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THE NICOTINAMIDE NUCLEOTIDE SPECIFICITY OF GLUTAMATE DEHYDROGENASE IN INTACT RAT-LIVER MITOCHONDRIA

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SUMMARY

1. The kinetics of oxidation of intramitochondrial reduced nicotinamide nucleotides by α-oxoglutarate plus ammonia in intact rat-liver mitochondria have been reinvestigated. It is demonstrated that the preferential oxidation of NADPH observed on addition of ammonia to mitochondria, preincubated under energized conditions in the presence of α-oxoglutarate, is due to a transhydrogenation catalysed by glutamate dehydrogenase rather than to an energy-dependent modification of the nicotinamide nucleotide specificity of the enzyme in intact mitochondria.

2. When mitochondria are preincubated at 25 °C under energized conditions in the presence of respiratory inhibitors with the substrates of glutamate dehydrogenase, an oxidation of NADPH, but not of NADH, is brought about by decreasing the reaction temperature. Both the rate of NADPH oxidation and the final steady-state mass-action ratio of nicotinamide nucleotides are dependent on the concentration of ammonia and on the final reaction temperature. A similar effect is observed when rhein is added to the reaction medium at 25 °C in order to inhibit the energy-linked transhydrogenase reaction.

3. In the presence of the substrates of glutamate dehydrogenase, intact rat-liver mitochondria catalyse an ATPase reaction due to the simultaneous activity of the energy-linked transhydrogenase and the non-energy-linked transhydrogenation catalysed by glutamate dehydrogenase.

4. These findings are discussed in relation to the nicotinamide nucleotide specificity of glutamate dehydrogenase and to a possible compartmentation of nicotinamide nucleotides in intact rat-liver mitochondria.

INTRODUCTION

Klingenberg and co-workers [1, 2] were the first to suggest that, in isolated rat-liver mitochondria, glutamate dehydrogenase (EC 1.4.1.3) reacts preferentially

Abbreviation: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

with NADP, in contrast to the isolated enzyme, which can react almost equally well with NADP and NAD. Borst [3] invoked the suggestion of Klingenberg in order to explain the observation that, in isolated rat-liver mitochondria, glutamate is oxidized mainly via the transamination pathway [4], and not via the deamination pathway, despite the high activity of glutamate dehydrogenase.

Experimental evidence in favour of the postulated NADP specificity of glutamate dehydrogenase was obtained from a series of studies by Tager and co-workers [5–10], and Papa and co-workers [11–14]. Oxidative deamination of glutamate was found to be closely related to the reduction level of NADP [6, 8, 9, 11, 12], which is, in turn, largely determined by the supply of reducing equivalents to NAD and, via the energy-linked transhydrogenase reaction [15–17], by the mitochondrial energy level. Studies on hydrogen transfer reactions involving glutamate dehydrogenase and other NAD- or NADP-linked dehydrogenases suggested that the energy-linked transhydrogenase plays a role in reactions where glutamate dehydrogenase is coupled to NAD-linked oxidoreductions but not when NADP-linked reactions are involved [5, 10, 12]. The most direct experimental evidence, however, was brought forward by Tager [7] and Papa et al. [12–14], who studied the kinetics of oxidation and reduction of intramitochondrial nicotinamide nucleotides on addition of α-oxoglutarate (plus ammonia) and glutamate, respectively. These authors observed that, in energized mitochondria, addition of the substrates of glutamate dehydrogenase mainly affects the oxidoreduction state of NADP, and not of NAD.

The postulated NADP-specificity of glutamate dehydrogenase in intact mitochondria was questioned by Krebs and co-workers [18, 19]. These authors measured the tissue levels in vivo of the substrates of glutamate dehydrogenase and (NAD-specific) β-hydroxybutyrate dehydrogenase (EC 1.1.1.30) and calculated that the mitochondrial free nicotinamide nucleotide pools reacting with these enzymes were at the same oxidoreduction state. We have subsequently observed [20] a similar phenomenon in isolated rat-liver mitochondria. Krebs and Veech [19] calculated that the redox potential of mitochondrial NAD under physiological conditions is sufficiently low to supply the reductive power required for glutamate synthesis. In other words, even at the relatively high oxidized state of mitochondrial NAD (as compared to NADP), very low levels of α-oxoglutarate and ammonia would be sufficient to inhibit effectively the oxidative deamination of glutamate (cf. ref. 21).

According to Krebs and Veech [19] glutamate dehydrogenase, being reactive with both NAD and NADP, should catalyse a non-energy-linked transhydrogenation. Furthermore, the combination of this reaction with the energy-linked transhydrogenase would result in a dissipation of energy, the physiological function of which is difficult to visualize.

This paper describes further studies on the nicotinamide nucleotide specificity of glutamate dehydrogenase in isolated rat-liver mitochondria, starting with a reinterpretation of the experimental results of Papa et al. [14] on the kinetics of oxidation of intramitochondrial NAD(P)H.

