# NAD-Dependent Methylenetetrahydrofolate Dehydrogenase–Methenyltetrahydrofolate Cyclohydrolase Is the Mammalian Homolog of the Mitochondrial Enzyme Encoded by the Yeast MIS1 Gene†

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ABSTRACT: The recombinant human bifunctional NAD-dependent methylenetetrahydrofolate dehydrogenase–methenyltetrahydrofolate cyclohydrolase is unique in its absolute requirement for $Mg^{2+}$ and inorganic phosphate. Both ions affect the affinity of the enzyme for NAD and have no effect on the binding of methylenetetrahydrofolate. The NAD cofactor can be replaced by NADP with a much higher $K_M$ and lower $V_{MAX}$. Kinetic investigation using NADP supports the role of $Mg^{2+}$ in dinucleotide binding and illustrates that the 2′-phosphate can substitute for phosphate in this process. The human NAD-dependent bifunctional enzyme has a 44% amino acid sequence identity with the dehydrogenase–cyclohydrolase domain of the yeast mitochondrial NADP-dependent trifunctional enzyme encoded by the MIS1 gene, compared to 37% identity with the corresponding domain of the cytosolic trifunctional enzyme. The sequence comparison and the kinetic properties suggest that the NAD bifunctional enzyme is the mammalian homolog of the yeast mitochondrial trifunctional enzyme, which has evolved a unique use of inorganic phosphate to change its dinucleotide specificity from NADP to NAD. Its role is proposed to be in providing formyltetrahydrofolate for the synthesis of formylmethionyl transfer RNA required for the initiation of protein synthesis in mitochondria.

Methylenetetrahydrofolate ($CH_2$-THF) dehydrogenases requiring either NADP or NAD as cofactor are involved in the interconversion of methylene, methenyl, and formyl derivatives of tetrahydrofolate (THF) in both prokaryotes and eukaryotes (MacKenzie, 1984). In mammals and yeast, the NADP-dependent $CH_2$-THF dehydrogenase, associated with methenyltetrahydrofolate ($CH^+$=THF) cyclohydrolase in the amino-terminal domain, is fused to a formyltetrahydrofolate (CHO-THF) synthetase domain, forming a trifunctional enzyme. Two such enzymes, encoded by two different nuclear genes, ADE3 and MIS1, are found in yeast cytosol and mitochondria, respectively (Shannon & Rabinowitz, 1986). It has been demonstrated that the cytosolic enzyme is involved in supplying formyl groups for purine synthesis in the cytoplasm since mutants which lack the cytosolic gene are purine auxotrophs (Jones, 1972), while the mitochondrial enzyme does not appear to be essential for cell growth (Shannon & Rabinowitz, 1988).

Barlowe and Appling proposed that, like yeast, mammalian cells contain two NADP-dependent trifunctional enzymes, cytosolic and mitochondrial, and hypothesized that both could be involved in the supply of cytoplasmic one-carbon units (Barlowe & Appling, 1988). This attractive hypothesis provides both a rational use for one-carbon units generated in mitochondria as well as a very significant role for the synthetase activity. However, it does not include a role for the $Mg^{2+}$- and NAD-dependent $CH_2$-THF dehydrogenase in mammalian cells. The NAD-dependent dehydrogenase activity, originally reported in extracts of ascites tumor cells (Scrimgeour & Huennekens, 1960), was later demonstrated to be present in transformed mammalian cells, as well as in embryonic, or nondifferentiated, tissues (Mejia & MacKenzie, 1985). The enzyme purified from ascites tumor cells was found to be a nuclear-encoded mitochondrial bifunctional dehydrogenase–cyclohydrolase (Mejia et al., 1986; Mejia & MacKenzie, 1988; Bélanger & MacKenzie, 1989). Although no enzyme activity is detectable in differentiated adult mouse tissues, the NAD-dependent dehydrogenase message was present at low levels in all tissues examined (Peri & MacKenzie, 1993).

