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MINI-REVIEW

The Proton-Translocating Nicotinamide Adenine Dinucleotide Transhydrogenase[1]

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Abstract

H^+-transhydrogenase couples the reversible transfer of hydride ion equivalents between NAD(H) and NADP(H) to the translocation of protons across a membrane. There are separate sites on the enzyme for the binding of NAD(H) and of NADP(H). There are some indications of the position of the binding sites in the primary sequence of the enzymes from mitochondria and *Escherichia coli*. Transfer of hydride ion equivalents only proceeds when a reduced and an oxidized nucleotide are simultaneously bound to the enzyme. When $\Delta p = 0$ the rate of interconversion of the ternary complexes of enzyme and nucleotide substrates is probably limiting. An increase in Δp accelerates the rate of interconversion in the direction of NADH $\rightarrow$ $NADP^+$ until another kinetic component, possibly product release, becomes limiting. The available data are consistent with either direct or indirect mechanisms of energy coupling.

Key words: Transhydrogenase; protonmotive force; proton translocation; energy coupling.

Introduction

H^+-nicotinamide nucleotide transhydrogenase is found in the inner membranes of animal (Danielson and Ernster, 1963) and plant (Carlenor *et al.*, 1988) mitochondria and in the cytoplasmic membranes of many bacteria (see Fisher and Earle, 1982). It catalyzes the reversible transfer of a hydride

[1] Abbreviations: DCCD, N N^1-dicyclohexylcarbodiimide; FSBA, 5^1-[*p*-(fluorosulfonyl)benzoyl] adenosine; FCCP, carbonylcyanide-*p*-fluoromethoxyphenylhydrazone; H^+-Thase, H^+-transhydrogenase; thio-$NADP^+$, thionicotinamide adenine dinucleotide phosphate; $AcPdAd^+$, 3-acetylpyridine adenine dinucleotide; Δp, proton electrochemical gradient; $\Delta\psi$, membane potential; ΔpH, pH difference across the membrane.

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ion equivalent between NAD(H) and NADP(H) and is coupled to the protonmotive force. The reaction can be summarized as

$$NADH + NADP^+ + nH_o^+ \rightleftharpoons NAD^+ + NADPH + nH_i^+ \quad (1)$$

where H_o^+ and H_i^+ signify the involvement of protons in the animal or plant cell cytoplasm and the mitochondrial matrix, respectively (or correspondingly, the aqueous phases external and internal to the bacterial cytoplasmic membrane).

Though the distribution of the enzyme is widespread and the activity in membranes is often high, there has been some interesting controversy over the function of H^+-Thase. It is generally thought that in bacteria the role of the enzyme is to generate NADPH for amino acid biosynthesis from NADH produced during catabolism (Bragg *et al.*, 1972), but for a more complex view, see Gerolimatos and Hanson (1978). The issue is not simple since other pathways for NADPH formation are also involved (see discussions in Hanson and Rose, 1980; Liang and Houghton, 1981). In eukaryotic cells the compartmentation of metabolism and of the nicotinamide nucleotide pools between the cytoplasm and the mitochondrial matrix may have permitted even more flexibility in function for H^+-Thase. Hoek and Rydstrom (1988) have summarized the NADPH-requiring reactions of detoxification and of intermediary metabolism in animal cells and have developed the thesis that the function of the enzyme in mitochondria is also mainly to generate NADPH. However, they proposed that the precise requirement for the NADPH is tissue-specific and that H^+-Thase is particularly important under conditions of oxidative stress. It is unlikely that H^+-Thase can operate as a powerful generator of Δp under physiological conditions [i.e., through the reversal of Eq. (1)], although even a very low protonmotive activity of the enzyme during conditions of anoxia could serve an important maintenance function (Rydstrom and Hoek, 1988).

The purpose of this review is to discuss plausible models for the protonmotive activity of H^+-Thase. The protein has been characterized in less detail than some of the other protonmotive enzymes of respiratory and photosynthetic membranes and yet it possesses features which make it very worthy of attention. First, H^+-Thase is structurally simpler than most other redox complexes and the H^+-ATPase. Second, the hydride transfer reaction can be measured in real time with good resolution, particularly with analogue substrates having modified optical properties; thio-$NADP^+$, an analogue of $NADP^+$, and acetylpyridine-AD^+, an analogue of NAD^+, are particularly useful. Third, there are no scalar protons associated with the reaction. Fourth, when $\Delta p = 0$, then the equilibrium constant of the reaction is close to unity and thus, with the membrane-bound enzyme and with the solubilized enzyme, it is a simple matter to drive the reaction in either direction.

It will be discussed below that, although the evidence is not unequivocal, the consensus view is that H^- transfer between the nucleotide substrates on H^+-Thase is direct and does not involve other redox centres. In this case the mechanism of the enzyme is unique in bioenergetics. Even so, it is not unreasonable to expect some unifying features between the protonmotive mechanisms of H^+-Thase and enzymes of the respiratory and photosynthetic electron transport chains and the ATP synthase. It will also be considered that, in formulating possible mechanisms of proton translocation by H^+-Thase, it may be of value (i) to make comparisons with soluble dehydrogenases, some of which have, of course, been characterized in great detail and (ii) to consider the solution chemistry of the nicotinamide nucleotides, especially those reactions which involve protons.

Earlier comprehensive reviews of the structure and properties of H^+-Thase can be found (Rydstrom, 1977; Rydstrom *et al.*, 1987; Fisher and Earle, 1982). Throughout this article the term H^+-transhydrogenase (H^+-Thase) refers specifically to the membrane-bound, "AB-specific," proton translocating enzyme found in mitochondria and bacteria. It is sometimes a matter of confusion in the older literature that the same enzyme was designated either "energy-linked" or "nonenergy-linked," depending on the level of energization of the membrane. It may be noted that there is another nicotinamide nucleotide transhydrogenase found in some bacteria which is a "BB-specific," flavin-containing, soluble protein and which is structurally unrelated to H^+-Thase (Rydstrom *et al.*, 1987).

Structure of the H^+-Thase

The amino acid sequence of H^+-Thase from *Escherichia coil* has been predicted from the nucleotide sequence of the gene (Clarke *et al.*, 1986). The sequence of the bovine mitochondrial enzyme was predicted from a cDNA clone (Yamaguchi *et al.*, 1988). The *E. coli* H^+-Thase is composed of two polypeptides, M_r 53906 (alpha) and 48667 (beta), the mitochondrial enzyme of one, M_r 109212. When the two polypeptides of the *E. coli* H^+-Thase are arranged contiguously (but including an extra 32 residues where the alpha polypeptide ends and the beta polypeptide begins) then there is approximately 50% sequence identity with the bovine enzyme (Yamaguchi *et al.*, 1988). The bovine H^+-Thase also has 13 N-terminal and 6 C-terminal residues in excess of the *E. coli* enzyme and there is an apparent frame shift affecting the sequence homology of 20 amino acids close to the middle of the alpha polypeptide.

The amino acid sequences of the H^+-Thase of *E. Coli* and bovine mitochondria reveal the membrane protein character of these enzymes. Each

protein can be seen to comprise three large domains. Domain I, approximately 400 residues at the N-terminus of the mitochondrial polypeptide (or the alpha polypeptide of the *E. coli* protein), is predominantly hydrophilic except for a central hydrophobic region which probably includes a nucleotide binding site (Yamaguchi *et al.*, 1988; Clarke *et al.*, 1986; and see below). Domain II, the central domain of approximately 400 residues, is strongly hydrophobic. Domain III, approximately 200 residues at the C-terminus of the mitochondrial polypeptide (or the beta polypeptide of the *E. coli* protein), is also relatively hydrophilic and may contain a second nucleotide binding site (Wakabayashi and Hatefi, 1987a; Yamaguchi *et al.*, 1988; Wakabayashi and Hatefi, 1987b; and see below). Since the nucleotide binding sites are probably exposed on the matrix side of the mitochondrial membrane (or on the cytoplasmic side of the bacterial membrane), then, in keeping with their hydrophilic character, Domains I and III are expected to protrude on that side. Hydropathy plots (Yamaguchi *et al.*, 1988; Clarke *et al.*, 1986) indicate the existence of a cluster of transmembrane helical segments in Domain II. Fourteen were suggested for the mitochondrial H^+-Thase and eleven for the *E. coli* enzyme (four associated with the alpha subunit and seven with the beta subunit).

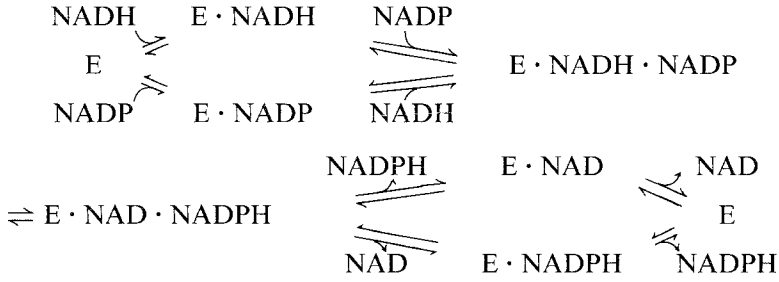
The H^+-Thases from bovine mitochondria and *E. coli* are the only ones with available sequence information and, until recently, were the only ones to have been solubilized and purified (Wu *et al.*, 1986; Persson *et al.*, 1984; Phelps and Hatefi, 1984; Clarke and Bragg, 1985). However, the enzyme from the photosynthetic bacterium, *Rhodobacter capsulatus*, has now been shown to have a subunit structure resembling that of *E. coli* (Lever *et al.*, 1990). H^+-Thase from another photosynthetic bacterium, *Rhodospirillum rubrum*, differs from the other enzymes that have been studied. It seems to be composed of both a membrane-bound and a soluble component (Fisher and Guillory, 1971; Fisher *et al.*, 1975). The soluble component can be removed by washing by centrifugation under very mild conditions. Neither the washed membranes nor the soluble protein factor have significant transhydrogenase activity, but activity is reconstituted upon mixing the two components. There is evidence that both components have nucleotide binding sites. The structural relationship, if any, between the *Rhs. rubrum* H^+-Thase and the enzymes from mitochondria, *E. coli* and *Rb. capsulatus* has yet to be established but the evidence, to date, suggests clear functional and mechanistic similarities (see the review by Fisher and Earle, 1982).

