allosteric ATP-inhibition of CcO during incubation of minced bovine heart tissue for 20 min at RT with weak decrease of the serine phosphorylation of CcO subunit-I
Bovine heart tissue was incubated without and with 200 mU/ml insulin (- and +) for 20 min at 25°C in Joklik medium with shaking. CcO kinetics were measured with ADP (blue) and ATP+PEP+PK (pink). Western blot analysis was performed after isolation of CcO by BN-PAGE and CcO subunit separation by SDS-PAGE with the antibodies against phosphserine (pSer), phosphothreonine (pThr), phosphotyrosine (pTyr) and CcO subunits I and IV (I+IV).

4.8 Isolation of CcO by BN-PAGE and specificity of Western blots

In many experiments, proceeded either with isolated mitochondria or bovine/ rat heart tissues, no clear relationship between the extent of allosteric ATP-inhibition and the phosphorylation pattern of CcO was found. In order to study if protein phosphatases in the mitochondria would change the phosphorylation pattern of CcO during BN-PAGE, I

investigated the effect of okadaic acid (a specific inhibitor of serine/threonine protein phosphatases PP1 and PP2a) on the phosphorylation pattern of CcO in mitochondria from frozen bovine heart. As presented in Fig. 36 with two independent experiments, the presence of 10 nM okadaic acid in the sample buffer for BN-PAGE prevented the loss of serine phosphorylation of CcO subunit-I of rat heart mitochondria, which exhibited a strong allosteric ATP-inhibition (see the kinetics of experiment 1 in the left part of Fig. 36).

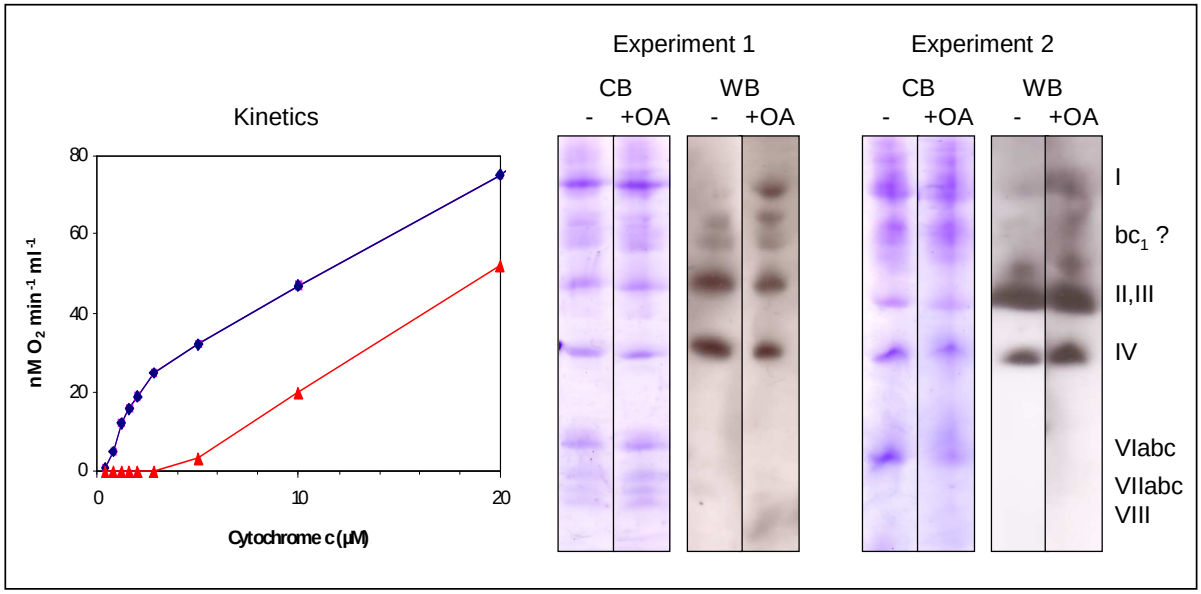


Fig. 36. Okadaic acid prevents the loss of serine phosphorylation of CcO subunit-I during BN-PAGE of bovine heart mitochondria
BN-PAGE gels were prepared with and without 100 nM okadaic acid in the gel mixture. Kinetics of CcO was measured in the presence of ADP (blue) and ATP+PEP+PK (pink). Coomassie blue staining and Western blot analysis with antibodies against phosphoserine from two different experiments are shown. CB = Coomassie blue staining, WB = Western blot, OA = okadaic acid. CB and WB were performed from two different SDS-PAGE gels.

In the Western blot of a BN-PAGE gel, performed with freshly isolated rat heart mitochondria of Fig. 19, only one band (complex IV, CcO) was found to react with the monoclonal antibody against CcO subunit IV (anti-Cox subunit IV, mouse IgG_{2a}). While in later studies, we observed the cross-reactivity of the monoclonal antibodies not only against CcO subunit-I and subunit-IV but also with other respiratory chain complexes i.e complex III and with complex V, as presented in Fig. 37. This cross-reactivity was found with each single monoclonal antibody. One BN-PAGE was performed with 10% DMSO in the gel, and a