METHODS

Rat-liver mitochondria were prepared by the method of Hogeboom [22]. Submitochondrial particles from rat liver were prepared by sonication in 0.03 M

Tris–acetate buffer, pH 7.4, as described by Danielson and Ernster [17]. The supernatant of the 120 000 $\times g$ centrifugation step was used as a source of glutamate dehydrogenase.

The standard reaction medium for the experiments with intact mitochondria contained (unless otherwise indicated) 15 mM KCl, 5 mM $MgCl_2$, 2 mM EDTA, 50 mM Tris–HCl, 5 mM P_i and 25 mM sucrose. The final pH was 7.4. Further additions are indicated in the legends to tables and figures.

Incubations were generally carried out in 10-ml reaction vessels with magnetic stirring. The final volume of the incubation medium was 5–7 ml. At different reaction times, 1-ml samples were taken with an Eppendorf automatic sampler, type B 315. The reaction vessels were surrounded by a water-jacket, connected to two constant-temperature baths at different temperatures. By means of three-way stopcocks the temperature of the circulating water could be rapidly changed. The temperature of the incubation medium re-equilibrated within 1–2 min after changing the temperature of the circulating water.

Reactions were stopped with $HClO_4$ (3.5% final concentration) and, after removal of the protein, the extracts were neutralized with KOH. $KClO_4$ was removed in the cold. For the determination of NAD(P)H, reactions were quenched with 0.5 ml 1 M KOH in alcohol; the alkaline extracts were neutralized with a buffer containing 0.5 M triethanolamine–HCl, 0.4 M KH_2PO_4 and 0.1 M K_2HPO_4 (see ref. 23).

Assays

NAD(P)H, $NAD(P)^+$ and α-oxoglutarate were determined enzymically by the methods described in ref. 24. Protein was determined by the biuret method.

Transhydrogenase activity was measured as described by Teixeira da Cruz et al. [25]. The transhydrogenation catalysed by glutamate dehydrogenase was measured by the same method; in this case the assay medium contained 100 mM glutamate. 0.6 mM α-oxoglutarate, 5 mM NH_4Cl, 0.2 mM NADPH and 0.25 mM NAD^+.

Glutamate dehydrogenase activity was measured in a medium containing 50 mM potassium phosphate buffer (pH 7.4) and either 10 mM NH_4Cl, 5 mM α-oxoglutarate and 0.2 mM NAD(P)H or 20 mM glutamate and 0.4 mM $NAD(P)^+$.

Enzymes were obtained from Boehringer und Soehne, Mannheim. Rhein was a gift from Sandoz Ltd, Basle.

RESULTS

Kinetics of oxidation of intramitochondrial nicotinamide nucleotides

As shown in Fig 1, we have repeated and extended the experiments of Papa et al. [14] on the kinetics of oxidation of intramitochondrial NAD(P)H. In the experiment of Fig. 1A, mitochondria were preincubated in the presence of α-oxoglutarate and rotenone in order to reduce the nicotinamide nucleotides. Due to the action of the energy-linked transhydrogenase, NADP was considerably more reduced than NAD. Subsequently, the reaction temperature was decreased to 0 °C in order to inhibit selectively the transhydrogenase reaction (which has been reported to have a relatively high temperature coefficient [26]). After 1 min further oxidation of α-oxoglutarate was prevented by adding arsenite. Addition of NH_4Cl under these conditions led to a rapid and preferential oxidation of NADPH, while NADH was oxidized

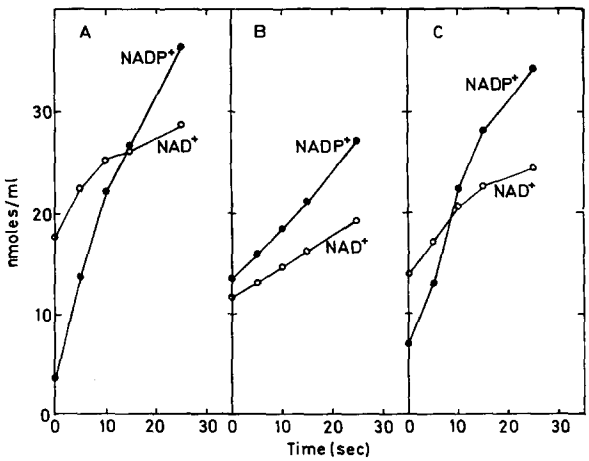

Fig. 1. Effect of FCCP plus oligomycin on the oxidation of intramitochondrial nicotinamide nucleotides in rat-liver mitochondria. Mitochondria (7.2 mg protein per ml incubation medium) were preincubated at 25.8 °C in the standard reaction medium plus 5 mM P_i, 2 mM ADP and 5 mM malonate. In Fig. 1B, 1.7 μM FCCP and 5 μg oligomycin were also present. After 2 min, 10 mM α-oxoglutarate was added, followed after 1 min by 10 μg rotenone. Subsequently, the reaction temperature was decreased to 2.5 °C and 1 mM arsenite was added. In Fig. 1C, 1.7 μM FCCP and 5 μg oligomycin were added 1 min after the addition of arsenite. Reactions were started 2 min after the addition of arsenite by adding 3 mM NH_4Cl. The final incubation volume was 6.0 ml. At the times indicated 1-ml samples were taken from the incubation medium for the determination of nicotinamide nucleotides.

