Book-Chapter 5: **Transport and ATP synthesis in mitochondria** (1978-1984)

III. Hypothesis: Mitochondrial ATP synthesis on the phosphate/proton symport system with oxidized glutathione as a catalyst

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Abbreviations: GSH, reduced glutathione; GSSG, oxidized glutathione; DNP, 2,4-dinitrophenol; NSPM, N'-[N''-n-nonyl-4-sulfamoylphenyl]-maleimide; NTU, n-nonylthiouracil; RCR, respiratory control ratio

Summary: We present as a hypothesis the mechanism for mitochondrial ATP synthesis in the 30 kDa phosphate/proton-symport system with oxidized glutathione as a catalyst. The effects of the uncoupler 2,4-dinitrophenol and arsenate in this mechanism are discussed. It is postulated that the mitochondrial F_0F_1-ATPase is actually, under normal physiological conditions, a K-Pump. Coupling between ATP synthase and ATPase is suggested. Our hypothesis represents the first description of a proton-driven build-up of high-energy intermediates (activated disulfides, sulfenyl phosphate) resulting in phosphoryl transfer or transport activities.
Key words: ATP-synthesis, uncoupling, K-pump, activated disulfide, sulfenyl phosphate.

Zusammenfassung: Wir präsentieren in einer Hypothese den Mechanismus für mitochondriale ATP-Synthese an dem 30 kDa Phosphat/Protonen-Symport-System mit oxidiertem Glutathion als Katalysator. Die Effekte der Entkoppler 2,4-Dinitrophenol und Arsenat in diesem Mechanismus werden diskutiert. Wir postulieren, daß die mitochondriale F_0F_1-ATPase unter normalen physiologischen Bedingungen eine K-Pumpe ist. Es wird angenommen, daß eine Kopplung zwischen ATP-Synthase und ATPase besteht. Unsere Hypothese ist die erste Beschreibung einer Protonen-getriebenen Bildung von Hochenergie-Intermediaten (aktiviertem Disulfid, Sulfenylphosphat) und dabei durchgeführtem Phosphoryl-Transfer oder -Transport.
Schlüsselwörter: ATP-Synthese, Entkopplung, K-Pumpe, aktiviertes Disulfid, Sulfenylphosphat.

Introduction

In 1970, Painter and Hunter [1] presented a model system containing cytochrome c, $NADP^+$, GSH, GSSG, P_i^-, ADP and $MgCl_2$ which produced high amounts of ATP. The disulfide GSSG was described as the essential catalyst. Bäuerlein [2] reinvestigated Hunter's model system using ^{14}C-ADP and thin-layer chromatographic analysis, instead of trapping the ATP and assaying it using a hexokinase system. In parallel experiments, the ATP-formation in the complete system was compared to a control without GSH and P_i, and approximately the same yields were obtained. The author concluded that Hunter's system leads to ATP-formation as a result of an adenylate kinase-like reaction. We propose that both conclusions [1, 2] are incorrect:

1. Hunter and Bäuerlein should have used $^{33}P_i$ for their experiments and
2. GSH is not necessary in the system to produce ATP, and the Hunter system may synthesize ATP from ADP and P_i, as well as via an adenylate kinase-like reaction (as shown by Bäuerlein [2]).

Since the reactions take place in a hydrophilic environment, proteins do not hinder the appropriate arrangements of the participating compounds.

Wieland and Bäuerlein formulated in 1968 [3] a mixed anhydride of sulfenic and phosphoric acid, $RSOPO_3H_2$, to explain the formation of ATP from ADP and P_i by oxidation of a thiol with bromine in anhydrous pyridine. Bäuerlein [4] showed in further model reactions ATP formation on oxidation of disulfides by iodine and postulated for these reactions also sulfenylphosphate as intermediate. However it was never possible to directly demonstrate the participation of sulfenylphosphate in the reactions, because this type of compound is extremely reactive.

During our attempt to elucidate the mechanism of oxidative phosphorylation in mitochondria, we came to the conclusion that a disulfide activated in the membrane had to be involved [5-7]. We proposed a phosphate reaction with ADP on this activated disulfide in a synchronous mechanism without the real build-up of a sulfenylphosphate intermediate [5].

