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NAD(P)-LINKED OXIDOREDUCTIONS AND THE NICOTINAMIDE NUCLEOTIDE SPECIFICITY OF GLUTAMATE DEHYDROGENASE IN RAT-LIVER MITOCHONDRIA

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SUMMARY

1. The oxidoreduction pattern of intramitochondrial NAD(P) during the transfer of hydrogens from α-oxoglutarate to α-oxoglutarate (+ ammonia), oxaloacetate, acetoacetate, and α-oxoglutarate (+ CO_2) in rat-liver mitochondria was studied. The intramitochondrial NAD(P) was first subjected to a cycle of oxidation by O_2 and reduction by α-oxoglutarate, either in the coupled state (ADP + P_i present) or in the uncoupled state (dicoumarol + oligomycin present), before adding the hydrogen acceptor.

2. When the cycle of oxidation and reduction was carried out in the coupled state, NADP was reduced to a greater extent than NAD. When it was done in the uncoupled state, NAD was more reduced than NADP.

3. In the coupled mitochondria, the oxidation rate of NADPH by α-oxoglutarate (+ ammonia) at 10° was higher than that of NADH. In the uncoupled mitochondria, the rate of oxidation of NADPH was greatly inhibited, and that of NADH only slightly.

4. With acetoacetate as hydrogen acceptor, NADH was oxidized rapidly and NADPH only slowly (at 10°), both in the coupled and in the uncoupled state. Similar results were obtained with oxaloacetate.

5. When the hydrogen acceptor was α-oxoglutarate (+ CO_2), both NADH and NADPH were rapidly oxidized (at 25°) in the coupled mitochondria. In uncoupled mitochondria, no oxidation of NADH occurred initially upon the addition of α-oxoglutarate (+ CO_2), and furthermore, the oxidation of NADPH was inhibited.

6. The effect of carrying out the oxidation part of the cycle in the uncoupled state was shown to be reversible. When dicoumarol was removed with albumin, and the addition of α-oxoglutarate could provide not only reducing equivalents, but also energy, the mitochondria behaved exactly like those preincubated in the coupled state.

7. It is concluded that when hydrogen is transferred from an NAD-linked substrate to α-oxoglutarate (+ ammonia or CO_2), energy is required not only to promote the transhydrogenation between NADH and $NADP^+$, but also for the reaction of NADPH with glutamate dehydrogenase or the NADP-linked isocitrate dehydro-

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genase. Energy is not required for the oxidation of NADH by acetoacetate or oxaloacetate. A possible mechanism is proposed.

8. The physiological significance of the results is discussed.

INTRODUCTION

The study of the redox pattern of mitochondrial nicotinamide nucleotides under various functional states and of their availability to the mitochondrial dehydrogenases is of basic importance for an understanding of the way in which the reducing power of mitochondria can be utilized either for the generation of ATP (or the energy equivalent) or for reductive synthesis. Investigations along these and related lines have led to the discovery of the occurrence in mitochondria of an energy-linked reversal of the respiratory chain[1,2] and of an energy-linked transhydrogenase reaction between NADH and $NADP^+$ (refs. 3–6). Also of relevance in this respect are the observations of KLINGENBERG and co-workers that in rat-liver mitochondria, endogenous $NADP^+$ is reduced to a greater extent by glutamate than by α-oxoglutarate or β-hydroxybutyrate[3], and that endogenous NADPH is oxidized to a greater extent by α-oxoglutarate (+ ammonia) than by oxaloacetate or acetoacetate[7]. On this basis KLINGENBERG and co-workers[3,4] postulated that glutamate dehydrogenase (EC 1.4.1.3) within the mitochondrion is specific for NADP, in contrast to the isolated enzyme which utilizes both NAD and NADP (see also ref. 8).

Direct evidence for this has recently been obtained by TAGER, PAPA and co-workers[9–11], who found that in coupled rat-liver mitochondria the rate of oxidation of NADPH by α-oxoglutarate (+ ammonia) is greater than that of NADH, and the rate of reduction of $NADP^+$ by glutamate greater than that of NAD^+. Furthermore, a direct correlation was observed between the level of $NADP^+$ and the rate of glutamate deamination in rat-liver mitochondria in various metabolic states[11,12].