Studies on the kinetic properties (Rios-Orlandi & MacKenzie, 1988; Yang & MacKenzie, 1992) and regulation of gene expression (Peri & MacKenzie, 1991, 1993) suggested that the bifunctional and trifunctional enzymes play different roles in cellular metabolism, with the level of the mitochondrial NAD-dependent enzyme being highly regulated. In this report, we describe properties of the bifunctional enzyme that support the hypothesis for its role in mammalian metabolism.

## MATERIALS AND METHODS

*Materials.* NAD, NADP, ATP, β-glycerophosphate and benzamidine were from Sigma Chemical Co. (St. Louis, MO), and 2-mercaptoethanol was from Eastman Kodak (Rochester, NY). (6*R*,*S*)-Tetrahydrofolate was prepared as described earlier (Drury et al., 1975). Sephadex G-25 and Blue Sepharose Fast Flow were products of Pharmacia Canada, Inc. (Montreal). $MgCl_2$ and KI were of the highest quality from Fisher Scientific Co. (Nepean, Ontario). Sodium orthovanadate was from Aldrich, and 4-morpholinepropanesulfonic acid (MOPS) was from Boehringer-Mannheim. Common chemicals were of the highest grade commercially available.

*Enzyme Purification.* The human enzyme was expressed and purified from *Escherichia coli* as previously described (Yang & MacKenzie, 1992). Aliquots of the purified enzyme were concentrated by elution from a small (2 mL) Blue

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Sepharose column with 1 M KCl in 50 mM potassium phosphate (pH 7.3), 30% glycerol, and 30 mM 2-mercaptoethanol. Protein concentration was measured by a modified Lowry method (Lowry et al., 1951; Cabib & Polacheck, 1984). To obtain phosphate-free enzyme for kinetic studies, 0.2 mL of enzyme solution was loaded onto a Sephadex G-25 column (0.5 × 6 cm) which was pre-equilibrated with 50 mM MOPS (pH 7.3) and 20% glycerol and eluted with the same buffer. Purified enzymes had dehydrogenase specific activities of 40–45 μmol/min/mg of protein.

*Enzyme Assay and Kinetic Experiments.* During the purification procedures, the dehydrogenase was assayed as described by Mejia et al. (1986). To carry out kinetic studies involving substrates as well as phosphate and magnesium, the standard conditions were defined as 25 mM MOPS (pH 7.3), 5 mM potassium phosphate (pH 7.3), 0.25 mM (6*R*,*S*)-tetrahydrofolate, 2.5 mM formaldehyde, 1.0 mM NAD, 5 mM $MgCl_2$, and 30 mM 2-mercaptoethanol. When initial velocity experiments were performed, one component was varied while all others were held constant at standard concentrations. Initial velocity measurements, where no more than 10% substrate conversion occurred, were fit to the Michaelis–Menten equation using the nonlinear regression analysis program, Enzfitter (R. J. Leatherbarrow, Biosoft, Cambridge, UK). Unless specified otherwise, units are expressed as micromoles/minute.

*Enzyme Stability.* Enzyme at 12 μg/mL in 50 mM MOPS (pH 7.3), 30% glycerol, and 50 mM KCl was incubated at 8 °C in the presence or absence of various ligands. Aliquots were removed at different times and assayed for dehydrogenase activity.

## RESULTS

The unusual requirement of $Mg^{2+}$ for the activity of the $CH_2$-THF dehydrogenase was earlier shown to be involved in the binding of NAD (Rios-Orlandi & MacKenzie, 1988). The proposal that $Mg^{2+}$ might bind to the enzyme in the absence of substrates was confirmed by subsequent experiments using fluorescence quenching (Yang & MacKenzie, 1992). We observed that the conformational change induced in the enzyme by $Mg^{2+}$, making the intrinsic fluorescence more susceptible to quenching by iodide, is actually dependent on the presence of phosphate (data not shown). This prompted us to examine the role of phosphate in more detail.