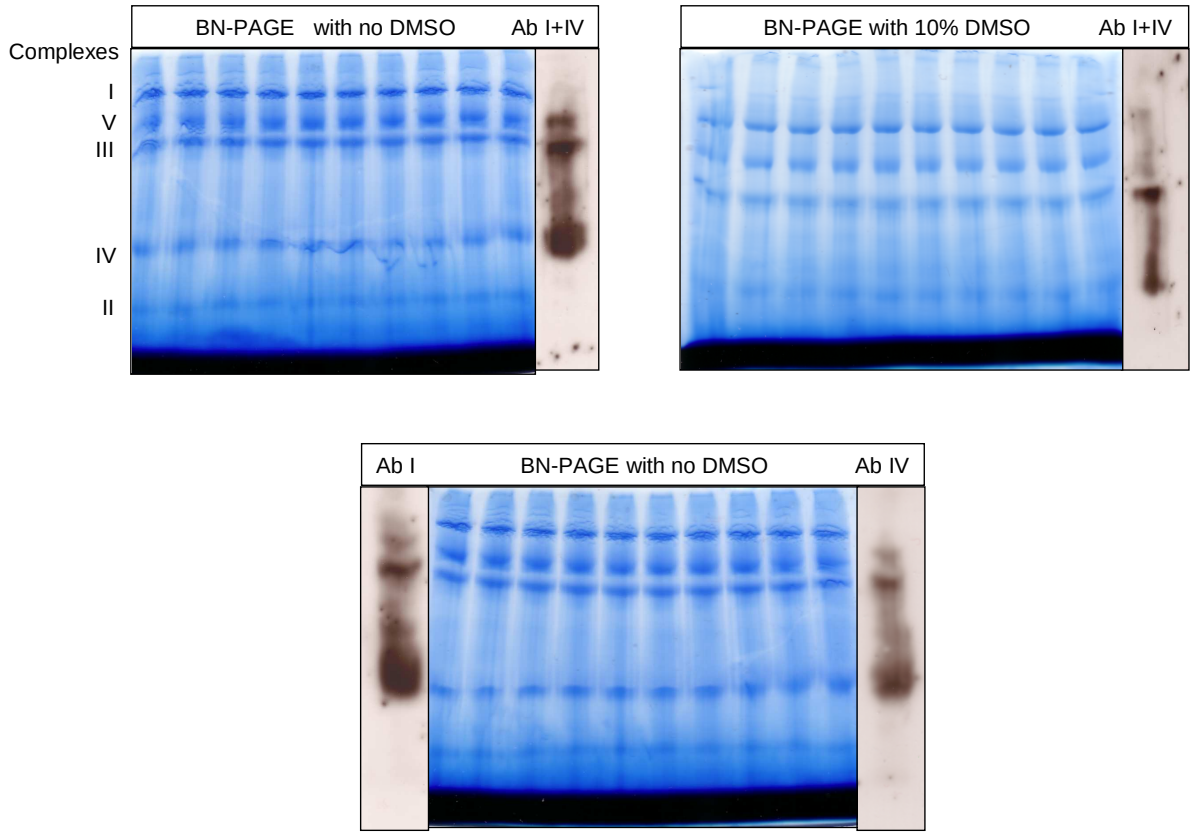


Fig. 37. Effect of DMSO in the BN-PAGE on the separation of respiratory chain complexes
Bovine heart mitochondria were prepared from frozen tissue with the isolation buffer-A. BN-PAGE gels were prepared without and with 10% DMSO in the gel mixture. Western blot analysis was performed with one lane each from a gel with and without DMSO, with antibodies to CcO subunit I and IV. In the lower gel, the reactivity with single antibodies against CcO subunit I and IV are shown.

different separation of the complexes was found (Fig. 19). The DMSO was used because okadaic acid was dissolved in DMSO. In the second gel, the effect of DMSO during BN-PAGE on the separation of complexes was studied. And the cross-reactivity of the antibodies was found to be the same. In Fig. 38, Western blots after SDS-PAGE with all complexes (separated by BN-PAGE) are shown. Complex IV bands from different lanes of one BN-PAGE gel were excised and run in different SDS-PAGE gels for Coomassie blue staining and Western blot analysis with antibodies against CcO subunit I + IV. The cross reactivity of these monoclonal antibodies was found extensively with almost all other complexes of the respiratory chain in both the BN-PAGE and SDS-PAGE. Although the same monoclonal antibody was used as in Fig. 19, one possibility of the cross reactivity could be based on a

mutation in the clones of the hybridoma cells producing the monoclonal antibodies which might result in a change of the antigen binding site.

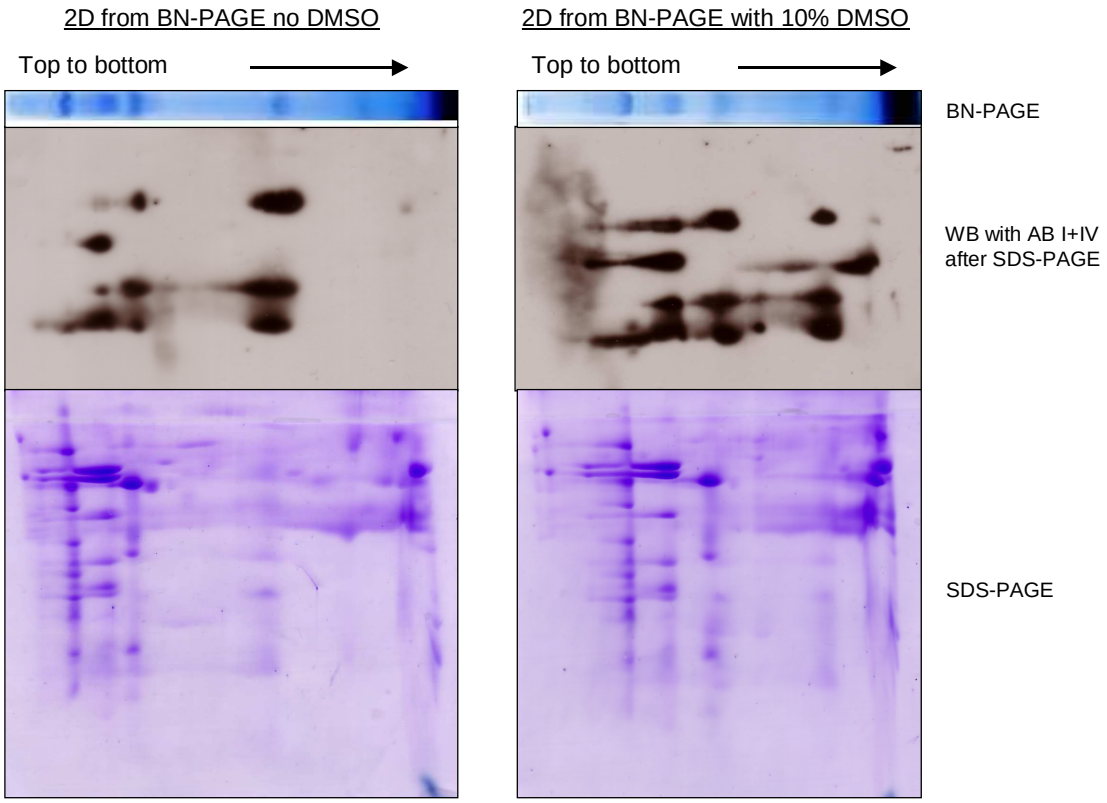


Fig. 38. SDS-PAGE from one lane each of BN-PAGE gels with and without DMSO was used for Western blots and Coomassie blue staining
From BN-PAGE gels, one whole lane from gels with and without DMSO of the Fig. 37, SDS-PAGE were performed for Coomassie staining and Western blot analysis with antibodies against CcO subunit I + IV. On the left are shown Western blot and Coomassie blue staining from a BN-PAGE gel without DMSO and on the right with 10% DMSO.