In native and reconstituted membranes (and in the solubilized enzyme at low concentrations of detergent) there is evidence from cross-linking studies (Anderson and Fisher, 1981; Wu and Fisher, 1983) and from radiation inactivation experiments (Perrson *et al.*, 1987) to suggest that the mitochondrial H^+-Thase exists as a dimer. At high concentrations of detergent the enzyme exists as inactive monomers (Persson *et al.*, 1987). Similar recent experiments

on H^+-Thase from *E. coli* have revealed that this enzyme too is effectively dimeric: it exists in cytoplasmic membrane vesicles and in its solubilized form as an $alpha_2$-$beta_2$ structure (Hou *et al.*, 1990).

Nucleotide Binding to H^+-Thase

Comprehensive descriptions of the steady-state kinetics of membrane-bound and of solubilized preparations of H^+-Thase from mitochondria, *E. coli* and *Rb. capsulatus* have been reported (Hanson, 1979; Enander and Rydstrom, 1982; Homyk and Bragg, 1979; Lever *et al.*, 1990). Following the fundamental analysis of Hanson (1979), there has been a clear measure of agreement on the interpretation of the data. Primary plots of the dependence of the rate of reaction upon the nucleotide concentration indicated that the reaction proceeds by a random addition of substrates to give a ternary complex:



The absence of any obvious curvature on the primary plots was taken as evidence that the interconversion of the ternary complexes was so slow that the substrate addition reactions reach equilibrium. Importantly, the data were inconsistent with a substituted enzyme (ping-pong) mechanism, a view which was supported by product inhibition experiments. These experiments showed, in addition, that "dead-end" ternary complexes could form, in which nucleotides on the enzyme were either both oxidized or both reduced. The possibility that H^+-Thase operates by way of the comparatively rare Theorell–Chance mechanism, in which the addition of substrates is ordered and the formation of the ternary complex is promptly followed by the dissociation of product, was ruled out by work with inhibitors (Hanson, 1979; Enander and Rydstrom, 1982; Homyk and Bragg, 1979; Lever *et al.*, 1990). Experiments have generally been carried out with H^+-Thase operating in the reverse direction, using $AcPdAD^+$ as an NAD^+ analogue and NADPH. It transpired that inhibitors such as 5^1AMP were competitive with respect to $AcPdAD^+$ and displayed mixed (formerly, "non competitive")

inhibition (see Cornish-Bowden, 1979) towards NADH, whereas inhibitors such as 2^1AMP were competitive relative to NADPH and mixed relative to $AcPdAD^+$.

Thus, the conclusion that emerges from the experiments to date on the kinetics of H^+-Thase in steady state is that the enzyme has separate binding sites for NAD(H) and for NADP(H). The site which preferentially binds NAD^+ and NADH can also bind $AcPdAD^+$ as a substrate and 5^1AMP as a competitive inhibitor. The site which preferentially binds $NADP^+$ and NADPH can also bind thio-$NADP^+$ as a substrate and 2^1AMP as a competitive inhibitor. The nucleotides can bind in any order and binding is fast relative to the subsequent turnover of the ternary complex. There is some evidence (Homyk and Bragg, 1979; Houghton *et al.*, 1976) that high concentration of nucleotide can lead to binding at the "wrong" site.

The idea of separate nucleotide binding sites on H^+-Thase fits comfortably with the observation that H^- transfer is from the *A* position (at C-4 of the dihydronicotinamide ring) of NADH to the *B* position in NADPH (Kawasaki *et al.*, 1964; Griffiths and Robertson, 1966; Lee *et al.*, 1965). Observations based on the crystal structures of a number of soluble dehydrogenases have led to the suggestion that binding of nucleotide in the *anti* configuration of the nicotinamide ring relative to the ribose tends to direct the H^- transfer to or from the *A* (or *pro-R*) position, whereas *syn* binding promotes transfer at the *B* (or *pro-S*) position (see Westheimer, 1987). Thus, it has been suggested that NADH might bind to H^+-Thase with an *anti* configuration and NADPH with a *syn* configuration (Kozlov, 1981; You *et al.*, 1978).

The prediction of the primary sequence of H^+-Thase from *E. coli* led to the identification of a segment of 20 amino acids in the α-subunit with strong homology to the FAD- and NAD(P)-binding folds of lipoyl dehydrogenase, glutathione, and mercuric reductases (Clarke *et al.*, 1986). It was proposed that this segment (which has the character of a $\beta\alpha\beta$ fold) may contain the binding site for one of the nucleotide substrates. Later, a strongly homologous structure was identified at an equivalent position in the predicted sequence of H^+-Thase from beef-heart mitochondria (Yamaguchi *et al.*, 1988). Using the convention adopted (Wierenga *et al.*, 1986), the fold begins with Lys-166 and Lys-186 in the *E. coli* and mitochondrial enzymes, respectively.

It had been shown previously that inhibition of the mitochondrial enzyme by DCCD was prevented by NAD(H) and 5^1AMP but not by NADP(H) and that treatment with this reagent prevented NAD^+ binding (Phelps and Hatefi, 1984). After incubation of mitochondrial H^+-Thase with radioactive DCCD and subsequent analysis of proteolytic fragments, a labelled peptide was identified whose sequence matched a region about 35–45

residues downstram from the $\beta\alpha\beta$ fold (Wakabayashi and Hatefi, 1987). Thus, these segments have been implicated in the NAD(H)-binding site. In support of this conclusion, modification of mitochondrial H^+-Thase by FSBA (which is also prevented by NAD(H) and by NAD^+ analogues) was found to occur at Tyr-245, just upstream from the Glu-257 which reacts with DCCD (Phelps and Hatefi, 1985; Wakabayashi and Hatefi, 1987b).

In the presence of NADH, FSBA was found to react more slowly with a second site on H^+-Thase from beef-heart mitochondria, close to the C-terminus of the protein at Tyr-1006 (Wakabayashi and Hatefi, 1987b). The region upstream of this residue shows especially close homology with the *E. coli* enzyme and was suggested to be a part of the NADP(H)-binding site.

Somewhat in contradiction with these conclusions, it has been found (Hu *et al.*, 1988) that 8-azido-AMP which, because of its 5^1 phosphate group is expected to be an NAD(H) analogue, binds at Tyr-1006 in mitochondrial H^+-Thase, i.e., in the putative NADP(H) site. Confusingly, *either* NAD(H) *or* NADP(H) protected against inhibition of enzyme activity by 8-azido-AMP. Experiments with the recently synthesized 8-azido-NADPH, an NADP(H) analogue, could help to resolve this dilemma (Hartog and Berden, 1990).

Although the putative binding sites for NAD(H) and NADP(H) are separated by several hundred residues in the primary sequence, it is entirely possible that in the protein they are in close proximity. It is interesting that the 7-nitrobenzofurazan-4-yl derivative of dephospho-CoA is a competitive inhibitor with respect to both NAD^+ and NADPH with the same K_i value, indicating that one molecule of inhibitor binds simultaneously to both the NAD(H) and NADP(H) sites (Kozlov *et al.*, 1984). In support of this conclusion, it was shown that binding of NADP(H) near the C-terminus of the mitochondrial H^+-Thase increased susceptibility to proteolysis by trypsin between Lys-410 and Thr-411, some 600 residues upstream in the primary sequence (Yamaguchi *et al.*, 1990).

Despite the fact that experiments on the steady-state kinetics of H^+-Thase generally indicate that the reaction proceeds through the random addition of nucleotide substrates, there have been some suggestions of a substituted enzyme mechanism. However, the observations leading to these suggestions can now be better explained. Thus, the finding that, in the presence of NADPH, NADH is able to reduce $AcPdAD^+$ in coupled, but not in uncoupled proteoliposomes, was first taken as evidence for the existence of a reduced enzyme intermediate (Wu *et al.*, 1981). In later publications (Fisher and Earle, 1982; Rydstrom *et al.*, 1981; Enander and Rydstrom, 1982) it was argued that the reaction more likely represents the simultaneous (Δp-generating) reduction of $AcPdAD^+$ by NADPH and the (Δp-consuming) reduction of $NADP^+$ by NADH. Chromatophores from *Rhs. rubrum* also catalyze the reduction of $AcPdAD^+$ by NADH, even in the absence of

NADP(H) (Fisher and Guillory, 1971; Jacobs and Fisher, 1979). Since this reaction was inhibited after removal of the soluble H^+-Thase component by washing the membranes and subsequently restored by addition of partially purified soluble component, it was suggested that it must arise from the H^+-Thase and that it indicates participation of a reduced intermediate during catalysis. However, there are some characteristics of the reaction which suggest that it is associated with an impurity in the fraction containing the soluble component (Lever *et al.*, 1990). Furthermore in chromatophores of *Rb. capsulatus*, which also catalyse NADP(H)-independent reduction of $AcPdAD^+$ by NADH, the reaction purifies *away* from H^+-Thase activity (Lever *et al.*, 1990).

Bioenergetics of H^+-Thase

In mitochondrial and bacterial membranes *both* the rate of the H^+-Thase reaction from left to right [Eq. (1)] *and* the mass action ratio of $[NADPH][NAD^+]/[NADP^+][NADH]$ are increased by generators of Δp such as the respiratory of photosynthetic electron transport systems or by ATP hydrolysis by the F_1-ATPase (Keister and Yike, 1966; Lee and Ernster, 1964; Danielson and Ernster, 1963) (and see Fisher and Earle, 1982). Alternatively, upon provision of NADPH and NAD^+, the H^+-Thase reaction can be made to proceed from right to left and generate Δp (Dontsov *et al.*, 1972; Oustroumov *et al.*, 1973).

