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REVIEW ARTICLE

Physiological roles of nicotinamide nucleotide transhydrogenase

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Introduction

Nicotinamide nucleotide transhydrogenase was first discovered in bacterial extracts in 1951 and, shortly afterwards, in beef heart membrane preparations (see [1–4] for reviews). The enzyme catalyses the reversible transfer of hydrogen between NAD and NADP according to the reaction.

$$NADH + NADP^+ \rightleftharpoons NAD^+ + NADPH$$

Since the two reactants have practically equal standard redox potentials, it would be expected that the enzyme equilibrates the redox levels of NAD and NADP. This function could indeed be demonstrated in certain species of bacteria, e.g. *Pseudomonas aeruginosa* and *Azotobacter vinelandii*. In these organisms, the rates of the forward and reverse reactions are regulated by several factors, such as Ca^{2+}, 2′-phosphate nucleotides, and by the prevailing substrate/product ratio (see [1] for a review of bacterial transhydrogenases). In some other micro-organisms and in higher organisms, the transhydrogenase is membrane-bound and is linked to the energy transfer system of the respiratory chain. In mammalian cells, the enzyme is an integral protein of the mitochondrial inner membrane.

The enzyme from beef heart has been purified to homogeneity [5–7]. It consists of a single polypeptide chain of M_r 120000 which forms a dimer both in the dispersed form and in reconstituted lipid vesicles. The reconstituted enzyme is a proton pump, acting in concert with the transfer of electrons from NADPH to NAD^+ [8,9]. Similarly, in mitochondria the reduction of $NADP^+$ by NADH is coupled to the translocation of protons from the C- (cytoplasmic) side to the M- (matrix) side of the mitochondrial inner membrane (see Fig. 1). The coupling of the redox reaction to proton translocation provides a mechanism to link the transhydrogenase reaction to other energy-linked processes in the mitochondrial membrane, presumably through a proton electrochemical gradient across the membrane ($\Delta\tilde{\mu}_{H^+}$).

The coupling of transhydrogenase to the mitochondrial energy conservation system has two consequences with important implications for the physiological function of the enzyme [1,2,4]. Energization of the mitochondria causes a marked change in the kinetic properties of the enzyme, probably associated with a conformational change of the protein [10,11]. The rate of the forward reaction (i.e. the reduction of $NADP^+$ by NADH) is increased several-fold and simultaneously the reverse reaction, the oxidation of NADPH by NAD^+, is inhibited. Furthermore, the stoichiometric coupling of a vectorial (proton transport) and a scalar (oxidoreduction) reaction results in a change of the apparent equilibrium

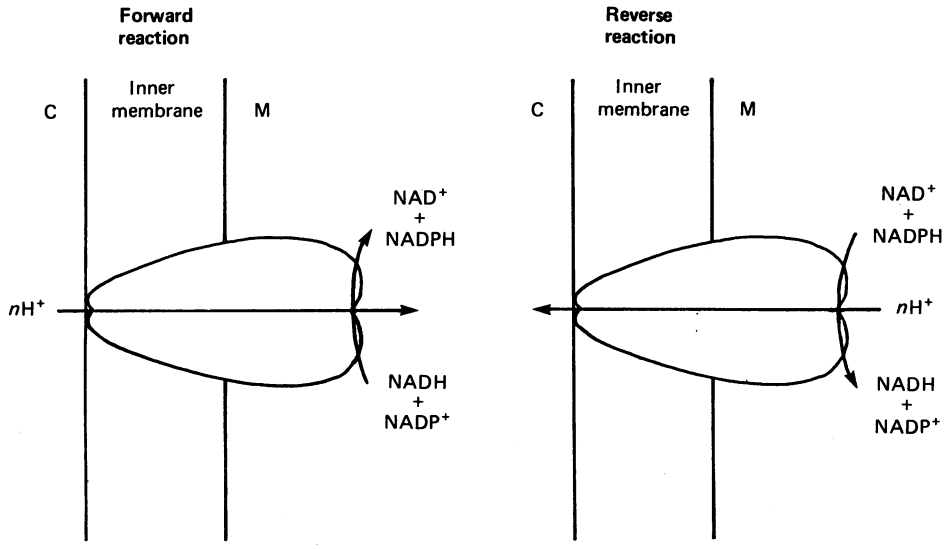

Fig. 1. Schematic representation of the proton translocating transhydrogenase

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constant of the redox system: on energization of the mitochondrial membrane, the mass action ratio (Γ) of the nicotinamide nucleotides:

$$\Gamma = [NADPH]\cdot[NAD^+]/[NADP^+]\cdot[NADH] \quad (1)$$

can reach a value of up to 500 [12,13]. The coupling of electron transfer and proton translocation reactions is reversible: not only can $NADP^+$ reduction be driven by a proton electrochemical gradient, $\Delta\tilde{\mu}_{H^+}$, but the formation of a membrane potential difference by the NAD^+-dependent oxidation of NADPH can be demonstrated in submitochondrial particles or reconstituted vesicles [8,14–17].

Ever since its discovery, the physiological role of the enzyme has been a source of speculation and sometimes a matter of controversy. Most authors have emphasized either the generation of a redox potential difference between NAD and NADP at the expense of energy, or the utilization of the redox potential difference for the maintenance of $\Delta\tilde{\mu}_{H^+}$. Since there are, in most mitochondria, one or more other reactions that can generate mitochondrial NADPH or a proton electrochemical gradient, there is a legitimate question if, and under what conditions, transhydrogenase is essential for the proper function of the cell. Traditionally, the most convenient approach to such questions is the use of inhibitors. Unfortunately, a selective inhibitor of transhydrogenase is not available that can be used conveniently in intact cells or mitochondria. Answers have to come from indirect experimental approaches and careful analysis of the properties of the enzyme and the metabolic situation in which it is positioned. In this paper, we take as our starting point a kinetic and thermodynamic consideration of the coupled electron transfer and proton translocation reaction, so as to define the conditions under which the transhydrogenase can be of significance. This approach leads us to propose that transhydrogenase is poised to provide a protective buffer against a dissipation of either the cellular redox power or of the mitochondrial energy supply. No attempt has been made in this paper to give an exhaustive coverage of the literature on this enzyme; interested readers are referred to earlier reviews [1–4] for a more complete discussion of other aspects of interest.