only slowly. When carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) plus oligomycin were included in the reaction medium during the preincubation, addition of NH_4Cl resulted in a slow oxidation of NADPH and NADH at approximately similar rates (Fig. 1B). According to Papa et al. [14], these findings convincingly demonstrate that, under energized conditions, glutamate dehydrogenase reacts preferentially with NADP and not with NAD.

In the experiment of Fig. 1C, the preincubation was carried out under the same conditions as in Fig. 1A, but FCCP and oligomycin were added after the reaction temperature was decreased to 0 °C. The preincubation was continued for 2 more min in order to ensure a complete deenergization of the mitochondria. Due to the low activity of the transhydrogenase reaction at this temperature [26], NADP remained more reduced than NAD. (The small increase in the level of $NADP^+$ in Fig. 1C as compared to Fig. 1A was presumably caused by the remaining transhydrogenase activity). On addition of NH_4Cl under these conditions, NADPH was again oxidized at a much higher rate than NADH.

In Fig. 2 the changes in the quotient of oxidized to reduced NAD and NADP (i.e. the mass-action ratio of the transhydrogenase reaction), calculated from the experiments of Fig. 1, are plotted. In Fig. 2A the addition of ammonia appears to result in a rapid equilibration of the reduction levels of NAD and NADP; after 25 s reaction time, the mass-action ratio of nicotinamide nucleotides approaches unity. In the experiment of Fig. 2B, where FCCP and oligomycin were included during the preincubation, the reduction levels of NAD and NADP were already equal at the time of addition of NH_4Cl. No significant changes in the mass-action ratio occurred

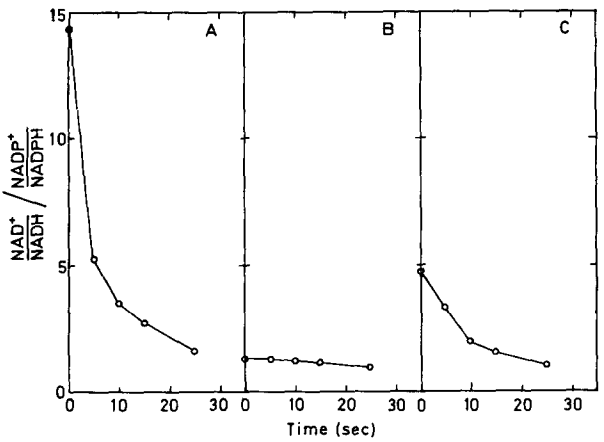

Fig. 2. Changes in the mass-action ratio of nicotinamide nucleotides in rat-liver mitochondria on addition of NH_4Cl. The levels of oxidized NAD and NADP were taken from the corresponding experiments of Fig. 1. Reduced nicotinamide nucleotides were obtained from parallel incubations where reactions were stopped with alkali.

throughout the reaction period.

When FCCP plus oligomycin were added after decreasing the reaction temperature (Fig. 2C), the addition of NH_4Cl again brought about a rapid decrease in the mass-action ratio of nicotinamide nucleotides until a value of one was approached.

These findings indicate that on addition of NH_4Cl under conditions where NADP is more reduced than NAD, glutamate dehydrogenase catalyses a transhydrogenation between NAD and NADP, which implies that the enzyme is reactive with both nucleotides.

Thus, it appears that the observed pattern of oxidation of nicotinamide nucleotides is not so much determined by an energy-dependent change in the nicotinamide nucleotide specificity of glutamate dehydrogenase, as proposed by Papa et al. [14], but rather is kinetic in origin, the preferential oxidation of NADPH being due to the high reduction level of NADP relative to that of NAD.

Temperature dependence of transhydrogenation reactions

A key role in the interpretation of the kinetic experiments, discussed above, is played by the difference in temperature coefficients of the transhydrogenation between NAD and NADP, catalysed by the energy-linked transhydrogenase and by glutamate dehydrogenase, respectively. This point was investigated in the experiment of Table I. Rat-liver mitochondria were sonicated for 30 s in Tris–acetate buffer as described by Danielson and Ernster [17] and centrifuged for 60 min at $120\,000 \times g$. The supernatant was carefully removed and used as a source of glutamate dehydrogenase. In this fraction the transhydrogenase activity in the absence of substrates of glutamate dehydrogenase was negligible. The pellet was washed twice in 0.03 M Tris–acetate buffer (pH 7.4) and used as a source of transhydrogenase. The final suspension contained no significant glutamate dehydrogenase activity. The transhydrogenation, catalysed by transhydrogenase and by glutamate dehydrogenase in the particle suspension and the supernatant fraction, respectively, was assayed in the temperature range from 8–30

