ARTIFICIAL ENERGY CONSERVATION IN THE RESPIRATORY CHAIN. NO NATIVE COUPLING SITE BETWEEN CYTOCHROME *c* AND OXYGEN

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1. Introduction

The study of partial reactions of the electron flow system in chloroplasts, as well as mitochondria, is one method to localize coupling sites. Usually an inhibitor of electron flow is employed and artificial electron acceptor or donor systems are added to restore electron flow, possibly coupled to ATP formation, in partial sequences before or after the inhibitor block. This way in chloroplasts two native energy conserving sites have been identified [1].

During these studies with chloroplasts the concept of artificial energy conservation has been introduced. This was to explain the finding that certain sequences in partial reactions of the photosynthetic electron transport chain are not coupled to ATP formation but that photophosphorylation may be restored onto it by an artificial electron donor, which is lipophilic and loses proton upon oxidation [2–4]. Based on the notion of vectorial electron transport in the chloroplast membrane and the chemiosmotic theory [5], the concept states that an artificial energy conserving loop might be constituted by the inward translocation of protons and electrons through the membrane via the artificial redox compound, and the back outward translocation of electrons via the reaction centers of the photosystems. In the presence of an electron transport inhibitor artificial proton translocation might replace native proton translocation step ocurring in the absence of the inhibitor.

In fig.1 the formulae and the redox reaction of TMPD and DAD, two efficient electron donors for photosystem I in chloroplasts are depicted. The former yields a stable radical upon oxidation losing only one electron, the latter loses two electrons and two protons during full oxidation. Photophosphorylation [6] and proton translocation [7] is catalyzed by DAD only – the key observation for our concept.

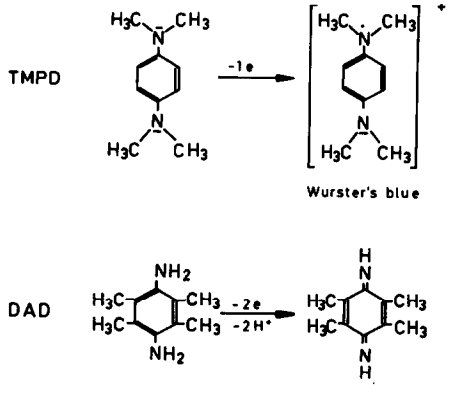

Fig.1. The redox reaction of TMPD and DAD.

Abbreviations: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine

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In respiration of mitochondria reduced TMPD in the presence of electron transport inhibitors is used to study the third site of energy conservation [8] which is linked to the terminal part of the chain from cytochrome *c* to oxygen. The P/O ratio observed in this partial reaction approaches 1.0. In this case TMPD needs not to cross the membrane to be oxidized and a proton potential of opposite polarity with regard to chloroplasts is generated by electron translocation in the cytochrome oxidase and proton consumption during water formation (see fig.4). However, in submitochondrial vesicles, which have the same polarity as chloroplasts, the reaction with TMPD should not be coupled to ATP formation, if our concept of artificial energy conservation is more generally applicable.

Indeed, the data in this paper demonstrate that if contribution from complex III can be excluded, oxidation of reduced TMPD is not coupled, but oxidation of reduced DAD is coupled. A similar conclusion is reached from a study of respiration in membranes from aerobically grown *Rhodopseudomonas capsulata.*

2. Methods

Mitochondria from beaf heart [9] and submitochondrial particles ([10] ETPH-Mg, Mn) were prepared and assayed for oxidative phosphorylation by standard methods. A Gilson oxigraph was employed. The assay conditions are given in detail in the legend to fig.2. Membrane vesicles from aerobically grown *Rhodopseudomonas capsulata* were obtained by the procedure of Baccarini-Melandri et al. [11]. Fluorescence of atebrine was measured as described in the same paper. The reaction mixture can be found in the legend to fig.3.

3. Results

In submitochondrial particles in the absence of antimycin A, the P/O ratio of DAD oxidation is about three times that of TMPD oxidation (fig.2a). At about 10^{-5} M antimycin A, however, phosphorylation with