In one report (Kay and Bragg, 1975) it was observed that in vesicles prepared from a mutant of *Salmonella typhimurium*, the transhydrogenase reaction could be driven by respiratory electron flow but not by ATP hydrolysis. This was despite the fact that ATP hydrolysis was coupled to the transport of amino acids. Those observations were considered to be irreconcilable with the chemiosmotic hypothesis. However, the possibility was not excluded that the vesicles contained a population which possessed transhydrogenase but no ATPase and a population which possessed both ATPase and amino acid transporter.

Solubilized and purified H^+-Thases from mitochondria (Rydstrom, 1979; Earle *et al.*, 1978) and *E. coli* (Clarke and Bragg, 1985) have been reconstituted into liposomes with recovery of electrogenic activity during H^- transfer from NADPH to NAD^+ (or its analogues) as demonstrated (i) by the uptake of H^+ from the medium (Wu *et al.*, 1986; Earle and Fisher, 1980), (ii) by the redistribution of lipid-soluble anions, indicating generation of $\Delta\psi$ (Rydstrom, 1979) and (iii) by the fluorescence change of 9-aminoacridine and related compounds, indicating the formation of ΔpH (Earle *et al.*, 1978; Earle and Fisher, 1980; Rydstrom, 1979). The rate of H^- transfer, either from

NADH to $NADP^+$ or from NADPH to NAD^+, in H^+-Thase proteoliposomes was stimulated upon addition of uncoupling agents, showing that the reactions are controlled by the buildup of Δp (Earle *et al.*, 1978). Experiments with combinations of K^+-ionophores (Fisher and Earle, 1980) revealed contributions from both $\Delta\psi$ and ΔpH in this apparently thermodynamic control of H^+-Thase. Co-reconstitution of H^+-Thase with either bacteriorhodospin or ATP synthase led to light-induced or ATP-induced H^+-Thase activity, respectively, apparently through the intermediate formation of Δp (Eytan *et al.*, 1987a, b, 1990).

The simple fact of Δp-driven (and Δp-controlled) reactions in reconstituted systems does not prove the physiological reality of the chemiosmotic hypothesis. Some authors have suggested that in natural membranes, under some circumstances, there may be a direct pathway for protons from the primary pumps (usually electron transport reactions) to the secondary pumps (such as H^+-ATP synthase and H^+-Thase), e.g., via membrane proteins, in addition to that through the bulk aqueous phases. In this view, delocalized or bulk-phase coupling operates in parallel with direct or localized coupling (Chiang and Dilley, 1987; Kell and Westerhoff, 1985). Two independent tests, the results of which have been taken to indicate localized interactions in electron transport-driven ATP synthesis, have only revealed *de*localized interactions in electron transport-driven transhydrogenase (Cotton *et al.*, 1987; Persson *et al.*, 1987). Whatever the nature of the localized interactions between the ATP synthase and the electron transport complexes, there is no similar concern for H^+-Thase: present evidence supports the assumption that H^+-Thase is driven only by Δp.

The H^+/H^- Ratio of H^+-Thase

A knowledge of the ratio of H^+ translocated across the coupling membrane of the H^- equivalents transfered between the nucleotide substrate by H^+-Thase is essential to our understanding of the mechanism of the enzyme. Although a range of values has been recorded, a consensus is emerging that the ratio is probably one.

In the first attempts to measure the H^+/H^- ratio, intact mitochondria were supplied with acetoacetate and isocitrate to provide matrix NAD^+ and NADPH, respectively, and the rate of H^+ efflux was measured with a glass electrode (Moyle and Mitchell, 1973). A ratio of 1.94 ± 0.12 was determined. However, the conclusion that matrix isocitrate dehydrogenase is specific for NADP(H) was later disputed (Hoek *et al.*, 1973; Smith and Plaut, 1979) and on the basis the H^+/H^- ratio determined by this method has been repeatedly challenged.

The most detailed attempts to measure the H^+/H^- ratio have been carried out by Fisher and colleagues using mitochondrial H^+-Thase reconstituted into liposomes. Proton uptake and H^- transfer following addition of NADPH and either $AcPdAD^+$ (Earle and Fisher, 1980) or NAD^+ itself (Fisher and Earler, 1982) were measured directly. Values of H^+/H^- in the range 0.35–0.9 were determined (Wu *et al.*, 1986). It was argued that the measured values indicate a real stoichiometry of one. It should be appreciated that in these estimates, the rate of H^+ uptake was recorded over a period of about 10 s and it was difficult to establish that backflow of protons was negligible during this time. In an attempt to compensate for this, the "true" H^+/H^- ratio was determined by extrapolation to zero time (Earle and Fisher, 1980; Wu *et al.*, 1986). However, it has to be said that the method of extrapolation was arbitrary. Even within about 5–10 s of substrate addition there was a pronounced curvature on the glass electrode trace (which probably indicates increasing rates of H^+ efflux) and the measured value, on this basis, should be considered to be a lower limit. Of course the proton uptake measurements were made in the presence of valinomycin to collapse the electrical component of the protonmotive force, but the H^- transfer rate used to calculate the H^+/H^- ratio was taken as the difference between the rates recorded in the presence and absence of ionophore. The justification for this was that the low rate of H^- transfer in the absence of valinomycin was thought to be due to H^+-Thase that was not functionally incorporated into liposomes (Wu *et al.*, 1986). However, it might also arise from the finite proton permeability of the vesicles, in which case the measured ratios would be overestimated. Further analysis of this interesting system, using more rapidly responding instrumentation, is required.

A new procedure to measure the H^+/H^- ratio in chromatophores from *Rb. capsulatus* was recently described (Cotton *et al.*, 1989). The rate of H^- transfer from NADH to thio-$NADP^+$ during photosynthetic illumination was measured spectrophotometrically and the accompanying rate of charge translocation across the chromatophore membrane was determined from the electrochromic absorbance changes of endogenous carotenoid pigments (Fig. 1). The H^+/H^- estimated in this way was 0.4 ± 0.5. The rather large error arose from the fact that the H^+-Thase-dependent proton current was only a small fraction of the total membrane ionic current. The major advantage of this procedure is that total charge fluxes are measured and thus the true ionic current accompanying H^- transfer is revealed. The disadvantages are (a) that the calibration of the electrochromic absorbance change into absolute units of charge is indirect and (b) that it underestimates the H^+/H^- ratio if the H^+-Thase "slips" protons in the absence of substrates (see below).

In principle the H^+/H^- can be estimated from equilibrium measurements of the value of Δp and the mass action ratio of the nucleotide substrates

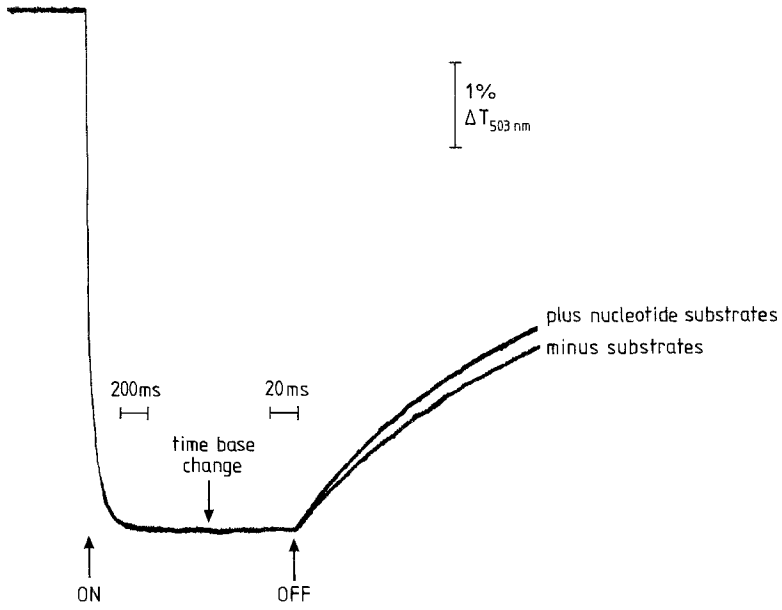


Fig. 1. The operation of the H^+-Thase increases the flow of ionic current across the membrane. Taken from Cotton *et al.* (1989). The two superimposed traces show the generation of $\Delta\psi$ during illumination of chromatophore membranes and its subsequent decay upon darkening in either the presence or absence of substrates of the H^+-Thase.

and products, although there has been only one systematic attempt to pursue this approach (Jackson *et al.*, 1990). Thus, illuminated chromatophores from *Rb. capsulatus* yielded an H^+/H^- ratio of 0.72. Though, in essence this is a simple procedure, technically there are a number of problems. First, equilibrium conditions must be confirmed by approaching the mass action ratio from both high substrate and high product concentrations [cf. Eq. (1)]. Then, at equilibrium, the substrate concentrations are very low and therefore subject to large error (although this problem is minimized to some extent by the fact that the H^+/H^- ratio depends on the logarithm of the mass action ratio). There also remains the possibility that the membrane preparation contains small fractions of either residual NADH dehydrogenase, which is uninhibited even by the high concentrations of rotenone used in the incubations, or "uncoupled" H^+-Thase, which is not properly inserted into a topologically closed membrane: each of these reactions, if kinetically significant, would lead to errors on the mass action ratio.

In summary, most recent determinations of the H^+/H^- ratio give values that are somewhat less than 1.0. On the basis that the measurements with the glass electrode and the equilibrium measurements could both be

underestimates and that there is a large error on the H^+-Thase-dependent membrane ionic current recorded by electrochromism, it is conceivable that the actual value is unity. Within the error of the ionic current measurements it is unlikely that the H^+/H^- ratio could be as high as 2.0. It is also significant that if the stoichiometry were 2.0, the mass action ratio of the nucleotides would be in excess of 10^6 for a $\Delta p > 180$ mV, whereas the measured value has never been quoted in excess of 10^3. Although precluded by a strictly chemiosmotic mechanism, it is not inconceivable that the H^+/H^- ratio of H^+-Thase is less than unity. In fact, the mean of the published H^+/H^- ratios is closer to 0.5 than to 1.0. This value could be consistent, for example, with some models of "conformational" coupling within a dimer of H^+-Thase (see Section 7.1).