TABLE I

ACTIVATION ENERGIES OF TRANSHYDROGENATION REACTIONS IN RAT-LIVER MITOCHONDRIAL FRACTIONS

A submitochondrial particle fraction P (19 mg protein/ml), containing transhydrogenase activity, and a soluble fraction S (3.7 mg protein/ml), containing glutamate dehydrogenase activity, were obtained from sonicated rat-liver mitochondria, as described in the text. Transhydrogenase and glutamate dehydrogenase activities were measured at different temperatures, ranging from 8–30 °C, as described under Methods. ATP (3 mM) and ADP (1 mM) were added to the assay medium where indicated. Arrhenius' activation energies (E_A) were calculated from a semilogarithmic plot of the reaction rate against the reciprocal of the absolute temperature. No deviations from linearity were observed for the reactions studied. Abbreviation: α-OG, α-oxoglutarate.

Fraction	Reaction	Further additions	E_A (kcal/mole)
P	$NADPH+NAD^+ \rightarrow NADP^+ + NADH$	–	26
P	$NADH+NADP^+ \rightarrow NAD^+ + NADPH$	ATP	29
S	$\alpha\text{-OG}+NH_3+NADPH \rightarrow Glu+NADP^+$ $Glu+NAD^+ \rightarrow \alpha\text{-OG}+NH_3+NADH$	–	1.4
S	$\alpha\text{-OG}+NH_3+NADPH \rightarrow Glu+NADP^+$ $Glu+NAD^+ \rightarrow \alpha\text{-OG}+NH_3+NADH$	ADP	5.4
S	$\alpha\text{-OG}+NH_3+NAD(P)H \rightarrow Glu+NAD(P)^+$	ADP	17.4
S	$Glu+NAD(P)^+ \rightarrow \alpha\text{-OG}+NH_3+NAD(P)H$	ADP	12.7

°C as described under Methods. As shown in Table I, the reverse and forward (ATP-supported) transhydrogenase reaction have an activation energy of 26 and 29 kcal/mole, respectively. This result agrees well with the value of 22 kcal/mole obtained by Hommes and Estabrook [26] in submitochondrial particles from beef heart. The supernatant fraction of sonicated rat-liver mitochondria catalysed a transhydrogenation, which was dependent on the substrates of glutamate dehydrogenase (cf. ref. 27) and was activated by ADP, especially at higher reaction temperatures. An activation energy of 1.4 and 5.4 kcal/mole was found in the absence and presence of ADP, respectively. The activation energy of the glutamate dehydrogenase-dependent transhydrogenation (in the presence of ADP) is approximately equal to the difference between the forward and reverse glutamate dehydrogenase reaction with either NAD or NADP.

Thus, it appears that glutamate dehydrogenase can, indeed, catalyse a transhydrogenation between NAD and NADP (in contrast to some other enzymes that are reactive with both NAD and NADP [28, 29]); under the assay conditions used, this reaction is characterized by a much lower activation energy than the reactions catalysed by the particle-bound nicotinamide nucleotide transhydrogenase.

The role of glutamate dehydrogenase and transhydrogenase in establishing the oxidoreduction state of NAD and NADP

In the experiment of Fig. 3, mitochondria were preincubated at 25.8 °C in the presence of rotenone (to inhibit electron transport through the respiratory chain), arsenite (to inhibit α-oxoglutarate oxidation) and malonate (to make the mitochondrial membrane freely permeable to α-oxoglutarate) [30]. ATP was added as a source

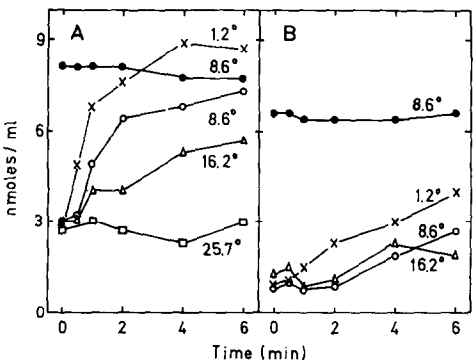

Fig. 3. Oxidation of NADPH in intact rat-liver mitochondria induced by decreasing the reaction temperature. Mitochondria (2.6 mg protein per ml incubation medium) were preincubated at 25.8 °C in the standard reaction medium plus 5 mM P_i, 2.5 mM ATP, 5 mM malonate, 2 mM arsenite and 2 μg/ml rotenone. After 2 min, 16 mM glutamate, 0.15 mM α-oxoglutarate and either 1.0 mM NH_4Cl (Expt A) or 0.1 mM NH_4Cl (Expt B) were added. The final incubation volume was 6.5 ml. The preincubation was continued for 3 min before the reaction temperature was decreased as described under Methods. The numbers at each curve indicate the final reaction temperature (which was established within 1–2 min). At the times indicated 1-ml samples were taken from the incubation medium for the determination of NAD^+ and $NADP^+$. ●–●, NAD^+ at 8.6 °C (the levels of NAD^+ at other reaction temperatures were not significantly different); other symbols, $NADP^+$.