In further experiments, we could demonstrate that the classical uncoupler 2,4-dinitrophenol (DNP) is bound and transported in mitochondria by the NSPM-sensitive P_i/H^+-symport system, but without direct uncoupling oxidative phosphorylation. The behavior of phosphate- or DNP-transport against inhibition by our lipophilic thiol reagent, NSPM, suggested the involvement of a regulatory factor in these transport activities [8]. Subsequently, glutathione could be identified as this regulatory factor. A mechanism has been formulated showing our P_i/H^+-symport system dependent on endogenous oxidized GSH and on P_i/H^+-ion-activated disulfides [5, 9].

Conclusions

The following conclusions can be drawn from earlier and recent work:

1. Lipophilic sulfenyl and thiol-group trapping compounds (NTU, NSPM, etc.) react in the membrane with activated mixed glutathione-protein disulfides, thereby abolishing RCR. The 30 kDa P_i/H^+-symport system is involved in this process. The high-energy compound, picrylacetate, acetylates RCR and dithiothreitol dependently, as well as DNP-sensitive 30 kDa membrane proteins including the P_i/H^+-symport system, thereby eliminating RCR. Our conclusions: RCR is dependent on glutathione bound to 30 kDa membrane proteins. DNP and its analogs, lipophilic NTU and its nucleophilic analog compounds, as well as lipophilic NSPM, and most probably picrylacetate, share the same reaction site on these proteins [9].

2. Binding of DNP could be seen in our system because phosphate had been omitted. This binding is probably not seen at normal concentrations of phosphate [8]. DNP and analog compounds may then falsely be seen in comparison with normal protonophors as proposed [10].

3. NSPM, by its structural similarity to the adenine moiety of nucleotides, interacts preferentially with high-affinity nucleotide binding sites (NADH- and ß-hydroxybutyrate-dehydrogenase, H^+-pumping and soluble ATPase, adenine nucleotide carrier and transhydrogenase) [11]. The binding of the lipophilic aromatic compounds, including NSPM, to the general binding site on the 30 kDa membrane proteins suggests to us also a possible binding of nucleotides, especially of ADP and ATP, on this site. Their binding would be dependent on energization. Our conclusion is supported by sequence data [12].

4. A significant net synthesis of ATP on clean membrane-bound ATPases (F_0F_1) [13], ATP-Pi-exchange complexes [14], or H^+-pumping ATPases reconstituted into phospholipid vesicles [15]

has never been shown. Moreover, ATP synthesis requires a hydrophobic protein fraction [15]. We conclude, therefore, that net ATP synthesis on clean complexes may never be shown simply because this is not their function.

Hypothesis

Our described mechanism for mitochondrial phosphate/proton and DNP/proton symport with glutathione as a regulatory factor [9], and the conclusions derived from this mechanism, lead to the question regarding the relationship of ATP synthesis to this mechanism. The connection of glutathione only to transport activities would be difficult to comprehend. The dependence of RCR on GSSG bound to 30 kDa membrane proteins is much more likely, and suggests direct involvement of these proteins in ATP synthesis. The nucleotide binding, which is possible in their general binding site, makes this suggestion even more attractive. Important in this aspect is our experimental differentiation between RCR on the one hand and P_i/H^+-symport as well as ADP/ATP-antiport on the other hand [8, 9].