4.9 Measurement of the mitochondrial membrane potential of isolated mitochondria

In various review articles, it was proposed that the allosteric ATP-inhibition of CcO keeps $\Delta\Psi_m$ in vivo at low values (< 240 mV) and thus prevents the formation of deleterious ROS [Kadenbach, 2003; Kadenbach et al., 1999; 2000; 2004; 2009; 2010; Lee et al., 2001; Ludwig et al., 2001]. In collaboration with Prof. Dr. Katrin Staniek in Vienna, we studied in her laboratory the $\Delta\Psi_m$ of isolated mitochondria from rat liver and rat heart by using a TPP^+-electrode. Surprisingly, we measured for the first time a reversible decrease of $\Delta\Psi_m$ in intact isolated rat liver mitochondria, which is not based on limited substrate supply, high F_0F_1-ATPsynthase activity, or uncoupling. As shown in Fig. 39, the mitochondria exhibit a $\Delta\Psi_m$

value of around 230 mV in the presence of glutamate plus malate. This $\Delta\Psi_m$ is decreased under state 3 conditions until ADP is converted to ATP (Fig. 39, upper graph). After addition of PEP and PK the $\Delta\Psi_m$ is slowly decreased to 178 mV and reaches 123 mV if the mitochondria were not supplemented with ADP before (Fig. 39, lower graph).

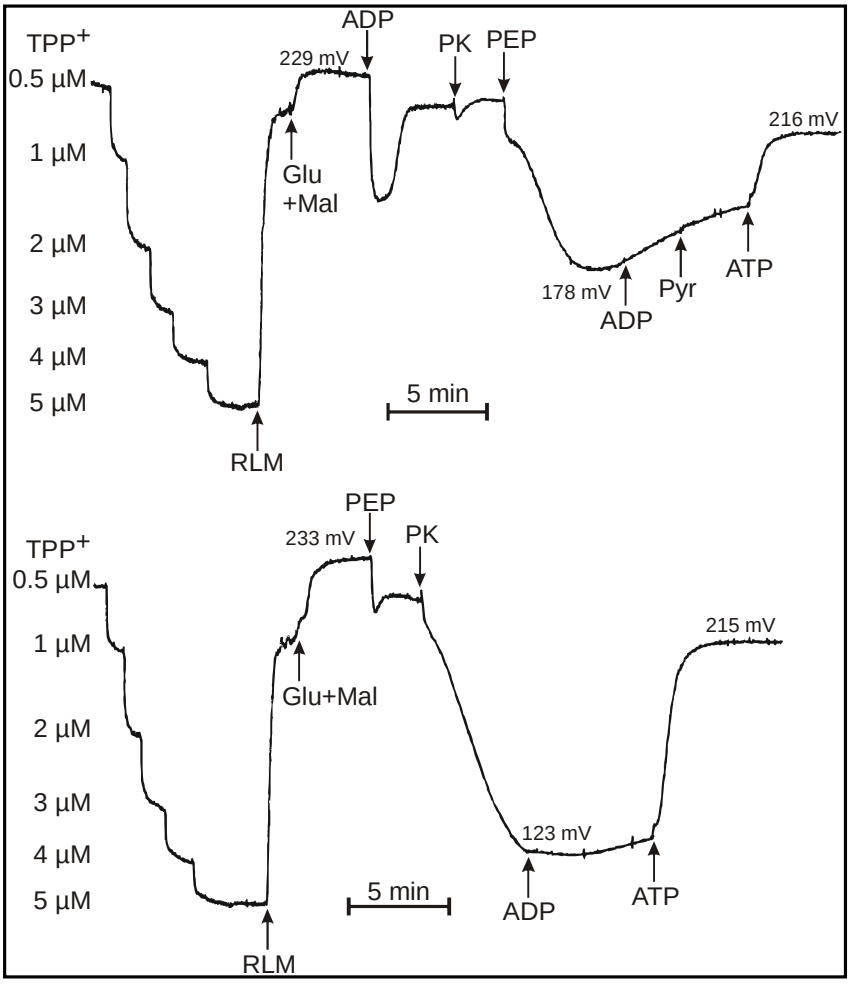


Fig. 39. Effect of ATP-regenerating system on the $\Delta\Psi_m$ of rat liver mitochondria
$\Delta\Psi_m$ was measured in the sucrose-buffer-B. $\Delta\Psi_m$ values (negative inside) calculated at the end of each kinetics are indicated in brackets after the additions: Upper graph: rat liver mitochondria (RLM, 1 mg/ml), 5 mM each glutamate/malate (Glu+Mal; 229 mV), 0.2 mM ADP (state 3: 199 mV, state 4: 222 mV), 160 U/ml PK (223 mV), 10 mM PEP (178 mV), 0.1 mM ADP (189 mV), 10 mM pyruvate (Pyr, 197 mV), 5 mM ATP (216 mV). Lower graph: rat liver mitochondria (RLM, 1 mg/ml), 5 mM each Glu+Mal (233 mV), 10 mM PEP (224 mV), 160 U/ml PK (123 mV), 0.2 mM ADP (139 mV), 5 mM ATP (215 mV).

Surprisingly, addition of 5 mM ATP reversed the $\Delta\Psi_m$ back to around 215 mV. The reversible decrease of $\Delta\Psi_m$ by PEP+PK is explained by the allosteric ATP-inhibition of CcO

due to increasing ATP/ADP ratios in the matrix of liver mitochondria by PEP which drives the anaplerotic and gluconeogenetic reactions backwords yielding GTP and ATP:

1) PEP + CO_2 + GDP → oxalacetate + GTP (PEP carboxykinase)

2) oxalacetate + ADP + phosphate → pyruvate + CO_2 +ATP (pyruvate carboxylase)

PEP carboxykinase and pyruvate carboxylase were shown to occur in the matrix of liver mitochondria ([Horn et al., 1997]; see discussion in [Stark et al., 2009]). PEP is transported into liver mitochondria by the citrate carrier [Robinson, 1971; Gnoni et al., 2009; Palmieri, 2008].

