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The NAD-linked isocitrate dehydrogenase activity in rat-liver mitochondria

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SUMMARY

A recent claim in the literature (Moyle, J. and Mitchell, P. (1973) *Biochem. J.* 132, 571–585) that the NAD-dependent isocitrate oxidation observed in extracts of rat-liver mitochondria proceeds entirely *via* the NADP-linked isocitrate dehydrogenase and nicotinamide nucleotide transhydrogenase, and that rat-liver mitochondria contain no NAD-linked isocitrate dehydrogenase, has been examined by using palmityl-CoA as a selective inhibitor of transhydrogenase and ADP as a selective activator of the NAD-linked isocitrate dehydrogenase. The results unambiguously demonstrate that the NAD-dependent oxidation of isocitrate observed under the conditions employed by Moyle and Mitchell proceeds predominantly *via* the NAD-linked isocitrate dehydrogenase. It is also shown that, by an unfortunate choice of assay conditions, these authors have considerably overestimated the rate of the transhydrogenase reaction.

The existence of a NAD-specific isocitrate dehydrogenase in animal tissues has been a matter of controversy for several years (*cf.* ref. 1). The question was resolved by the recognition of the specific kinetic properties of the enzyme (as distinct from the NADP-specific isocitrate dehydrogenase) (see ref. 2 for a review) and by the complete separation of the NAD- and NADP-linked isocitrate dehydrogenases from rat- and rabbit-liver mitochondria[3]. Further purifications of the NAD-linked isocitrate dehydrogenases from porcine liver[4] and bovine heart mitochondria[5] have been reported.

Recently, however, the existence of a NAD-specific isocitrate dehydrogenase in rat-liver mitochondria was questioned again, by Moyle and Mitchell[6]. On the basis of a number of indirect experimental observations, they concluded that the NAD-linked isocitrate dehydrogenase activity, found in the supernatant fraction of sonicated rat-liver mitochondria, is the result of a combined action of the NADP-specific isocitrate

dehydrogenase and the nicotinamide nucleotide transhydrogenase.

The experimental evidence brought forward by Moyle and Mitchell[6] in favour of this postulate is three-fold: (1) the NAD(P) transhydrogenase activity was found to be faster than the NAD-linked isocitrate dehydrogenase activity in soluble fractions of sonicated rat-liver mitochondria; (2) the rate of the total isocitrate dehydrogenase in the

TABLE I

ENZYME ACTIVITIES IN SOLUBLE FRACTIONS OF SONICATED RAT-LIVER MITOCHONDRIA

Reactions were performed in a medium containing 250 mM sucrose, 50 mM Tris–acetate, pH 7.5 and 5 μg rotenone in a final volume of 3 ml. The assay system for isocitrate dehydrogenase contained in addition 330 μM NAD^+ or $NADP^+$, 20 μl of the soluble fraction and, where indicated, 7.5 mM $MgCl_2$, 0.5 mM ADP and 10 μM palmityl-CoA. Reactions were started by addition of 2 mM isocitrate. The assay system for transhydrogenase contained, in addition to the standard components 100 μM NADPH, 330 μM NAD^+, 10 mM pyruvate and 0.1 μg lactate dehydrogenase. $MgCl_2$, ADP and palmityl-CoA were added where indicated. Reactions were started by addition of 20 μl of the soluble fraction. Changes in absorbance were followed at 366 nm in an Aminco-Chance dual-wavelength spectrophotometer.

