Note

Submitochondrial Location and Some Properties of NAD⁺- and NADP⁺-Linked Malate Dehydrogenase in *Euglena*

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NAD⁺-linked malate dehydrogenase [L-malate: NAD⁺ oxidoreductase, EC 1.1.1.37] (NAD⁺-MDH) is distributed in mitochondria, microbodies and cytosol of most organisms,^{1~4)} while the NADP⁺-linked enzyme [L-malate: NADP⁺ oxidoreductase, EC 1.1.1.82] (NADP⁺-MDH) is found only in chloroplasts of higher plants.^{5,6)} In *Euglena*, the occurrence, subcellular location and properties of NAD⁺-MDH have been reported,^{1~3)} but no information is available on NADP⁺-MDH in this protozoan. In the present paper are reported the occurrence, submitochondrial location and some properties of NAD⁺- and NADP⁺-MDH in *Euglena gracilis*.

E. gracilis SM-ZK, a streptomycin-bleached strain, was cultured for 5 days at 27°C under illumination (2,000 lux) in the Oda medium.⁷⁾ The activity of NADP⁺-MDH was assayed in 2 ml of reaction mixture containing 50 mM potassium phosphate buffer, pH 6.3, 0.5 mM oxaloacetate, 0.1 mM NADPH, 0.025% Triton X-100 and enzyme. The reaction mixture without oxaloacetate was preincubated at 30°C for 1 min and after starting the reaction by adding oxaloacetate, activity was determined by measuring the

absorption at 340 nm. NAD⁺-MDH was assayed in the same manner as described for NADP⁺-linked enzyme using 50 mM Tris-HCl buffer, pH 8.0, and 0.1 mM NADH in place of phosphate buffer and NADPH. Glutamate dehydrogenase (EC 1.4.1.4),⁸⁾ succinate semialdehyde dehydrogenase (EC 1.2.1.16),⁹⁾ adenylate kinase (EC 2.7.4.3),¹⁰⁾ lactate dehydrogenase (cytochrome) (EC 1.1.2.3),¹¹⁾ rotenone-insensitive NADPH-cytochrome reductase (EC 1.6.2.4),¹²⁾ and glucose-6-phosphatase (EC 3.1.3.9),¹³⁾ marker enzymes for cytosol, mitochondrial matrix, intermembrane space, inner membrane, outer membrane and microsomes, respectively, were assayed by the methods of references cited. Protein was determined by the method of Lowry *et al.*¹⁴⁾

A cell homogenate of *Euglena* was obtained by partial trypsin digestion of the pellicle followed by mild mechanical disruption,¹⁵⁾ and fractionated by differential centrifugation by the method of Shigeoka *et al.*¹⁶⁾ Table I shows the distribution of the MDH activity with NAD⁺ or NADP⁺ as an electron acceptor and the activities of marker enzymes in the subcellular fractions from differential centrifugation of a homogenate of *E. gracilis*. The activities of NAD⁺-MDH in mitochondria and cytosol, after correction for leakage of succinate-semialdehyde dehydrogenase, were 12.9 and 91.1%, respectively, and those of NADP⁺-linked enzyme were 15.9 and 81.0%, respectively. Both NAD⁺- and NADP⁺-MDH activities were virtually absent in microsomes. Although Davis and Merrett¹⁷⁾ have reported detection of NAD⁺-MDH in the *Euglena* microbody fraction, neither NAD⁺- nor NADP⁺-linked isozyme was present in this fraction in the present experiments, probably because the vitamin B₁₂-limited *Euglena* cells here employed contained few microbodies (data not shown).

Mitochondria were purified as follows: The suspension of washed mitochondria was layered on the top of a discontinuous Percoll (Pharmacia Co., Ltd., Lot No. 4634) gradient and centrifuged in a Hitachi RPRS 6-2 swinging bucket rotor for 30 min at 5,000 × *g*. The gradient was prepared by layering successively 1.5 ml each of 50, 30 and 10% Percoll containing 25 mM glycylglycine-KOH

TABLE I. SUBCELLULAR DISTRIBUTION OF MALATE DEHYDROGENASE ISOZYMES AND MARKER ENZYMES IN *Euglena gracilis*

Enzyme	Enzymic activity (with percent distribution)			
	Crude homogenate	Mitochondria	Microsomes	Cytosol (100,000 × <i>g</i> sup)
Malate dehydrogenase (NADP ⁺ -linked)	228.9* (100) [†]	32.5* (14.2) [†]	0* (0) [†]	189.5* (82.7) [†]
Malate dehydrogenase (NAD ⁺ -linked)	733.0 (100)	84.3 (11.5)	2.9 (0.4)	678.1 (92.5)
Succinate semialdehyde dehydrogenase	10.53 (100)	9.40 (89.3)	0.02 (2.3)	1.13 (10.7)
Glutamate dehydrogenase (NADP ⁺ -linked)	10.95 (100)	0.07 (0.6)	0 (0)	10.88 (99.4)
Glucose-6-phosphatase	1.58 (100)	0.12 (7.9)	1.34 (85.1)	0.16 (10.0)

* Activity in $\mu\text{mol/min}$.