*Effect of Phosphate on Dehydrogenase Activity.* Using phosphate-free enzyme, we could demonstrate that the dehydrogenase not only requires $Mg^{2+}$ for activity (Figure 1A) but also has an absolute requirement for inorganic phosphate (Figure 1B). The enzyme shows simple saturation kinetics with $Mg^{2+}$ and phosphate with relatively low values of apparent $K_M$ of 0.18 and 0.17 mM, respectively. The phosphate can be replaced by its analog, arsenate, but not by pyrophosphate, vanadate, sulfate, 2-glycerophosphate, or ATP (data not shown).

The possible role of the phosphate in turnover and/or substrate binding was investigated by kinetic studies. When NAD is varied at different fixed levels of phosphate, the initial velocity patterns shown in Figure 2 were obtained. Intersection on the ordinate is indicative of a rapid equilibrium-ordered addition of phosphate prior to NAD (Cleland, 1970; Segel, 1975; Morrison & Ebner, 1971). A replot of slope vs $[phosphate]^{-1}$ (inset to Figure 2) shows the marked dependency of the $K_M$ for NAD on phosphate concentration. The initial velocity pattern obtained from experiments where the phosphate was varied at increasing fixed levels of NAD yields a series of lines that intersect to the left of the ordinate. A replot of the slope vs $[NAD]^{-1}$ appears to intersect at zero (data not shown). In similar experiments with $CH_2$-THF as the variable substrate, the straight lines intersected at a common point on the abscissa (data not shown), indicating that the $K_M$ value for this substrate is independent of the phosphate concentration.

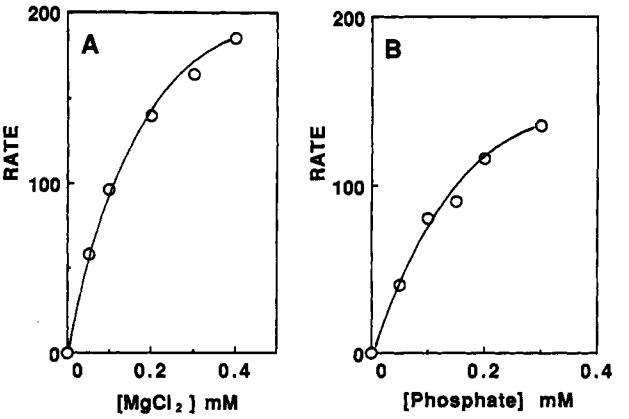



FIGURE 1: $Mg^{2+}$ and phosphate dependence of the dehydrogenase activity. Assay conditions were as described in Materials and Methods, except that $Mg^{2+}$ (A) or phosphate (B) was varied through the concentration ranges shown.

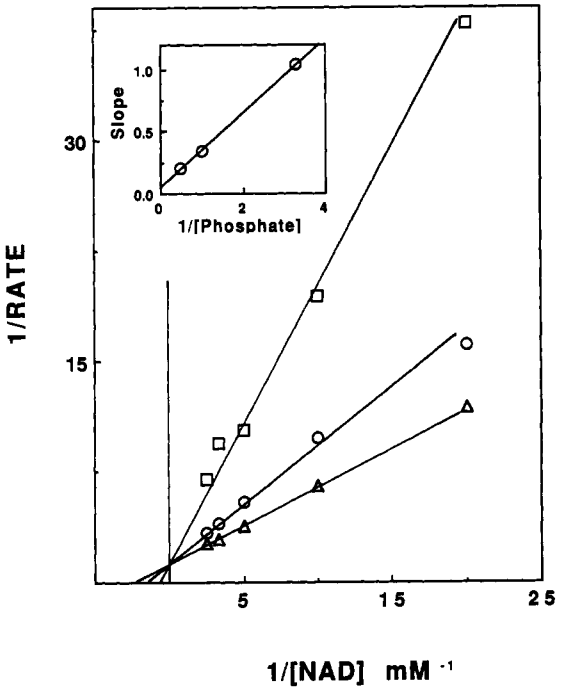



FIGURE 2: Double-reciprocal plot of initial velocity of the dehydrogenase reaction as a function of NAD concentration at fixed phosphate concentrations of 0.3 (□), 1.0 (○), and 2.0 mM (△). The inset is a replot of slope as a function of $[phosphate]^{-1}$.