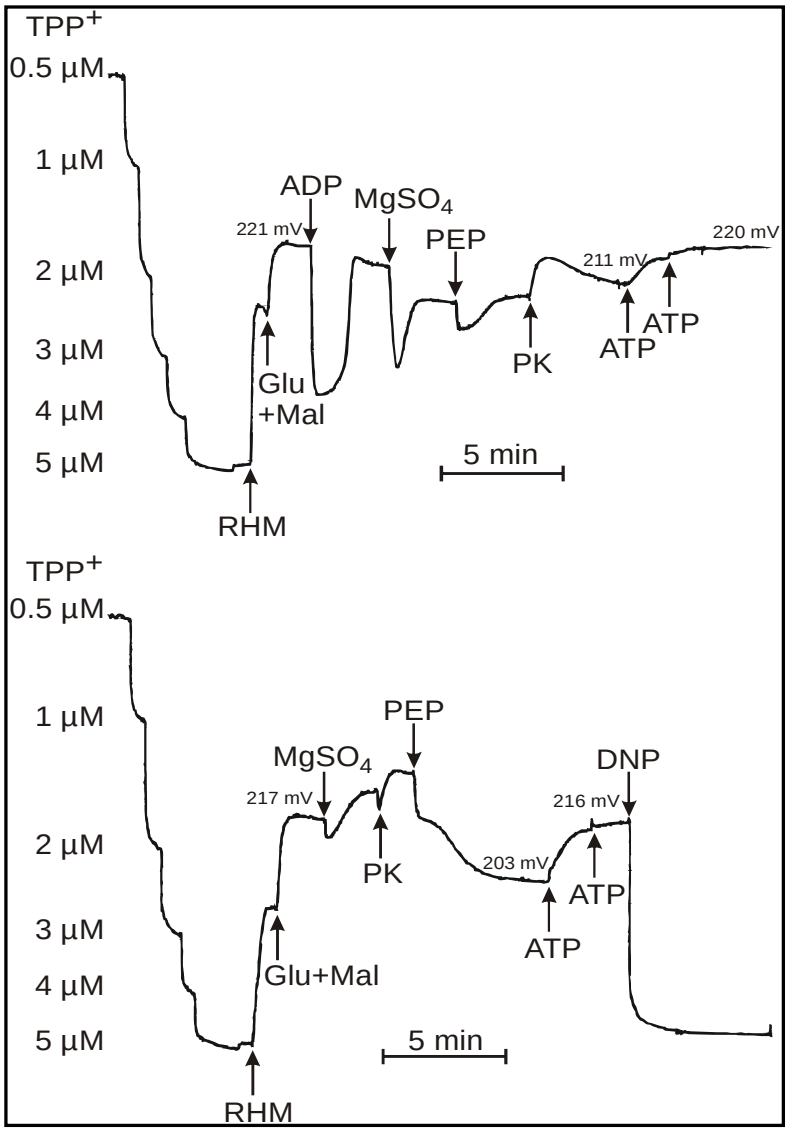


Fig. 40. Effect of ATP-regenerating system on the $\Delta\Psi_m$ of rat heart mitochondria

$\Delta\Psi_m$ was measured in the KCl-buffer; $\Delta\Psi_m$ values (negative inside) calculated at the end of each kinetics are indicated in brackets. Upper graph: rat heart mitochondria (RHM, 0.5 mg/ml), 5 mM each glutamate/malate (Glu+Mal; 221 mV), 0.2 mM ADP (state 3: 180 mV, state 4: 217 mV), 5 mM $MgSO_4$ (208 mV), 10 mM PEP (209 mV), 160 U/ml PK (211 mV), 1 mM ATP (217 mV) + 4 mM ATP (220 mV). Lower graph: rat heart mitochondria (RHM, 0.5 mg/ml), 5 mM each Glu+Mal (217 mV), 5 mM $MgSO_4$ (222 mV), 160 U/ml PK (225 mV), 8

mM PEP (203 mV), 1 mM ATP (214 mV) + 4 mM ATP (216 mV), 0.4 mM 2,4-dinitrophenol (DNP; 0 mV).

However, the decrease of $\Delta\Psi_m$ after addition of PEP and PK was not obtained in rat liver mitochondria using 5 mM succinate as substrate ($\Delta\Psi_m$ decreased from 234 mV to 222 mV only; data not shown), probably due to the removal of the allosteric ATP-inhibition of CcO at high substrate supply (high ferrocytochrome c/ferricytochrome c ratio with succinate) as a consequence of the sigmoidal kinetics of ATP-inhibition of CcO [Arnold and Kadenbach, 1997]. Succinate has in fact been shown to increase the steady state ferro-/ferri-cytochrome c ratio in mitochondria [Chance and Williams, 1955b]. Also with rat heart mitochondria no decrease of $\Delta\Psi_m$ by PEP and PK was obtained, as presented in Fig. 40. This can be explained by the lack of PEP carboxykinase in the matrix of heart mitochondria [MacDonald et al., 1978; Wiese et al., 1991].

5. Discussion

5.1 Two mechanisms of the control of mitochondrial respiration

The allosteric ATP-inhibition of CcO ('second mechanism of respiratory control') [Kadenbach and Arnold, 1999] was discovered more than ten years ago [Arnold and Kadenbach, 1997]. Although it was clearly shown that CcO is completely inhibited at intramitochondrial ATP/ADP ratios > 50 and low cytochrome c concentrations [Arnold and Kadenbach, 1999], it was not generally accepted as a mechanism for regulation of respiration. This was partly due to the lacking proof for its function in intact isolated mitochondria, since previous experiments had been done mainly with the purified CcO or with detergent solubilized mitochondria. Therefore, the first intention of the present dissertation was to prove the allosteric ATP-inhibition in intact isolated mitochondria.

Generally, the regulation of mitochondrial respiration is considered to be based on the "respiratory control", i.e. stimulation of respiration by ADP (state 3 respiration rate) followed by its inhibition after conversion of ADP into ATP (state 4 respiration rate). The Mitchell Theory [Mitchell, 1966; Nicholls and Ferguson, 2002] explains this behaviour by a decrease of $\Delta\Psi_m$ when the F_0F_1-ATPsynthase consumes the proton gradient across the inner mitochondrial membrane for the synthesis of ATP, followed by the increase of $\Delta\Psi_m$ when all ADP is converted into ATP. The decrease of respiration after conversion of ADP into ATP is based on the inhibition of proton pumps (complexes I, III, and IV) at high $\Delta\Psi_m$ values (200-230 mV).

