Vol. 155, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1988 Pages 1-6

NAD-DEPENDENT METHYLENETETRAHYDROFOLATE DEHYDROGENASE-METHENYLTETRAHYDROFOLATE CYCLOHYDROLASE IN TRANSFORMED CELLS IS A MITOCHONDRIAL ENZYME

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Recevied July 8, 1988

Transformed mammalian cells express a unique bifunctional NAD-dependent methylenetetrahydrofolate dehydrogenase-methenyltetrahydrofolate cyclohydrolase in addition to the usual NADP-dependent dehydrogenase-cyclohydrolase-synthetase. Subcellular fractionation of murine cell lines revealed that the NAD-dependent dehydrogenase activity is located predominantly in mitochondria, while the NADP-dependent trifunctional dehydrogenase appears to exist only in the cytosol of these cells. Western analysis using monospecific polyclonal antisera confirms the subcellular distribution of these two proteins.

Methylenetetrahydrofolate is an important precursor for the synthesis of methionine, thymidylate and purines. In normal mammalian cells a trifunctional NADP-dependent methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5) - methenyltetrahydrofolate cyclohydrolase (EC 3.5.4.9)-formyltetrahydrofolate synthetase (EC 6.3.4.3) enzyme converts methylene - to formyltetrahydrofolate, the donor of one-carbon units in purine biosynthesis. We demonstrated earlier that most if not all transformed mammalian cells contain an NAD-dependent methylenetetrahydrofolate dehydrogenase (EC 1.5.1.15) activity (1). This activity, first detected in extracts of Ehrlich ascites tumor cells (2), was found on purification to be a property of a bifunctional dehydrogenase-cyclohydrolase similar in size and function to the amino-terminal dehydrogenase-cyclohydrolase domain of the NADP-dependent trifunctional enzyme (3). However, the two murine proteins are immunologically distinct (4) and show significant kinetic differences in the dehydrogenase-cyclohydrolase activities (5). Although we have postulated

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0006-291X/88 $1.50

that the expression of this enzyme could support increased purine biosynthesis, its actual metabolic role has not been established. Here we demonstrate that the NAD-dependent dehydrogenase is located largely, if not entirely in the mitochondria of transformed cells, while the NADP-dependent enzyme can be detected only in the cytosol of the same cells.

MATERIALS AND METHODS

Cell culture: Materials for cell culture were obtained from Gibco Laboratories and three murine cell lines, YAC, P815 and Ehrlich ascites tumor cells were cultured in vitro as described previously (1). Ehrlich ascites cells and P815 were seeded at 2.5×10^6 and YAC at 5×10^5 cells per 150 mL of medium.

Preparation of mitochondria: Isolation of mitochondria from cell lines followed the general method of Freeman (6). Cells were harvested by centrifugation and all subsequent steps were at 0-4°C. The cells were washed 2x with PBS, and once with medium I; 300 mM sucrose, 2 mM EDTA, 30 mM nicotinamide and 1 mM potassium phosphate pH 7.3. After suspending in 50 mL of medium I, the cells ($\sim 2 \times 10^9$) were allowed to stand 15 min at 4°C. The cell suspension was divided into 10 mL aliquots and homogenized with a Brinkman Polytron at the lowest speed setting for 2-3 x 15 sec. Cell breakage was monitored by nigrosin staining until approximately 80-95% of the cells were nigrosin positive. Then 2 M KCl, 1 M benzamidine and 2-mercaptoethanol were added to give final concentrations of 100 mM, 1 mM and 25 mM respectively. After centrifugation at 450 x g for 10 min, the supernatant was retained and the pellet was resuspended in 10 mL of medium II (250 mM sucrose, 100 mM KCl, 1 mM potassium phosphate pH 7.3, 1 mM benzamidine and 25 mM mercaptoethanol) and homogenized 15 sec as before. After centrifugation for 10 min at 450 xg, the supernatants were combined. A crude mitochondrial fraction was obtained by centrifugation at 5200 x g for 10 min. This pellet was resuspended in 3 mL of medium III (800 mM sucrose, 1 mM EDTA, 100 mM KCl, 1 mM benzamidine, 1 mM potassium phosphate pH 7.3 and 25 mM 2-mercaptoethanol) and purified by equilibrium sedimentation in a sucrose gradient (6). At each step of this procedure an aliquot was prepared for enzyme assay by adjusting the concentration of potassium phosphate to 100 mM and PMSF to 1.2 mM to stabilize the enzymes and reduce proteolysis. The aliquots of extract, crude and purified mitochondria were homogenized at high speed for 30 sec. The samples were assayed for enzyme activities and a portion was frozen immediately for SDS-PAGE/Western analysis.