We attempted to investigate the roles of phosphate and magnesium by treating them as substrates. Initial velocity patterns when $Mg^{2+}$ was varied at different phosphate concentrations and when phosphate was varied at different $Mg^{2+}$ concentrations were obtained (Figures 3 and 4). The results cannot identify whether $Mg^{2+}$ or phosphate binds to the enzyme first, but indicate that binding of $Mg^{2+}$ to the enzyme is influenced by the concentration of phosphate and vice versa. Linear replots of slope or intercepts vs fixed ligand concentrations indicate the existence of a single class of binding site for $Mg^{2+}$ and for phosphate.

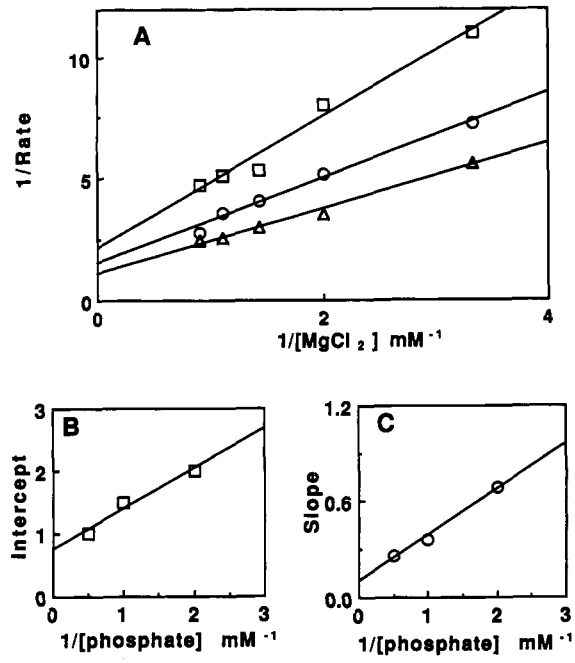



FIGURE 3: (A) Effect of the concentration of $Mg^{2+}$ on initial velocity at fixed phosphate concentrations of 0.5 (□), 1.0 (○), and 2.0 mM (△). Also shown are replots of intercepts (B) and slopes (C) as a function of [phosphate]$^{-1}$.

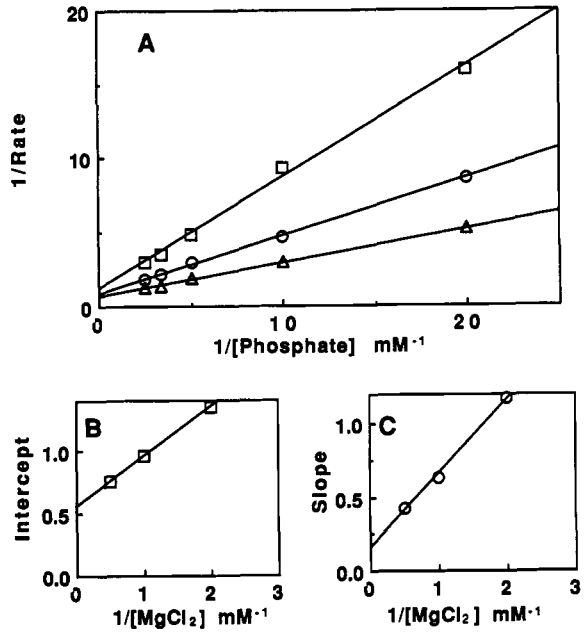



FIGURE 4: (A) Effect of the concentration of phosphate on initial velocity at fixed $Mg^{2+}$ concentrations of 0.5 (□), 1.0 (○), and 2.0 mM (△). Also shown are replots of the intercepts (B) and slopes (C) as a function of $[Mg^{2+}]^{-1}$.