I have shown for the first time the presence and functioning of both control mechanisms within the same intact isolated mitochondria. In Figures 4-6, the 'first mechanism of respiratory control' is seen by the addition of ADP (except with the uncoupled bovine heart mitochondria, Fig. 4, lower graph). After addition of the uncoupler of oxidative phosphorylation CCCP, which abolishes $\Delta\Psi_m$, further addition of ATP causes some inhibition of respiration which is abolished by ADP because it decreases the ATP/ADP ratio (Fig. 4, upper graph). I interpret this inhibition as the 'second mechanism of respiratory control' (allosteric ATP-inhibition of CcO). Interestingly, in some mitochondrial preparations, the $\Delta\Psi_m$-independent ATP-inhibition of respiration was not found (Fig. 7). This may be explained by the reversible switching on and off of the allosteric ATP-inhibition of CcO via reversible phosphorylation [Bender and Kadenbach, 2000; Lee et al., 2001, 2002; Helling et al., 2008]. In fact, I found variability in the extent of allosteric ATP-inhibition of CcO in different mitochondrial preparations, which have been isolated and measured under identical conditions. This is presented in the percentage inhibition of the allosteric ATP-inhibition of

CcO in 42 mitochondrial rat heart preparations (Fig. 26) varying between 0 and 100%. This variability is proposed to be based on switching off of the allosteric ATP-inhibition under stress, i.e. the psychological situation of the animal at the time of killing determines the extent of allosteric ATP-inhibition.

5.2 High ATP/ADP ratios are required to measure the CcO kinetics of ATP inhibition of CcO

Previous studies with purified and reconstituted CcO have demonstrated full allosteric ATP-inhibition of CcO at an intraliposomal ATP/ADP ratio > 50 and no ATP-inhibition at ATP/ADP < 16 (Fig. 41 [Arnold and Kadenbach, 1999]).This regulatory mechanism of CcO is suggested to establish a constant high ATP/ADP ratio in living cells (homeostasis) as illustrated in Fig. 42. Polarographic measurements of the kinetics of ATP-inhibition of CcO in isolated mitochondria require titration of oxygen consumption at increasing substrate concentrations (ferrocytochrome c). And this is achieved by using excess ascorbate which reduces ferricytochrome c continuously [Ferguson and Margoliash, 1978]. The detergent Tween-20 has to be added to make all CcO complexes accessible to cytochrome c. The full extent of allosteric ATP-inhibition, however, was only measured in the presence of an ATP-regenerating system (PEP + PK + Mg^{2+} ions) because various ATPases in the mitochondrial preparation decrease continuously the ATP/ADP ratio below the value for maximal allosteric ATP-inhibition (> 50). This problem of isolated mitochondria is shown in Fig. 16, where full inhibition of respiration by ATP is found at the beginning of titration with cytochrome c, but later on, this inhibition is completely lost with time (~2 min) unless the ATP-regenerating system (PEP + PK + Mg^{2+} ions) is present.

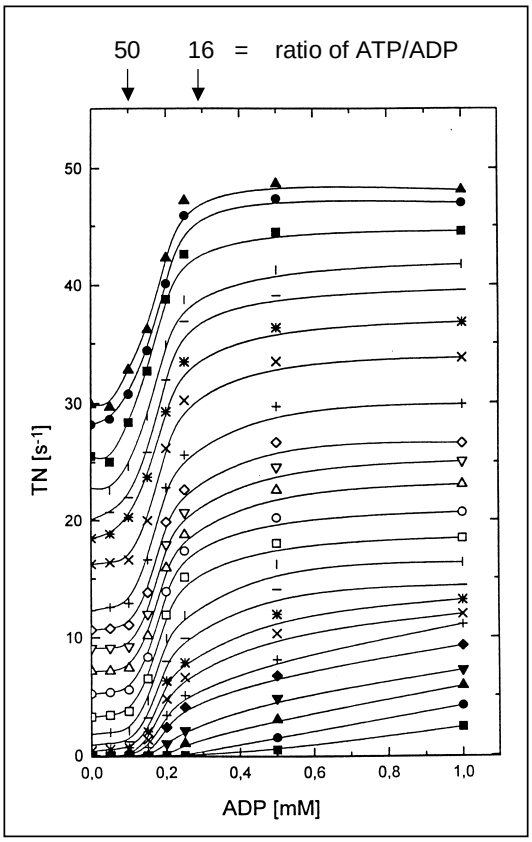


Fig. 41. Allosteric ATP inhibition of reconstituted bovine heart CcO
The curves represent the activity of reconstituted CcO from bovine heart between 0.25 µM (lower curve) and 60 µM (upper curve) of cytochrome c in the presence of various intraliposomal ADP concentrations where ADP + ATP = 5 mM (taken from Arnold and. Kadenbach [1999]).

In previous studies a 4-5-fold increased capacity of CcO activity for the rate of respiration was measured in isolated mitochondria by "metabolic control analysis" [Groen et al., 1982; Tager et al., 1983; Letellier et al., 1993; Rossignol et al., 1999]. However, in later studies with intact cells, using the same metabolic control analysis, a low reserve of CcO capacity was found [Villany and Attardi, 1997, 2000, 2001; Villani et al., 1998; Piccoli et al., 2006; Dalmonte et al., 2009]. The results of this dissertation explain this difference by the allosteric ATP-inhibition of CcO (Fig. 42). Only small changes in intramitochondrial free ATP concentrations switch on or off the allosteric ATP-inhibition. Therefore, it is proposed that the difference in the “control strength” of CcO in isolated mitochondria and intact cells is based on the different ATP/ADP ratios in vitro and in vivo.

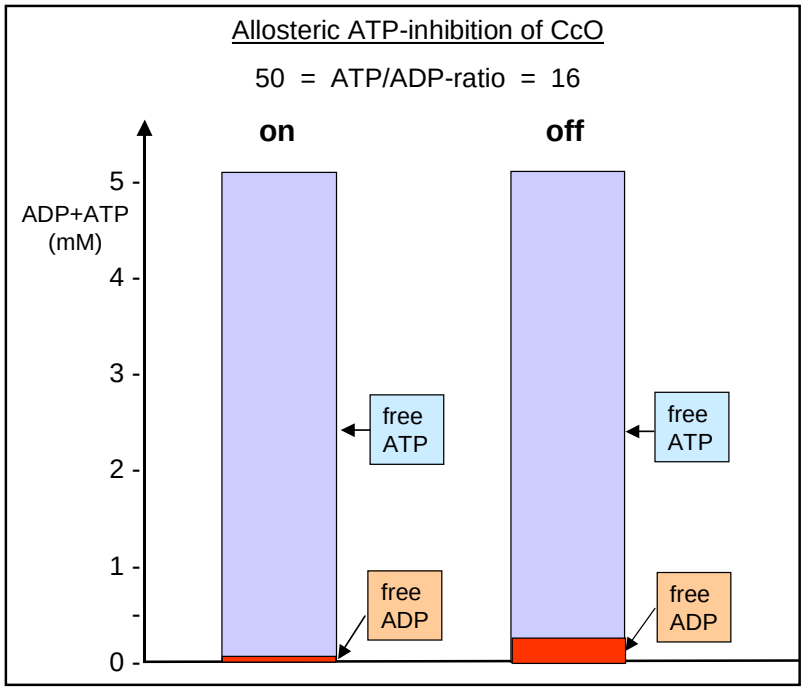


Fig. 42. The allosteric ATP-inhibition of CcO contributes to the homeostasis of high ATP/ADP ratios in living cells
The figure is based on the data of Arnold and. Kadenbach [1999].