[†] % distribution given in parentheses.

TABLE II. SUBMITOCHONDRIAL DISTRIBUTION OF MALATE DEHYDROGENASE ISOZYMES AND MARKER ENZYMES IN *Euglena gracilis*

Fraction	Protein (mg)	Malate dehydrogenase		Lactate dehydrogenase (cytochrome <i>c</i>)	NADPH-cyt C reductase	Succinate semialdehyde dehydrogenase	Adenylate kinase
		NADP ⁺ -linked	NAD ⁺ -linked				
Purified mitochondria	9.80 (100) [†]	8.62 (100) [†]	21.07 (100) [†]	0.70 (100) [†]	25.19 (100) [†]	2.33 (100) [†]	6.43 (100) [†]
Outer membrane	0.32 (3.3)	0 (0)	0 (0)	0 (0)	10.45 (41.5)	0.02 (0.7)	0 (0)
Intermembrane space	4.95 (50.5)	4.42 (51.2)	0.25 (1.1)	0 (0)	0.30 (1.2)	0.16 (6.9)	0.89 (13.8)
Inner membrane	1.33 (13.6)	0.54 (6.3)	0 (0)	0.25 (36.1)	0.27 (1.1)	0 (0)	0 (0)
Matrix	4.50 (45.9)	36.5 (42.4)	17.28 (82.1)	0 (0)	0.13 (0.5)	1.70 (72.9)	0 (0)

Activity in $\mu\text{mol/min}$ for all enzymes.[†] % distribution given in parentheses.

buffer, pH 7.4, 3% Ficoll and 0.25 M sucrose into centrifuge tubes. After centrifugation the isopically banded mitochondria were recovered from the gradient, washed and resuspended with 25 mM glycylglycine buffer, pH 7.4, containing 0.25 M sucrose. Purified mitochondria recovered at the interface of the 30 and 50% Percoll solutions were completely free from contamination by other organelles as shown by no detection of marker enzymes. The recoveries of succinate semialdehyde dehydrogenase, a marker enzyme of mitochondrial matrix, in the purified mitochondria fraction ranged from 65 to 75% of crude homogenate, indicating that the inner membrane is little damaged during purification. Purification of mitochondria with Percoll brought about a 50% increase of the specific activity of succinate semialdehyde dehydrogenase. The specific activity of adenylate kinase, a marker enzyme of mitochondrial intermembrane space, was increased about 10%. However, the activity of adenylate kinase was almost completely lost from the mitochondria during Percoll gradient centrifugation without 3% Ficoll, showing protection of the mitochondrial outer membrane by this reagent. The P/O ratio and respiratory control ratio of the purified mitochondria were similar to those reported by Tokunaga *et al.*¹⁵⁾ The present method is free from osmotic interference, unlike a sucrose density gradient, and gave purer mitochondria with negligible contamination by other organelles with shorter procedures.

Subfractionation of mitochondria was achieved by the method of Bandlow and Bauer¹⁸⁾ for yeast mitochondria using glycylglycine-KOH buffer, pH 7.4. The vesicles of inner membrane and matrix were recovered at equilibrium density of 1.253 g/cm³. As shown in Table II, the activities of adenylate kinase and lactate dehydrogenase were located only in the intermembrane space and inner membrane, respectively, indicating that these fractions did not contaminate other submitochondrial fractions. Since the recovery of succinate semialdehyde dehydrogenase from purified mitochondria was 0.7, 6.9, and 72.9% in the outer membrane, intermembrane space and matrix fraction, respectively, contaminations of matrix fraction into intermembrane space and outer membrane fractions were calculated to be 8.6 and 0.9%, respectively. The activity of rotenone-insensitive NADPH-cytochrome reductase was scarcely detected in other submitochondrial fractions. The recoveries of marker enzymes in subfractions were at low levels, suggesting that these enzymes were unstable. Table II shows the distribution of activities of the NAD⁺- and NADP⁺-MDH in the submitochondrial fractions of purified *Euglena* mitochondria together with activities of the marker enzymes. Most of the NAD⁺-linked enzyme was located in matrix. NADP⁺-MDH was found in the matrix and intermembrane space and slightly in inner membrane.