Although we could not kinetically determine the order of addition of $Mg^{2+}$ and phosphate binding to the enzyme, it was possible to determine whether these ligands alone, or in combination, affected the stability of the enzyme. The protection of the enzyme by substrates and ions is shown in Figure 5. The effects can be divided roughly into three groups: no protection, $Mg^{2+}$ and $SO_4^{2-}$; weak protection, NAD, phosphate, and $Mg^{2+}$/phosphate; and good protection, $Mg^{2+}$/phosphate/NAD. From these results it is clear that phosphate can bind to the enzyme in the absence of other ligands, including $Mg^{2+}$.

*Dinucleotide Specificity.* Previous studies suggested that this dehydrogenase is specific for NAD and does not utilize

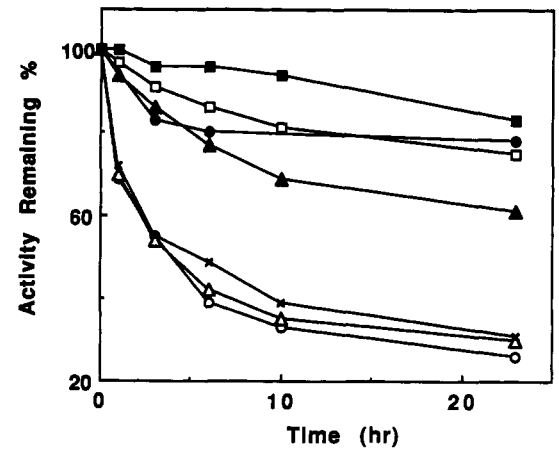



FIGURE 5: Effects of substrates and ions on the stability of the dehydrogenase. Conditions were as described in Materials and Methods. The ligands used were as follows: none (○); 5 mM potassium sulfate (×); 1 mM $Mg^{2+}$ (△); 5 mM potassium phosphate (▲); 1 mM $Mg^{2+}$ + 5 mM phosphate (●); 1 mM NAD (□); 1 mM NAD + 1 mM $Mg^{2+}$ + 5 mM phosphate (■).

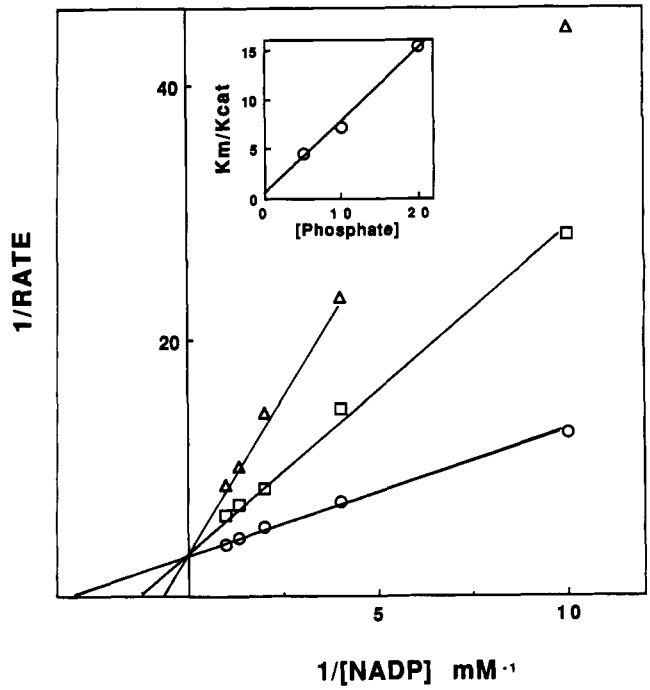



FIGURE 6: Competitive inhibition of the NADP-dependent dehydrogenase by phosphate. Concentrations of phosphate were 5 (○), 10 (□), and 20 mM (△) at a single fixed $Mg^{2+}$ concentration of 5 mM. The inset is a plot of slope as a function of [phosphate].

NADP. The unusual requirement for inorganic phosphate prompted us to reconsider this, using phosphate-free assay conditions. Under these conditions NADP is a substrate, although the activity is only 20% that observed with NAD. Magnesium is required for activity with NADP as well as with NAD (data not shown). However, phosphate, absolutely required for the NAD-dependent reaction, inhibits the NADP-dependent activity and, as shown in Figure 6, is actually a competitive inhibitor toward NADP.