Thermodynamics of energy-linked transhydrogenase

The proton-pumping capacity of the transhydrogenase (see Fig. 1) introduces a connection between the nicotinamide nucleotide redox potential difference (ΔE_h) and the mitochondrial proton electrochemical potential ($\Delta\tilde{\mu}_{H^+}$). Assuming the redox couple equilibrates with the bulk-phase proton gradient on either side of the mitochondrial membrane, the quantitative expression of this interconnection is given by the free energy change, ΔG, of the coupled reaction:

$$n\,H^+_c + NADH + NADP^+ \rightleftharpoons n\,H^+_m + NAD^+ + NADPH$$
$$\Delta G = n\,\Delta\tilde{\mu}_{H^+} + 2\,F\,\Delta E_h \quad (2)$$

The stoichiometry factor n refers to the number of protons translocated across the mitochondrial membrane per pair of electrons transferred between NAD and NADP; subscripts m and c refer to the intramitochondrial and cytosolic compartments, respectively.

Both the electrical membrane potential difference ($\Delta\psi$, M-side negative) and the pH difference (ΔpH, M-side minus C-side) contribute to the proton electrochemical potential:

$$\Delta\tilde{\mu}_{H^+} = F\,\Delta\psi - 2.303\,\boldsymbol{RT}\,\Delta pH \quad (3)$$

In isolated mitochondria maximally energized by substrate oxidation a $\Delta\tilde{\mu}_{H^+}$ (in electrical units) has been measured in the range of 180–220 mV (negative inside), composed of a membrane potential difference of 130–170 mV and a ΔpH varying between 30 and 60 mV (0.5–1.0 pH units) [18–20]. Similar values for the membrane potential have been obtained in intact hepatocytes from ion distribution measurements [21,22].

The redox potential difference between NAD and NADP (ΔE_h) is determined almost exclusively by the redox ratios of the two nucleotides, since their midpoint potentials are very close:

$$\Delta E_h = E_h(NAD) - E_h(NADP) = \frac{\boldsymbol{RT}}{2F}\ln\left(\frac{a_{NAD^+}\cdot a_{NADPH}}{a_{NADH}\cdot a_{NADP^+}}\right) \quad (4)$$

where a_{NAD^+}, a_{NADPH}, a_{NADH} and a_{NADP^+} are the activities ('free' concentrations) of the respective nucleotides in the mitochondrial matrix.

Early studies on isolated mitochondria [23] demonstrated that, upon energization, the NADP redox ratio can be maintained more reduced than that of NAD by a factor of 20–50, corresponding to a redox potential difference of about 40–50 mV. These estimates could not take into account the preferential binding of the oxidized or reduced form of either nucleotide to intramitochondrial constituents (see [24,25] for a discussion of nicotinamide nucleotide binding and compartmentation). Estimates of the redox potential differences of the 'free' mitochondrial nicotinamide nucleotides by indicator metabolite systems [24,25] indicate a ΔE_h of up to 80–100 mV, in isolated mitochondria or in intact hepatocytes, with the NADP being maintained at the more negative redox potential. These estimates correspond well with the degree of NADP reduction catalysed by transhydrogenase in isolated membrane systems. In experiments on submitochondrial particles, where binding of nucleotides is not a matter of concern, the energy-linked transhydrogenase was found to maintain NADP up to 500 times more reduced than NAD [12,13], corresponding to a ΔE_h of about 80 mV. In similar particles, a $\Delta\mu_{H^+}$ of the order of 160–180 mV can be maintained [18–20].

Less certain is the value of the stoichiometry factor n in eqn. (2). Initial estimates of the stoichiometry of the ATP-driven transhydrogenase by Lee & Ernster [26] indicated the hydrolysis of one ATP per pair of electrons, after correcting for non-energy-linked transhydrogenase reactions and suppressing non-specific ATP hydrolysis. Similar figures were obtained by other investigators [27]. Current consensus indicates a stoichiometry of 3 protons/ATP of the ATPase system [28]; a similar stoichiometry of 3 H^+ per pair of electrons would therefore be indicated for the transhydrogenase reaction. Skulachev and coworkers [14,16] observed a linear relationship between ΔE_h and the distribution of lipophilic anions (an indicator for $\Delta\psi$) in submitochondrial particles with a slope that would be indicative of a much lower stoichiometry; they noted that transhydrogenase was much less efficient than ATPase or succinate oxidation in generating a membrane potential. Moyle &

Mitchell [29] proposed a $H^+/2e^-$ stoichiometry of 2, based on proton pulse measurements in intact mitochondria oxidizing isocitrate. It is difficult, however, to assess the contributions of NAD-linked and NADP-linked isocitrate oxidation in this system [30]. Moreover, these estimates are subject to potential interference by other proton-coupled ion movements across the mitochondrial membrane [31,32].

With the purification and reconstitution of the transhydrogenase, new estimates of the proton stoichiometry of the reaction have been made. Earle & Fisher [33] used a kinetic approach, measuring the initial rates of proton translocation and electron transfer reactions. A stoichiometry of less than one proton per pair of electrons was obtained. However, their experimental design is highly sensitive to proton leak, especially under conditions of variable $\Delta\tilde{\mu}_{H^+}$ induced by the proton pumping activity. Eytan *et al.* [34] compared ATP hydrolysis and $NADP^+$ reduction in co-reconstituted liposomes containing purified ATPase and transhydrogenase. With varying ATP concentration, the ratio of transhydrogenase to oligomycin-sensitive ATPase activity approached a limiting value of 3. On the basis of an ATPase stoichiometry of 3 protons/ATP, these data suggest a minimum stoichiometry of one $H^+/2e^-$ for transhydrogenase. This approach is probably less subject to interfering ion movements than earlier methods and is less dependent on corrections for variable $\Delta\tilde{\mu}_{H^+}$ (see [32,35] for a discussion of the merits of comparable approaches to stoichiometry measurements during electron transport).