This finding of NADP⁺-MDH located in cellular sites other than chloroplasts is the first time that has been reported. Davis and Merrett¹⁷⁾ reported that no measurable activity of NADP⁺-linked enzyme in *Euglena gracilis* was detected at pH 7.5. The optimum pH of this enzyme

both in the intermembrane space and matrix of *Euglena* mitochondria was 6.3. The chloroplast enzyme of higher plants has an optimum pH of about 8.0.¹⁹⁾ The NADP⁺-linked enzyme from higher plants was activated by dithiothreitol,^{19,20)} while the *Euglena* enzyme was not. Maximum activity of the *Euglena* enzyme was found at 40°C, and the activation energy for the enzyme reaction calculated from the Arrhenius equation was 3.7 kcal per mol. The *K_m* values of NADP⁺-MDH in the intermembrane space of *Euglena* mitochondria for oxaloacetate and NADPH was 14 and 35 μ M, respectively, at optimum pH, showing higher affinities than those of spinach and maize.^{5,6)} The enzyme in the mitochondrial matrix had *K_m* values of 37 μ M for oxaloacetate and of 66 μ M for NADPH; these values are similar to those of the enzymes in chloroplasts.^{5,6)}

NAD⁺-MDH from *Euglena* mitochondria was optimum at pH 7.2 and the *K_m* value for oxaloacetate was 36 μ M and for NADH 63 μ M. Davis and Merrett¹⁷⁾ have reported that the corresponding values are 30 and 107 μ M, respectively. They also reported two isozymes in cytosol and peroxisomes, which have different properties from that in mitochondria. These results suggest that NAD⁺- and NADP⁺-MDH are different molecular species. NAD⁺-linked malate dehydrogenase in the matrix is generally considered to take part in the mitochondrial side of the "malate-aspartate shuttle" providing a means of transporting reducing power, NADH,²¹⁾ but the physiological significance of NADP⁺-linked enzyme remains uncertain at the present time.

REFERENCES

- 1) N. Begin-Heick, *Biochem. J.*, **134**, 607 (1973).
- 2) M. J. Peak, J. G. Peak and I. P. Ting, *Biochim. Biophys. Acta*, **284**, 1 (1972).
- 3) N. Collins and M. J. Merrett, *Biochem. J.*, **148**, 321 (1975).
- 4) L. J. Banaszak and R. A. Bradshaw, "The Enzyme," Vol. 11, ed by P. D. Boyer, Academic Press, New York, San Francisco and London, 1975, p. 369.
- 5) I. P. Ting and V. Rocha, *Arch. Biochem. Biophys.*, **147**, 156 (1971).
- 6) C. R. Slack, M. D. Hatch and D. J. Goodchild, *Biochem. J.*, **114**, 489 (1969).
- 7) Y. Oda, Y. Nakano and S. Kitaoka, *J. General Microbiol.*, **128**, 853 (1982).
- 8) M. Tokunaga, Y. Nakano and S. Kitaoka, *J. Protozool.*, **26**, 471 (1979).
- 9) S. Kitaoka, Y. Nakano, K. Miyatake and A. Yokota, "The Biology of *Euglena*," Vol. 4, ed. by D. E. Buetow, Academic Press, Inc., New York and London, 1983, in press.
- 10) M. Chiga and G. W. G. Plant, *J. Biol. Chem.*, **248**, 1778 (1960).
- 11) K. Hiromi and J. M. Sturtevant, *J. Biol. Chem.*, **240**, 4662 (1965).

- 12) A. Yokota and S. Kitaoka, *Agric. Biol. Chem.*, **43**, 855 (1979).
 - 13) C. de Duve, B. C. Pressman, R. Gianetto, T. G. Wattiaux and F. Appelman, *Biochem. J.*, **60**, 604 (1955).
 - 14) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
 - 15) M. Tokunaga, Y. Nakano and S. Kitaoka, *Agric. Biol. Chem.*, **40**, 1439 (1976).
 - 16) S. Shigeoka, Y. Nakano and S. Kitaoka, *Agric. Biol. Chem.*, **43**, 2187 (1979).
 - 17) B. Davis and M. J. Merrett, *Plant Physiol.*, **51**, 1127 (1973).
 - 18) W. Bandlow and P. Bauer, "Methods in Cell Biology," Vol. 12, ed. by D. M. Prescott, Academic Press, Inc., New York, San Francisco and London, 1975, p. 311.
 - 19) R. A. Wolosiuk, B. B. Buchanan and N. A. Crawford, *FEBS Lett.*, **81**, 253 (1977).
 - 20) H. S. Johnson, *Biochem. Biophys. Res. Commun.*, **43**, 703 (1971).
 - 21) A. L. Lehninger, "Biochemistry," Worth Publishers, Inc., New York, 1975, p. 533.
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