The occurrence in rat-liver mitochondria of the energy-linked transhydrogenase reaction as well as of a preferential reaction of glutamate dehydrogenase with NADP has prompted our study of the pathways and mechanisms of hydrogen transfer at the level of the nicotinamide nucleotides in rat-liver mitochondria. We have examined the properties of a number of hydrogen-transfer reactions between NAD(P)-linked substrates, and the energy control of the reductive amination of α-oxoglutarate in rat-liver mitochondria[13]. In the present paper the oxidoreduction pattern of NAD and NADP during various NAD(P)-linked oxidoreduction reactions in rat-liver mitochondria has been investigated. In particular, the effect of the energy level on the kinetics of oxidation of NADH and NADPH by α-oxoglutarate (+ ammonia) has been examined. The results obtained show that the nicotinamide adenine nucleotide specificity of glutamate dehydrogenase is energy dependent. Possible mechanisms for the energy control of hydrogen transfer at the level of the nicotinamide nucleotides are discussed. A preliminary account of part of these investigations has been presented[14,15].

METHODS

The materials and experimental procedure used are described in the preceeding paper[13]. The standard reaction mixture (final volume, 1 ml; final pH, 7.5) contained

15 mM KCl, 5 mM $MgCl_2$, 2 mM EDTA, 50 mM Tris, 10 mM potassium phosphate, 2 mM ADP and 25 mM sucrose (derived from the mitochondrial suspension). Other additions and the reaction temperatures are given in the legends to the figures. For these short-time experiments, the reaction was carried out in small beakers with rapid stirring (see refs. 11 and 16 for details).

RESULTS

The oxidoreduction pattern of endogenous NAD and NADP during hydrogen transfer from α-oxoglutarate to α-oxoglutarate (+ ammonia), oxaloacetate, acetoacetate and α-oxoglutarate (+ CO_2) was studied, with particular reference to the kinetics of oxidation of NADH and NADPH by the different hydrogen acceptors. Crucial factors in this investigation have been the small amount of intramitochondrial NAD(P), so that complete oxidation or reduction can occur in a few seconds, and the presence of endogenous substrates and nucleoside phosphates. The former difficulty was overcome by adopting the rapid-sampling techniques described by VAN DAM[16], using sensitive spectrophotometric methods, and lowering the reaction temperature. The latter difficulty was circumvented by choosing the proper conditions to eliminate interfering NAD(P)-linked reactions.

Fig. 1 shows an analysis of the oxidoreduction pattern of NAD and NADP during hydrogen transfer from α-oxoglutarate to α-oxoglutarate (+ ammonia). In Expt. A, mitochondria were preincubated aerobically with ADP and P_i. After the preincubation, the percentage reduction of NADP had decreased from 97 to 20%, and that of NAD from 60 to 10%. Oxoglutarate was then added, allowing the first step of the dismutation to take place. On addition of rotenone (1 min later), α-oxoglutarate caused extensive reduction of nicotinamide nucleotides. NADP was reduced to a greater extent than NAD, due to operation of the energy-linked transhydrogenase (see refs. 3, 5, and 17), the equilibrium of which is towards NADP reduction[18]. In a control experiment where no α-oxoglutarate was added, the reduction of NAD and NADP (after the addition of rotenone) was negligible. Thus, the only hydrogen donor in the system was α-oxoglutarate. Once NAD(P) had become maximally reduced, arsenite was added to arrest the oxidation of α-oxoglutarate. The addition of ammonia made it possible, under these conditions, to determine the absolute rate of oxidation of NADH and NADPH by α-oxoglutarate (+ ammonia). The rate of NADPH oxidation after the addition of ammonia was much faster than that of NADH (Expt. A). In Expt. B the incubation was carried out in the presence of dicoumarol and oligomycin. This treatment gave a complete oxidation of both endogenous NADH and NADPH, and brought the mitochondria into a low-energy state. α-Oxoglutarate was then added, together with rotenone. Under these conditions, NAD became reduced to a greater extent than NADP, indicating suppression of the energy-linked transhydrogenase. On addition of ammonia, a striking inhibition of the rate of NADPH oxidation was observed. The rate of NADH oxidation was inhibited to a lesser extent, so that the ratio between the rates of oxidation of the two nucleotides approached 1.