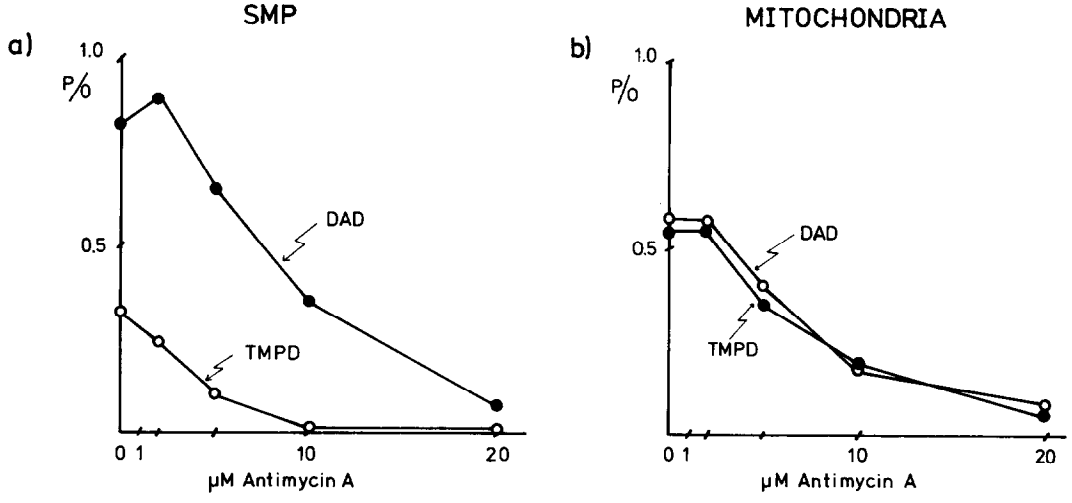

Fig.2. P/O ratios in the third region of energy conservation in mitochondria and submitochondrial particles (SMP), measured with DAD or TMPD. The assay mixture in a 1.1 ml reaction cell of a Gilson oxigraph contained 0.25 M sucrose, 40 mM glucose, 20 mM Tris–HCl, pH 7.5, 5 mM potassium phosphate, pH 7.5, 2 mM ADP, 2 mM $MgCl_2$, about 3 IEU hexokinase, 10^{-4} M TMPD or 2×10^{-4} M DAD, 10 mM ascorbate, about 1 mg protein of mitochondria or submitochondrial particles, the indicated concentrations of antimycin A, and water which had been saturated with air. In addition 10^6 dpm $^{32}P/\mu$mole P_i were present. The temperature was set to 30°C. The reaction was started by addition of mitochondria or particles. The rate of electron transport with DAD or with TMPD was about 0.09 and 0.05 μmol O_2 consumed/mg protein/min, in submitochondrial particles and mitochondria, respectively.