of energy. The substrates of glutamate dehydrogenase were added and the preincubation was continued for 3 min. Under these conditions, NADP is highly reduced compared to NAD, due to the energy-linked transhydrogenase reaction. As demonstrated previously [20], the intramitochondrial concentration ratio of substrates of glutamate dehydrogenase presumably is close to equilibrium with the oxidoreduction state of NAD (cf. refs 18, 19). After the preincubation the reaction temperature was changed to various levels (approximately 1–2 min were required for establishing the temperature indicated). The levels of NAD^+ and $NADP^+$ were measured after different reaction times. As shown in Fig. 3A, when the preincubation was carried out in the presence of 16 mM glutamate, 0.15 mM α-oxoglutarate and 1 mM NH_4Cl, the decrease in reaction temperature brought about an oxidation of NADPH. In contrast, no significant changes were observed in the level of NAD^+. The rate of oxidation of NADPH and the final steady-state reduction level are dependent on the reaction temperature. Control experiments (not shown) demonstrated that on subsequently increasing the reaction temperature the original reduction level of $NADP^+$ was restored. When the preincubation was carried out in the presence of 0.1 mM NH_4Cl under otherwise identical conditions (Fig. 3B), changing the reaction temperature had much less effect: a significant oxidation of NADPH was obtained only when the temperature was decreased to 1.2 °C.

The effect of the ammonia level was investigated further in the experiment of Fig. 4. After preincubation under similar conditions with various concentrations of NH_4Cl, the reaction temperature was decreased to 0.2 °C. The degree of oxidation of NADPH, brought about by decreasing the temperature, was dependent on the concentration of ammonia. No significant oxidation of NADPH was observed when NH_4Cl was omitted from the reaction medium or when the temperature was kept at

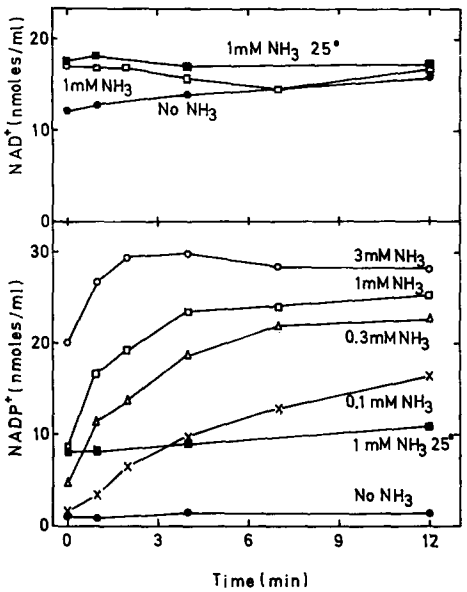

Fig. 4. Oxidation of NADPH induced by decreasing the reaction temperature, at different concentrations of ammonia. Reaction conditions as described in the legend to Fig. 3; 7.0 mg mitochondrial protein were added per ml incubation medium (final volume, 7.5 ml). After 2 min preincubation, 20 mM glutamate, 0.15 mM α-oxoglutarate and different concentrations of ammonia were added as indicated. The preincubation was continued for 3 min before decreasing the temperature to 0.2 °C. (In a control experiment the temperature was kept at 25 °C throughout the reaction time). The levels of NAD^+ found at other concentrations of ammonia were not significantly different from the values obtained at 1 mM NH_4Cl. The mitochondria contained 19.8 nmoles $NAD^+ + NADH$ and 31.7 nmoles $NADP^+ + NADPH$ (mean values from five incubations in which both oxidized and reduced NAD(P) were determined).

25.8 °C (in the presence of 1 mM NH_4Cl). Again, no significant changes in the level of NAD^+ are caused by the decrease in temperature.

In Fig. 5 the values of $NAD^+/NADH$ to $NADP^+/NADPH$ (i.e. the mass-action ratio of the transhydrogenase reaction), calculated from the experiment of Fig. 4, are plotted. It appears that the steady-state mass-action ratio of nicotinamide nucleotides, obtained after equilibration at the low temperature, approaches the equilibrium constant of the non-energy-linked transhydrogenase reaction (0.6 at ionic strength 0.25; ref. 31) when increasing concentrations of ammonia are present. A similar tendency can be observed at 25 °C, as appears in the initial values measured after the preincubation, but before the temperature is changed; here, however, the effect is apparent only at the higher levels of ammonia.

These results suggest that, under the conditions used here, the oxidoreduction state of NADP relative to that of NAD is determined by the simultaneous action of the energy-linked transhydrogenase and the (non-energy-linked) transhydrogenation catalysed by glutamate dehydrogenase. The activity of the latter reaction is dependent on the concentration of ammonia (the K_m for ammonia of glutamate dehydrogenase is about 3 mM; ref. 24), whereas the relative activity of the energy-linked transhydrogenase is decreased at lower temperatures.