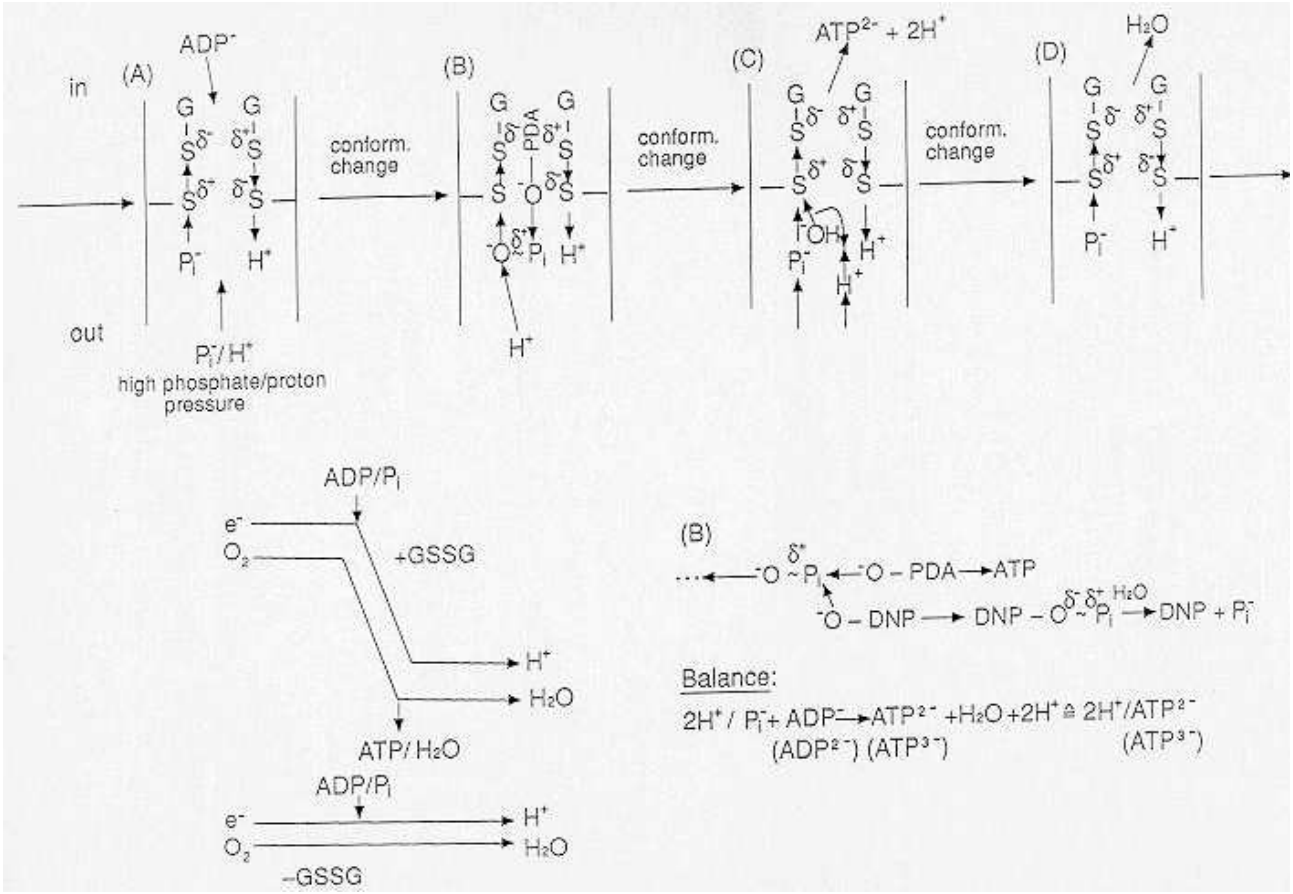

Fig.1. ATP-synthesis in the glutathione dependent P_i/H^+-symport system

Fig.1 shows the mechanism described by us for phosphate/proton symport [9] completed for ATP synthesis. The whole mechanism would then correcly be ascribed as „glutathione dependent proton/phosphate symport-driven ATP synthesis". P_i^- and H^+ activate the disulfides. A further H^+ from the outside, as well as ADP^- from the inside, lead to the transfer of $\sim Pi^{\delta+}$ (synchronous build-up of a sulfenyl phosphate) onto the nucleophilic oxygen of ADP^-. ATP^{2-} and 2 H^+ are released to the inside. P_i and H^+, again from the outside, liberate H_2O from the activated

disulfides (sulfenic acid) for release (presumably) to the inside allowing a new reaction sequence to start. This cycle is repeated as long as ADP is present. Otherwise, the system works as a proton/phosphate symporter [9]. The result is that 2 protons are pumped from the outside to the inside during synthesis of one ATP.