Assays: Substrates for enzyme assays, pyruvate, α-ketoglutarate, ATP, NAD, NADH and NADP were from Sigma, and (6-ambo)-tetrahydrofolate was prepared as described previously (1). NAD and NADP-dependent methylenetetrahydrofolate dehydrogenases were assayed as before (1), glutamate dehydrogenase according to McDaniel et al. (7), lactate dehydrogenase by the decrease in absorbance of NADH at 340 nm (Worthington Enzymes) and acid phosphatase using p-nitophenylphosphate as substrate at pH 4.9 and measuring product formation at 410 nm after addition of NaOH (8).

Protein content was determined by the dye-binding method using bovine serum albumin as standard and reagents from Bio-Rad Laboratories.

SDS-PAGE was carried out by the method of Laemmli (9) using 9% gels and applying 50 µg of protein to each well. Transfer to nitrocellulose and Western analysis was performed using both anti-NAD and anti-NADP-dehydrogenase antisera, diluted 1/2500 following the procedure previously described(4).

RESULTS

Preparation of intact mitochondria from cultured cells was highly dependent upon the extent of homogenization. Gentle homogenization which did

TABLE 1
Subcellular localization of NAD and NADP-dependent methylenetetrahydrofolate dehydrogenases

ENZYME ACTIVITY	EXTRACT		CYTOSOL		CRUDE MITOCHONDRIA		PURIFIED MITOCHONDRIA	
	Total	S.A.	Total	S.A.	Total	S.A.	Total	S.A.
NADP-DH	495	5.5	495	6.9	5	0.6	N.D.	-
NAD-DH	513	5.7	199	2.8	195	24.5	128	97
GDH	713	7.9	156	2.2	219	27.4	293	218
LDH	822	9.1	471	6.6	15	1.9	0.4	0.3
AP	6738	74	4390	62	715	90	31	23

Total enzyme units are nanomoles min^{-1} and specific activities (S.A.) are nanomoles $min^{-1}mg^{-1}$ except for lactate dehydrogenase where the units are μmoles. Abbreviations: NADP-DH and NAD-DH represent the methylenetetrahydrofolate dehydrogenases; GDH, glutamate dehydrogenase; LDH, lactate dehydrogenase; AP, acid phosphatase. N.D. not detected.

not provide extensive cell breakage yielded apparently unbroken mitochondria, but in much poorer yield. The conditions selected in this study resulted in some breakage of the mitochondria as evidenced by the appearance of some glutamate dehydrogenase in the cytosolic fraction. However, less than 0.1% of the lactate dehydrogenase and 0.5% of the acid phosphatase were present in the purified mitochondria. As illustrated in Table I, purified mitochondria did not contain detectable NADP-dependent methylenetetrahydrofolate dehydrogenase, but were enriched in the NAD-dependent dehydrogenase as indicated by a 17-fold increase in specific activity over that of extract.

In Figure 1, Western analysis of the various subcellular fractions is presented, using identical amounts of protein in each lane. In Figure 1B, anti-NADP-dehydrogenase antiserum detected the 100 kDa band representing the trifunctional protein both in crude extract and the cytosolic fraction but not in purified mitochondria. The anti-NAD-dehydrogenase antiserum detects the 34 kDa band corresponding to the bifunctional dehydrogenase-cyclohydrolase and its enrichment in the mitochondrial fractions is apparent (Fig. 1C). This analysis was extended to the cytosolic and mitochondrial fractions of two additional cell lines, shown in Figure 2. In each case, the NADP-dependent methylenetetrahydrofolate dehydrogenase is observed in the cytosol, while the NAD-dependent enzyme is found predominantly in the mitochondrial fraction.