A summary of the kinetic properties is given in Table I. $K_M$ values for NAD at $[P_i] \rightarrow 0$ and $[P_i] \rightarrow \infty$ were obtained from the replots of $K_M$ vs $1/[P_i]$ and $1/K_M$ vs $[P_i]$, respectively.

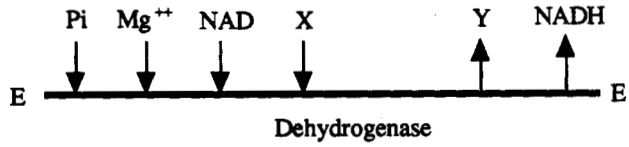
*Amino Acid Sequence Comparison.* The published amino acid sequences (Bélanger & MacKenzie, 1989; Hum et al., 1988; Peri et al., 1989; Shannon & Rabinowitz, 1988; Staben & Rabinowitz, 1986) of the $CH_2$-THF dehydrogenases from

Table I: Kinetic Properties of the NAD-Dependent Enzyme

| substrate[a] | $K_M$ (μM) | conditions: nucleotide | conditions: phosphate |
|---|---|---|---|
| $CH_2$-THF | 4.6 ± 2.1 | NAD | 25 mM |
| $Mg^{2+}$ | 171 ± 26 | NAD | 25 mM |
| $Mg^{2+}$ | ~200 | NAD | →∞ |
| $Mg^{2+}$ | 470 ± 27 | NADP | 0 |
| $P_i$ | 190 ± 36 | NAD | 0 |
| NAD | 63 ± 20 | | 25 mM |
| NAD | ~40[b] | | →∞ |
| NAD | ~2500[c] | | →0 |
| NADP | 403 ± 10 | | 0 |
| NADP | 1070 ± 130 | | 25 mM |

[a] Fixed substrate concentrations were as described in Materials and Methods. [b] Determined from a plot of $K_m$ vs 1/[phosphate]. [c] Determined from a plot of $1/K_m$ vs [phosphate].

Scheme I: Kinetic Mechanism of the Dehydrogenase[a]




[a] X represents 5,10-$CH_2$-THF, and Y represents 5,10-$CH^+$-THF.

different sources were compared. The human NAD-dependent bifunctional enzyme has 44% sequence identity with the dehydrogenase–cyclohydrolase domain of the yeast mitochondrial NADP-dependent trifunctional enzyme encoded by the MIS1 gene, compared to 36–37% identity with the corresponding domain of the cytosolic trifunctional enzymes from yeast, human, and rat.

## DISCUSSION

Most methylenetetrahydrofolate dehydrogenases are NADP-dependent. The known NAD-dependent enzymes from *Clostridium formicoaceticum* (Moore et al., 1974), *Acetobacterium woodii* (Ragsdale & Ljungdahl, 1984), and *Saccharomcyes cerevisiae* (Barlowe & Appling, 1990a,b) are monofunctional while the mammalian enzyme is bifunctional (Mejia et al., 1986; Bélanger & MacKenzie, 1989). Perhaps the most unusual characteristics of this mammalian enzyme are its requirements for $Mg^{2+}$ and phosphate. Magnesium was found by Rios-Orlandi et al. (1988) to be involved in dinucleotide binding; the role of phosphate in activating the enzyme was less clear and suggested that it might play a role in enzyme stability. We have demonstrated here that the phosphate requirement for dehydrogenase activity is absolute and that it, too, is involved in promoting the binding of NAD and not methylenetetrahydrofolate. However, NAD can bind to the enzyme in the absence of phosphate, but with weak affinity. Extrapolation of the values of $K_M$ for NAD to zero phosphate concentration yields a value of 2.5 mM. This conclusion is supported by the stabilization of the enzyme by high concentrations of NAD in the absence of phosphate and $Mg^{2+}$. However, this weak binding of NAD is not productive, in that the enzyme is not active unless both phosphate and $Mg^{2+}$ are present.