Mitochondria are decoupled without GSSG and no ATP is synthesized: RCR = 1. Maximal coupling is seen with sufficient GSSG and high amounts of ATP may be obtained: RCR = ∞ (Fig. 1).

DNP and its analogs are bound and transported by our system. These compounds compete with phosphate on the specific binding site (A) and they may lead to inhibition of ATP synthesis by blocking ADP reaction (B). The build-up of phosphorylated compounds by competition with ADP on the sulfenyl phosphate (B) is not very likely since the affinity of ADP should be much higher. On the other hand, one should consider that the compounds are rapidly taken up and not available for this kind of reaction, a reaction which at sufficient extern steady state concentration of the compounds and than consequently also phosphorylated compounds, as well as energy dissipation by their hydrolysis inside mitochondria, would lead to uncoupling. A sufficient external steady-state concentration may be obtained by release of the accumulated anions [8]. Reaction of the compounds to the sulfenic acid (C) should result in their transport.

Thiophosphate [9] does block phosphate transport and ATP synthesis, most likely by functioning as a suicide inhibitor: This sulfur compound substitutes phosphate on the activated disulfide (A) leading to a stable sulfur analog of the "masked" sulfenyl phosphate (B). ATP synthesis is then blocked at this stage (B) because P_i/H^+ and, of course, H_2O are not able to release the inhibition.

Anyone discussing oxidative phosphorylation should realize by now why no one has been able to detect a covalent intermediate in ATP formation (irregardless of method used). Based on the results and the mechanism described, we will attempt to provide more direct proof of sulfenylphosphate participation, although this should already be clear.

The described mechanism for ATP synthesis leads to the question of the proton-pumping ATPase (F_0F_1). As outlined at the beginning of our hypothesis, the real function of this complex may not be the synthesis of ATP. We would like, therefore, to direct the research endeavers into a new direction by stating: mitochondrial F_0F_1 is under normal physiological conditions nothing more than a K-pump (Fig. 2). The literature in the past has included numerous results pointing to this statement, including our own (see for instance [14, 16-21]). The separation of ATPase- and ATP-P_i-exchange activities in complex V using various energy transfer inhibitors and uncouplers [18, 19], and our measurements of mitochondrial K-fluxes (part IV) imply the presence of such a pump in order to maintain a high K-gradient (low K-concentrations inside, but high outside).

However, some points (part IV) should be outlined:

1. The oligomycin- and aurovertin-sensitive stimulation of mitochondrial ATPase activity by valinomycin [21], that is in vesicularized structures where K-concentration gradients can be established.
2. The inability of valinomycin to influence ATPase activity in soluble ATPase complexes [19] where K-concentration gradients do not exist, but where direct binding of valinomycin [16-18]

with inhibition of ATP-P_i-exchange [14] and elimination of localized membrane potential (oxonol VI response [19]) could be demonstrated.
3. The modulation of isolated ATPase conformations by ATP, ADP/Mg (Ca)/ K [20] which are strong indicators of the proposed K-pump. Further convincing indicators are
4. the anti-swelling activity of ATP and the contraction of swollen mitochondria by ATP [21, 22] as is
5. the uncoupling of mitochondria after removal of F_1 from the mitochondrial membrane.

Aurovertin and oligomycin, two inhibitors for ATP synthesis bind at F_1 and F_0, respectively, thus demonstrating a direct coupling between ATPase and ATP synthase (Fig. 2). Aurovertin may block a passing of ADP by $F_{1\alpha}$ to the ATP synthase and thereby also enhance DNP effects [19]. Coupling between F_0 and the phosphate/ arsenate(Ca)-transport using oligomycin has been shown [23, unpublished], and may also explain the oligomycin- and aurovertin-sensitive uncoupling by arsenate [24]. The synthesis of ADP-arsenate [25] should be blocked, a compound hydrolyzed in the inside of mitochondria. The cyclic movement of arsenate should normally complete the uncoupling mode in our proposed mechanism (Fig. 1). The different sensitivities to inhibitors (NSPM/oligomycin-aurovertin) in the uncoupling actions of DNP or arsenate, are then best explained by DNP or arsenate substituting ADP or phosphate, respectively. The primary target of another ATPase inhibitor, namely dicyclohexylcarbodiimide, for abolishment of RCR in mitochondria are most likely the phospholipids (~ 3 to 3,5 nmol/mg mitochondria) [5].