DISCUSSION

In normal mammalian cells the NADP-dependent dehydrogenase-cyclohydrolase activities of the trifunctional enzyme presumably act to

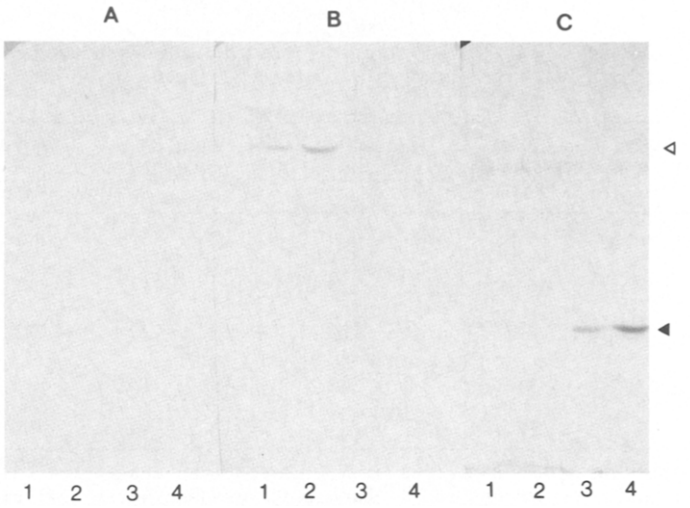

Fig. 1. Western analysis of subcellular fractions prepared from the murine line YAC. Each lane was loaded with 50 µg of protein: 1 extract; 2 cytosol; 3 crude mitochondria; 4 purified mitochondria. Immunoblots were developed using different antisera: Panel A, non-immune serum; Panel B, anti-NADP-dehydrogenase antiserum; Panel C, anti-NAD-dehydrogenase antiserum. Migrations of the 100 kDa trifunctional (◁) and 34 kDa bifunctional (◀) enzymes are indicated.

convert methylenetetrahydrofolate to formyltetrahydrofolate for purine biosynthesis. The expression of a second enzyme, NAD-specific, in transformed cells was proposed by us to further support purine biosynthesis

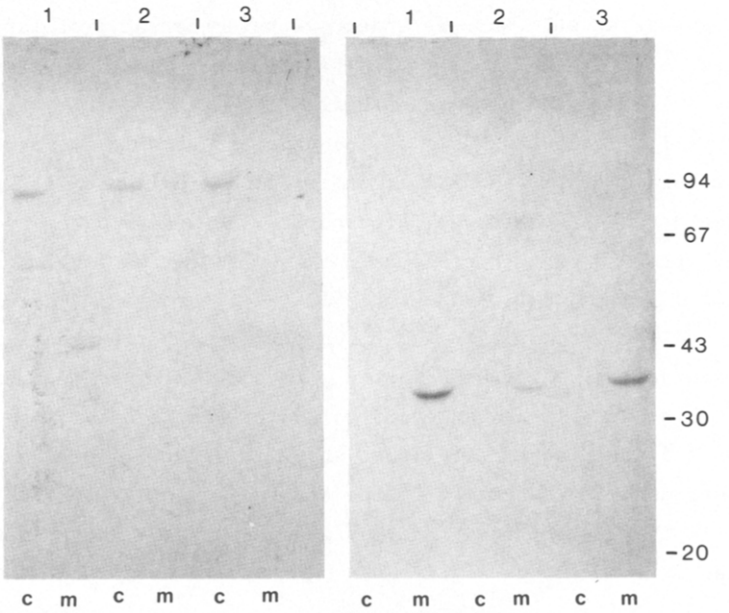

Fig. 2. Immunoblots of the cytosolic (c) and mitochondrial (m) fractions of three murine cell lines: 1, YAC, a lymphoma; 2, P815 and 3, Ehrlich ascites tumor cells, both mastocytoma lines. Fifty µg of protein were applied to each lane. The left panel was developed using anti-NADP-dependent dehydrogenase antiserum, and the right panel with anti-NAD-dehydrogenase antiserum.