Preparation	*Reductant*	*Oxidant*	*Further additions*	*Activity (nmoles/min per mg protein)*
GSH–P_i–ADP	NADPH	NAD^+	None	5.4
	NADPH	NAD^+	$MgCl_2$, ADP	5.0
	NADPH	NAD^+	Palmityl-CoA	0.4
	NADPH	NAD^+	$MgCl_2$, ADP, palmityl-CoA	0.1
	Isocitrate	NAD^+	$MgCl_2$	14.1
	Isocitrate	NAD^+	$MgCl_2$, ADP	19.8
	Isocitrate	NAD^+	$MgCl_2$, palmityl-CoA	15.9
	Isocitrate	NAD^+	$MgCl_2$, ADP, palmityl-CoA	19.4
	Isocitrate	$NADP^+$	$MgCl_2$	100
	Isocitrate	$NADP^+$	$MgCl_2$, ADP	91
	Isocitrate	$NADP^+$	$MgCl_2$, palmityl-CoA	81
	Isocitrate	NAD^+ *plus* $NADP^+$	$MgCl_2$, ADP	114
	Isocitrate	NAD^+ *plus* $NADP^+$	$MgCl_2$, ADP, palmityl-CoA	97
Sucrose–Tris	NADPH	NAD^+	$MgCl_2$, ADP	8.5
	NADPH	NAD^+	$MgCl_2$, ADP, palmityl-CoA	0.4
	Isocitrate	NAD^+	$MgCl_2$	8.9
	Isocitrate	NAD^+	$MgCl_2$, palmityl-CoA	11.0
	Isocitrate	NAD^+	$MgCl_2$, ADP, palmityl-CoA	18.6
	Isocitrate	$NADP^+$	$MgCl_2$	114
	Isocitrate	$NADP^+$	$MgCl_2$, palmityl-CoA	120

presence of NAD^+ *plus* $NADP^+$ was found to be equal to that in the presence of $NADP^+$ alone; (3) in a preparation in which the NAD-linked isocitrate dehydrogenase activity was decreased, either by aging in the absence of added ADP, or by treatment with nucleotide pyrophosphatase, addition of 80 μM NADPH restored the original NAD-linked isocitrate dehydrogenase activity.

A reinvestigation of these points, using palmityl-CoA as a specific inhibitor of the transhydrogenase reaction[7] has led us to the conclusion that this reaction is not involved in the NAD-linked isocitrate dehydrogenase activity measured in the soluble fraction from sonicated rat-liver mitochondria.

Rat-liver mitochondria, prepared by the method of Hoogeboom[8], were suspended in a medium containing either 250 mM sucrose *plus* 10 mM Tris–HCl, pH 7.5 (sucrose–Tris preparation), or 100 mM potassium phosphate buffer, pH 7.2, 10 mM GSH and 1 mM ADP (GSH–P_i–ADP preparation). Mitochondrial suspensions (30 mg protein/ml) were sonicated at 0 °C for 2 min, using a Schollinger sonicator. Membrane fragments were removed by centrifugation for 60 min at 75 000 × *g*. The protein content of the supernatant fraction was 23 mg/ml. Protein was determined by the biuret method.

In Table I the activities of the transhydrogenase and NAD- and NADP-linked isocitrate dehydrogenase reactions are given, as measured in the soluble fractions of rat-liver mitochondria, sonicated in sucrose–Tris or GSH–P_i–ADP media. The following points emerge:

(1) The NAD-linked isocitrate dehydrogenase activity amounts to 7–20% of the activity of the NADP-linked enzyme, depending on the sonication medium and the assay conditions. This result corresponds well with the findings of Moyle and Mitchell, if one takes into account that these authors measured the NAD-linked isocitrate dehydrogenase activity under slightly suboptimal conditions (*i.e.* in the absence of the activator ADP, and in the presence of 0.4 mM KCN; this concentration of KCN was found to inhibit the NAD-linked activity by 15% (*cf.* ref. 9).

(2) The transhydrogenase activity was generally lower than the NAD-linked isocitrate dehydrogenase activity; only in the sucrose–Tris preparation was the transhydrogenase activity found to be approximately equal to the NAD-linked isocitrate dehydrogenase activity, measured in the absence of ADP.