The implications of the proton stoichiometry for the interpretation of transhydrogenase function in intact cells and mitochondria are significant. As shown in eqn. (2), if $n = 2$ or 3, the maximal redox potential difference ΔE_h of 80–100 mV that has been detected between mitochondrial NAD and NADP is insufficient to generate a proton electrochemical gradient of a magnitude compatible with that maintained across mitochondrial membranes by substrate oxidation. Under those conditions $\Delta G < 0$; hence, it would be impossible for the transhydrogenase to contribute to the generation and/or maintenance of $\Delta\tilde{\mu}_{H^+}$. The reaction would be irreversibly locked into NADPH generation, unless the $\Delta\tilde{\mu}_{H^+}$ decreases to less than 100 mV or ΔE_h is increased to more than 180 mV.

A different picture emerges, however, from the recent estimates indicating a stoichiometry of one proton per pair of electrons. The free energy change of the proton-coupled reaction, under conditions of a $\Delta\tilde{\mu}_{H^+}$ of 180–200 mV and a ΔE_h of 80–100 mV, is then close to zero and relatively small fluctuations in either $\Delta\tilde{\mu}_H$ or ΔE_h can generate conditions favourable to either the forward or the reverse reaction. If the conditions in the mitochondria are thermodynamically favourable to support the flux of the reaction in the direction of either NADPH formation or NADPH oxidation, the kinetic characteristics of the enzyme will then determine the actual activity of the enzyme at the prevailing level of substrates, products and energetic conditions.

Kinetic characteristics of energy-linked transhydrogenase

As a first approximation, the actual rates of transhydrogenation in the intact mitochondrion under different conditions, including varying degrees of energization and redox levels of NAD and NADP, can be estimated by analysing the individual effects of these parameters on the kinetics of the transhydrogenase reaction. A suitable model system for kinetic studies has been found in beef heart submitochondrial particles [10,13,36].

Both the forward and the reverse reactions catalysed by the mitochondrial transhydrogenase are inhibited by the products NADPH and NAD^+, and NADH and $NADP^+$, respectively [2,4,10,13,36]. The inhibition by the nicotinamide nucleotide products is site-specific, i.e., even at a high concentration of inhibitor and low concentrations of substrates, NADP(H) does not interact with the NAD(H)-binding site and *vice versa* [10,36]. Under coupled conditions, the extent of the inhibition is influenced by the prevailing $\Delta\tilde{\mu}_{H^+}$, due to the fact that the affinities of the transhydrogenase for its substrates are dependent upon $\Delta\tilde{\mu}_{H^+}$ [10]. Thus, the affinity for $NADP^+$ is increased 5-fold and that for NAD^+ decreased 5-fold in the presence of an energy source, e.g. succinate. Presumably, these affinity changes reflect altered dissociation constants for the transhydrogenase–$NADP^+$ and transhydrogenase–NAD^+ complexes. Less pronounced affinity changes are observed for NADH and NADPH. Consequently, the inhibition of the forward reaction by NADPH or NAD^+ decreases several-fold with an increase in $\Delta\tilde{\mu}_{H^+}$, whereas the inhibition of the reverse reaction by NADH or $NADP^+$ shows a corresponding increase by energization.

In a more recent investigation, the influence of energization on the product-dependent inhibition of the forward and reverse transhydrogenase reactions was investigated in the presence of subsaturating concentrations of substrates [37]. $\Delta\tilde{\mu}_{H^+}$ was varied by adding increasing concentrations of malonate to submitochondrial particles which were oxidizing succinate in the presence of oligomycin. A replot of these data illustrates the effect of the substrate/product ratios on the activity of the forward (Figs. 2*a* and 2*b*) and reverse (Figs. 2*c* and 2*d*) transhydrogenase reactions. The forward reaction is strongly product-inhibited by NADPH and NAD^+ in a manner that varies with $\Delta\tilde{\mu}_{H^+}$. An NADPH/$NADP^+$ ratio of 20 gives an inhibition of 70% and 92%, respectively, in energized and de-energized systems (Fig. 2*a*); at higher ratios (NADPH/$NADP^+$ > 100), inhibition is essentially complete, irrespective of the degree of energization. An NAD^+/NADH ratio of 100 gives an inhibition of 75% and 97%, respectively, under conditions of high and low $\Delta\tilde{\mu}_{H^+}$ (Fig. 2*b*). The reverse reaction is similarly inhibited by its products NADH and $NADP^+$; at an $NADP^+$/NADPH ratio of 2.5, the inhibition amounts to 75% and 63%, respectively, in energized and de-energized systems (Fig. 2*c*). Correspondingly, an NADH/NAD^+ ratio of 1.65 inhibits the reverse reaction by 72% at low $\Delta\tilde{\mu}_{H^+}$ and close to 100% at high $\Delta\tilde{\mu}_{H^+}$ (Fig. 2*d*).

Using metabolite indicator methods discussed in [24,25], mitochondrial redox potentials of NAD and NADP in an intact liver cell have been estimated to be −318 mV and −415 mV, respectively, corresponding to an NADH/NAD^+ ratio of approx. 0.2 and NADPH/$NADP^+$ ratio of about 200. In view of the pronounced product inhibition of the transhydrogenase it is conceivable that its activity under these conditions is kinetically limited in both directions of the reaction. The rate of the forward reaction would be close to zero at

