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Isolation and Characterization of a Novel Eukaryotic Monofunctional NAD$^+$-Dependent 5,10-Methylenetetrahydrofolate Dehydrogenase†

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ABSTRACT: An NAD$^+$-dependent 5,10-methylenetetrahydrofolate (THF) dehydrogenase has been purified to homogeneity from the yeast *Saccharomyces cerevisiae*. The purified enzyme exhibits a final specific activity of 5.4 units mg^{-1} and is represented by a single protein of apparent M_r = 33 000–38 000 as determined by sodium dodecyl sulfate gel electrophoresis. A native M_r = 64 000 was determined by gel filtration, suggesting a homodimer subunit structure. Cross-linking experiments with dimethyl suberimidate confirmed the dimeric structure. The enzyme is specific for NAD$^+$ and is not dependent on Mg^{2+} for activity. The forward reaction initial velocity kinetics are consistent with a sequential reaction mechanism. With this model, K_m values for NAD$^+$ and (6*R*,*S*)-5,10-methylene-THF are 1.6 and 0.06 mM, respectively. In contrast to all other previously described eukaryotic 5,10-methylene-THF dehydrogenases, the purified enzyme is apparently monofunctional, with undetectable 5,10-methenyl-THF cyclohydrolase and 10-formyl-THF synthetase activities. Subcellular fractionation of yeast indicates the enzyme is cytoplasmic, with no NAD$^+$-dependent 5,10-methylene-THF dehydrogenase detectable in mitochondria. The activity was found in all yeast strains examined, at all stages of growth from the lag phase through the stationary phase.

Folate-mediated one-carbon metabolism plays an essential role in several major cellular processes including nucleic acid biosynthesis, mitochondrial and chloroplast protein biosynthesis, amino acid biosynthesis and conversions, and vitamin metabolism. The variety of pathways that utilize these one-carbon units is dependent upon the ability of the organism to vary the oxidation state of the carbon unit attached to the coenzyme, tetrahydrofolate (THF).[1] In most organisms, the major source of one-carbon units is the 3-carbon of serine, derived from glycolytic intermediates (Schirch, 1978). The one-carbon unit is transferred to THF in a reaction catalyzed

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[1] Abbreviations: THF, tetrahydrofolate; Tris, tris(hydroxymethyl)-aminomethane; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; K-HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid potassium salt; PMSF, phenylmethanesulfonyl fluoride.

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by serine hydroxymethyltransferase (EC 2.1.2.1), generating 5,10-methylene-THF and glycine. This form of the coenzyme is required for de novo dTMP synthesis by thymidylate synthase (EC 2.1.1.45). 5,10-Methylene-THF may also be reduced to 5-methyl-THF or oxidized to 10-formyl-THF depending on the needs of the cell. In rapidly growing cells, the synthesis of purines is a critical folate-dependent pathway, requiring 2 mol of 10-formyl-THF/mol of purine ring. 5,10-Methylene-THF is oxidized to 10-formyl-THF via the sequential enzymes 5,10-methylene-THF dehydrogenase (EC 1.5.1.5) and 5,10-methenyl-THF cyclohydrolase (EC 3.5.4.9) (reactions 1 and 2, respectively). 5,10-Methylene-THF dehydrogenase is thus located at an important branch point of folate-mediated one-carbon metabolism.

5,10-methylene-THF + $NAD(P)^+ \leftrightarrow$ 5,10-methenyl-THF + NAD(P)H (1)

5,10-methenyl-THF + $H_2O \leftrightarrow$ 10-formyl-THF (2)