(1). It is now clear from the results presented here that the transformation - associated enzyme is located at least largely in the mitochondria, while the NADP dependent enzyme cannot be detected in the mitochondria of the same cells. This interpretation based on enzyme activities is supported by immunological detection of the enzymes in the subcellular fractions. The Western analyses help to rule out the presence of inactivated forms of the trifunctional enyme in mitochondria. It is difficult to establish that the NAD-dependent activity is entirely mitochondrial, although this is likely. Isolation of cDNA clones of the murine NAD-dependent enzyme and DNA sequencing reveals the presence of an amino terminal presequence (C. Bélanger, unpublished).

In yeast there are two NADP-dependent dehydrogenase-cyclohydrolase-synthetase enzymes, one cytosolic and the other found in mitochondria (10). The physiological role of the mitochondrial enzyme is not clear since it has recently been shown to be completely dispensible (11). The mitochondrial enzyme found in transformed cells differs markedly from that of yeast, being only bifunctional and having an NAD and Mg^{++} dependent methylenetetra-hydrofolate dehydrogenase activity. In whatever way this enzyme contributes to folate-mediated metabolism in mitochondria, it is clear that the synthetase activity is not required. Methylenetetrahydrofolate in mitochondria can be converted by this enzyme to formyltetrahydrofolate, which in turn can be used in the production of methionyl-$tRNA^{fmet}$ to support mitochondrial protein synthesis. However, if the role of the enzyme is to support purine biosynthesis, the one-carbon group must exit the mitochondrion. Since it is unlikely that folates are readily transported, the one-carbon group must be released as formate, or participate in some type of shuttle. To date we have not been successful in detecting any type of 10-formyltetrahydrofolate hydrolase activity in the mitochondria from these cell lines. The role played by this NAD-dependent methylenetetrahydrofolate dehydrogenase in transformed mammalian cells is still not resolved, but now must take into account it's occurrence in mitochondria.

ACKNOWLEDGEMENTS

This work was supported by an operating grant from the National Cancer Institute of Canada. The authors appreciate discussions with Dr. K.B. Freeman concerning preparation of mitochondria from cell lines and thank Josie D'Amico for typing this manuscript.

REFERENCES

1. Mejia, N.R., and MacKenzie, R.E. (1985) J. Biol. Chem. 260, 14616-14620.
2. Scrimgeour, K.G., and Huennekens, F.M. (1960) Biochem. Biophys. Res. Commun. 128, 148-154.

3. Mejia, N.R., Rios-Orlandi, E.M., and MacKenzie, R.E. (1986) J. Biol. Chem. 261, 9509-9513.
4. Gardam, M.A., Mejia, N.R., and MacKenzie, R.E. (1988) Biochem. Cell Biol. 66, 66-70.
5. Rios-Orlandi, E.M., and MacKenzie, R.E. (1988) J. Biol. Chem. 263, 4662-4667.
6. Freeman, K.B. (1965) Biochem. J. 94, 494-501.
7. McDaniel, H., Bosing-Schneider, R., Jenkins, R., Rasched, I., and Sund, H. (1986) J. Biol. Chem. 261, 884-888.
8. Chemistry for Medical Technologists - by W.L. White, M.M. Erickson and S.C. Stevens. Third Edition. C.V. Mosby, St. Louis 1970 pages 290-291.
9. Laemmli, U.K. (1970) Nature 277, 680-685.
10. Shannon, K.W., and Rabinowitz, J.C. (1986) J. Biol. Chem. 261, 12266-12271.
11. Shannon, K.W., and Rabinowitz, J.C. (1988) J. Biol. Chem. 263, 7717-7725.