The kinetic mechanism of the enzyme was reported by Rios-Orlandi and MacKenzie (1988) to involve the binding of NAD before methylenetetrahydrofolate with obligatory binding of $Mg^{2+}$ prior to NAD. Our kinetic studies treating phosphate as a substrate demonstrate that it also must bind prior to NAD, as shown in Scheme I. These data indicate a bireactant-ordered mechanism where the addition of the first substrate is at thermodynamic equilibrium (Cleland, 1970). In this case, when the second substrate is varied at fixed levels of the first (phosphate), the double-reciprocal plot intersects on the ordinate as in Figure 2. Similar kinetic results were obtained with $Mg^{2+}$ as substrate (Rios-Orlandi & MacKenzie, 1988), but initial velocity experiments could not determine the order of phosphate binding relative to that of $Mg^{2+}$. Fluorescence quenching experiments demonstrated that conformational changes in free enzyme induced by $Mg^{2+}$ require phosphate, but again this cannot resolve the order of binding or, in fact, determine whether the ions bind as a $Mg^{2+}$–phosphate complex. It is clear, however, that $Mg^{2+}$ and phosphate can bind in the absence of substrates. This is confirmed by the effects of the ions on the stabilization of the enzyme. Because phosphate provides stabilization whereas $Mg^{2+}$ alone has no effect, it is likely that phosphate binds prior to $Mg^{2+}$.

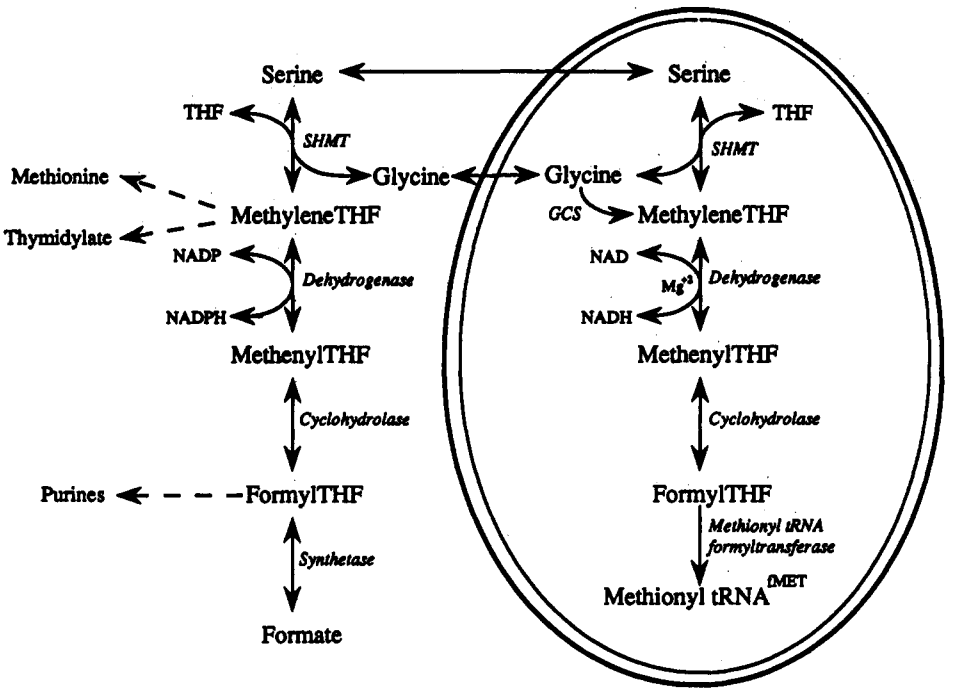
From sequence homology it is clear that the human $Mg^{2+}$-/phosphate-dependent dehydrogenase is more closely related to the yeast mitochondrial NADP-dependent trifunctional enzyme than the cytosolic form or, for that matter, any other known eukaryotic methylenetetrahydrofolate dehydrogenase. Careful analysis in phosphate-free conditions showed that the NAD-dependent bifunctional enzyme actually has low affinity and turnover with NADP. We suggest that this enzyme is the mammalian homolog of the yeast trifunctional mitochondrial dehydrogenase–cyclohydrolase–synthetase that, through evolution, became NAD-dependent. This alteration apparently occurred by substituting inorganic phosphate for the 2′-phosphate of NADP. We propose that the role of $Mg^{2+}$ is to assist phosphate binding to play this role. The kinetic experiments with NADP support this proposal; NADP requires $Mg^{2+}$ to be active, but the $K_m$ for $Mg^{2+}$ is more than double that seen with inorganic phosphate and NAD. Because phosphate is competitive with NADP binding (but promotes NAD binding), it must bind to the same subsite as the 2′-phosphate of NADP. The alteration of the enzyme to involve $Mg^{2+}$ in the phosphate binding is retained in its requirement for binding the 2′-phosphate of NADP.