There is still controversy as to whether respiratory chain complexes and ATP synthase from various sources can "slip," i.e., catalyze chemical reaction with diminished ratios of proton translocation or, conversely, conduct protons in the absence of chemical transformation. There is some evidence for slip in mitochondrial H^+-Thase although it is not compelling. The key observation was that treatment of H^+-Thase proteoliposomes with DCCD led to stronger inhibition of proton uptake [measured either with a glass electrode (Pennington and Fisher 1981) or by the quenching of 9-aminoacridine fluorescence (Persson *et al.*, 1984)] than of hydride transfer. It was implied that DCCD binds to an H^+-binding domain in the enzyme and causes decoupling from the H^- transfer reaction. It was appreciated (Pennington and Fisher, 1981) that the observation could also be explained by increased passive proton conductance due to the DCCD treatment although experiments to test this were not rigorous. Thus, the inhibition of proton uptake was monitored after a period of preincubation with DCCD, whereas increase of proton conductance was examined immediately after addition of the reagent and, even so, was only compared with a rather high concentration (3.6 μM) of the very efficient protonophore, FCCP. It is also significant that later work with DCCD (Phelps and Hatefi, 1984) suggested that this reagent does not inhibit H^+-Thase in the way that it inhibits other proton translocators, by interacting with acidic residues in the proton-conducting domain of the enzyme. Rather it reacts with a glutamate residue close to the NAD(H) binding site (Wakabayashi and Hatefi, 1987a). It was also shown that DCCD depressed the response of Oxonol VI (an indicator of $\Delta\psi$) in parallel with the inhibition of H^- transfer in proteoliposomes containing H^+-Thase (Phelps and Hatefi, 1984).

The finding that the H^+/H^- ratio, measured with a glass electrode, decreased on either side of neutral pH has also been taken to indicate slip in H^+-Thase (Persson, 1988). However, it is not clear that this interesting observation does not also result from pH-dependent changes in the passive

proton conductance of the liposomes. The general conclusion at present must be that, although slip in H^+-Thase cannot be ruled out, evidence for the quantitative significance of the process (relative to catalysis) is still required.

Kinetic Components of H^+-Thase: Interaction with Δp

Our understanding of the interaction of H^+-Thase with Δp is rudimentary. Only a little information is available on the level at which Δp is involved in catalytic turnover. When chromatophores, catalyzing the H^+-Thase reaction (slowly) in the dark, were subjected to a period of photosynthetic illumination, there was a burst ($t_{1/2}$ = 5 ms) of reaction before establishment of the steady-state rate (Palmer and Jackson, 1990a). The burst probably represented a single turnover of H^+-Thase. In accordance with conclusions from steady-state kinetics (see above), the observation was interpreted as evidence for the existence in the dark ($\Delta p = 0$) of a rate-limiting step following the rapid equilibrium addition of substrates to the enzyme. Upon illumination (and a sharp increase in the value of Δp), that rate-limiting step was accelerated by Δp until another component, possibly product release, became rate limiting:

$$\mathrm{E} + \mathrm{NADH} + \mathrm{NADP} \rightleftharpoons \mathrm{E} \cdot \mathrm{NADH} \cdot \mathrm{NADP} \xrightarrow{k(\Delta p)} \mathrm{E} \cdot \mathrm{NAD} \cdot \mathrm{NADPH}$$

$$\longrightarrow \mathrm{E} + \mathrm{NAD} + \mathrm{NADPH}$$

where $k(\Delta p)$ indicates that the rate constant is dependent on the value of Δp.

Another way to investigate the energy dependence of component reactions in H^+-Thase is to study the dependence of the steady-state rate of reaction on the values of Δp. This can be considered to be equivalent to studies on the dependence of the steady-state rate on the concentration of substrates. However, it has to be recognized that away from equilibrium, ΔpH and $\Delta\psi$ may not have equivalent effects on the rate of catalysis by a protonmotive enzyme (Hansen *et al.*, 1981; Lauger, 1984) but in fact, it is often easy experimentally to eliminate the contribution from ΔpH such that $\Delta\psi$ is the sole contributor to Δp.

Despite suggestions in early literature, it is unlikely that Δp serves simply to convert H^+-Thase from a relatively inactive to a relatively active conformation because this would not account for the effect of Δp on the equilibrium of the reaction. Moreover, in chromatophores there is no evidence for acceleration of the rate of reduction of $AcPdAD^+$ by NADH as a result of incremental increases in the value of $\Delta\psi$ (Palmer and Jackson, 1990b).

It is likely, therefore, that $\Delta\psi$ increases the rate of the H^+-Thase reaction by interacting with a component of the catalytic process which involves the

translocation of charge, either wholly or partly across the membrane. Translocation of charge might be coupled indirectly to chemical reaction by way of conformational changes in the protein or it may involve the participation of charged ligands directly in catalysis (see below). The charge translocation step might reach equilibrium with $\Delta\psi$, in which case the overall rate of reaction could be accelerated through an increase in concentration of an intermediate state of the enzyme. Alternatively, if the charge translocation step is itself rate-limiting in catalysis, then the effect of $\Delta\psi$ on the rate of charge translocation would directly increase the overall rate of the reaction.

If the translocated charge is a bound proton which is freely accessible only to one aqueous phase adjacent to the membrane, and if the translocation process is in equilibrium with $\Delta\psi$, then the structure is described as a "proton well" (Mitchell, 1969). The drop in the electrical potential (which depends on the depth of the well and on the local dielectric properties of the protein) results in an increased proton concentration in the well. It may be shown that

$$[H^+]_w = [H^+]_b \exp(hF\Delta\psi/RT)$$

where $[H^+]_w$ and $[H^+]_b$ are the proton concentration in the well and in the bulk phase, respectively, and h is the dielectric depth of the well (see Apell *et al.*, 1987). If a protolytic reaction involved in catalysis is sited in the well, and if this reaction is limiting in catalysis, then increase in $\Delta\psi$ would lead to an accelerated rate of reaction. This concept has also been discussed in terms of absolute rate theory (Lauger and Stark, 1970; Apelle *et al.*, 1987; Lauger, 1984). In this case, the well is described as a series of diffusion barriers that are of low enough energy to ensure that the bulk aqueous phase and the domain at the bottom of the well are at electrochemical equilibrium. The effect of a trans-membrane electric field across the series of energy barriers is to raise the probability of proton occupancy in the well. When there are no other $\Delta\psi$-dependent steps in catalysis, the proton-well hypothesis predicts that the relationship between the rate of reaction and $\Delta\psi$ (for $\Delta pH = 0$) is highly dependent on the bulk phase pH. However, for H^+-Thase this prediction is not matched by experiment, particularly in the region of neutral pH (Cotton *et al.*, 1989).

The idea that displacement of charge through a protein in a direction normal to the plane of the membrane by the membrane potential can be coupled kinetically to chemical reaction has been developed at length by Lauger and coworkers (Lauger and Stark, 1970; Apell *et al.*, 1987; Lauger, 1984; and see Hansen *et al.*, 1981). Absolute rate theory again provides a useful vehicle with which to describe the concept. Thus, the translocation of charge across the membrane proceeds over an energy barrier and the height of the barrier is affected by the electrostatic energy of the trans-membrane electric potential. It can be shown that the rate constant for charge

translocation down an electric potential gradient across a single symmetrical barrier is given by

$$k_1 = k_0 \exp(dF\Delta\psi/2RT)$$

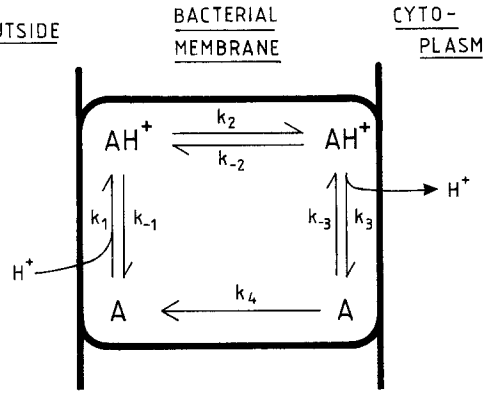
where k_1 and k_0 are the rate constants in the presence and absence, respectively, of membrane potential and d is the dielectric depth of the energy barrier. In its simplest form this model predicts that the relation between the rate of the reaction and $\Delta\psi$ should be independent of the bulk-phase pH. In order to fit experimental data for H^+-Thase to such a scheme, it was necessary arbitrarily to assume pH-dependent rate constants (Cotton *et al.*, 1989). A more satisfactory model is shown in Fig. 2, in which good agreement with the data is achieved by including a proton release step to account for the dependence of bulk-phase pH. To reach the best fit the value of d was set to 0.74. Interestingly, an even better fit to the data was obtained (not shown) when the H^+-binding reaction (k_1) was set in a proton well with $h = 0.26$. Thus, the reality of this model of H^+-Thase would be a protein with a proton well on the lumenal side of the chromatophore membrane and a slower charge translocation step extending across the remaining three-quarters of the membrane dielectric.