Proof for direct interaction of dicyclohexylcarbodiimide with F_0 (proteolipids) and, consequently, inhibition of ATP synthase therefore does not exist based on the preceeding results. The proof for

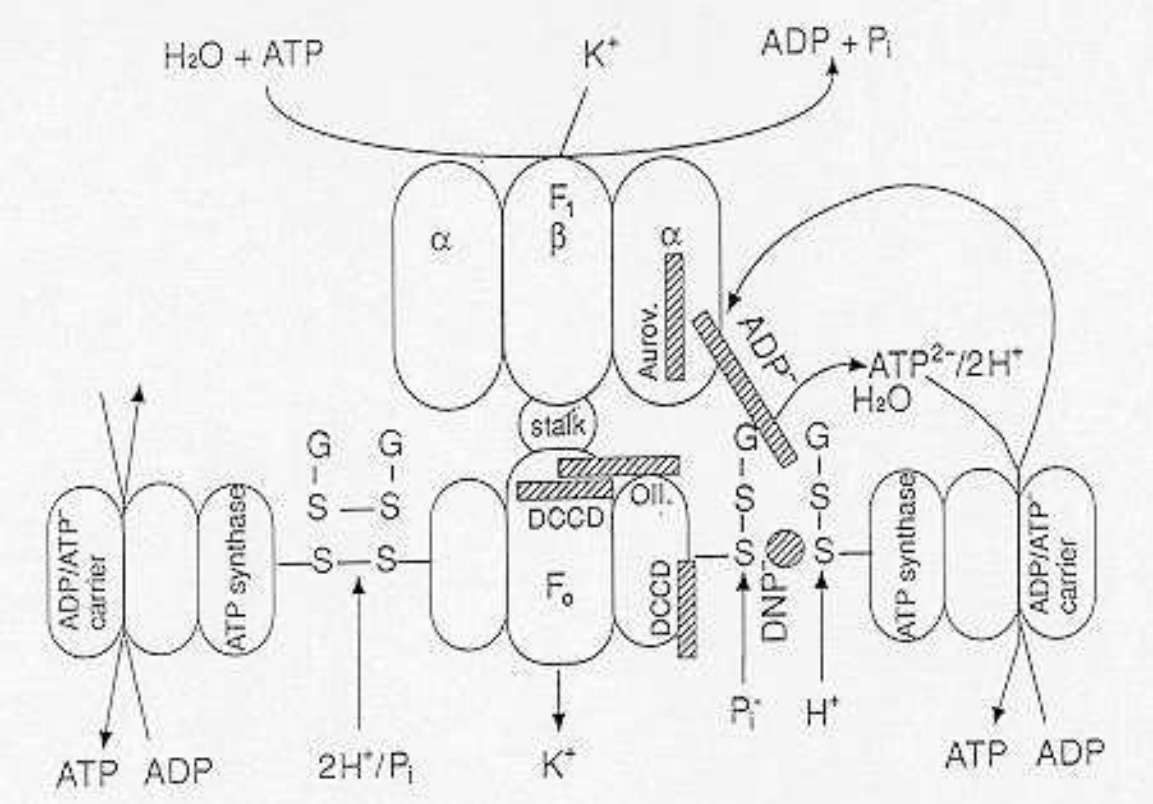

Fig.2. Coupling between ATP synthase, membrane bound ATPase (F_0F_1) and ATP/ADP-translocator

participation of a special phospholipid in the ATP synthetic activity is thus very important.