Fig. 2 shows the oxidoreduction pattern of NAD(P) during hydrogen transfer from α-oxoglutarate to acetoacetate. The experimental procedure was exactly the same as that described in Fig. 1. When acetoacetate was added to coupled mitochondria (after the cycle of oxidation and reduction of the nicotinamide nucleotides),

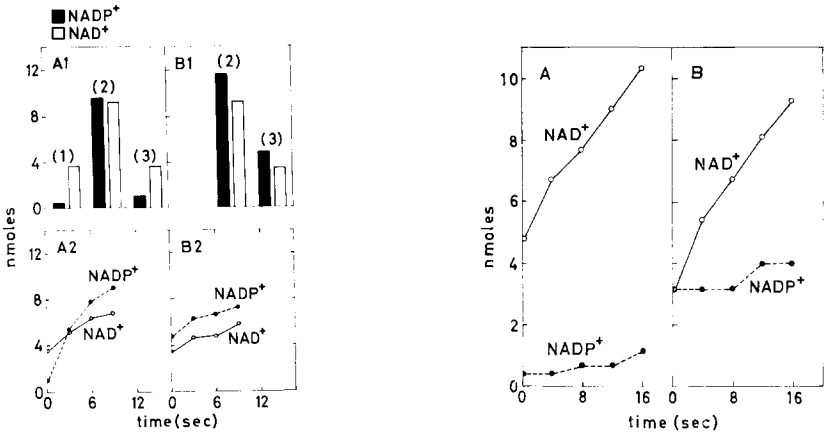

Fig. 1. Effect of preincubation with dicoumarol *plus* oligomycin on the oxidoreduction pattern of the intramitochondrial nicotinamide nucleotides during the Krebs–Cohen dismutation in rat-liver mitochondria. In A, mitochondria (3 mg protein) were preincubated at 25° in the standard reaction mixture. In B, 20 μM dicoumarol and 15 μg oligomycin were also present. After 2 min, 10 μmoles α-oxoglutarate were added in Expt. A, followed 1 min later by 1 μg rotenone. In Expt. B, α-oxoglutarate and rotenone were added together after the 2-min preincubation. In both cases 2 min after the addition of α-oxoglutarate, the incubation beakers were transferred to an ice bath (in A2 and B2). After 1 min, 1 μmole arsenite was added, and 1 min later, when the temperature of the reaction mixture was 10°, 10 μmoles NH_4Cl were added. The reaction was stopped at the times indicated with $HClO_4$ or KOH. The bars (in A1 and B1) show the NAD^+ and $NADP^+$ levels in mitochondria (1) before preincubation; (2) after 2 min preincubation; (3) 1 min after the addition of arsenite. The mitochondria contained 9.5 nmoles NAD^+ + NADH and 12 nmoles $NADP^+$ + NADPH.

Fig. 2. Effect of preincubation with dicoumarol *plus* oligomycin on the oxidoreduction pattern of the intramitochondrial nicotinamide nucleotides during the transfer of hydrogens from α-oxoglutarate to acetoacetate. Experimental conditions as described in Fig. 1, except that the amount of mitochondrial protein was 3.5 mg, and 10 μmoles acetoacetate were added instead of the NH_4Cl. In B, the reaction mixture contained 20 μM dicoumarol and 15 μg oligomycin.