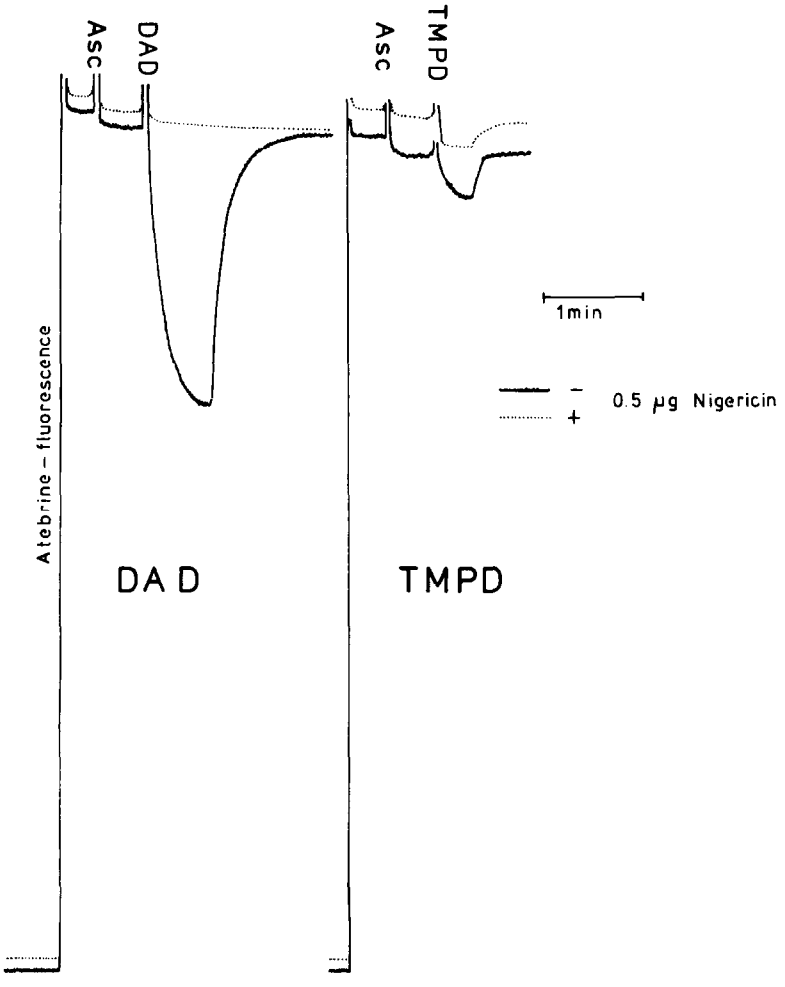

Fig.3. Energy conservation of the third region in membrane vesicles from aerobically grown *Rhodopseudomonas capsulata*, with TMPD or DAD, measured by the quench of atebrine fluorescence. The assay mixture contained in 2 ml of a fluorescence cuvette, 20 mM glycylglycine, pH 7.2, 50 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 3 mM ascorbate, 10^{-4} M DAD or TMPD, 2×10^{-6} M antimycin A, 4 µg valinomycin, 2 mg protein of membranes and 5 µM atebrine. The rate of electron transport was determined independently in the oxigraph. It was about 0.05 and 0.03 µmol O_2 consumed/mg protein/min, for TMPD and DAD, respectively. The dotted trace was run in the presence of 0.5 µg nigericin. The measurement was carried out at room temperature.

TMPD as electron donor is abolished, while the reaction with DAD is still coupled to an appreciable extent. At higher concentration the uncoupling effect of antimycin prevails [12] (at still higher concentrations interaction of antimycin with the phenylenediamines interferes). The rate of oxidation with either phenylenediamine was about 0.09 µmol O_2 consumed/mg protein/min, and was not influenced by addition of antimycin.

Figure 2b shows that in mitochondria the oxidation of both compounds is coupled with the same stoichiometry, and is inhibited by antimycin to the same extent. Also in this case antimycin had no effect on the electron transport rate, which was about 0.05 µmol O_2 consumed/mg protein/min.

Baccarini-Melandri et al. [11] have used DAD to study the third coupling site in the respiratory chain of *Rhodopseudomonas capsulata*. Oxidative phosphorylation with reduced DAD in the presence of antimycin was found, but the P/O value was rather low.

A more convenient test for energy conservation is the quench of atebrine fluorescence. Figure 3 shows this test again, with DAD compared to TMPD, in the presence of 2×10^{-6} M antimycin A. After oxygen is consumed the fluorescence returns to the original level. Only very little quench, if any, is observed with TMPD. The small change is not fully sensitive to nigericin, the insensitive part possibly reflecting the formation of the blue colored, oxidized TMPD. This experiment suggests that also in membrane vesicles from *Rhodopseudomonas capsulata* like in submitochondrial particles, only the oxidation of DAD, not of TMPD, is appreciable coupled to energy conservation.

4. Discussion

Redox potential and solubility of DAD and TMPD are rather similar. It is feasible to assume that the reaction of both compounds with the respiratory chain involves the same electron carrier – most likely cytochrome *c* in mitochondria as well as in submitochondrial vesicles. The only obvious difference between DAD and TMPD is that only the former liberates protons upon oxidation (fig.1).

Therefore it seems to us that our observation on the coupling of terminal electron transport in submitochondrial vesicles depending on the chemistry of the donor is best explained in terms of the chemiosmotic theory of Mitchell [5], as depicted in fig.4. DAD, being oxidized by cytochrome *c* inside the vesicles, with the electrogenic action of cytochrome oxidase, constitutes an artificial loop of energy conservation (fig.4a). TMPD does yield an electron flow loop across the membrane, but not a proton translocating loop because it does not liberate protons (fig.4b). In mitochondria no such loop exists at site three, because compounds are oxidized at the external surface (fig.4c, d). No actual translocation of protons occurs, but protons are consumed on the inner side during water formation, and are liberated artificially on the external side during net oxidation of ascorbate/DAD as well as of ascorbate/TMPD (consult [13]). From fig.4 it becomes clear why in mitochondria, the oxidations of DAD as well as of TMPD are coupled