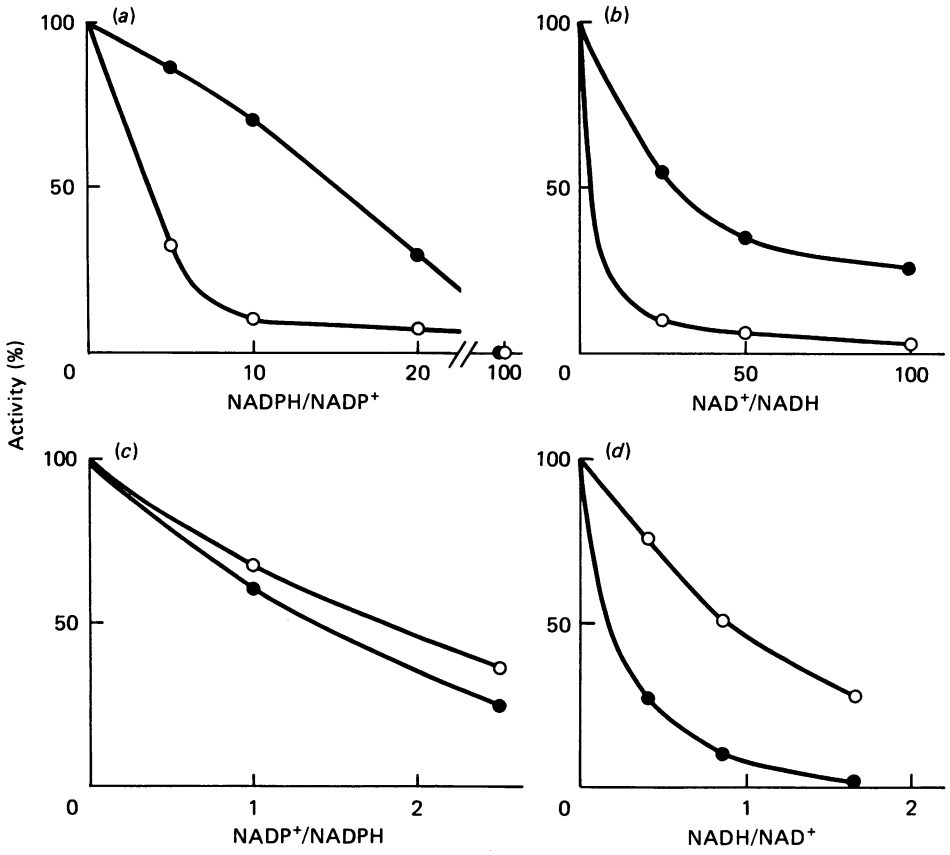

Fig. 2. Effect of varying product/substrate ratios on the activities of the forward (*a*,*b*) and reverse (*c*,*d*) transhydrogenase reactions catalysed by beef heart submitochondrial particles, at low and high levels of energization

The forward and reverse transhydrogenase reactions were assayed in the presence of the product/substrate ratios indicated, and in the presence of 3 mM-succinate and oligomycin (1 μg/ml) (●), or 3 mM-succinate, oligomycin (1 μg/ml) and 5 mM-malonate (○). Replotted from [37].

an NADPH/NADP$^+$ ratio of > 100 (cf. Fig. 2*a*). In addition, at these high NADPH/NADP$^+$ ratios the influence of the mitochondrial energy state on the extent of the inhibition is small. Provided the kinetic properties of transhydrogenase in beef heart submitochondrial particles are similar to those of the enzyme in intact mitochondria from liver and other tissues, the forward transhydrogenase reaction would therefore be essentially inactive under conditions where the NADP is highly reduced. The mitochondrial NADH/NAD$^+$ ratio in intact cells varies from 0.1 to 1.0 [24,25], a range where it has substantially less influence on the activity of the forward reaction, especially when the $\Delta\tilde{\mu}_{H^+}$ is high (Fig. 2*b*). The reverse reaction, by contrast, is relatively insensitive to variations in the redox potential of NADP over the physiologically relevant range (Fig. 2*c*), but may respond critically to changes in the NADH/NAD$^+$ ratio (Fig. 2*d*). In energized mitochondrial membrane preparations, the degree of inhibition varies from 45% to 95% for a NADH/NAD$^+$ ratio between 0.1 and 1.0. A decrease in the $\Delta\tilde{\mu}_{H^+}$, moreover, can further stimulate the activity by a factor of 2–8 (Fig. 2*d*).

Consequently, the kinetic characteristics indicate that, under the conditions likely to be encountered in intact cells, the enzyme activity is controlled in the forward direction by the NADPH/NADP$^+$ ratio and in the reverse direction by the combined effects of the NADH/NAD$^+$ ratio and in the reverse direction by the combined effects of the NADH/NAD$^+$ ratio and the $\Delta\tilde{\mu}_{H^+}$.

Energy-linked transhydrogenase as a redox buffer

A flexible picture emerges from the kinetic and thermodynamic characteristics of the transhydrogenase. In the 'resting' cell (i.e., in the absence of a major drain on the supply of NADPH and energy), the redox potential difference of mitochondrial nicotinamide nucleotides may well be set in close agreement with the mitochondrial $\Delta\tilde{\mu}_{H^+}$; assuming a $H^+/2e^-$ stoichiometry of 1, the ΔG of the overall reaction is close to zero. Yet, the transhydrogenase could not properly be considered a near-equilibrium reaction, since these conditions impose strict limitations on the kinetic capabilities of the system. The activity of the forward reaction would be almost completely dependent on the oxidation of NADPH. The reverse reaction would be controlled by the combined action of the NADH/NAD$^+$ ratio and the $\Delta\tilde{\mu}_{H^+}$; a significant decrease in either of these would result in an activation of NADPH oxidation through NAD$^+$. Thus, the flux through the transhydrogenase in either direction will be determined to a large extent by the degree of energization and the redox state prevailing under the specific conditions in the mitochondria. A disturbance in the resting state will tend to activate the reaction and increase the flux in a direction which counteracts the

changes imposed on the system. According to the characteristics illustrated in Fig. 2, the flux rate will increase in proportion to the magnitude of the disturbance imposed on the system. Hence, the transhydrogenase will act as a buffer counteracting either NAD(P)H oxidation or depletion of $\Delta\tilde{\mu}_{H^+}$. In the following sections, we will consider some implications of an energy-linked transhydrogenase functioning as a redox buffer which may throw some new light on its role in cellular metabolism.