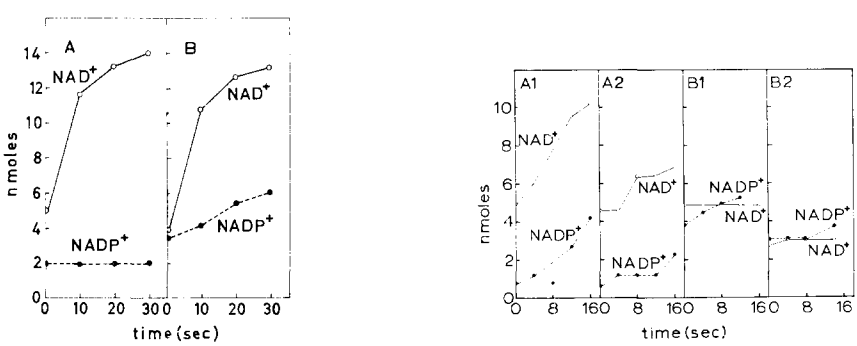

Fig. 3. Effect of preincubation with dicoumarol *plus* oligomycin on the oxidoreduction pattern of the intramitochondrial nicotinamide nucleotides during the transfer of hydrogen from α-oxoglutarate to oxaloacetate. Experimental procedure as in Fig. 1, except that the amount of mitochondrial protein was 4 mg, and 10 μmoles oxaloacetate were added instead of the NH_4Cl. In B, the reaction mixture contained 20 μM dicoumarol and 15 μg oligomycin.

Fig. 4. Effect of preincubation with dicoumarol *plus* oligomycin on the oxidoreduction pattern of the intramitochondrial nicotinamide nucleotides during the transfer of hydrogen from α-oxoglutarate to α-oxoglutarate (+ CO_2). Experimental procedure as in Fig. 1, except that the incubation temperature was maintained at 25° throughout the experiment, the amount of mitochondrial protein was 6 mg, and 10 μmoles $KHCO_3$ were added instead of the NH_4Cl. In Expts. A1 and B1, 95 % O_2–5 % CO_2 was bubbled through the reaction mixture from the time of addition of $KHCO_3$. In Expts. B1 and B2, the reaction mixture contained 20 μM dicoumarol and 15 μg oligomycin.

NADH was oxidized rapidly and NADPH only very slowly (Fig. 2A). This slow oxidation of NADPH must have been mediated by the nicotinamide nucleotide transhydrogenase. Fig. 2B shows that preincubation with dicoumarol *plus* oligomycin had no significant effect on the rate of NADH (or NADPH) oxidation by acetoacetate. In a parallel experiment with the same mitochondrial preparation, preincubation with dicoumarol *plus* oligomycin caused a marked inhibition of NADPH oxidation by α-oxoglutarate (+ ammonia).

Fig. 3 shows that in coupled mitochondria oxaloacetate oxidized NADH only, and that with this substrate, too, preincubation with dicoumarol (*plus* oligomycin) had no effect on the rate of NADH oxidation. It should be noted that since the oxidation of intramitochondrial NADH by added oxaloacetate is slow, the reaction was followed over a longer time interval than in Figs. 1 and 2. After the preincubation with dicoumarol, a slow oxidation of NADPH was observed on the addition of oxaloacetate; this was probably due to suppression of the energy-linked transhydrogenase.

Fig. 4 shows the effect of the energy level of the mitochondria on the oxidoreduction pattern of NAD(P) during hydrogen transfer from α-oxoglutarate to α-oxoglutarate (+ CO_2). Since the rate of this dismutation was found[13] to be considerably lower than that of the other reactions studied, the temperature during the last phase of the experiment was kept at 25° instead of being lowered to 10° (*cf.* Figs. 1–3). The oxidation of the nicotinamide nucleotides was initiated by the addition of $KHCO_3$ (Expts. A2 and B2) or $KHCO_3$ *plus* CO_2 (Expts. A1 and B1). When the cycle of oxidation and reduction of the nicotinamide nucleotides was carried out in the presence of ADP and P_i, so that the mitochondria remained coupled, the addition of $KHCO_3$ (± CO_2) caused oxidation of both NADPH and NADH (Fig. 4, Expts. A1 and A2), the former by the NADP-linked isocitrate dehydrogenase, and the latter by $NADP^+$ *via* the energy-linked transhydrogenase*. When the mitochondria were preincubated with dicoumarol *plus* oligomycin, so that the mitochondria were uncoupled and the energy-linked transhydrogenase was suppressed, no oxidation of NADH occurred upon the addition of $KHCO_3$ (± CO_2), at least during the first 16 sec. Furthermore, the oxidation of NADPH by α-oxoglutarate (+ CO_2) was inhibited in the uncoupled mitochondria, exactly like the oxidation of NADPH by α-oxoglutarate (+ ammonia)**.