(3) Addition of palmityl-CoA inhibits the transhydrogenase activity by more than 90%. A slight inhibition of the NADP-linked isocitrate dehydrogenase activity was also observed. The NAD-linked isocitrate dehydrogenase activity was not affected when assayed in the presence of ADP; in the absence of ADP some activation by palmityl-CoA was observed. This result demonstrates clearly that the transhydrogenase is not involved in the NAD-linked isocitrate dehydrogenase activity.

(4) ADP and $MgCl_2$ have been found to inhibit the transhydrogenase activity[10]. In contrast, these compounds activate the NAD-linked isocitrate dehydrogenase[1,2]. Under the conditions used here, ADP and $MgCl_2$ have only a small effect on the transhydrogenase activity. The NAD-linked isocitrate dehydrogenase activity, in particular in the sucrose–Tris preparation, is stimulated strongly by ADP; the activity in the presence of ADP is

considerably higher than the transhydrogenase activity, and comparable to the activity of the GSH–P_i–ADP preparation.

(5) In contrast to the findings of Moyle and Mitchell[6], the total (NAD-linked *plus* NADP-linked) isocitrate dehydrogenase activity, as measured in the presence of NAD^+ *plus* $NADP^+$, was approximately equal to the sum of the NAD-linked and NADP-linked activities, when measured under comparable conditions. Additivity was also observed when palmityl-CoA was present. However, in view of the relatively small contribution of the NAD-linked isocitrate dehydrogenase, even when measured in the presence of ADP, too much weight cannot be attributed to the argument of additivity.

A crucial difference between our results and those reported by Moyle and Mitchell[6] is to be found in the relative activities of the transhydrogenase and NAD-linked isocitrate dehydrogenase activities. Since we used pyruvate *plus* added lactate dehydrogenase as the NAD^+-regenerating system, while Moyle and Mitchell[6] made use of the endogenous malate dehydrogenase activity of the soluble fraction for the reoxidation of NADH, we have investigated the effect of the NAD^+-regenerating system on the transhydrogenase assay. As shown in Table II, when added oxaloacetate and the endogenous malate dehydrogenase activity are used as NAD^+-regenerating system, the apparent transhydrogenase activity is three times higher than that measured with added lactate dehydrogenase and pyruvate. Palmityl-CoA inhibits the latter activity by 88%; in contrast, the activity measured in the presence of oxaloacetate is inhibited only 25%. It is noteworthy that the absolute decrease in activity is equal in both systems. This result suggests that reactions other than the transhydrogenase contribute to the oxidation of NADPH,

TABLE II

COMPARISON OF PYRUVATE *PLUS* LACTATE DEHYDROGENASE AND OXALOACETATE AS NAD^+-REGENERATING SYSTEMS IN THE TRANSHYDROGENASE ASSAY

Reactions were studied in the GSH–P_i–ADP preparation, stored for 72 h at –20 °C. Transhydrogenase activity was determined as described in the legend to Table I. Where indicated, pyruvate *plus* lactate dehydrogenase (LDH) were omitted, and reactions were started by addition of 1 mM oxaloacetate. Malate and lactate dehydrogenase activities in the soluble fraction were determined in the standard medium *plus* 100 μM NADH and 1 mM oxaloacetate or 10 mM pyruvate, respectively.

Reductant	*Oxidant*	*NAD-regenerating system*	*Further additions*	*Activity (nmoles/min per mg protein)*
NADPH	NAD^+	pyruvate *plus* LDH	–	3.2
NADPH	NAD^+	pyruvate *plus* LDH	palmityl-CoA (10 μM)	0.4
NADPH	NAD^+	oxaloacetate	–	11.3
NADPH	NAD^+	oxaloacetate	palmityl-CoA	8.4
NADH	oxaloacetate	–	–	2200
NADH	pyruvate	–	–	100