Models for Proton Translocation by H^+-Thase

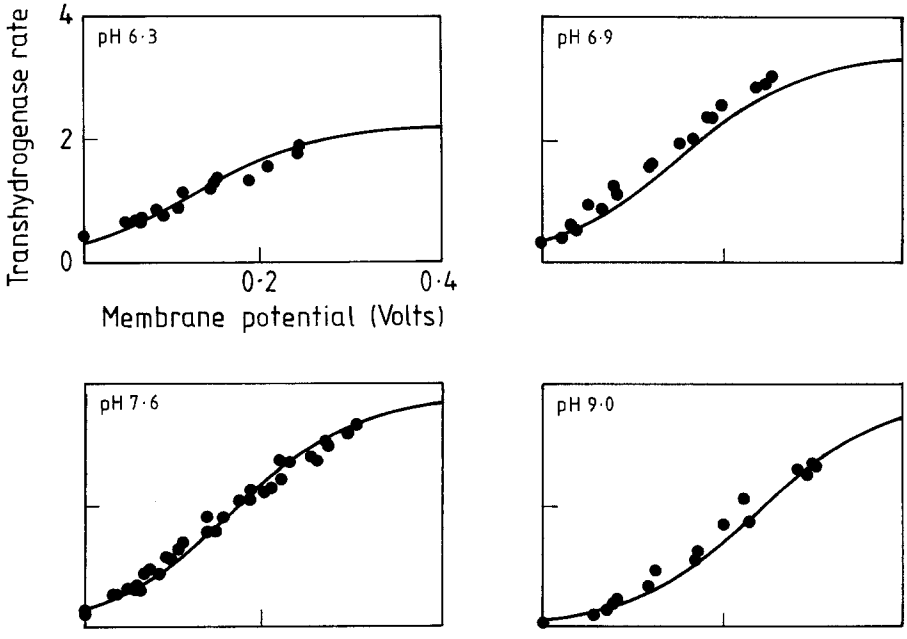
Conformational Coupling

Most of the research groups active in the field of H^+-Thase have adopted a variation of "conformational" or "indirect" coupling as a working model (Fisher and Earle, 1982; Rydstrom *et al.*, 1981; Yamaguchi and Hatefi, 1989). The seminal description of conformational coupling, as applied to H^+-Thase, arose from Skulachev (1974). The key element is that proton conduction through the enzyme is not directly involved in the chemistry of H^- transfer but is coupled to that reaction by way of a conformational change in the protein. It is supposed that a component in the process of proton translocation through the enzyme, across the membrane, leads to conformational strain which can relax and drive unfavorable H^- transfer between the nucleotides. Because the hypothesis remains at the "black box" stage, there are no difficulties in accommodating the findings that H^- transfer between the nucleotides is direct and takes place in the ternary complex. A version of the conformational coupling model is shown in Fig. 3. In principle, the free energy may be transmitted by the conformational change over large distances within the protein, or even between proteins, and thus, the translocation pathway may be remote from the catalytic centre.

(a)


OUTSIDE
BACTERIAL MEMBRANE
CYTO-PLASM
AH+
AH+
k2
k-2
H+
k1
k-1
k-3
k3
H+
A
k4
A

(b)


Transhydrogenase rate
4
2
0
pH 6·3
0·2
0·4
Membrane potential (Volts)
pH 6·9
pH 7·6
pH 9·0

There is good evidence (see above) that the H^+-Thases from mitochondria and *E. coli* function effectively as dimeric units. It may be that the two units of the dimer operate independently. However, there is an indication that the enzyme displays half-of-the sites reactivity with respect to inhibition DCCD (Phelps and Hatefi, 1984) and FSBA (Phelps and Hatefi, 1985). Thus, at any point on the inhibition curve the incorporation of reagent into the protein was only half of that expected on a mole per mole basis, which suggests that there is a cooperative interaction between the subunits. Since the FSBA and (uncharacteristically for this reagent) the DCCD seem to interfere with substrate binding in a hydrophilic part of the enzyme, it was suggested that this may indicate cooperative effects with respect to nucleotide binding. On this basis, the possibility was raised that the catalytic sites in the H^+-Thase dimer operate alternately and that conformational interactions between subunits are critical to the energy coupling mechanism, in a manner similar to that described for the H^+-ATPase (Boyer, 1988). In general, work on the kinetics of H^+-Thase in steady state does not support the concept of cooperative binding of substrates to the enzyme. In the reverse reaction [Eq. (1)], except at very high nucleotide concentrations, Michaelis–Menten behavior is observed (Houghton *et al.*, 1976; Homyk and Bragg, 1979). In the forward direction, there *was* evidence of cooperative behavior of H^+-Thase in *E. coli* membranes (Hanson, 1979) but not in mitochondria (Teixeira *et al.*, 1971; Rydstrom *et al.*, 1971).

Undoubtedly there are large conformational changes involved in the catalytic turnover of H^+-Thase. These are best documented in many types of chemical modification experiments in which nucleotide substrates either protect against, or enhance, the effect of proteolytic enzymes (Blazyk *et al.*, 1976; Fisher *et al.*, 1975; Jacobs *et al.*, 1977; Yamaguchi *et al.*, 1990), or inhibitors (Phelps and Hatefi, 1984; Phelps and Hafeti, 1981; Phelps and Hatefi, 1984; Wakabayashi and Hatefi, 1987a, b; Yamaguchi and Hatefi, 1985; Yamaguchi and Hatefi, 1989; Persson *et al.*, 1988; Persson and Rydstrom, 1987; Wu and Fisher, 1982; Pennington and Fisher, 1981; Modrak *et al.*, 1988; Earle *et al.*, 1978; O'Neal and Fisher, 1977; Jacobs and Fisher, 1979; McFadden and Fisher, 1978). Despite assertions to the contrary,

Fig. 2. A kinetic model to describe the dependence on bulk phase pH of the relation between the rate of H^+-Thase and $\Delta\psi$ in chromatophores. (a) Description of the model adapted from Cotton *et al.* (1989). It is intended to be general and not to imply either a direct or an indirect coupling mechanism. k_2 and k_{-2} are the rate constants for charge translocation. The charge need not be protonic charge. (b) The data are taken from Cotton *et al.* (1989). The relation for the dependence of k_2 and k_{-2} on $\Delta\psi$ (see text) was substituted into the steady-state rate equation. The solid lines show the best fit and were achieved (assuming 1 H^+-Thase per 100 bulk bacteriochlorophyll) with the following parameters: k_1, $6.0 \times 10^{10}\,M^{-1}\,s^{-1}$; k_{-1}, $120\,s^{-1}$; k_2^0 $(=k_{-2}^0)$, $0.36\,s^{-1}$; k_3, $3.8 \times 10^4\,s^{-1}$; k_{-3}, $6.0 \times 10^{10}\,M^{-1}\,s^{-1}$; k_4, $4.0\,s^{-1}$; d, 0.74. The superscript on k_2^0 indicates that the value applies when $\Delta\psi = 0$ (see text). (In collaboration with A. M. Bickerton.)

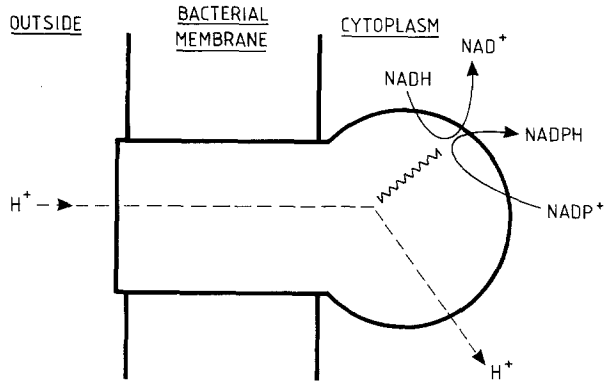


Fig. 3. Model to illustrate conformational coupling by H^+-Thase. The zig-zag line indicates the conformational interaction between proton translocation and H^- transfer. The pathway of the proton through the enzyme may be remote from the site of H^- transfer.

the existence of these conformational changes should not be taken as evidence favoring "conformational" or "indirect" coupling—conformational changes are expected also in strictly chemiosmotic coupling (see also Scarborough, 1985). However, it is interesting to speculate that they might be analogous to "loop closure" around the nucleotide-binding site of some soluble dehydrogenases which is responsible for excluding water from the catalytic centre and, by changing the local environment, may be important in altering the effective redox potential of the substrate—cf. the oil-charge repulsion effect postulated in lactate dehydrogenase (Parker and Holbrook, 1977). Surprisingly, in view of the emphasis given to the role of conformational changes in the coupling mechanism, there are few clear indications of conformational transitions in H^+-Thase accompanying changes in the level of Δp. Experiments to date (Anderson *et al.*, 1981; Blazyk and Fisher, 1975) unfortunately do not rule out the possibility that effects on the "energy-linked transhydrogenase" are, in fact, effects on the generators of Δp rather than H^+-Thase itself. Even where such conformational changes can be attributed directly to the H^+-Thase, it will be difficult to establish whether they are directly involved in the process of energy transduction.

An influential suggestion in the development of the concept of conformational coupling between H^+ translocation and chemical reaction was that, in catalysis, the enzyme makes use of changes in ligand-binding energy derived from the energy of the protonmotive force; see, e.g., Hill (1977) and Jenks (1980). In H^+-Thase it can be supposed that proton translocation through the enzyme promotes either the binding of NADH and $NADP^+$, or the release of NAD^+ and NADPH, or both. The K_m^{app} values for nucleotide substrates in the forward direction of H^+-Thase

from mitochondria (Galante *et al.*, 1980; Rydstrom, 1977) and from bacterial chromatophores (Cotton *et al.*, 1989) decreased when the membranes were energized. In mitochondria the K_m^{app} values for the nucleotide substrates in the reverse direction increased during energization (Galante *et al.*, 1980; Rydstrom, 1977). These observations were taken as evidence for an increased affinity of the enzyme for the substrates and a decreased affinity for the products upon imposition of Δp and was used in support of the view that conformational changes mediate the energy transduction process (Galante *et al.*, 1980; Rydstrom, 1977; Fisher and Earle, 1982). However, it is comparatively rare that K_m^{app} values provide information about the substrate affinity (see Cornish-Bowden, 1979), and the situation with an enzyme in which Δp effectively operates as a third substrate is particularly complex since terms in Δp will enter the expression for K_m^{app}. The conclusion (Galante *et al.*, 1980; Rydstrom, 1977; Fisher and Earle, 1982) should be treated with caution. It should also be noted that Δp-dependent changes in the apparent binding affinity are not at all incompatible with direct mechanisms of coupling (Mitchell, 1987).

Chemiosmotic Coupling

An attractive feature of the chemiosmotic hypothesis is that it attempts to explain how proton (or hydroxide ion) translocation across biological membranes is directly coupled to protolytic reactions involved in chemical catalysis. However, of the three original proposals of Mitchell to explain energy coupling in H^+-Thase (Mitchell, 1966), two can now be ruled out. First, the "type II loop" not only requires an H^+/H^- ratio of 2 (compare Section 5) but, in contradiction with established experimental evidence (Lee *et al.*, 1965), it predicts that there should be isotopic exchange between the 4A H atom of the nicotinamide ring of NADH and water. Second, it now seems unlikely that transhydrogenase is coupled to Δp by way of a metallic cation exchange since (a) the purified enzyme behaves as a proton translocator (Earle *et al.*, 1978; Earle and Fisher, 1980; Rydstrom, 1979; Wu *et al.*, 1986; Clarke and Bragg, 1985) and (b) transhydrogenase, in membranes and in purified state, does not have a specific metal ion requirement (T. Lever, N. P. J. Cotton, and J. B. Jackson, unpublished).