Fig. 4 also shows that in the coupled mitochondria, the rate of oxidation of the nicotinamide nucleotides was faster when $KHCO_3$ *plus* CO_2 was added than when CO_2 was omitted (*cf.* Expts. A1 and A2), in accordance with the suggestion of CHAPPELL AND CROFTS[29] that mitochondria are more permeable to CO_2 than to the bicarbonate ion. Furthermore, inhibition of the oxidation of the nicotinamide nucleotides in uncoupled mitochondria was found even when CO_2 was added together with $KHCO_3$ (*cf.* Expts. A1 and B1).

In the preceding paper[13], it was shown that the inhibition of the oxidation of NADPH by α-oxoglutarate (+ ammonia), brought about by the preincubation with dicoumarol *plus* oligomycin, could be released by removing dicoumarol with albumin

* Reduction of α-oxoglutarate (+ CO_2) by the NAD-linked isocitrate dehydrogenase has not been demonstrated (see DISCUSSION).

** There was only negligible interference of the nicotinamide nucleotide transhydrogenase with the pattern of oxidation of NAD(P)H by α-oxoglutarate (+ ammonia), since the latter reaction was studied at 10°; the transhydrogenase reaction, which is highly temperature-dependent[19], is much slower at 10° than at 25°.

and allowing energy generation to take place. This was further investigated in the experiment of Fig. 5. Mitochondria were preincubated in the presence of dicoumarol and oligomycin. After 2 min, albumin and α-oxoglutarate were added in Expts. A1 and B1. Since albumin binds dicoumarol and restores the coupled state[21], α-oxoglutarate oxidation could provide not only reducing equivalents but also energy. This resulted in a restoration of the energy-linked transhydrogenase, as revealed by the higher extent of reduction of NADP with respect to that of NAD. On addition of ammonia (preceded by rotenone and arsenite), NADPH was quickly and preferentially oxidized. In Expts. A2 and B2, rotenone and sulphide were added together with the albumin and α-oxoglutarate, so that the latter could provide reducing equivalents, but because of the inhibition of the respiratory chain, neither energy generation nor the energy-linked transhydrogenase could be restored. On the addition of ammonia under these conditions, the oxidation of NADPH was strongly inhibited, that of NADH was little affected, and the rates of oxidation of the two nucleotides were equal.

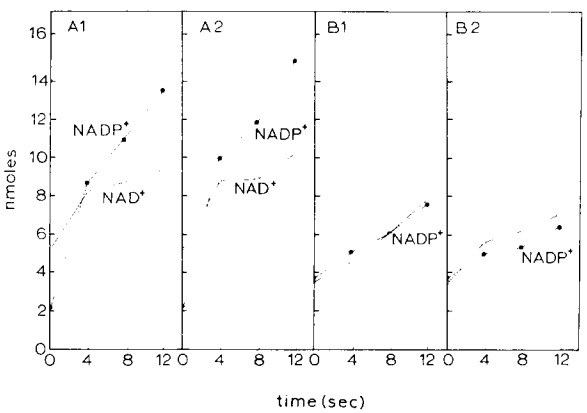

Fig. 5. Effect of energy supply and of malonate on the oxidoreduction pattern of the intramitochondrial nicotinamide nucleotides during the Krebs–Cohen dismutation in rat-liver mitochondria. Mitochondria (4 mg protein) were preincubated in 1 ml of a reaction mixture containing, in addition to the basic components, 20 μM dicoumarol and 15 μg oligomycin. After 2 min, 10 μmoles α-oxoglutarate and 8 mg dialyzed bovine serum albumin were added in Expts. A1 and A2, followed 1 min later by 1 μg rotenone. In Expts. B1 and B2, α-oxoglutarate and albumin were added together with 1 μg rotenone and 1 μmole Na_2S. In both cases, 2 min after the addition of α-oxoglutarate, the incubation beakers were transferred to an ice-water bath. After 1 min, 1 μmole arsenite was added, and 1 min later, 10 μmoles NH_4Cl were added in Expts. A1 and B1, and NH_4Cl *plus* 10 μmoles malonate in Expts. A2 and B2. For other experimental details, see Fig. 1.