Two types of 5,10-methylene-THF dehydrogenase have been identified to date in eukaryotes. The major activity is found as a trifunctional enzyme, termed C_1-THF synthase, which includes 5,10-methenyl-THF cyclohydrolase and 10-formyl-THF synthetase activities as well (Paukert et al., 1976, 1977; Tan et al., 1977; Caperelli et al., 1978; Schirch, 1978; Cheek & Appling, 1989). In the yeast *Saccharomyces cerevisiae*, two isozymes of C_1-THF synthase exist, one in mitochondria and the predominate one in the cytoplasm (Shannon & Rabinowitz, 1986). In both of these trifunctional enzymes, the 5,10-methylene-THF dehydrogenase activity is $NADP^+$-dependent (Paukert et al., 1977; Shannon & Rabinowitz, 1986). It is not known whether higher eukaryotes also possess a trifunctional mitochondrial isozyme, but all three activities of C_1-THF synthase have been demonstrated in rat liver mitochondria (Barlowe & Appling, 1988). The second type of 5,10-methylene-THF dehydrogenase found in eukaryotic cells is an NAD^+-dependent activity that exists as part of a bifunctional protein containing a 5,10-methenyl-THF cyclohydrolase activity (Mejia et al., 1986). This enzyme is found in spleen, thymus, bone marrow, and many mammalian cell lines, but not in other adult tissues (Mejia & MacKenzie, 1985), and is localized to mitochondria (Meijia & MacKenzie, 1988). The metabolic function of this enzyme is not known. A third type of 5,10-methylene-THF dehydrogenase has been found in certain prokaryotes. Some anaerobic bacteria possess a monofunctional 5,10-methylene-THF dehydrogenase, specific for either NAD^+ (Moore et al., 1974; Ragsdale et al., 1984) or $NADP^+$ (Uyeda & Rabinowitz, 1967).

We report here the discovery of a monofunctional NAD^+-dependent 5,10-methylene-THF dehydrogenase in the yeast *S. cerevisiae*. Recently, we constructed a strain of yeast that expresses a catalytically inactive cytoplasmic C_1-THF synthase. To our surprise, this strain retained the ability to synthesize purines at normal rates (Barlowe and Appling, submitted for publication). We reasoned that the required 10-formyl-THF could be supplied by an additional cytoplasmic activity. Examination of several yeast strains revealed the existence of a novel NAD^+-dependent 5,10-methylene-THF dehydrogenase, the first observation of a monofunctional form of the enzyme in eukaryotes. In the present work, we have purified the enzyme to homogeneity and characterized it with respect to kinetics, subunit structure, and subcellular location.

Experimental Procedures

Materials and Strains. Dimethyl suberimidate, NAD^+, β-glucuronidase, folic acid, and 5-formyl-THF were purchased from Sigma Chemical Co. (6*R,S*)-Tetrahydrofolate was prepared by the hydrogenation of folic acid over platinum oxide (Matheson Coleman and Bell) in neutral aqueous solution (Blakley, 1957) and was purified by chromatography on DEAE-cellulose (Curthoys & Rabinowitz, 1971). The stock solution contained 10 mM (6*R,S*)-tetrahydrofolate, 0.2 M Tris-HCl, pH 7.0, and 0.5 M 2-mercaptoethanol. (6*R,S*)-5,10-Methylene-THF was chemically prepared from formaldehyde and (6*R,S*)-tetrahydrofolate at a molar ratio of 1.5:1 (Delk et al., 1976). (6*R,S*)-5,10-Methenyl-THF and (6*R,S*)-10-formyl-THF were prepared from 5-formyl-THF (Rabinowitz, 1963). *S. cerevisiae* strains KSY8 (**a** *ser1 ade3 mis1*::*URA3*) and KSY9 (**a** *ser1 mis1*::*URA3*) were used in these studies (Shannon & Rabinowitz, 1988). Growth medium (YPD) contained 1% yeast extract, 2% bactopeptone, and 2% dextrose.

