Absence of direct coenzyme transfer in an A–B dehydrogenase system

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Direct transfer of NADPH between two NADP-dependent dehydrogenases, isocitrate dehydrogenase and glutamate dehydrogenase, has been investigated. These enzymes have opposite stereospecificity for hydrogen transfer to the coenzyme. In contrast with the general direct-transfer mechanism postulated for NAD-dependent dehydrogenases [Srivastava & Bernhard (1986) *Science* **234**, 1081–1086], no evidence for direct transfer in either direction was found for these NADP-dependent dehydrogenases.

INTRODUCTION

Srivastava & Bernhard (1986) have advanced the intriguing hypothesis that direct transfer of metabolites between enzymes plays a significant role in metabolic processes under physiological conditions. In particular, they concluded that direct transfer of coenzyme is a general mechanism for pairs of dehydrogenases having opposite stereospecificity for hydrogen transfer to carbon-4 of nicotinamide. We have been interested in the interaction of nucleotides with mitochondrial NADP-dependent isocitrate dehydrogenase (Ehrlich & Colman, 1975, 1985). One possible recipient of reduced coenzyme from the isocitrate dehydrogenase reaction would be glutamate dehydrogenase, which is also a mitochondrial enzyme. The decreased production of glutamate in rat liver mitochondria when incubated with an inhibitor of isocitrate dehydrogenase, α -methyl isocitrate (Smith & Plaut, 1979), might suggest a co-ordination in roles of the two enzymes.

NADP-dependent isocitrate dehydrogenase stereospecifically adds hydrogen to the A-side of NADP and binds NADP in the *anti* conformation (Ehrlich & Colman, 1985; Nakamoto & Vennesland, 1960). Glutamate dehydrogenase, a 'B' enzyme (Nakamoto & Vennesland, 1960), utilizes both NADPH and the product of the isocitrate dehydrogenase reaction, 2-oxoglutarate. I have done experiments to test whether direct transfer can be observed between isocitrate dehydrogenase and glutamate dehydrogenase, as would be predicted by the hypothesis of Srivastava & Bernhard (1986).

EXPERIMENTAL

Isocitrate dehydrogenase was purified by the method of Bacon *et al.* (1981). Glutamate dehydrogenase was obtained from Boehringer Mannheim as an $(\text{NH}_4)_2\text{SO}_4$ suspension and dialysed into 0.06 M-triethanolamine chloride buffer, pH 7.4. NADPH, nicotinamide mononucleotide and 2-oxoglutarate were from Sigma. Nicotinamide riboside was prepared by the method of Kaplan & Stolzenbach (1957).

The rate of NADPH oxidation was measured at 340 nm by using a Perkin–Elmer 553 spectrophotometer with the cuvette maintained at 25 °C. The glutamate dehydrogenase reaction was assayed in triethanolamine/

0.05 M-acetate, pH 8.0, containing 0.1 mM-EDTA, 2% (v/v) glycerol and 0.05 M- NH_4Cl . The reductive carboxylation of 2-oxoglutarate by isocitrate dehydrogenase was measured in 0.06 M-triethanolamine chloride containing 20 mM-2-oxoglutarate, 40 mM- NaHCO_3 and 2 mM- MnSO_4 . Because of absorption by 2-oxoglutarate, the measurements were performed at 350 nm. Nicotinamide mononucleotide and nicotinamide monoriboside were tested as coenzymes in the isocitrate dehydrogenase reaction in 0.03 M-triethanolamine chloride, pH 7.4, containing 2 mM- MnSO_4 , 4 mM-DL-isocitrate and either 1 mM-adenosine 2',5'-bisphosphate or 2'-AMP. The A_{340} was measured on the addition of enzyme.

RESULTS

Table 1 shows the results of experiments designed to examine direct transfer of NADPH between isocitrate dehydrogenase and glutamate dehydrogenase. When isocitrate dehydrogenase is present at concentrations in excess of NADPH, the observed rate of oxidation in the glutamate dehydrogenase reaction is decreased drastically (Expts. 1 and 2). This is not due to sequestering of 2-

Table 1. Comparison of the rate of glutamate dehydrogenase catalysis of 2-oxoglutarate (2-OG) reduction by NADPH in the presence or absence of isocitrate dehydrogenase (ICDH)

The glutamate dehydrogenase concentration was constant (1 nM). In addition, the assay mixture contained 0.05 M- NH_4Cl , 0.1 mM-EDTA, 2% (v/v) glycerol and triethanolamine/0.05 M-acetate, pH 8.0.

Expt.	[ICDH] (μM)	[2-OG] (μM)	[NADPH] (μM)	Rate (nmol/min)
1	0	40	45	7.36
2	50	40	45	< 0.02
3	50	80	45	< 0.02
4	0	80	90	7.88
5	50	80	90	7.21

Table 2. Rate of NADPH oxidation by isocitrate dehydrogenase in the presence or absence of glutamate dehydrogenase (GDH)

The decrease in NADPH was measured at 350 nm in 0.06 M-triethanolamine chloride, pH 7.4, containing 2 mM-MnSO₄, 20 mM-2-oxoglutarate and 40 mM-NaHCO₃. The reaction was initiated by the addition of 15 nM-isocitrate dehydrogenase. Where indicated, 24 μ M-glutamate dehydrogenase was added. Predicted rates were determined by using constants given in the text.