Alteration of the enzyme to use NAD rather than NADP clearly shifts the equilibrium position of substrates toward CHO-THF. From data reviewed by Sies (1982) for liver cells, the NADPH/NADP ratio is 1.4 for the cytosol and 15 for mitochondria. The NADH/NAD ratio is 0.05 for cytosol and 1 for mitochondria. Thus, the shift from a cytosolic NADP system to a mitochondrial NAD-dependent system maintains a more favorable equilibrium in favor of CHO-THF. On the basis of estimated redox potentials (rather than total dinucleotide levels), this change represents about +75 mV, while maintenance of the NADP specificity in mitochondria would result in a system poised at an additional −20 mV over the cytosolic system (Sies, 1982).

These properties of the enzyme argue strongly for its relationship with the yeast mitochondrial enzyme and perhaps for an analogous function in mammalian cells. This function clearly does not involve formyltetrahydrofolate synthetase, the third activity of the yeast enzyme. Whether this represents a function lost or whether evolution of this bifunctional domain occurred prior to gene fusion to form the trifunctional enzyme is an open question.

Appling and co-workers (Appling, 1991; Barlowe & Appling, 1988) have proposed that two NADP trifunctional enzymes might exist in mammalian cells: one in mitochondria and one in the cytosol, as seen in yeast (Shannon & Rabinowitz, 1986). Careful subcellular fractionation revealed very low but detectable activities of the dehydrogenase and synthetase in mitochondria. Moreover, they demonstrated that liver

Scheme II: Serine Hydroxymethyl Transferase (SHMT) and Glycine Cleavage System (GCS)[a]




[a] Adapted from Barlowe and Appling (1988).

mitochondria can produce formate and that this can be incorporated into the cytosolic one-carbon pool. The role of mitochondria in folate-mediated metabolism clearly is important, as emphasized by Appling (1991), and is still poorly understood. Our proposal is that the $Mg^{2+}$/phosphate requiring NAD-dependent dehydrogenase–cyclohydrolase, rather than being considered an "oncodevelopmental" protein, is actually the enzyme normally found in all mammalian mitochondria, as illustrated in Scheme II. We have shown that it is highly regulated—in fact, its overexpression in transformed cells, which led to its discovery (Scrimgeour & Huennekens, 1960; Mejia & MacKenzie, 1985) and characterization (Mejia et al., 1986; Rios-Orlandi & MacKenzie, 1988), and its elevated levels in fetal tissues actually misdirected our understanding of its role. We have shown more recently that the mRNA can be detected in all normal cells at very low levels and that mitogenic stimuli alter its steady-state level in cells in culture (Peri & MacKenzie, 1993). This regulation was found not to affect the levels of mRNA encoding the NADP-dependent trifunctional enzyme (Peri & MacKenzie, 1991), which plays a housekeeping role in providing CHO-THF for purine synthesis.

The NAD-dependent enzyme appears to represent the mammalian homolog of the yeast NADP-dependent trifunctional enzyme and is involved in mitochondrial biogenesis, presumably by providing the one-carbon units for the synthesis of fmet–tRNA required for initiation of mitochondrial protein synthesis. Very low levels of the enzyme would satisfy this requirement in adult tissues, and its complex regulation allows for increased expression in cells carrying out more extensive mitochondrial biogenesis.

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