5.3 The allosteric ATP-inhibition of CcO is switched on and off by reversible phosphorylation

Purified CcO from bovine heart exhibited no phosphorylation of subunit I at serine, threonine or tyrosine. Treatment of the isolated enzyme with PKA, cAMP and ATP turned on the allosteric ATP-inhibition accompanied by the phosphorylation of CcO subunit-I at serine and threonine [Helling et al., 2008]. In isolated mitochondria from rat heart, the allosteric ATP-inhibition and phosphorylation of CcO subunit-I at serine and threonine was found (Fig. 20 [Helling et al., 2008]). In previous experiments, the allosteric ATP-inhibition of CcO was switched on by incubation of bovine liver mitochondria with cAMP [Bender and Kadenbach, 2000], but I could not find this effect with mitochondria from rat or bovine heart. On the other hand, incubation of purified CcO from bovine heart and kidney with cAMP + PKA + ATP induced the allosteric ATP inhibition of CcO [Lee et al., 2002]. With purified bovine heart CcO the allosteric ATP-inhibition was induced by treatment with PKA and ATP and switched off by subsequent treatment with the serine/threonine-specific protein phosphatase PP1 [Lee et al., 2001], indicating that this mechanism is reversibly switched on by serine and/or threonine phosphorylation of CcO. The presence and absence of the cAMP effect can be

explained on the basis of tissue differences, i.e. in liver and heart occur different signalling pathways.

It was postulated that the allosteric ATP-inhibition is based on phosphorylation of Ser^{441} in CcO subunit I [Lee et al., 2001]. This conclusion was based on 3 observations:

1) Treatment of isolated CcO from bovine heart with PKA, cAMP and ^{32}P-ATP resulted mainly in phosphorylation of subunit I [Bender and Kadenbach, 2000, see also Lee et al., 2001].

2) In isolated and in liposome-reconstituted CcO from bovine heart, the allosteric ATP-inhibition could only be switched on by phosphorylation from the outer (cytosolic) side [Lee et al., 2001, 2002].

3) Only one consensus sequence for phosphorylation by PKA ($RRYS^{441}$) was found on the cytosolic side of CcO subunit I.

Extensive search by mass spectrometry to identify a phosphorylated fragment containing Ser^{441} was so far unsuccessful. A fragment containing Ser^{441} was isolated (see Fig. 43) but in samples of CcO with or without allosteric ATP-inhibition, until now, the phosphorylated fragment was not found. This may be due to a highly sensitive dephosphorylation mechanism.

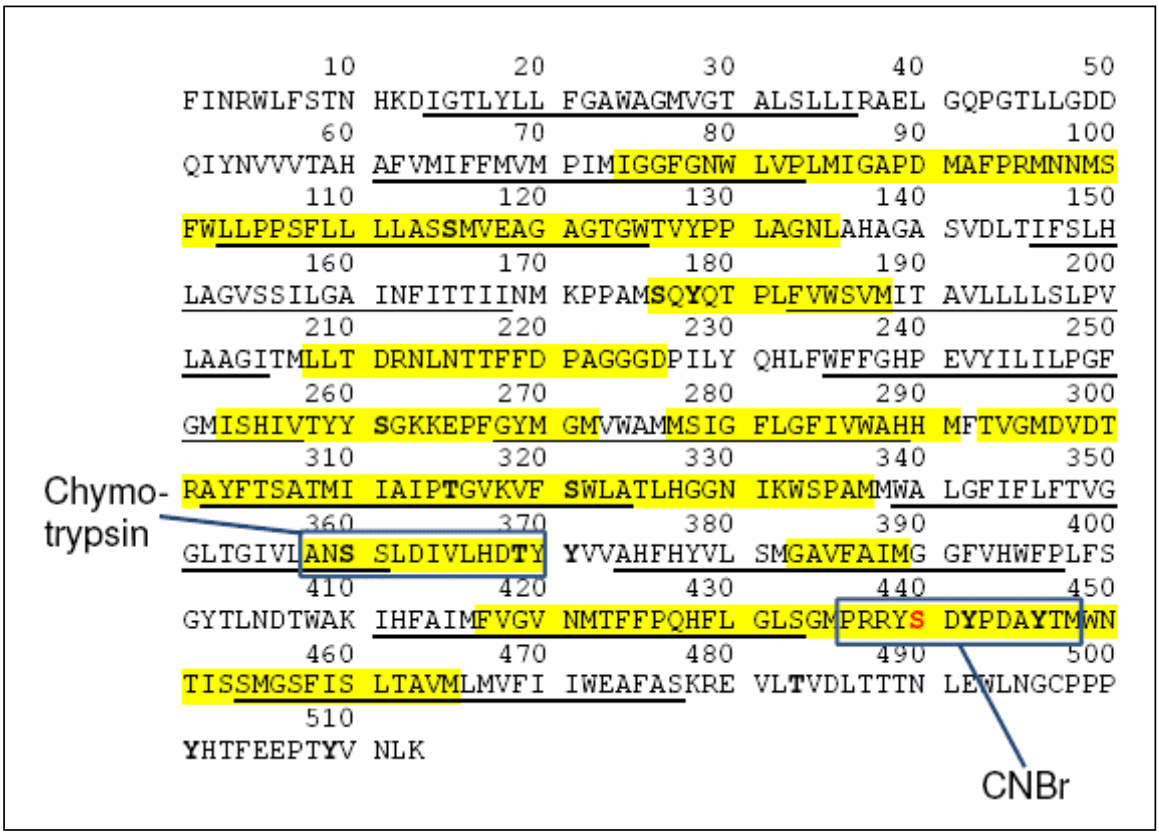


Fig. 43. Identified polypeptides of the CcO subunit I-sequence from bovine heart

The sequence of bovine heart subunit I at fully oxidized state is shown (gi|40889823 Chain A). Polypeptides identified by mass spectrometry are shown in yellow. In bold are presented predicted phosphorylation sites (NetPhos 2.0: http://www.cbs.dtu.dk/services/NetPhos) and underlined are the predicted transmembrane helices (TMHMM 2.0: http://www.cbs.dtu.dk/services/TMHMM-2.0). (S. Helling, personal communication).