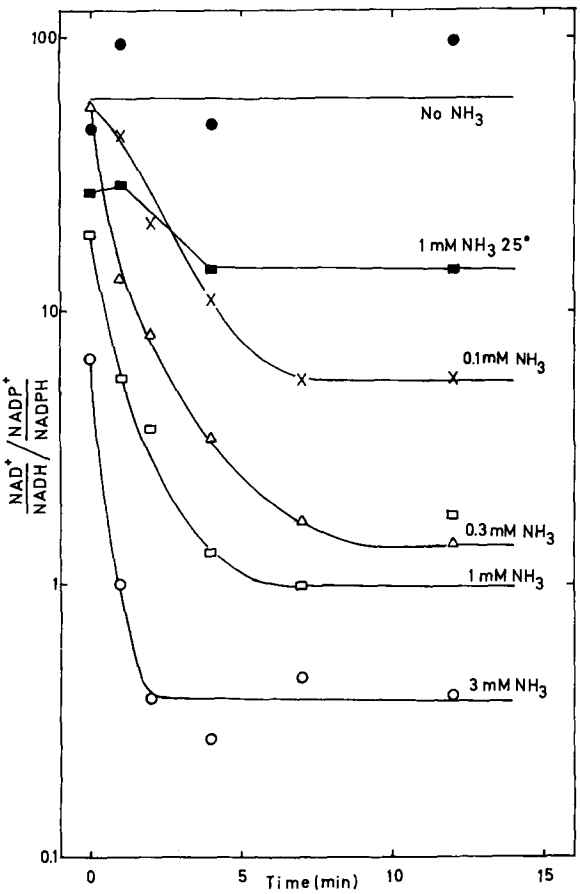

Fig. 5. Changes in the mass-action ratio of nicotinamide nucleotides induced by decreasing the reaction temperature. Mass-action ratios were calculated from the data of Fig. 4, using the mean values for total NAD and NADP, indicated in the legend.

Further support for this interpretation was obtained by using rhein as an inhibitor of the transhydrogenase reaction in intact mitochondria (cf. ref. 32). As shown in Fig. 6, when mitochondria were preincubated in the presence of glutamate, α-oxoglutarate and ammonia, addition of 100 mM rhein resulted in a rapid oxidation of NADPH, particularly at the higher level of ammonia, while the reduction level of NAD was unaffected. In the absence of rhein the level of NAD^+ and $NADP^+$ remained constant (not shown). In the control experiment in the absence of added ammonia (Fig. 6C), the addition of rhein resulted in an oxidation of NADH. The reason for this is not clear. However, in contrast to the experiments of Fig. 6A and 6B, the NADP remained considerably more reduced than NAD under these conditions.

Effect of substrates of glutamate dehydrogenase on ATPase activity in rat-liver mitochondria

The simultaneous activity of the ATP-supported energy-linked transhydrogen-

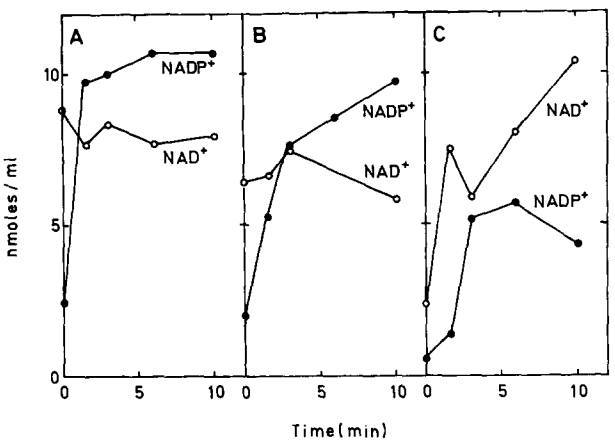

Fig. 6. Effect of rhein on the reduction level of NAD(P) in intact rat-liver mitochondria. Reaction conditions as described in the legend to Fig. 3; 2.2 mg mitochondrial protein were added per ml incubation medium (final volume, 6.6 ml). After 2 min preincubation, a mixture containing (final concentrations) 20 mM glutamate, 0.15 mM α-oxoglutarate and 1 mM NH_4Cl (Expt A), 0.3 mM NH_4Cl (Expt B) or no NH_4Cl (Expt C) was added and the preincubation was continued for 3 min. Reactions were started by addition of 100 μM rhein (added from a 5-mM stock solution at pH 8.5).

ase reaction and the glutamate dehydrogenase-dependent transhydrogenation should result in a continuous dissipation of energy, and hence an ATPase activity dependent on the presence of the substrates of glutamate dehydrogenase.