measured in the presence of oxaloacetate. In view of the high activity of malate dehydrogenase in the soluble fraction, even a low activity of this enzyme with NADPH may cause severe interference with the transhydrogenase assay. In separate experiments, using purified malate dehydrogenase, it was found that the activity with NADPH may amount to 1–2% of the activity with NADH under the reaction conditions used here. This would be more than sufficient to explain the palmityl-CoA-insensitive oxidation of NADPH in the presence of oxaloacetate. A similar unspecific oxidation of NADPH is observed with lactate dehydrogenase. In this case, however, the contribution of this activity is determined in each incubation before addition of the soluble fraction. (The lactate dehydrogenase activity in the soluble enzyme fraction is too low to interfere strongly with the transhydrogenase assay.) Unfortunately, such a correction is not possible when oxaloacetate is used: the presence of considerable amounts of endogenous NAD^+ in the soluble fraction (*cf.* ref. 6) makes it impossible to study the NADPH oxidation by malate dehydrogenase in the absence of NAD^+. Thus, the use of oxaloacetate as NAD^+-regenerating system can lead to a considerable overestimation of the transhydrogenase activity.

In the experiment of Table III the effect of added NADPH on the NAD-linked isocitrate dehydrogenase activity was investigated in preparations that were aged for 72 h at −20 °C. The activity in the GSH–P_i–ADP preparation was only slightly affected, but in the sucrose–Tris preparation only 25% of the original activity was left. When the reaction was assayed in the presence of 100 μM NADPH, the initial rate of NAD^+ reduction was strongly increased in both preparations. However, in the sucrose–Tris preparation this stimulation was largely prevented when palmityl-CoA was included in the reaction medium, suggesting that the increased rate of NAD^+ reduction under these conditions is

TABLE III

EFFECT OF ADDED NADPH ON NAD-LINKED ISOCITRATE DEHYDROGENASE IN SOLUBLE FRACTIONS OF SONICATED RAT-LIVER MITOCHONDRIA AFTER AGING

The preparations used in Table I were stored at −20 °C for 72 h. Isocitrate dehydrogenase activity was determined in the standard assay medium *plus* 0.5 mM ADP and 7.5 mM $MgCl_2$.

Preparation	*Additions*	*Activity (nmoles/min per mg protein)*
Sucrose–Tris	None	4.3
	NADPH (100 μM)	12.5*
	Palmityl-CoA (10 μM)	4.3
	NADPH + palmityl-CoA	6.0
GSH–P_i–ADP	None	17.1
	NADPH	24.6
	Palmityl-CoA	16.0
	NADPH + palmityl-CoA	24.6

*Initial rate; the rate decreased rapidly within 3–4 min to about 6 nmoles/min per mg.

due to transhydrogenase activity, and not to NAD-linked isocitrate dehydrogenase. In the GSH–P_i–ADP preparation no effect of palmityl-CoA was observed. In this case, however, the assays were complicated by a slow oxidation of NADPH before addition of isocitrate. This may be caused by a small activity of glutathione reductase in the soluble fraction (to which glutathione was added in the sonication medium). This result cannot, of course, explain the inhibition of NAD-linked isocitrate dehydrogenase that Moyle and Mitchell[6] observed on addition of nucleotide pyrophosphatase to the assay medium. However, it cannot be excluded that degradation products of NAD or NADPH, formed by the massive amounts of nucleotide pyrophosphatase (or the contaminating phosphomono- and phosphodiesterases[11]) inhibit the NAD-linked isocitrate dehydrogenase, an enzyme known to possess regulatory sites for adenine nucleotides.

In conclusion, the present results demonstrate that the experiments of Moyle and Mitchell[6] do not provide any reason to doubt the occurrence of a NAD-linked isocitrate dehydrogenase in rat-liver mitochondria (the separate existence of which had already been demonstrated[3]). The degree to which this enzyme contributes to the oxidation of isocitrate in intact mitochondria under the conditions employed by Moyle and Mitchell[6] remains a matter of discussion.

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