5.4 Identification of new phosphorylation sites in CcO

A list of phosphorylation sites in subunits of the complexes of oxidative phosphorylation has been published recently [Kadenbach et al., 2010]. But only in very few cases a correlation between the reversible phosphorylation of a specific amino acid in subunits of the complexes and its biological function was given. Several preparations of CcO from bovine heart mitochondria with allosteric ATP-inhibition, which by Western blot analysis clearly exhibit phosphorylation of CcO subunit I at serine/threonine, were isolated by BN-PAGE and separated into subunits by SDS-PAGE. The dried samples of complex IV and of isolated subunits were analysed by mass spectrometry in collaboration with Dr. Helling in Bochum. In no case, any phosphorylation site within CcO subunit-I was identified. Additionally, the physiological function of the 5 newly identified phosphorylated amino acids in CcO subunits (Ser^{34} in subunit IV, Ser^{4} in subunit Va, Thr^{35} in subunit Va [Helling et al. 2008], Ser^{126} in subunit II, Tyr^{118} in subunit II, Ser^{1} in subunit VIIc) has not been elucidated yet. This may be due to 1) incomplete sequence coverage of subunit-I fragments for mass spectrometry (Fig. 43), 2) incomplete and highly variable phosphorylation of the particular amino acids in different mitochondrial preparations, and last but not the least 3) the loss of phosphorylation of the specific amino acid involved in allosteric ATP-inhibition during BN-PAGE and fragmentation of the samples for mass spectrometry (see below).

5.5 Effectors switching on and off the allosteric ATP-inhibition of CcO

Numerous experiments with sliced bovine or rat heart, incubated for various times with different effectors, including $CaCl_2$, forskolin (specific stimulator of adenylyl cyclase), IBMX (inhibitor of phosphodiesterases), acetylcholine (neurotransmitter), PP2 (inhibitor of c-Src) and Wortmanin (inhibitor of phosphatidylinositol-3-phosphate kinase), did not clearly depict the effects on the allosteric ATP-inhibition, as measured after isolation of mitochondria from the incubated slices. Also, incubation of isolated mitochondria for various times with cAMP, Br-cAMP (membrane permeable activator of PKA), H89 (inhibitor of PKA), metal ions, etc. did not give any clear result concerning the signal pathways turning on or off the allosteric ATP-inhibition. It is possible that protein kinases or phosphatases of the signal pathways which switch on and off the allosteric ATP-inhibition via reversible

phosphorylation of CcO are not permanently localized in mitochondria. In fact, it has been shown that many protein kinases and phosphatases are translocated into mitochondria after their activation by phosphorylation (reviewed in [Vogt et al., 2007]). Stimulation of a variety of cell types with insulin-like growth factor-1 or insulin leads to phosphorylation and translocation of Akt (protein kinase B) into mitochondria where it phosphorylates subunit-b of the ATP synthase [Bijur and Jope, 2003]. Also, binding of EGF to its receptor EGFR (epidermal growth factor receptor) results in the phosphorylation of EGFR by c-Src and translocation of the phosphorylated receptor into mitochondria, where it binds to CcO subunit-II [Boerner et al., 2004]. Another pathway has been suggested by Lee et al. [2005] for phosphorylation of CcO subunit-I at Tyr^{304}. They found this phosphorylation after starvation or treatment of HepG2 cells with glucagon or forskolin (activator of adenylyl cyclase) and suggested that cAMP activates PKA bound to AKAP121 (A-kinase anchor protein 121) localized to the mitochondrial outer membrane, where the signal is transmitted to a bound tyrosine kinase (c-Src).

I have found a decrease of the allosteric ATP-inhibition of CcO and serine-phosphorylation of subunit-I after incubation of bovine heart tissue slices with insulin (Fig. 34 and 35). If this effect of insulin is based on the translocation of Akt into mitochondria, that already has been shown for different cells [Bijur and Jope, 2003] and recently also for cardiomyocytes [Yang et al., 2009], one possibility would be that Akt phosphorylates and activates a protein phosphatase which dephosphorylates CcO and switches off the allosteric ATP-inhibition.

The variable (and often not reproducible) results on the allosteric ATP-inhibition of CcO in different mitochondrial preparations of this dissertation may depend on the signalling situation of the animal at the time of death, i.e. if the specific protein kinase or phosphatase has or has not been translocated into the mitochondria. Therefore, it is suggested that the psychological situation of the animal during killing determines the function or extent of the allosteric ATP-inhibition of CcO in the isolated mitochondria.

5.6 The role of the allosteric ATP-inhibition in the regulation of cell energy metabolism

As already explained the allosteric ATP-inhibition of CcO was originally described with the isolated enzyme and with detergent solubilized mitochondria. Its function in the living cell was proposed in a hypothesis which was described in 8 review articles [Kadenbach

et al., 1999; 2000; 2004; 2009; 2010; Lee et al., 2001; Ludwig et al., 2001; Kadenbach, 2003]. The main proposal was the decrease of $\Delta\Psi_m$ in living cells to the values below 140 mV by feedback inhibition of CcO at high ATP/ADP ratios, because the F_0F_1-ATPsynthase is saturated and has maximal activity at about 130 mV [Kaim and Dimroth, 1999]. Low $\Delta\Psi_m$ values (< 140 mV) have in fact been measured in living cells [Wan et al., 1993; Mollica et al., 1998; Zhang et al., 2001; Berkich et al., 2003], which contrast or oppose the 180-230 mV determined in isolated mitochondria [Fox et al., 1993; Nicholls and Ferguson, 2002; O'Brien et al., 2008]. At $\Delta\Psi_m$ values above 140 mV the formation of ROS [Liu, 1997; Korshunov et al., 1997; Rottenberg et al., 2009] and the proton permeability of the inner mitochondrial membrane [O'Shea et al., 1984] increase exponentially. Two further published observations supported the hypothesis:

1) In living cells various stress factors resulted in hyperpolarisation of $\Delta\Psi_m$ to high values, The stress factors include oxidative stress (e.g. H_2O_2), irradiation, cytostatica, fungicides, high glucose, increased cytosolic calcium and receptor stimulation (for review see [Kadenbach, Ramzan, Wen and Vogt, 2010]).