With only a minor modification, the third of Mitchell's original proposals (a "type I loop") remains a viable model (Fig. 4). In this scheme the oxidation of NADH on one side is coupled to the translocation of a hydride ion equivalent across the membrane. The uptake of H^+ on the other side is accompanied by the translocation of 2 H atom equivalents back across the membrane and the subsequent reduction of $NADP^+$. The model must be constrained to ensure that H atoms from the NADH are transferred to the

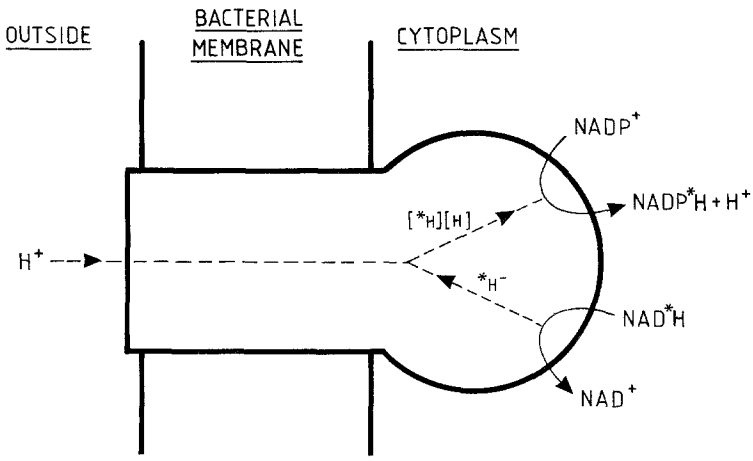


Fig. 4. A "type I loop" to explain the mechanism of energy coupling in H^+-Thase, modified from Mitchell (1966). The starred symbols show the pathway for the transfer of H^- and H equivalents between the nucleotide substrates. Protons from the external phase cannot equilibrate with the H^-- and 2[H]-translocating pathways unless both substrates are bound to the enzyme.

$NADP^+$ without coming into equilibrium with the water (see Fig. 4). The H^+/H^- ratio is unity. There are some features which are not immediately obvious from the original description (Mitchell, 1966) and this may explain why this model has received little attention. First, the $[H^-]$ and [2H] translocations do not need to span the entire membrane, for example, as depicted (Fisher and Earle, 1982). In fact, the movement of the $[H^-]$ and [2H] need traverse only a part of the membrane dielectric; some of the electrogenic reaction could be associated with H^+ translocation. In a limiting case H^+ translocation could comprise the entire electrogenic reaction. The model formulated in Mitchell (1966) does not define the chemical nature of the $[H^-]$-carrying and [2H]-carrying arms of the loop but they *need not* be electron carriers and nucleotide substrates, respectively, as supposed (Fisher and Earle, 1982; Rydstrom, 1977). Although it is established that purified H^+-Thase has no identifiable strong chromophores in the visible and near u.v., there is an unfortunate lack of information on the existence, or non-existence, of prosthetic groups in the enzyme—work is needed in this area. However, the $[H^-]$- and [2H]-carrying arms could be either relatively simple prosthetic groups, such as a bound pyruvate and lactate (compare the enzymes histidine decarboxylase and proline reductase) or an imine or even amino acid residues, such as cysteine, in the protein itself.

Independently, Skulachev (1970) and Mitchell (1972) suggested mechanisms by which the nucleotide substrates themselves could serve to translocate

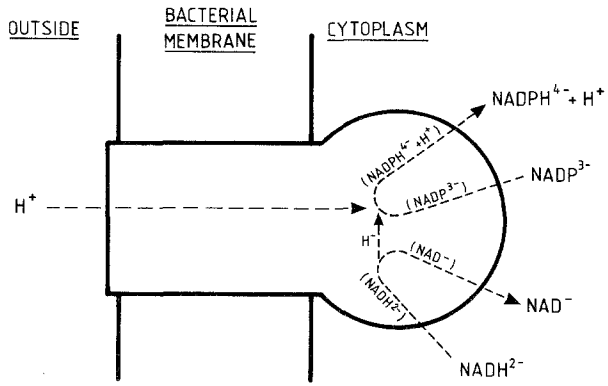


Fig. 5. A group translocation model to explain the mechanism of energy coupling in H^+-Thase, modified from Mitchell (1972). The nucleotides in the cytoplasm are shown in their actual ionization states at pH 8.0. The brackets enclose the species that are translocated through the membrane barrier. Alternatively the barrier could be considered to move across the substrate binding site (Mitchell, 1987).

charge during chemiosmotic coupling of H^+ translocation to H^- transfer in transhydrogenase. Both authors drew parallels with a possible coupling mechanism for H^+-ATPase. It was proposed (Skulachev, 1970; Mitchell, 1972) that nucleotides, but only in certain ionization states, can be conducted through the protein, to and from the active center at which H^- transfer takes place. The appropriate ionization states might be achieved, it was argued, through protonation of the nucleotide phosphate groups. Kozlov (1981) proposed an ingenious variation on this mechanism in which it was argued that reduced nucleotides, but because of the quaternary N atoms of the nicotinamide rings, not oxidized nucleotides, can serve to conduct protons to and from the catalytic center across the membrane dielectric. A modification of Mitchell's original model, which takes into account the more likely H^+/H^- ratio of 1.0, is shown in Fig. 5. As with other chemiosmotic models, group translocation mechanisms have been neglected in favor of indirect coupling and again, there has been some misrepresentation of the original concept. Thus, despite objections raised (Fisher and Earle, 1982; Rydstrom, 1977), the nucleotides do not need fully to traverse the membrane, and the membrane does not become permeable to the nucleotides. They are conducted through the protein, a part of the way across the membrane dielectric. As described above, electrogenic H^+ conduction from the phase at relatively positive protonic potential to the catalytic centre could also contribute to the energetics of the reaction. It is worth considering that it is not necessary that the bulky and hydrophilic nucleotide should have to slide through the osmotic barrier. It is conceivable that the barrier region in the protein switches or rolls across the bound nucleotide (Mitchell, 1987). Some of the protein conformational

changes which are contingent upon nucleotide binding to H^+-Thase (references cited above) could indeed reflect these events.

Realistic Models of Direct Coupling

A special case of a chemiosmotic model for H^+-Thase in which H^+ transfer through the protein is the sole electrogenic reaction is shown in Fig. 6. For illustrative purposes, the model is shown with a disulfide bond as the intermediate acceptor [cf. evidence for involvement of vicinal disulfides in the H^+-Thase reaction (Persson and Rydstrom, 1987; Wu and Fisher 1982)], but a keto group or an imine would serve equally well. It is interesting that many soluble dehydrogenases possess relay systems to transfer the protons needed for substrate reduction from the aqueous medium to the catalytic

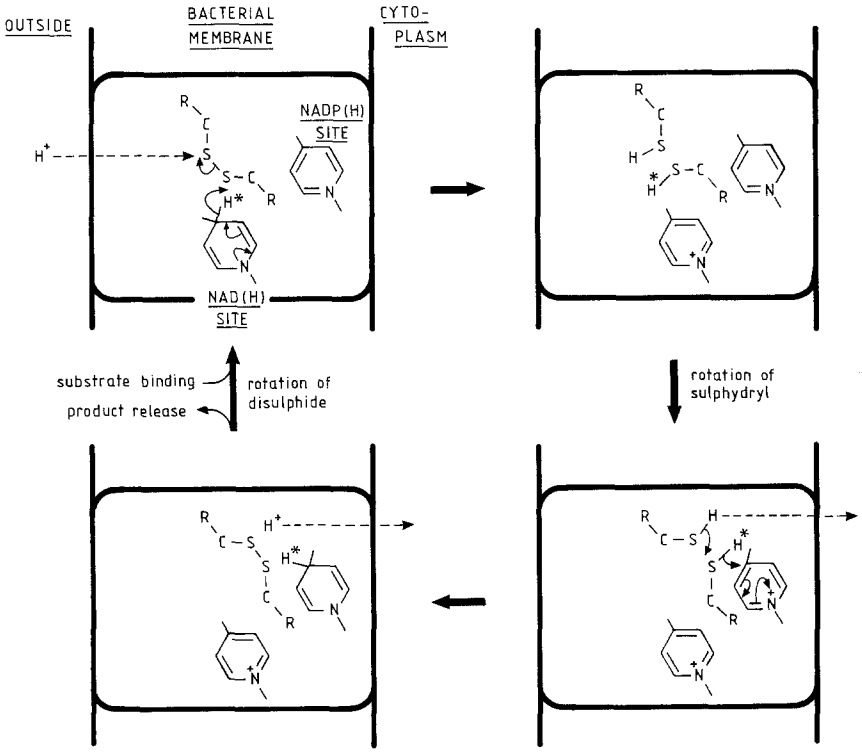


Fig. 6. Model to illustrate direct coupling in H^+-Thase by way of a reduced enzyme intermediate. The intermediate can only form within the ternary complex and so exchanges of water protons with the nucleotides are forbidden. The starred symbols show the pathway of H^- transfer from NADH to $NADP^+$.

center (e.g., Hennecke and Plapp, 1983). Thus, in the model for H^+-Thase shown in Fig. 6, the proton required during reduction of the disulfide bond by NADH is relayed to the catalytic center only fom the aqueous phase at high protonic potential, and the proton released during oxidation of the sulfydryl group by $NADP^+$ is relayed from the catalytic center only to the aqueous phase at low protonic potential. Interestingly, it has been suggested on the basis of crystallographic evidence, that in liver alcohol dehydrogenase, the 2^1OH group of the NAD^+-ribose itself participates in the proton relay system (Branden and Eklund, 1980). Were such a device operative in H^+-Thase, it would minimize the conduction of H^+ through the enzyme in the absence of substrate and thus prevent any slip. A critical feature in Fig. 6 is that, following reduction of the disulfide bond, there is a rotation step which ensures that H^- from C4 of the nicotinamide ring of NADH is transferred to C4 of $NADP^+$ without equilibrating with water protons. Although the model in Fig. 6 postulates the existence of a reduced enzyme intermediate, it should be stressed that all the H^- transfer steps take place during the interconversion of the ternary complexes. Therefore there is no conflict with the kinetic evidence ruling out a substituted enzyme mechanism nor with repeated failure to find evidence of a reduced enzyme intermediate which is stable in the absence of nucleotides (see Section 3).