NADPH (μ M)	Rate (nmol/min)		
	No GDH	+ GDH	
		Observed	Predicted
6		<0.05	0.07
8	1.55	0.12	0.11
16	1.89	0.20	0.30
26		0.54	0.70
35	2.33	1.23	1.30
43		1.64	1.43
52	2.70	1.70	1.77
83		2.37	2.32
91	3.19	2.44	2.40

oxoglutarate, since increasing 2-oxoglutarate to a concentration in excess of enzyme still results in a low rate of reaction (Expt. 3). The presence of an inhibitor in the isocitrate dehydrogenase preparation is ruled out because an increase in NADPH to concentrations above that of enzyme results in full restoration of activity (Expts. 4 and 5). In the absence of glutamate dehydrogenase, no oxidation of NADPH is observed, since metal is required for the reductive carboxylation reaction by isocitrate dehydrogenase (Colman, 1972).

To rule out the possibility that direct transfer can still occur in the direction from glutamate dehydrogenase to isocitrate dehydrogenase, the reductive carboxylation of 2-oxoglutarate was measured in the presence or absence of 24 μ M-glutamate dehydrogenase (Table 2). In the absence of glutamate dehydrogenase, the dependence of the rate of NADPH oxidation upon NADPH concentration was hyperbolic, with a Michaelis constant of 11 μ M. In the presence of glutamate dehydrogenase, Michaelis-Menten kinetics were not observed. At 16 μ M-NADPH the velocity in the presence of glutamate dehydrogenase was only 10 % of that in its absence. Aqueous NADPH concentrations in the presence of glutamate dehydrogenase were calculated by using a dissociation constant of 2.3 μ M (Chalabi *et al.*, 1987). The dissociation constant in the presence of 2-oxoglutarate has not been measured, but carboxylic acids are known to lower the dissociation constant (Bell *et al.*, 1985), and small differences would not alter the conclusions. The measured velocity of NADPH oxidation by isocitrate dehydrogenase can be predicted (Table 2) when expressed in terms of aqueous NADPH. The Michaelis constant, 30 μ M, is slightly higher than in the absence of glutamate dehydrogenase. These experiments indicate that direct transfer does not occur from glutamate dehydrogenase to isocitrate dehydrogenase.

The molecular mechanism of coenzyme transfer

proposed by Srivastava *et al.* (1985) for several NAD-dependent dehydrogenases includes an initial step of nicotinamide transfer between dehydrogenases. The independent interaction of nicotinamide fragments with liver alcohol dehydrogenase has been demonstrated (Sicsic *et al.*, 1984). Attempts were made to determine if isocitrate dehydrogenase could accept nicotinamide mononucleotide or nicotinamide monoriboside as coenzymes, either alone or with 2'-AMP and adenosine 2',5'-bisphosphate. No enzymic reaction was found for concentrations up to 1 mM. Nicotinamide mononucleotide is not an inhibitor of the isocitrate dehydrogenase reaction at 10 mM. The failure of isocitrate dehydrogenase to participate in direct coenzyme transfer could result from this failure to show appreciable independent binding of the nicotinamide moiety.

DISCUSSION

In the absence of direct transfer of NADPH between isocitrate dehydrogenase and glutamate dehydrogenase, the inability to observe a reaction when isocitrate dehydrogenase was present in concentrations in excess of NADPH is not unexpected. Under similar conditions (with the omission of 2-oxoglutarate), the dissociation constant for NADPH has been estimated as 1 nM or less (Reynolds *et al.*, 1978; Mas & Colman, 1985). The calculated free concentration of NADPH in Expt. 3 (Table 1) is 9 nM, whereas the Michaelis constant for glutamate dehydrogenase is 20 μ M (Frieden, 1963). The dissociation rate of NADPH from isocitrate dehydrogenase is less than 7 s⁻¹ (Fatania *et al.*, 1982a; Ehrlich & Colman, 1985). The comparable dissociation rates from the binary isocitrate dehydrogenase-NADPH complex and from transient kinetics of the isocitrate dehydrogenase reaction (Dalziel *et al.*, 1978; Fatania *et al.*, 1982b) in triethanolamine buffer suggest that 2-oxoglutarate does not significantly affect NADPH dissociation.

The tight binding and slow dissociation of NADPH in the presence of isocitrate dehydrogenase need not preclude coenzyme transfer to glutamate dehydrogenase. Turnover of 1,3-diphosphoglycerate is rapid in the phosphoglycerate kinase-glyceraldehyde-3-phosphate dehydrogenase system, although dissociation from phosphoglycerate kinase is slow, and the dissociation constant is less than 10 nM (Weber & Bernhard, 1982). Moreover, Srivastava & Bernhard (1987) have shown that transfer rates between the dehydrogenases they have studied may exceed by more than an order of magnitude the dissociation rate of NADH from the individual enzymes.

The possibility remains that direct transfer of NADPH does occur, but the catalytic rate in the two enzyme-NADPH complex is reduced. For glutamate dehydrogenase this rate must be diminished to the extent where it cannot be distinguished from the rate of utilization of aqueous NADPH. Similarly, for isocitrate dehydrogenase in the presence of glutamate dehydrogenase, all of the observed flux may be attributed to aqueous NADPH (Table 2).

These results lead to the conclusion that direct transfer of coenzyme between dehydrogenases is not a necessary correlate of their stereospecificity. Direct transfer may require particular molecular adaptation of the two enzymes so that their surfaces are complementary (Srivastava *et al.*, 1985). Observation of direct transfer

between two enzymes may indicate specific adaptation for a metabolic role and emphasizes the importance of studies such as those of Srivastava & Bernhard (1986).

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