2) In living muscle ATP/ADP ratios of 100 – 1000 have been calculated from ^{31}P-NMR measurements [From et al., 1990; Kupriyanov et al., 1993; Himmelreich and Dobson, 2000; Fenning et al., 2003]. In contrast, by chemical analysis ATP/ADP ratios of about 1 (mitochondrial matrix) and 10 (cytosol) were measured in liver [Soboll et al., 1978]. Since in living cells ADP is bound with high affinity to non-catalytic sites of many proteins, the concentration of free ADP is much lower [Veech et al., 1979; Mörikofer-Zwez and Walter, 1989] and the ATP/ADP ratio of free nucleotides much higher, as determined by ^{31}P-NMR.

A second aspect of the hypothesis was the reversible switching on and off (under stress) of the allosteric ATP-inhibition of CcO by protein phosphorylation (see Fig. 44).

Two main observations of this dissertation prove the validity of the hypothesis and contribute to current studies on "Molecular System Bioenergetics" [Weiss et al., 2006; Saks et al., 2009]:

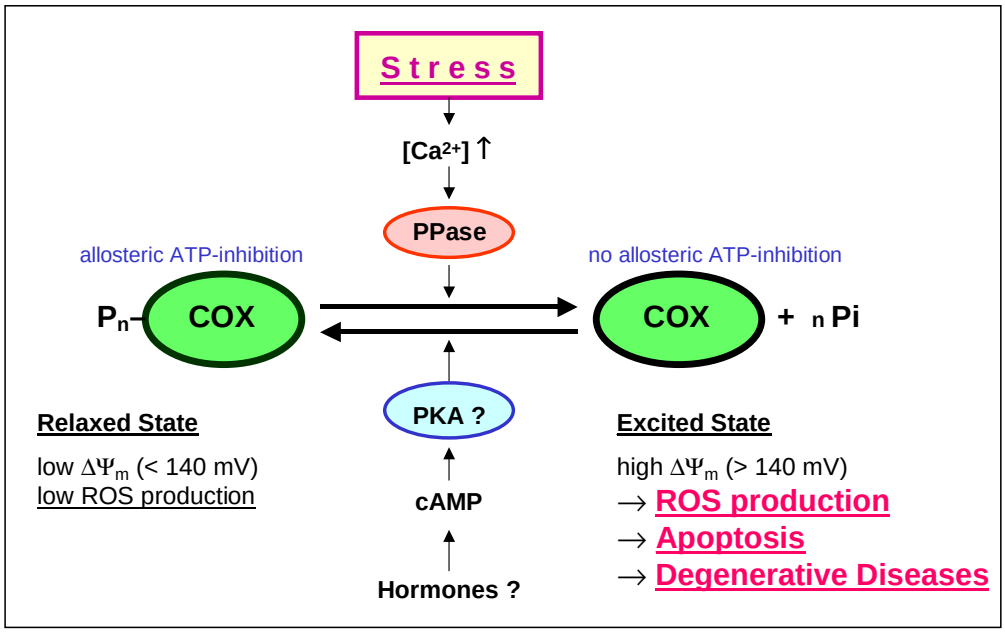


Fig. 44. Hypothesis on regulation of $\Delta\Psi_m$ and ROS formation in mitochondria
(Taken from Kadenbach, Ramzan and Vogt, 2009).

1) Measurement of the allosteric ATP-inhibition of CcO in mitochondria requires very high ATP/ADP ratios (> 50, see Fig. 41) which are difficult to establish in vitro. Therefore, previous measurements of the respiration of isolated mitochondria or the activity of CcO may not reflect the in vivo situation, unless they have been performed at high ATP/ADP ratios.
2) The reversible decrease of $\Delta\Psi_m$ in isolated rat liver mitochondria by PEP and PK (Fig. 39), that is suggested to be based on the allosteric ATP-inhibition of CcO at high ATP/ADP ratios, can explain the discrepancy between high $\Delta\Psi_m$ values (180-230 mV) measured in isolated mitochondria [Fox et al., 1993; Nicholls and Ferguson, 2002; O'Brien et al., 2008] and low $\Delta\Psi_m$ values (100-140 mV) observed in living cells/tissues [Wan et al., 1993; Mollica et al., 1998; Zhang et al., 2001; Berkich et al., 2003].

5.7 Conclusions

To understand the complex regulation of respiration in living cells by the 'second mechanism of respiratory control', two aspects have to be distinguished:
a) The allosteric ATP-inhibition of CcO is only active at high ratios of free ATP/ADP (> 16).
b) The allosteric ATP-inhibition of CcO is switched on and off by reversible phosphorylation of CcO (independent of the ATP/ADP ratio).

The results of this dissertation support 4 proposed physiological functions of the 'allosteric ATP-inhibition of CcO' in living eukaryotic organisms:

1. It regulates respiration according to the requirement of ATP. At ATP/ADP ratios > 50, respiration is switched off. At ATP/ADP ratios < 16, the rate limiting function of CcO is abolished and respiration and oxidative phosphorylation are increased.
2. It maintains the homoeostasis of high ATP/ADP ratios (> 50) in living cells. Since turning on and off occurs by a change of the low free ADP concentrations, the amount of ATP remains high and almost constant.
3. It keeps $\Delta\Psi_m$ at low values, as found in living cells/tissues (< 140 mV), and prevents inefficient oxidative phosphorylation, based on increased proton permeability of membranes at high $\Delta\Psi_m$ values (180 – 230 mV).
4. It prevents the formation of ROS in mitochondria (at high $\Delta\Psi_m$ values) that on the other hand, have been demonstrated to stimulate aging and the formation of degenerative diseases.

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7. Abbreviations

Ab	antibody
AKAP 121	A-kinase anchor ptotein
Akt	protein kinase B
CcO	cytochrome c oxidase
c-Src	nonreceptor tyrosine kinase
$\Delta\Psi_m$	mitochondrial membrane potential
D.W.	distilled water
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ID	first dimension
2D	second dimension
IGF-1	insuline-like growth factor-1
MALDI-TOF	matrix-assisted laser desorption ionization time-of-flight mass spectrometry
PDGF	platelate derived growth factor
PEP	phosphoenol pyruvate
PK	pyruvate kinase
PKA	protein kinase A
PVDF	polyvinyldifluoride
ROS	reactive oxygen species
RT	room temperature

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