The place of energy-linked transhydrogenase in the network of redox relationships in the cell

Beginning with the early work of Bücher & Klingenberg [38], a large number of studies have analysed the relationship between redox potentials of NAD and NADP in different compartments of the cell (see [24,25,38,39] for reviews). Much of the detailed analytical work in this area has focused on liver, both for technical reasons and because liver is a particularly interesting and complex organ from the point of view of redox interactions. The major conclusions may be equally applicable to other tissues, although a thorough analysis of the mitochondrial nicotinamide nucleotide redox state is generally not available.

Under resting conditions, NAD and NADP in the liver cell are maintained at widely different redox potentials, both in the mitochondrial and cytosolic cell compartments; and, while there is relatively little difference in the redox potential of NADP in cytosol and mitochondria, NAD is kept at a much more reduced level in the mitochondria than in the cytosol. These differences are relatively unaffected by changes in the metabolic conditions [25] and therefore appear to be carefully controlled parameters of cellular metabolism. These redox relationships are maintained by the network of nicotinamide nucleotide dependent dehydrogenases interlinked by sets of shared substrates [39] and by the selective permeability characteristics of the mitochondrial membrane for metabolites that interconnect the redox systems in cytosol and mitochondria. The substrate shuttle systems linking mitochondrial and cytosolic NAD are all characterized by the input of energy; for example, aspartate is transported by an electrogenic exchange with glutamic acid which causes aspartate to be preferentially extruded from the mitochondrial matrix [40]. In this way, the malate–aspartate shuttle does not interfere with, and in fact helps maintain, the redox potential difference between mitochondrial and cytosolic NAD [25,40]. The electroneutral (iso)citrate–malate transport system interconnects the almost equipotential pools of NADP in cytosol and mitochondria through NADP-linked isocitrate dehydrogenases in both compartments [24,25,40]; it is not influenced by the membrane potential, but responds only to the pH difference across the mitochondrial membrane.

Any enzyme activity that allows the transfer of reducing equivalents between NAD and NADP within one compartment must be controlled to maintain these differences in redox potential. The energy input into the transhydrogenase reaction fulfils exactly that purpose: it prevents the dissipation of the redox potential of both mitochondrial and cytosolic NADP into the mitochondrial pool of NAD and subsequently into the respiratory chain [see 24,25].

An exchange of reducing equivalents between NAD and NADP which is not controlled by the energy level can also be achieved by combinations of enzymes that react with both NAD and NADP and share their other reactants. For instance, glutamate dehydrogenase reacts about equally well with either NAD or NADP as coenzyme. As pointed out by Krebs & Veech [39], the combination of reactions catalysed by this enzyme can bring about a (non-energy linked) 'transhydrogenation', provided adequate concentrations of its substrates and products are present. A combination of NAD-linked and NADP-linked isocitrate dehydrogenases could achieve the same effect [41]. Cytosolic processes catalysing a similar substrate-dependent exchange of reducing equivalents between NAD and NADP have also been described, e.g., associated with glutathione metabolism [42] and with oestradiol metabolism [43]. Any of these combinations of enzymes catalyse the following series of reactions:

$$\begin{array}{r} SH_2 + NAD^+ \rightleftharpoons S + NADH + H^+ \\ S + NADPH + H^+ \rightleftharpoons SH_2 + NADP^+ \\ \hline NAD^+ + NADPH \rightleftharpoons NADH + NADP^+ \end{array}$$

The combination of these processes with an energy-linked transhydrogenase activity will result in an energy-consuming cycling of electrons. In isolated mitochondria, it has been demonstrated that this cycle does, indeed, occur in the presence of adequate substrate levels of glutamate dehydrogenase [44]. In practice, however, the rate of such dissipating cycling is prevented by a careful kinetic control of at least one of the two partial reactions. In the case of glutamate dehydrogenase in mitochondria, the control appears to be predominantly at the level of the partial reaction catalysing the reduction of α-oxoglutarate ($+NH_3$) with NADPH [44,45]. Under resting conditions, the metabolites of glutamate dehydrogenase therefore equilibrate primarily with NAD and not with NADP, even though a net formation of glutamate will be driven by the high level of NADPH [44,45]. In the case of isocitrate dehydrogenase, the kinetic control is primarily at the level of the NAD-linked enzyme [41]; the NADP-linked activity predominates in a resting cell and the ratio of metabolite levels of isocitrate dehydrogenase therefore reflects primarily the redox state of NADP [46].

Thus, as for transhydrogenase, a kinetic control must be an essential element of all enzyme systems that interlink the NAD and NADP redox systems. In the resting cell, the redox state of NAD and NADP will be set primarily by the more active dehydrogenase activities that are not subject to such kinetic constraints. The activation of one of the controlled elements of these reactions will dissipate the redox potential difference between NAD and NADP and relieve the kinetic constraints on the energy-linked transhydrogenase. A new steady state will be set with a smaller change in the redox potential difference, but with an increased rate of utilization of energy. The role of transhydrogenase therefore would be to provide a buffer against an uncontrolled dissipation of the redox potential difference between NAD and NADP, while avoiding a large input of energy in maintaining that difference under resting conditions.

The role of the energy-linked transhydrogenase in the supply of reducing equivalents

From the point of view of NADP-related metabolism, the hepatocyte is an unusually complex cell type. It has an exceptionally high level of mitochondrial NADP, combined with a high activity of one of the major mitochondrial NADPH-utilizing enzymes, glutamate dehydrogenase. Several decades ago, the work of Tager [47] and of Klingenberg [48] demonstrated that conditions could be created in isolated mitochondria where transhydrogenase is involved in the synthesis of glutamate from ammonia and α-oxoglutarate, utilizing NAD-linked substrates as a source of reducing equivalents. Sies *et al.* [49] later suggested that the same situation may hold in intact hepatocytes; these authors showed that rhein, an inhibitor of transhydrogenase, decreases the rate of urea synthesis from ammonia, a process which requires the synthesis of glutamate in the mitochondria. Isocitrate synthesis can be driven with NADPH generated by transhydrogenase activity, by way of the NADP-linked isocitrate dehydrogenase activity [50,51]. This activity has the capacity to utilize the transhydrogenase to the maximal extent [51]. Transhydrogenase can also be utilized to drive the mitochondrial NADPH-dependent glutathione reduction with NAD-linked substrates [52,53]. In addition, the liver is extremely active in cytosolic NADPH-utilizing processes, most notably fatty acid synthesis and endoplasmic reticular hydroxylation reactions involved in detoxification of xenobiotics. The latter processes, although localized outside the mitochondrial matrix, have been shown to depend to a large extent on an effective supply of reducing equivalents from the mitochondria, transferred across the mitochondrial membrane by way of substrate shuttle systems [54–56]. Transhydrogenase may be involved in these activities, as shown in model systems [50,51], but in intact cells alternative pathways are also available to transfer reducing equivalents to cytosolic NADP [55].