This experiment indicates that high-energy intermediates of oxidative phosphorylation are required for maximal and preferential oxidation of NADPH by α-oxoglutarate (+ ammonia).

DE HAAN AND TAGER[22,23] have presented evidence that malonate stimulates the penetration of α-oxoglutarate into rat-liver mitochondria. Fig. 5 illustrates that although malonate slightly stimulated the oxidation of NADPH (and NADH) by α-oxoglutarate (+ ammonia) in Expt. A, it had no effect at all on the inhibition of NADPH oxidation in Expt. B. This shows that, in the uncoupled mitochondria, the penetration of α-oxoglutarate was not the rate-limiting step in the oxidation of NADPH (see also ref. 13).

DISCUSSION

In rat-liver mitochondria, energy promotes the transfer of hydrogens from α-oxoglutarate to α-oxoglutarate (*plus* ammonia)[13,24,25] and to α-oxoglutarate (*plus* CO_2)[13,26]. Although isolated glutamate dehydrogenase reacts with both NAD and NADP, the enzyme within the mitochondrion reacts preferentially with NADP (see INTRODUCTION and Figs. 1 and 5). The reductive carboxylation of α-oxoglutarate is catalysed by the NADP-linked isocitrate dehydrogenase[27,28], but has not been demonstrated[28] with the NAD-linked enzyme isolated from bovine heart*. In intact mitochondria, when the energy-linked transhydrogenase is suppressed, only NADPH is oxidized by α-oxoglutarate (*plus* CO_2).

The fact that both glutamate and isocitrate synthesis specifically require NADPH implies that the energy-linked transhydrogenation between NADH and $NADP^+$ will promote hydrogen transfer from NAD-linked substrates to α-oxoglutarate (*plus* ammonia or CO_2). However, closer examination[13] showed that energy promotes the oxidation of intramitochondrial reduced nicotinamide nucleotides by α-oxoglutarate (*plus* ammonia). This energy requirement is not related to the action of uncouplers as competitive inhibitors of the uptake of anionic substrates (see refs. 30–33).

A clue to this puzzling phenomenon was obtained by examining the effect of the energy level of the mitochondria on the oxidoreduction pattern of NAD and NADP during various NAD(P)-linked oxidoreductions. When the nicotinamide nucleotides were first oxidized by O_2, and subsequently reduced by α-oxoglutarate under coupled conditions, there was a preferential oxidation of NADPH by α-oxoglutarate (*plus* ammonia) and of NADH by acetoacetate or oxaloacetate. α-Oxoglutarate (*plus* CO_2) caused oxidation of both nucleotides (at 25°). When the cycle of oxidation and reduction was carried out under uncoupled conditions, the following facts emerged.

(1) The rate of NADPH oxidation by α-oxoglutarate (*plus* ammonia) was inhibited, and that of NADH was only slightly affected, if at all. Thus, the energy level of the mitochondria is directly involved in the oxidation of NADPH by α-oxoglutarate (*plus* ammonia). If the energy level were affecting only the penetration of α-oxoglutarate, it is difficult to understand why there should be a selective inhibition of NADPH oxidation.

(2) The rate of oxidation of NADPH by α-oxoglutarate (*plus* CO_2) was markedly inhibited and that of NADH suppressed (at 25°).

(3) The rate of NADH oxidation by acetoacetate or oxaloacetate was not affected.

It can be concluded that when hydrogen is transferred from a NAD-linked substrate to α-oxoglutarate (+ ammonia or CO_2) energy is required not only to promote the transhydrogenation between NADH and $NADP^+$, but also for the reaction of the NADPH, formed in this way, with glutamate dehydrogenase or the NADP-linked isocitrate dehydrogenase.