It is evident that if a reaction pathway exists in which proton translocation shares intermediates with H^- transfer between nucleotides, then energy coupling can occur. Of course, the energy levels of the intermediate states must be matched to allow rapid turnover, and specificity rules must ensure that coupling is tight (see Jenks, 1987). The solution chemistry of the nicotinamide adenine dinucleotides is extensive and many of the reactions involve protons. It is therefore possible to devise feasible energy coupling pathways for H^+-Thase based on such protolytic reactions. The formation of adducts between ligands such as –SH compounds, carbonyl compounds, amines and imidazoles, and the C-2 and C-6 positions of the nicotinamide ring of NAD^+ have all been observed experimentally in nonenzymic systems (Oppenheimer, 1987). Each of these reactions is expected to involve H^+. Thus, by way of illustration, it can be imagined that interaction of the functional group of an amino acid in H^+-Thase with the nicotinamide ring of (say) bound $NADP^+$ could lead to the release of H^+ into the aqueous phase at low protonic potential. H^- transfer from NADH to $NADP^+$ on the enzyme, (simultaneous) transfer of the amino acid ligand to NAD^+ and subsequent breakdown of this adduct by proton uptake from the aqueous phase at high potential could therefore serve as the basis of an effective coupling device. Were any of these adducts to occur in H^+-Thase, they might be detectable by spectroscopy. A related model can be considered on the basis that only the reduced nicotinamide ring is likely to bind a proton at

physiological values of pH. A simple scheme is shown in Fig. 7. Note that the most readily protonatable group of the dihydronicotinamide ring is the carbon atom at position 5 (due to the fact that the structure is effectively a cross-conjugated vinylogous urea, the nitrogen atom at position 1 has a $pK \ll 1$ and is unlikely to protonate under physiological conditions (Oppenheimer, 1987). In the context of the model shown in Fig. 7, it is interesting that the hydration product of the protonated NADH intermediate *is* generated enzymically, though probably as a side reaction, in glyceraldehyde-3-phosphate dehydrogenase (Chaykin *et al.*, 1956).

It is emphasized that in models for the mechanism of action of H^+-Thase is which ligand conduction plays a central role (e.g., Figs. 4–7), conformational changes have an essential function, first to limit the conduction of specific ligands to appropriate states of the enzyme (and hence

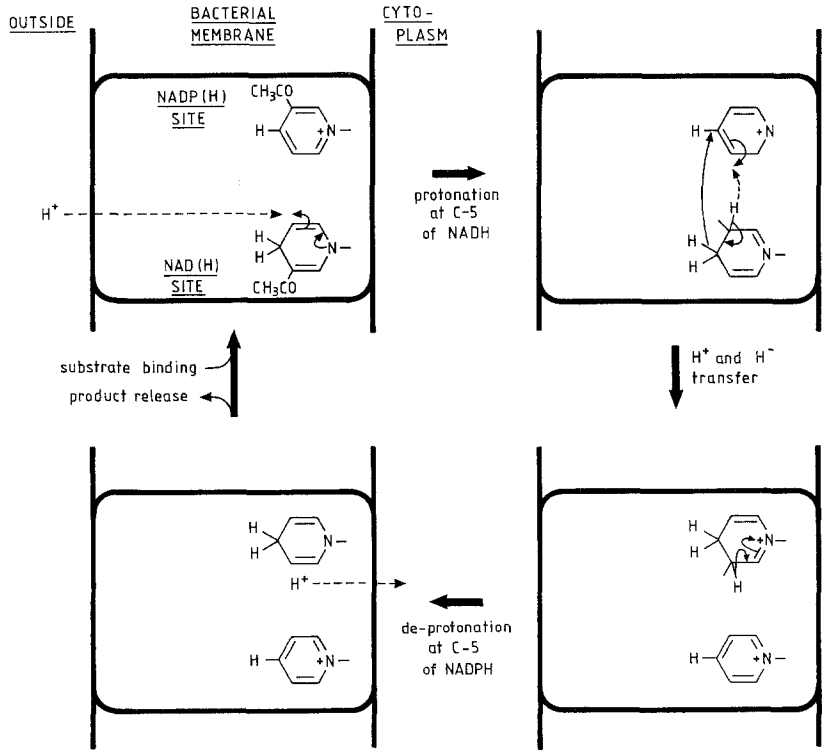


Fig. 7. Model to illustrate direct coupling in H^+-Thase involving protonation of the reduced nicotinamide rings. The transfer of H^- from NADH to $NADP^+$ can be more easily envisaged if the nicotinamide rings are stacked. The constraints governing exchange with water protons (legend to Fig. 6) also apply here.

eliminate or control slip) and second (possibly) to provide the appropriate local environment in the vicinity of the nucleotides for the correct orientation of the redox-active groups to promote transfer of the H^- equivalent. Moreover, conformational changes in the protein would also be a consequence of alterations in the redox or protonation states of the nucleotides and other moieties involved in the coupling mechanism.

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References

Anderson, W. M., Fowler, W. T., Pennington, R. M., and Fisher, R. R. (1981). *J. Biol. Chem.* **256**, 1888–1895.
Anderson, W. M., and Fisher, R. R. (1981). *Biocim. Biophys. Acta* **635**, 194–199.
Apell, H. J., Borlinghaus, R., and Lauger, P. (1987). *J. Membr. Biol.* **97**, 179–191.
Blazyk, J. F., and Fisher, R. R. (1975). *FEBS Lett.* **50**, 227–232.
Blazyk, J. F., Lam, D., and Fisher, R. R. (1976). *Biochemistry* **15**, 2843–2848.
Boyer, P. D. (1988). *Trends Biochem. Sci.* **13**, 5–7.
Bragg, P. D., Davis, P. L., and Hou, C. (1972). *Biochem. Biophys. Res. Commun.* **47**, 1248–1255.
Branden, C.-I., and Eklund, H. (1980). In *Dehydrogenases Requiring Nicotinamide Coenzymes* (Jeffery, J., ed.), Birkhauser Verlag, Basel, pp. 40–84.
Carlenor, E., Persson, B., Glaser, E., Andersson, B., and Rydstrom, J. (1988). *Plant Physiol.* **88**, 303–308.
Chaykin, S., Meinhart, J. O., and Krebs, E. G. (1956). *J. Biol. Chem.* **220**, 811–820.
Chiang, G., and Dilley, R. A. (1987). *Biochemistry* **26**, 4911–4916.
Clarke, D. M., and Bragg, P. D. (1985). *Eur. J. Biochem.* **149**, 517–523.
Clarke, D. M., Loo, T. W., Gillam, S., and Bragg, P. D. (1986). *Eur. J. Biochem.* **158**, 647–653.
Cornish-Bowden, A. (1979). *Fundamentals of Enzyme Kinetics*, Butterworths, London.
Cotton, N. P. J., Myatt, J. F., and Jackson, J. B. (1987). *FEBS Lett.* **219**, 88–92.
Cotton, N. P. J., Lever, T. M., Nore, B. F., Jones, M. R., and Jackson, J. B. (1989). *Eur. J. Biochem.* **182**, 593–603.
Danielson, L., and Ernster, L. (1963). *Biochem. Biophys. Res. Commun.* **10**, 91–96.
Dontsov, A. B., Grinius, L. L., Jasaites, A. A., Severina, I., and Skulachev, V. P. (1972). *J. Bioenerg.* **3**, 277–303.
Earle, S. R., and Fisher, R. R. (1980). *J. Biol. Chem.* **255**, 827–830.
Earle, S. R., O'Neal, S. G., and Fisher, R. R. (1978a). *Biochemistry* **17**, 4683–4690.
Earle, S. R., Anderson, W. M., and Fisher, R. R. (1978b). *FEBS Lett.* **91**, 21–24.
Enander, K., and Rydstrom, J. (1982). *J. Biol. Chem.* **257**, 14760–14766.
Eytan, G. D., Persson, B., Ekebacke, A., and Rydstrom, J. (1987a). *J. Biol. Chem.* **262**, 5008–5014.
Eytan, G. D., Eytan, E., and Rydstrom, J. (1987b). *J. Biol. Chem.* **262**, 5015–5019.
Eytan, G. D., Carlenor, E., and Rydstrom, J. (1990). *J. Biol. Chem.* **265**, 12949–12954.
Fisher, R. R., and Earle, S. R. (1980). *Biochemistry* **19**, 561–569.