In other tissues, specialized functions often determine the need for mitochondrial NADPH supply [1]. Intramitochondrial NADPH-dependent mono-oxygenation reactions involved in steroid synthesis have long been known to occur, for instance, in adrenal, kidney and other cells. In these mitochondria, the requirement for NADPH can be met by substrates for NAD-linked dehydrogenases when the transhydrogenase is operative; however, such mitochondria in general have a relatively high level of NADP-linked dehydrogenases, specifically isocitrate dehydrogenase and malic enzyme, which can accommodate the need for NADPH without involving the transhydrogenase, provided the substrate requirements can be met.

The general pattern emerging from the majority of these studies is one of flexibility: it is possible, in most systems where transhydrogenase activity is present, to impose conditions which require its involvement to transfer reducing equivalents from NAD-linked substrates to NADPH for synthetic purposes. It is not clear that any of these NADPH-dependent processes depends on transhydrogenase at the exclusion of other NADP-linked reactions. Rather, the kinetic characteristics of the energy-linked transhydrogenase would indicate that the enzyme is called upon only under conditions of high demand for NADPH, characterized by a decrease in the reduction level of this coenzyme. Several studies of drug hydroxylation in liver have indicated a significant contribution of reducing equivalents from the mitochondrial compartment; these conditions were always accompanied by a significant oxidation of NADPH [54–56]. Similarly, a high demand for glutamate synthesis, e.g., during urea synthesis from ammonia, leads to oxidation of mitochondrial NADPH [49]. Plaut and coworkers [57,58] assessed the role of NADP-linked isocitrate dehydrogenase by the use of the specific inhibitor α-methyl isocitrate. These studies support the interpretation that the NADP-linked isocitrate dehydrogenase is predominant in the provision of reducing equivalents when NADP is highly reduced. Inhibition of this enzyme causes an oxidation of the NADPH and diminishes the rate of urea synthesis in intact hepatocytes [58]. These data are indicative of a system in which transhydrogenase, and consequently the NAD-linked pool of reducing equivalents, is called upon only under conditions of stress, as characterized by an elevated NADP redox potential.

Heart cells are of interest in that they contain a high activity of transhydrogenase, in combination with a high level of NADP-specific isocitrate dehydrogenase; yet, major intramitochondrial NADPH-requiring synthetic processes have not been identified in the heart. Moreover, the system lacks an obvious capacity for substrate shuttling to support extramitochondrial NADPH-dependent processes: most or all of the NADP-linked isocitrate dehydrogenase activity is intramitochondrial [59] and the activity of the tricarboxylate carrier is relatively low [60]. This system, even more than liver, therefore indicates that transhydrogenase activity fulfils other functions than being a high-capacity source of low redox potential NADPH for synthetic processes.

Protective effects of the mitochondrial NADP redox state

There is much evidence that a highly reduced state of NADP has a protective role in many different cell types. At least three different processes involve NADPH in the protection against oxidative stress or other sources of trouble. Firstly, NADPH is a substrate for endoplasmic reticular mono-oxygenation reactions, a major pathway for the metabolism of xenobiotics. As discussed above, a large part of the reducing equivalents for this process may be derived from the mitochondrial compartment. Secondly, the two-electron NADP-dependent reduction of quinones, catalysed by NAD(P)H:quinone oxidoreductase (menadione reductase, DT-diaphorase) may be a factor in avoiding the formation of semiquinone free radicals and associated lipid peroxidation [61]. Mitochondria from different tissues contain a substantial activity of this enzyme [62]. Thirdly, a major protective function is associated with the glutathione redox couple [42,63]. Glutathione is involved in the protection against free radical-induced damage, through the action of glutathione peroxidase. It can also assist in the removal of foreign compounds by the formation of glutathione conjugates. Glutathione may play a role in maintaining protein-bound sulphydryl functions in a reduced state or regulate the function of specific enzymes by the formation of mixed disulphides. All these activities depend on the reduced form of glutathione. Under conditions of oxidative stress, the GSH/GSSG ratio falls rapidly and

damage ensues (by mechanisms that have not been adequately defined) unless the GSH is regenerated by glutathione reductase; the latter process requires NADPH as the source of reducing equivalents. Inhibition of glutathione reductase greatly enhances the vulnerability of various cell types to oxidative stress and other injuries [64,65]. Although most of the cellular glutathione is cytosolic, a relatively independent glutathione pool is present in the mitochondrial compartment, where it is presumed to fulfill similar protective functions [52,66].

For the most part, a role of transhydrogenase in the protective actions of NADPH is subject to the same limitations as discussed above for the synthetic processes: the supply of NADPH must have the capacity to keep up with demand. A significant quantitative contribution of transhydrogenase to this process, which calls on the supply of reducing equivalents from NAD-linked substrates to maintain the protective action of NADPH, would depend on activation of transhydrogenase by a decrease in the NADPH/$NADP^+$ ratio.

A somewhat different situation may exist for the processes involved in the glutathione-dependent maintenance of protein sulphydryl groups [67,68]. In principle, the reactions involved are all readily reversible; thus, the modification of protein sulphydryl groups by the formation of mixed disulphides may reflect the redox potential of glutathione and/or NADP. This provides for a mechanism by which a change in the redox state of NADP could result in a reversible covalent modification of a target protein.