In the experiment of Table II, mitochondria were incubated at 25 °C under similar reaction conditions as used in the previous sections, with different concentrations of NH_4Cl. In addition, phosphoenolpyruvate and pyruvate kinase (EC 2.7.1.40)

TABLE II

EFFECT OF SUBSTRATES OF GLUTAMATE DEHYDROGENASE ON ATPase ACTIVITY IN ISOLATED RAT-LIVER MITOCHONDRIA

The incubation medium (3 ml) contained the standard components, plus 2 mM arsenite, 5 mM malonate, 5 μg rotenone, 2.5 mM ATP, 1 mM phosphoenolpyruvate, 0.1 mM NADH, 20 μg lactate dehydrogenase and 40 μg pyruvate kinase. The enzyme preparations were made ammonia-free by dialysis against 0.01 M phosphate buffer, pH 7.4. Further additions or omissions from the standard medium as indicated. Reactions were performed in a cuvette in the Aminco-Chance dual-wavelength spectrophotometer (wavelength pair 350–375 nm) at 30 °C. After 2 min preincubation to remove endogenous ADP and pyruvate, reactions were started by addition of 0.34 mg mitochondrial protein. Abbreviation: βOHB, β-hydroxybutyrate.

Glutamate (mM)	α-Oxoglutarate (mM)	Further additions or omissions	ATPase activity (nmoles/min per mg protein) at NH_4Cl concentration of:			
			0 mM	0.3 mM	3.3 mM	33 mM
16	0	None	42	47	44	48
16	0.2	None	47	54	71	89
0	0.5	None	46	53	52	51
0	0.2	βOHB (32 mM)	51	53	67	91
16	0.2	No malonate	31	35	42	58
0	0	FCCP (1.7 μM)	154	–	–	–

were added as an ATP-regenerating system, and pyruvate was trapped with lactate dehydrogenase (EC 1.1.1.27) and NADH. The rate of NADH oxidation, followed spectrophotometrically, was taken as a measure of the ATPase activity. No oxidation of NADH was observed in the absence of pyruvate kinase or ATP (not shown). As shown in Table II, the addition of increasing concentrations of NH_4Cl resulted in a considerable increase in the ATPase activity under these conditions. The ammonia-induced ATPase was dependent on the presence of α-oxoglutarate. No significant differences were observed when the α-oxoglutarate concentration was varied between 0.15 and 2.0 mM. When malonate (required for a free permeability of the mitochondrial membrane to α-oxoglutarate) [30] was omitted from the reaction medium, the ammonia-dependent ATPase activity was partially inhibited.

The presence of glutamate was also required for the stimulation of ATPase activity by NH_4Cl. This demonstrates that the effect is due neither to a direct oxidation of added NADH by α-oxoglutarate plus ammonia, catalysed by glutamate dehydrogenase from damaged mitochondria, nor to a reduction of α-oxoglutarate plus ammonia by endogenous hydrogen donors. The requirement for glutamate indicates that an effective transhydrogenation between NAD and NADP can only be catalysed by glutamate dehydrogenase when all substrates of the reaction are present. In addition, glutamate may be required to ensure that the reduction level of NAD is sufficiently high to allow an effective reaction of the energy-linked transhydrogenase. When glutamate was replaced by β-hydroxybutyrate, the presence of NH_4Cl gave rise to a similar increase in ATPase activity. In this case, however, the reaction involves a net synthesis of glutamate, as appears from the disappearance of α-oxoglutarate (see Fig. 7). In contrast, in the presence of glutamate the ammonia-induced ATPase activity was not accompanied by a disappearance of α-oxoglutarate. This demonstrates that, under these conditions, the substrates of glutamate dehydrogenase have a catalytic function in the ammonia-induced ATPase activity.

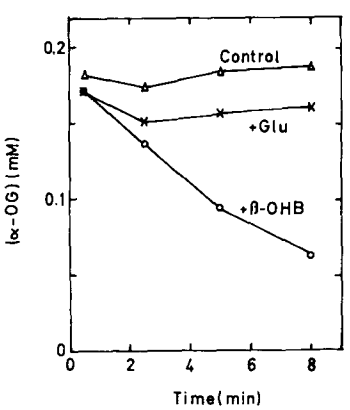

Fig. 7. Concentration of α-oxoglutarate during ammonia-dependent ATPase activity in intact rat-liver mitochondria. Reaction conditions as described in the legend to Table II, except that the concentration of NADH was 200 μM, lactate dehydrogenase was 80 μg/ml and pyruvate kinase was 160 μg/ml. In addition, NH_4Cl (1 mM) and α-oxoglutarate (α-OG, 0.2 mM) were present. Glutamate (Glu, 16 mM) and DL-β-hydroxybutyrate (β-OHB, 33 mM) were added as indicated. At different reaction times, 1-ml samples were acidified with $HClO_4$, and, after neutralization as described under Methods, assayed for α-oxoglutarate.

DISCUSSION

The data presented in this paper allow the following conclusions to be drawn:

(1) In intact rat-liver mitochondria, under the conditions used here, glutamate dehydrogenase can react with both NAD and NADP, irrespective of the mitochondrial energy state.