The arrangements of the functional important parts of the ATP synthesizing machinery can be described as follows [Fig.2]. The nucleotide carrier should be near the ATP synthase. The ATP synthase may surround, as 3 dimers [9], the F_0-part (6 proteolipids) of the ATPase (3 α, 3 ß subunits) [9, 13] and participate in the catalytic-site cooperation [26] with one active dimer at a time [9].

Our hypothesis represents the first description of a proton-driven build-up of high energy intermediates (activated disulfides, sulfenyl phosphate) resulting in phosphoryl transfer or transport activities. The continued debate about Mitchel's chemiosmotic theory [27] can be best resolved by our formulated hypothesis [5-7].

It should be added that the hypothesis presented in no way contradicts the isotope exchange data of Boyer [26], or the results of others. One needs to be aware of the coupling between ATP synthase and ATPase, and should, for instance, compare the data with the SR-Ca^{2+}-pump-ATPase (ATP synthase) [28], and one should read [20, 29]. The nucleotide binding is dependent not only on Mg^{2+}, but also more importantly on K^+: K^+ modulates bound ATP concentrations, which resembles the Ca^{2+}-pump of SR. The presented methods in [20, 28, 29] are ideal for the exploration of the involved K^+-pump activity. It should be kept in mind that Boyers experiments were done at high cation concentrations (including K^+) [26].

The ATP synthase and ATPase, as already mentioned, are under normal physiological conditions coupled. ADP from the cytosol is able to disturbe this coupled system. Masked sulfenylphosphate is unstable and under high phosphate and proton pressure, in the presence of ADP (α-sub. or cytosol), results in the immediate formation of ATP. Sulfenylphosphate involvement is then not seen under the normal conditions used, and therefore not comparable to other high-energy intermediates. One must undertake some modifications in order to trap this highly reactive "intermediate" (as described in this manuscript). There is, of course, a stoichiometric relationship between P_i/H^+/ADP-ATP/H_2O, but no such relationship between these compounds and the sulfenyl groups or the ATPase molecules involved. Consequently, there is no incompatibility with Jagendorf's experiments (on chloroplasts) [30]. The hypothesis and results of our hypothesis have already been presented [31-34].

Finally, the ATP-synthesizing or P_i-transporting mosaic enzyme described by us, very much resembles the immune system function of the hypervariable part of the immunoglobulins or the glutathione-thioether-carrier. Based on this example, researchers become aware of the difficulties involved in the investigation of the function and mechanism of unknown enzymes, and realize the importance and necessity of enhanced interdisciplinary research efforts.

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Transport and ATP synthesis in mitochondria 1
III. Hypothesis: mitochondrial ATP synthesis of the phosphate/proton symport system with oxidized glutathione as catalyst

by

Kiehl Reinhold

Editor: Böck

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I regret to inform you that the Editor responsible for your manuscript has advised me that it cannot be accepted for publication in the Journal. The referees' reports are enclosed for your information.

The top copy of your manuscript is being returned to you by separate printed-matter mail.

Yours sincerely,

Philipp Christen

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This paper describes a mechanism for ATP synthesis in mitochondria which depends on glutathione as regulatory factor and involves the Pi/H^+ symport as a key component in this system. There is no serious experimental evidence for such a mechanism.

Report 2

Re: MS No. 92-1073 AB

I just have read the manuscript by R. Kiehl entitled: "Transport and ATP synthesis in mitochondria. III. hypothesis: Mitochondrial ATP synthesis on the phosphate/proton symport system with oxidized glutathione as catalyst".

The paper presenting no experimental data but referring to rather old (and in part non-reviewed) publications or to submitted manuscripts was hard to read - in part due to the really poor English. The author postulates a new mechanism for mitochondrial ATP synthesis occuring on the phosphate/proton symporter. I hesitate to refuse this manuscript as pure speculation not supported by any experimental evidence but rather suggest you to consult a real expert on the field of bioenergetics e.g. Prof. Klingenberg (Munich) or Prof. Kröger (Frankfurt).
Sorry for this. I return the MS by seperate mail. Nevertheless, I would appreciate if you could keep me informed on the final evaluation of the MS.