Although a wide variety of enzymes can be modulated by the formation of a mixed disulphide [67,68], it is not clearly established to what extent this process is used for purposes of regulating the flux through a particular metabolic pathway. Some of the evidence seems to indicate that the formation of mixed disulphides and the consequent conformational changes imposed upon the protein may be part of protein degradative pathways [67]. These are obviously of interest from the point of view of control of protein turnover and may contribute to the long-term regulation of metabolic pathways. The protection against NADPH oxidation which the transhydrogenase provides at the expense of energy may constitute an important safeguard against excessive acceleration of such degradative mechanisms in the mitochondrial compartment and, indirectly, in the cytosol.

In addition, short-term adjustments in the NADP redox state could potentially be used to impose regulatory features on proteins which are subject to such thiol–disulphide interchange [68]. Of particular interest in this connection may be transport proteins, several of which can be affected by a change in the thiol status [69]. Orrenius and coworkers [70,71] have demonstrated that the $Ca^{2+}+Mg^{2+}$-ATPase in the plasma membrane of liver cells can be inactivated in conjunction with the formation of a mixed disulphide. These authors attribute a significant role in the disturbances of cellular calcium homeostasis after oxidative stress to the inactivation of the Ca^{2+} pump [71]. Jocelyn [53] provided evidence that adducts of mitochondrial transport proteins with diamide could be substrates for intramitochondrial glutathione. On a more speculative level, there is considerable evidence that mitochondrial calcium efflux is increased under conditions where NADPH oxidation occurs [72]. The mechanism of this phenomenon has been the subject of a long-standing debate [72–75]. Among several alternative mechanisms, the activation of a specific Ca^{2+} efflux system in the mitochondrial inner membrane has been proposed [74–76], which could be regulated by the status of critical thiol groups on the protein [75], or by covalent modification through ADP-ribosylation [74]. Thiol reagents can protect against the activation of Ca^{2+} efflux that is induced by NADPH oxidation in mitochondria, but it is not clear that this action is exerted at the level of the Ca^{2+} efflux, or indeed, that the efflux activity has any specificity for Ca^{2+} [77,78]. Pfeiffer and coworkers [73,77,79] suggest that thiol protection is exerted at the level of phospholipid reacylation. Moreover, we have observed that oxidation of mitochondrial glutathione can be dissociated from the activation of calcium efflux: in mitochondria which had their glutathione reductase activity inhibited by 1,3-bis(2-chlorethyl)-1-nitrosourea (BCNU), oxidation of glutathione with butyl hydroperoxide was not associated with NADPH oxidation and did not induce the efflux of accumulated Ca^{2+} (J. B. Hoek, unpublished work). This does not exclude the possibility that the NADP redox state is mediated to protein sulphydryl functions by a different intermediate than glutathione. Alternative mechanisms would also be compatible with these observations, e.g., involving a role for NAD as substrate for ADP ribosylation [80]. For the present time, the possible mechanism by which the NADP redox state can control mitochondrial calcium fluxes remains speculative.

Utilization of the redox potential difference between NAD and NADP

The kinetic properties of the transhydrogenase and the stoichiometry of proton extrusion are compatible with an activation of the reverse transhydrogenase reaction by a decrease in the mitochondrial $\Delta\tilde{\mu}_{H^+}$. Under these conditions, the transhydrogenase could act to utilize the redox potential difference between NAD and NADP as a buffer against the change in $\Delta\tilde{\mu}_{H^+}$. In isolated mitochondria, Nicholls & Garland [81] demonstrated that isocitrate oxidation can occur through the NADP-dependent isocitrate dehydrogenase with transhydrogenase operating in reverse, provided the mitochondrial membrane potential is dissipated with uncouplers. The significance of this process in a physiological system is more difficult to demonstrate. A recent study by Anderson *et al.* [82], however, describes conditions in which we speculate a reversal of transhydrogenase could have played a role.

In this study, isolated hepatocytes were subjected to anoxia and the ensuing changes in mitochondrial membrane potential were assessed by the distribution of triphenylmethylphosphonium ($TPMP^+$), essentially by the method of Hoek *et al.* [21]. The authors observed a rapid but partial decrease in membrane potential, which was maintained at a level of over 130 mV, despite the complete cessation of electron transport and a dramatic drop in ATP/ADP ratio in the cells. Nicotinamide nucleotides were highly reduced under these conditions, due to the inhibition of the respiratory chain. The authors excluded glycolytic ATP as a source of energy for maintaining the $\Delta\tilde{\mu}_{H^+}$ and suggested that another unknown source of energy may be responsible. We suggest that these conditions appear suitable for the energy-linked transhydrogenase to fulfil this role.

Malate and citrate levels were found to decrease during anoxia [83], and may have been utilized to provide a source of low-potential NADPH via malic enzyme and aconitate hydratase/NADP-linked isocitrate dehydrogenase, respectively. Assuming a proton stoichiometry of 1 $H^+/2e^-$, transhydrogenase would require a redox potential difference of 60–70 mV to sustain the measured $\Delta\mu_{H^+}$. It would be of great interest to further investigate this system by assessing the mitochondrial NAD and NADP redox states from the distribution of metabolite levels in these experiments.

Thus, there is an interesting possibility that transhydrogenase might, indeed, provide a buffering capacity to maintain cellular ion distributions and membrane potential levels for a prolonged period of time under conditions of anoxia. This would provide protection against one of the most prevalent forms of insult the cells are exposed to under physiological conditions. It would specifically provide a significant advantage to heart cells, where transhydrogenase is high in activity, despite the lack of quantitatively significant NADPH-requiring reactions.