(2) When the substrates of the reaction are present in sufficiently high concentration, the enzyme catalyses a transhydrogenation that tends to equalize the oxidoreduction state of NAD and NADP.

(3) Under conditions where both the energy-linked transhydrogenase and the glutamate dehydrogenase-dependent transhydrogenation occur simultaneously, and when the contributions of other NAD(P)-linked oxidoreduction reactions are limited, the reduction level of NADP relative to that of NAD is determined by the relative activities of the two competing reactions. (The reduction level of NAD is set mainly by the concentration ratio of oxidized and reduced substrates of glutamate dehydrogenase under the experimental conditions used here; cf. ref. 20).

(4) The simultaneous occurrence of the two competing transhydrogenation reactions constitutes an energy-dissipating cycle, apparent as an increased ATPase activity, in which the substrates of glutamate dehydrogenase act catalytically.

As shown in the experiments of Figs 1 and 2, the relative rate of glutamate synthesis with NADH or NADPH is determined by the difference in reduction level between NAD and NADP. Thus, under conditions where NADP is more reduced than NAD, the reducing equivalents for reduction of α-oxoglutarate plus ammonia are mainly supplied by NADPH. Similarly, the ammonia-dependent ATPase presumably takes place by the coupling of an NADPH-linked reduction of α-oxoglutarate plus ammonia with an NAD^+-linked oxidation of glutamate and the energy-consuming reduction of $NADP^+$ by NADH via the transhydrogenase. Apparently then, the occurrence of the energy-linked transhydrogenase brings about a preferentially NADP-linked reaction of glutamate dehydrogenase with respect to glutamate synthesis. In contrast, on similar kinetic considerations the enzyme may be expected to react preferentially with NAD^+ in glutamate oxidation. Thus, the results presented here are in agreement with a model in which the action of the energy-linked transhydrogenase results in a functional "compartmentation" of the nicotinamide nucleotides in their reactivity with glutamate dehydrogenase, although the enzyme itself is reactive with both NAD and NADP. Most of the experimental evidence that has been brought forward in favour of a postulated NADP specificity of glutamate dehydrogenase in intact mitochondria [5-14] can be accommodated in this model (cf. ref. 21). Moreover, it also provides a suitable explanation for the previously reported observations [20] that both the glutamate and β-hydroxybutyrate redox couples in intact mitochondria are found to be close to equilibrium with the mass-action ratio of total mitochondrial NAD under conditions where the total NADP is at a much higher reduction level (cf. refs 18, 19).

The fact that both the rate of the glutamate dehydrogenase-dependent ATPase reaction (Table II) and the rate of oxidation of NADPH occurring on decreasing the temperature (Figs 3–5) or on addition of rhein (Fig. 6) is greatly dependent on the concentration of ammonia present in the reaction medium indicates that the oxidation of NADPH by α-oxoglutarate plus ammonia is the rate-limiting step in the over-all

processes. In view of the high potential capacity of the glutamate dehydrogenase reaction in rat-liver mitochondria (in sonicated rat-liver mitochondria the rate of NADPH oxidation in the presence of 10 mM NH_4Cl and 2 mM α-oxoglutarate is in the order of 100 nmoles/min per mg protein and 800 nmoles/min per mg protein in the absence and presence of 1 mM ADP, respectively) it is surprising that the rate of the ammonia-dependent ATPase is limited to no more than about 40 nmoles/min per mg protein (Table II). However, since glutamate dehydrogenase is known to be subject to a complex allosteric regulation (see ref. 33), the activity of the enzyme may be considerably lower under the conditions prevailing in the mitochondrial matrix. Thus it is difficult to interpret these results on a quantitative basis.

Nevertheless, the present results do not exclude the possibility that, in addition to the suggested kinetic model of compartmentation of nicotinamide nucleotides, binding or complexing of part of the mitochondrial $NAD(P)^+$ or NAD(P)H occurs (cf. refs 32, 34, 35) that affects their reactivity with glutamate dehydrogenase. At present, however, no direct evidence for this type of compartmentation is available.

The finding that the glutamate dehydrogenase and energy-linked transhydrogenase activities in intact mitochondria can be coupled to produce an energy-dissipating cycle raises two important questions. Firstly, although the present results were obtained with isolated mitochondria under highly artificial conditions, it may not be immediately evident how the simultaneous activity of the two reactions could be prevented in vivo. The most likely explanation is that the K_m for ammonia in the glutamate dehydrogenase reaction is so high, and the concentration of ammonia under normal conditions so low, that no significant ATPase activity occurs (cf. Table II). Indeed, one of the causes of ammonia toxicity may be a dissipation of energy; this possibility is being investigated.

The second question is, has the process described in this paper physiological significance? It may be possible that the functional separation of the processes of glutamate synthesis and glutamate oxidation with respect to nicotinamide nucleotide specificity (which is reminiscent of comparable functional differentiation in microorganisms; cf. ref. 33) provides an advantage to the organism that counterbalances a certain dissipation of metabolic energy.

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