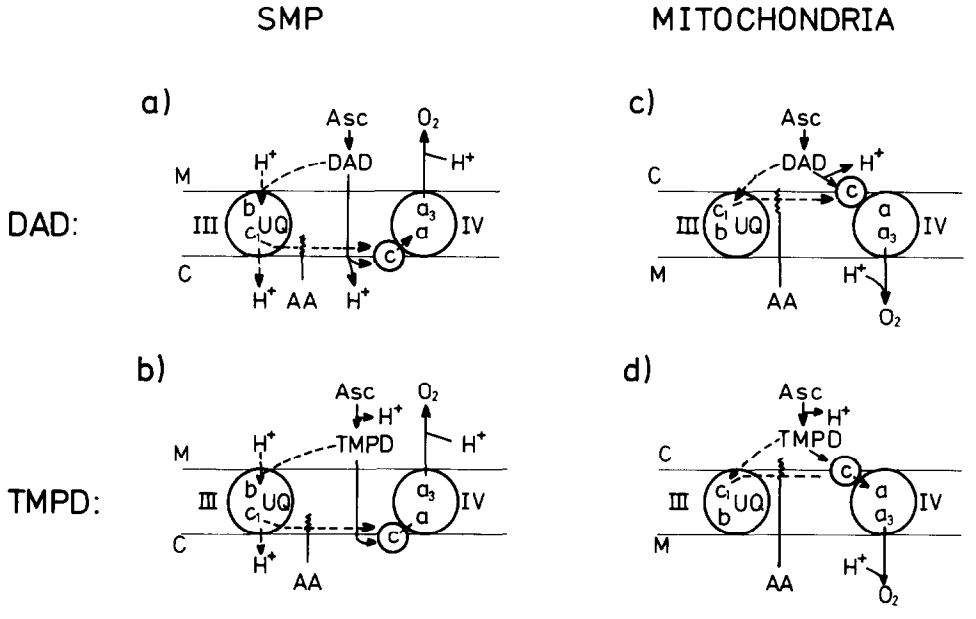

Fig.4. Schemes for electron and proton transport in the third region of energy conservation with DAD and TMPD. The symbols used are: SMP for submitochondrial particles; M and C for the surfaces of the membrane which face the matrix or carry cytochrome *c*, respectively; Asc for ascorbate; AA for antimycin A; b,c,c_1, a and a_3, for cytochromes *b*, *c*, c_1, *a* and a_3, respectively; III and IV for complex III and IV, respectively.

to energy conservation. Both lead to the formation of an electrochemical proton potential across the membrane.

In the absence of antimycin some phosphorylation is observed during TMPD oxidation also in submitochondrial vesicles. This can be accounted for by a reduction of complex III (see [14]), possibly of ubiquinone by TMPD (dashed arrows in fig.4) which results in proton translocation. The reaction of TMPD with complex III seems to be reversible since it is known that TMPD can bypass the inhibition of succinate or NADH oxidation by antimycin in mitochondria [15], and in submitochondrial vesicles (in preparation). In this reaction energy conservation at site 2 is lost, which could reflect reduction of TMPD by a component before the antimycin block on the M-side and oxidation of TMPD after the block on the C-side, abolishing proton translocation in complex III. This possibility is not included in the simple schemes of fig.4. TMPD also in bacterial photosynthetic electron transport bridges inhibition by antimycin, but phosphorylation is fully retained in this system (see [16]). In chloroplasts a similar bypass by TMPD of electron transport inhibition by dibromothymoquinone has been found [17]. In this reaction photophosphorylation is also restored in part, and it has been suggested that TMPD transfers electrons from plastoquinone to photosystem I remaining on the inner side of the chloroplast membrane.

The interaction of TMPD with the respiratory chain in submitochondrial particles has been studied previously [14,18,19] and the low coupling efficiency of TMPD oxidation compared to oxidation of *N*-methylphenazonium methosulfate has been noticed without providing an explanation. Energization of submitochondrial particle by reduced TMPD, on the other hand, has been reported by Skulachev et al. [20]. Their conditions of assay with low antimycin and succinate present, however, did not allow to study coupling site three alone.

Our own experiments with *Rhodopseudomonas capsulata* and experiments of other laboratories with *Paracoccus denitrificans* [21,22] and with *Pseudomonas saccharophila*, demonstrate that also in bacterial respiration TMPD oxidation is not coupled, but oxidation of H-carrying redox compounds is coupled. However, coupling with TMPD is found with preparations from *Mycobacterium phlei* [23] and *Azotobacter vinlandii* [24]. We think that this discrepancy might reflect the different polarities of vesicular membrane preparations: the former would correspond to submitochondrial vesicles, while the later cases should resemble mitochondria.

If our interpretation is correct, there is no complete coupling site in the respiratory chain between cytochrome *c* and oxygen, e.g., at cytochrome a_3, as has been repeatedly suggested [25,26]. Native energy conservation in the third region of the respiratory chain must be dependent on a component before the entry of electrons from reduced TMPD after the antimycin block, and this could very well be ubiquinone in complex III, as has been suggested recently again by Mitchell in a detailed scheme [27]. According to him the third region of energy conservation comprises proton translocation by ubiquinone and electron translocation in opposite direction by cytochrome oxidase. According to our concept artificial energy conservation in the third region is possible if native proton translocation by ubiquinone is replaced by artificial translocation with a H-carrying artificial redox compound like DAD in the case of submitochondrial vesicles, or by artificial proton liberation by ascorbate/DAD, as well as by ascorbate/TMPD at the external surface in the case of mitochondria.

Our result might also be explained by the assumption that a local proton potential, not a transmembrane potential, is the energy transmitter in respiration [28]. This local potential could be dependent on protons from DAD, when antimycin is present. However, we then find it difficult to explain, why coupling with TMPD should depend on the polarity of the membrane. If protons from DAD are required, no coupling with TMPD also in mitochondria should occur. Alternatively, if the protons are provided by action of cytochrome oxidase, coupling with TMPD should be found in both systems.

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