It is proposed that:

(i) Glutamate dehydrogenase and the NADP-linked isocitrate dehydrogenase

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* A slow reductive carboxylation of α-oxoglutarate has been observed with the NAD-linked isocitrate dehydrogenase from yeast, at high enzyme concentrations and low pH (ref. 29).

are in the same mitochondrial compartment, and α-oxoglutarate dehydrogenase forms NADH that is not freely available to the former two enzymes.

(ii) Energy promotes translocation of NADPH.

(iii) The energy-linked removal of NADPH from the site of the transhydrogenase gives a higher rate of NADPH formation.

(iv) The energy-linked translocation of NADPH is to the site of glutamate dehydrogenase and the NADP-linked isocitrate dehydrogenase. This promotes oxidation of NADPH by α-oxoglutarate (+ ammonia or CO_2).

The question remains of how energy could promote the translocation of NADPH from the site of the transhydrogenase to the site of glutamate dehydrogenase. It can be thought of as an energy-dependent translocation system that is distinct from the transhydrogenase enzyme. Alternatively, it is possible that the energy-rich bond imposes directionality on the catalytic activity of the transhydrogenase, perhaps by modifying the configuration of the enzyme, or by imposing a particular axis of rotation on the enzyme molecule (*cf.* ref. 34).

Our mechanism is similar in some respects to that postulated by KLINGENBERG[26] in 1963 (and since abandoned[35]). However, in KLINGENBERG's scheme the energy involvement was restricted to the movement of NADH. Our results on the nicotinamide nucleotide specificity of glutamate dehydrogenase can not be explained simply in terms of purine nucleotide effects (*cf.* FRIEDEN[36]). Firstly, the purine nucleotides have a greater effect on the reaction of isolated glutamate dehydrogenase with NAD than with NADP[36]. Secondly, in our experiments, conditions were chosen so as to change the level of the energy-rich bond in the mitochondria with minimal changes in the endogenous purine nucleotides. However, our mechanism and a regulation of dehydrogenase activity by purine nucleotide effects do not exclude one another, and may even cooperate in controlling metabolism.

In considering the possible physiological significance of the phenomena described in this paper, it should be remembered that glutamate and isocitrate synthesis in the mitochondria are important reductive reactions at the junction between the major terminal pathway of cellular oxidations (the tricarboxylic acid cycle) and anabolic processes in cells. The mechanism that switches the glutamate dehydrogenase and isocitrate dehydrogenase reactions from oxidation to synthesis are, therefore, of great relevance.

A central role in this metabolic event is played by the nicotinamide nucleotide transhydrogenase. This reaction constitutes a device by which reducing equivalents can be diverted from oxidation *via* the respiratory chain and transferred to $NADP^+$. A competitive relationship exists between the energy-linked transhydrogenase and oxidative phosphorylation[37,38], the competition being in favour of the transhydrogenase as the $NADH/(NADH+NAD^+)$ ratio and the phosphate potential ($ATP/ADP \cdot P_i$) increase. The increase in the $NADPH/(NADPH + NADP^+)$ ratio, brought about by the energy-linked transhydrogenase, and the translocation of NADPH to the matrix, promote the synthesis of isocitrate and/or glutamate. It is known that isocitrate oxidation by the isolated NAD-linked isocitrate dehydrogenase is activated by ADP and inhibited by ATP[39,40]. Furthermore, this reaction is inhibited by NADH, the effect being potentiated by NADPH[39]. Thus, conditions leading to increased synthesis of isocitrate *via* the NADP-linked isocitrate dehydrogenase would also lead to decreased isocitrate oxidation *via* the NAD-linked enzymes (see ref. 40). The result is that iso-

citrate and citrate accumulate and can be transported out of the mitochondria and utilized for fatty acid synthesis[41].

Glutamate (+ aspartate) may also accumulate. Indeed, no significant oxidative deamination of glutamate is observed when the level of NADPH is high[11,12]. Thus, the conditions that promote glutamate and isocitrate synthesis *via* NADPH prevent their oxidation *via* NAD^+ and the respiratory chain. This cooperation between equilibrium and regulation effects confers a physiological significance on the energy-linked transhydrogenase; otherwise the latter would be simply part of an energy-dissipating cycle.

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