Fisher, R. R., and Earle, S. R. (1982). In *The Pyridine Nucleotide Coenzymes* (Everse, J., Andersson, B., and You, K.-S., eds.), Academic Pres, New York, pp. 279–324.
Fisher, R. R., and Guillory, R. J. (1971a). *J. Biochem. Chem.* **246**, 4687–4693.
Fisher, R. R., and Guillory, R. J. (1971b). *J. Biol. Chem.* **246**, 4679–4684.
Fisher, R. R., Rampey, S. A. Sadighi, A., and Fisher, K. (1975). *J. Biol. Chem.* **250**, 819–825.
Galante, Y. M., Lee, Y., and Hatefi, Y. (1980). *J. Biol. Chem.* **255**, 9641–9646.
Gerolimatos, B., and Hanson, R. L. (1978). *J. Bacteriol.* **134**, 394–400.
Griffiths, D. E., and Robertson, A. M. (1966). *Biochim. Biophys. Acta* **118**, 453–464.
Hansen, U.P., Gradmann, D., Sanders, D., and Slayman, C. L. (1981). *J. Membr. Biol.* **63**, 165–190.
Hanson, R. L. (1979). *J. Biol. Chem.* **254**, 888–893.
Hanson, R. L., and Rose, C. (1980). *J. Bacteriol.* **141**, 401–404.
Hartog, A. F., and Berden, J. A. (1990). *FEBS Lett.* **261**, 161–164.
Hennecke, M., and Plapp, B. V. (1983). *Biochemistry* **22**, 3721–3728.
Hill, T. L. (1977). *Free Energy Transduction in Biology*, Academic Press, New York.
Hoek, J. B., Rydstrom, J., and Ernster, L. (1973). *Biochim. Biophys. Acta* **305**, 669–674.
Homyk, M., and Bragg, P. D. (1979). *Biochim. Biophys. Acta* **571**, 201–217.
Hou, C., Potier, M., and Bragg, P. D. (1990). *Biochim. Biophys. Acta* **1018**, 61–66.
Houghton, R. L., Fisher, R. J., and Sanadi, D. R. (1976). *Arch. Biochem. Biophys.* **176**, 747–752.
Hu, P.-S., Persson, B., Carlenor, E., Hartog, A. F., Hoog, J.-O., Jornvall, H., Berden, J. A., and Rydstrom, J. (1988). 5th Eur. Bioenergetics Conf., Short Reports Vol. 5, p. 65 (Abstract).
Jackson, J. B., Cotton, N. P. J., Lever, T. M., Cunningham, I. J., Palmer, T., and Jones, M. R. (1990). In *Molecular Biology of Membrane-Bound Complexes in Phototrophic Bacteria* (Drews, G., and Dawes, E. A., eds.), Plenum Press, New York, pp. 415–424.
Jacobs, E., and Fisher, R. R. (1979). *Biochemistry* **18**, 4315–4322.
Jacobs, E., Heriot, K., and Fisher, R. R. (1977). *Arch. Microbiol.* **115**, 151–156.
Jenks, W. P. (1980). *Adv. Enzymol.* **51**, 75–106.
Jenks, W. P. (1983). *Curr. Top. Membr. Transp.* **19**, 1–18.
Kawasaki, T., Satoh, K., and Kaplan, N. O. (1964). *Biochem. Biophys. Res. Commun.* **17**, 648–654.
Kay, W. W., and Bragg, P. D. (1975). *Biochem. J.* **150**, 21–29.
Keister, D. L., and Yike, N. J. (1966). *Biochem. Biophys. Res. Commun.* **24**, 519–525.
Kell, D. B., and Westerhoff, H. V. (1985). In *Organized Multienzyme Systems*, Academic Press, New York, pp. 63–139.
Kozlov, I. A. (1981). *Curr. Top. Membr. Transp.* **16**, 383–392.
Kozlov, I. A. Milgrom, Y. M., Saburova, L. A., and Sobolev, A. Y. (1984). *Eur. J. Biochem.* **145**, 413–416.
Lauger, P. (1984). *Biochim. Biophys. Acta* **779**, 307–341.
Lauger, P., and Stark, G. (1970). *Biochim. Biophys. Acta* **211**, 458–466.
Lee, C.-P., and Ernster, L. (1964). *Biochim. Biophys. Acta* **81**, 187–190.
Lee, C.-P., Simard-Duquesne, N., Ernster, L., and Hoberman, H. D. (1965). *Biochim. Biophys. Acta* **105**, 397–409.
Lever, T. M., Palmer, T., Cunningham, I. J., Cotton, N. P. J., and Jackson, J. B. (1990). *Eur. J. Biochem.* **197**, 247–255.
Liang, A., and Houghton, R. L. (1981). *J. Bacteriol.* **146**, 997–1002.
McFadden, B. J., and Fisher, R. R. (1978). *Arch. Biochem. Biophys.* **190**, 820–828.
Mitchell, P. (1966). *Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation*, Glynn Res. Publ., Bodmin, U.K.
Mitchell, P. (1969). *Theor. Exp. Biophys.* **2**, 159–215.
Mitchell, P. (1972). *J. Bioenerg. Biomembr.* **3**, 5–24.
Mitchell, P. (1987). In *Integration and Control of Metabolic Processes; Pure and Applied Aspects* (Kon, O.L., ed.), ICSU Press, Cambridge, U.K., pp. 231–245.
Modrak, D. E., Wu, L. N. Y., Alberta, J. A., and Fisher, R. R. (1988). *Biochemistry* **27**, 7665–7671.
Moyle, J., and Mitchell, P. (1973). *Biochem. J.* **132**, 571–585.

O'Neal, S. G., and Fisher, R. R. (1977). *J. Biol. Chem.* **252**, 4552–4556.
Oppenheimer, N. J. (1987). In *Pyridine Nucleotide Coenzymes* (Dolphin, D., Poulson, R., and Avramovic, O., eds.), Wiley, New York, pp. 323–365.
Oustroumov, S. A., Samuilov, V. D., and Skulachev, V. P. (1973). *FEBS Lett.* **31**, 27–30.
Palmer, T., and Jackson, J. B. (1990a). *FEBS Lett.* **277**, 45–48.
Palmer, T., and Jackson, J. B. (1990b), unpublished.
Parker, D. M., and Holbrook, J. J. (1977). In *Pyridine Nucleotide-Dependent Dehydrogenases* (Sund, H., ed.), De Gruyter, Berlin, pp. 485–501.
Pennington, R. M., and Fisher, R. R. (1981). *J. Biol. Chem.* **256**, 8963–8969.
Persson, B. (1988). *Mitochondrial Nicotinamide Nucleotide Transhydrogenase*, University of Stockholm, Sweden.
Persson, B., and Rydstrom, J. (1987). *Biochem. Biophys. Res. Commun.* **142**, 573–578.
Persson, B., Enander, K., Tang, H. L., and Rydstrom, J. (1984). *J. Biol. Chem.* **259**, 8626–8632.
Perrson, B., Ahnstrom, G., and Rydstrom, J. (1987a). *Arch. Biochem. Biophys.* **259**, 341–349.
Persson, B., Berden, J. A., Rydstrom, J., and van Dam, K. (1987b). *Biochim. Biophys. Acta* **894**, 239–251.
Persson, B., Hartog, A. F., Rydstrom, J., and Berden, J. A. (1988). *Biochim. Biophys. Acta* **953**, 241–248.
Phelps, D. C., and Hatefi, Y. (1981). *J. Biol. Chem.* **256**, 8217–8221.
Phelps, D. C., and Hatefi, Y. (1984a). *Biochemistry* **23**, 6340–6344.
Phelps, D. C., and Hatefi, Y. (1984b). *Biochemistry* **23**, 4475–4480.
Phelps, D. C., and Hatefi, Y. (1984c). *Biochemistry* **23**, 4475–4480.
Phelps, D. C., and Hatefi, Y. (1985). *Biochemistry* **24**, 3503–3507.
Rydstrom, J., Teixeira Da Cruz, A., and Ernster, L. (1971). *Eur. J. Biochem.* **23**, 212–219.
Rydstrom, J. (1977). *Biochim. Biophys. Acta* **463**, 155–184.
Rydstrom, J. (1979). *J. Biol. Chem.* **254**, 8611–8619.
Rydstrom, J., and Hoek, J. B. (1988). *Biochem. J.* **254**, 1–10.
Rydstrom, J., Lee, C.-P., and Ernster, L. (1981). In *Chemiosmotic Proton Circuits in Biological Membranes* (Skulachev, V. P., and Hinkle, P. C., eds.), Addison-Wesley, Reading, Massachusetts, pp. 483–508.
Rydstrom, J., Persson, B., and Carlenor, E. (1987). In *Pyridine Nucleotide Coenzyme: Chemical Biochemical, and Medical Aspects*, Vol. 2B (Dolphin, D., Poulson, R., and Avramovic, O., eds.), Wiley, New York, pp. 433–460.
Scarborough, G. A. (1985). *Microbiol. Rev.* **49**, 214–231.
Skulachev, V. P. (1970). *FEBS Lett.* **11**, 301–308.
Skulachev, V. P. (1974). *Ann. N.Y. Acad. Sci.* **227**, 188–202.
Smith, C. M., and Plaut, G. W. E. (1979). *Eur. J. Biochem.* **97**, 283–295.
Teixeira Da Cruz, A., Rydstrom, J., and Ernster, L. (1971). *Eur. J. Biochem.* **23**, 203–211.
Wakabayashi, S., and Hatefi, Y. (1987a). *Biochem. Int.* **15**, 667–675.
Wakabayashi, S., and Hatefi, Y. (1987b). *Biochem. Int.* **15**, 915–924.
Westheimer, F. H. (1987). In *Pyridine Nucleotide Coenzymes: Chemical, Biochemical, and Medical Aspects*, Vol. 2A (Dolphin, D., Poulson, R., and Avramovich, O., eds.), Wiley, New York, pp. 253–322.
Wierenga, R. K., Terpstra, P., and Hol, W. G. J. (1986). *J. Membr. Biol.* **187**, 101–107.
Wu, L. N. Y., and Fisher, R. R. (1982). *Biochim. Biophys. Acta* **681**, 388–396.
Wu, L. N. Y., and Fisher, R. R. (1983). *J. Biol. Chem.* **258**, 7847–7851.
Wu, L. N. Y., Earle, S. R., and Fisher, R. R. (1981). *J. Biol. Chem.* **256**, 74017408.
Wu, L. N. Y., Alberta, J. A., and Fisher, R. R. (1986). *Methods Enzymol.* **126**, 353–360.
Yamaguchi, M., and Hatefi, Y. (1985). *Arch. Biochem. Biophys.* **243**, 20–27.
Yamaguchi, M., and Hatefi, Y. (1989). *Biochemistry* **28**, 6050–6056.
Yamaguchi, M., Hatefi, Y., Trach, K., and Hoch, J. A. (1988). *J. Biol. Chem.* **263**, 2761–2767.
Yamaguchi, M., Wakabayashi, S., and Hatefi, Y. (1990). *Biochemistry* **29**, 4136–4143.
You, K., Arnold, L. J., Jr., Allison, W. S., and Kaplan, N. O. (1978). *Trends Biochem. Sci.* **3**, 265–268.