For a further understanding of the significance of this mechanism of protection, it would be of interest to compare the maintenance of a mitochondrial $\Delta\tilde{\mu}_{H^+}$ during anoxia with the response to oxidative stress. Under the latter conditions, the injury is typically associated with a rapid depletion of cellular NADPH levels, often in combination with an increased rate of electron transport due to uncoupling or Ca^{2+} cycling. Starke *et al.* [84] reported that oxidative stress is associated with a rapid decrease of the mitochondrial membrane potential as indicated by a loss of $TPMP^+$, but these authors did not do a quantitative analysis of the associated decrease in $\Delta\tilde{\mu}_{H^+}$. Careful quantitative studies of these systems may provide further information on the possible protective action of the energy-linked transhydrogenase in these and other experimental conditions.

Hormonal effects on transhydrogenase activity

The ability of transhydrogenase to contribute to the control of the nicotinamide nucleotide redox potential and the maintenance of the mitochondrial energy state depends not only on the kinetic features of the enzyme, but also on its maximal activity. The latter parameter can vary in response to hormonal stimulation. For instance, a short-term (5–15 min) treatment of rats with glucagon [85] or catecholamines [86,87] *in vivo* or *in vitro* was found to cause a 30–50% increase in the activity of the enzyme as measured in mitochondrial inner membrane preparations or in solubilized mitochondria. The effects of catecholamine treatment were observed both in liver and heart preparations and were specifically inhibited by antagonists for β-adrenergic, but not α-adrenergic, receptors [87]. The results of Medvedev *et al.* [87] indicate that these changes in transhydrogenase activity represent an increase in $V_{max.}$ with little significant effect on the K_m for substrates of the reverse reaction (NADPH oxidation). It would be predicted, therefore, that the sensitivity of the forward reaction to inhibition by NADPH also would be unaffected. In liver mitochondria, this short-term hormone treatment protocol also results in a wide range of other alterations in mitochondrial characteristics, including an activation of mitochondrial ATPase and electron transport, and a decreased sensitivity to calcium overloading and peroxide challenge [88]. However, in contrast to the report by Medvedev *et al.* [87] on transhydrogenase, the latter mitochondrial effects of hormone treatment are mimicked by α_1-adrenergic agonists and by vasopressin [88], agents which do not raise the level of cyclic AMP, but which operate through an activation of polyphosphoinositide breakdown. An increase in transhydrogenase activity has also been reported in liver mitochondrial preparations from hypothyroid rats [89], in combination with a substantial increase in mitochondrial levels of NAD and NADP [90,91]. In contrast with the response after glucagon treatment, hypothyroidism is associated with a decreased activity of electron transport and a lower ADP/O ratio as detected in isolated mitochondria; these alterations can also be reversed by a short-term treatment with thyroid hormones [91]. It has been suggested [91] that an increased transhydrogenase activity, associated with a higher rate of intrinsic NADPH oxidation, could account for the lower phosphorylation efficiency in these mitochondria.

Functional implications of an increased capacity of transhydrogenase would be evident primarily by a shift in the relationship between the flux through the enzyme and the parameters that control its activity. The predominant parameters are the $NADPH/NADP^+$ ratio and the $\Delta\tilde{\mu}_{H^+}$; an increased efficiency of its protective action would be detectable under conditions of an increased drain on the mitochondrial NADPH supply, or when the $\Delta\tilde{\mu}_{H^+}$ is substantially decreased. A more effective protection of mitochondrial integrity associated with a higher mitochondrial NADPH level has indeed been reported in mitochondria from glucagon-treated rats in response to challenges by t-butylhydroperoxide and calcium overloading [92]. It remains to be investigated whether such protective actions also are exerted in intact cells. Mitochondrial preparations from hypothyroid rat liver also exhibit substantial differences in the response of NADPH to State 3–State 4 transitions [90,91]. It should be noted that, in this system, the increased transhydrogenase activity and higher apparent NADPH level are associated with a decrease in the respiratory activity and no apparent change in the $\Delta\tilde{\mu}_{H^+}$ maintained under State 4 conditions.

The mechanism by which a relatively short (5–15 min) treatment with glucagon, thyroid hormone or other agonists can affect the transhydrogenase (or other membrane-bound mitochondrial enzymes) remains to be established. Since the alterations in activity are preserved in submitochondrial particle preparations [85], or even when the mitochondria are solubilized with detergent [86,87], it appears that the enzyme itself may be affected, or a constitutent that is tightly bound to the protein. Interestingly, both the soluble and the reconstituted transhydrogenase could be reversibly inactivated by GSSG [93], indicating that the enzyme has critical sulphydryl groups that are accessible to this reagent. In addition, prolonged exposure to GSSG also had an uncoupling effect. The reactivity with GSSG and GSH was affected by the availability of substrates of the enzyme [93]. These findings suggest the interesting potential for a regulation of transhydrogenase activity by *S*-thiolation and, conceivably, this property might play a role in the hormonally induced alterations in activity. The potential of the hormonal modulation of transhydrogenase activity suggests this might be a

rewarding area for the study of its role in the mitochondrial control of the nicotinamide nucleotide redox state as well as the conservation of the energy level in the intact cell.

Summary

From the foregoing considerations, the energy-linked transhydrogenase reaction emerges as a powerful and flexible element in the network of redox and energy interrelationships that integrate mitochondrial and cytosolic metabolism. Its thermodynamic features make it possible for the reaction to respond readily to challenges, either on the side of NADPH utilization or on the side of energy depletion. Yet, the kinetic features are designed to prevent a wasteful input of energy when other sources of reducing equivalents to NADP are available, or to deplete the redox potential of NADPH in other than emergency conditions. By virtue of these characteristics, the energy-linked transhydrogenase can act as an effective buffer system, guarding against an excessive depletion of NADPH, preventing uncontrolled changes in key metabolites associated with NADP-dependent enzymes and calling on the supply of reducing equivalents from NAD-linked substrates only under conditions of high demand for NADPH. At the same time, it can provide an emergency protection against a depletion of energy, especially in situations of anoxia where a supply of reducing equivalents through NADP-linked substrates can be maintained. The flexibility of this design makes it possible that the functions of the energy-linked transhydrogenase vary from one tissue to another and are readily adjustable to different metabolic conditions.

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