Enzyme Assays. NAD^+-dependent 5,10-methylene-THF dehydrogenase was assayed by incubation in 0.1 M KCl, 0.02 M 2-mercaptoethanol, 0.05 M K-HEPES (pH 8.0), 0.5 mM (6*R,S*)-5,10-methylene-THF, and 2 mM NAD^+ at 37 °C for 5 min. Production of 5,10-methenyl-THF was determined by measuring the increase in absorbance at 350 nm after adjusting to a final concentration of 0.25 M HCl. Enzymic activity was calculated from the extinction coefficient at 350 nm of 24900 M^{-1} for 5,10-methenyl-THF. This assay is linear for at least 10 min when a background value (minus NAD^+) was subtracted and is linear with respect to protein concentration. 5,10-Methenyl-THF cyclohydrolase activity was measured in both directions by monitoring the decrease in absorption at 355 nm when starting with 5,10-methenyl-THF or the increase in absorption due to conversion of 10-formyl-THF to 5,10-methenyl-THF (Paukert et al., 1976). Reactions were incubated at 30 °C and contained 0.1 M potassium maleate (pH 7.4), 0.02 M 2-mercaptoethanol, 0.1 mM (6*R,S*)-5,10-methenyl-THF, or (6*R,S*)-10-formyl-THF. Methods for assay of 10-formyl-THF synthetase (Appling & Rabinowitz, 1985) and fumarase (Racker, 1950) have been described. Protein concentrations were determined by a dye-binding assay (Bradford, 1976) with bovine serum albumin as the standard.

Purification of NAD^+-Dependent 5,10-Methylene-THF Dehydrogenase. Strain KSY8 was grown at 30 °C to late log phase in 4 L of YPD. The following steps were performed at 4 °C. Cells were harvested by centrifugation and washed with deionized water. The cell pellet (40 g wet weight) was resuspended in 2 volumes of buffer A [25 mM Tris-HCl (pH 7.6), 2 mM EDTA, 10 mM 2-mercaptoethanol, 1 mM PMSF] and disrupted with glass beads. This lysate was centrifuged at 25000*g* for 30 min, and the resulting supernatant fluid was filtered through cheese cloth and then adjusted to a pH of 5.2 with dilute acetic acid. After centrifugation at 20000*g* for 30 min, the soluble fraction was adjusted to a pH of 7.6 with 1 N KOH. This extract was diluted with 10 mM 2-mercaptoethanol and 1 mM PMSF until the conductivity was below 1 mmho and applied to a 2.5 × 50 cm TSK-DEAE 650 (EM Science) column equilibrated with buffer A. The column was washed with 1 L of buffer A, and activity was eluted with 0.075 mM KCl in buffer A. Fractions that contained activity were pooled and loaded directly onto a 2.5 × 20 cm Matrix Gel Orange A (Amicon) affinity column equilibrated with buffer A. This column was washed with 200 mL of buffer A and then 400 mL of 0.025 M KCl in buffer A prior to elution with a 600-mL gradient from 0.025 M KCl to 0.80 M KCl in buffer A. The fractions containing peak amounts of activity were pooled, and solid KCl was slowly added with mixing to achieve a final concentration of 1.5 M. The re-

mainder of the purification was performed at room temperature.

The pooled fractions were loaded onto a 1 × 10 cm phenyl-Sepharose CL-4B (Sigma) column and washed with 100 mL of 0.025 M Tris-HCl (pH 7.6), 1.5 M KCl, and 1 mM EDTA, followed by 100 mL of buffer B [0.015 M Tris-HCl (pH 7.6), 1 mM EDTA]. Activity was eluted from the hydrophobic matrix with a step gradient containing 40% ethylene glycol in buffer B. The peak activity fractions were pooled and loaded directly onto a 0.46 × 25 cm Synchropak AX500 HPLC column (anion exchanger) equilibrated with buffer B at a flow rate of 1 mL/min. The absorbance at 260 nm returned to base line after washing with 40 mL of buffer B, activity was eluted with a 20-mL linear gradient from zero to 0.2 M KCl in buffer B, and 1-mL fractions were collected. Peak activity fractions were analyzed by SDS–PAGE, and fractions containing pure protein were pooled.

Gel Electrophoresis and Gel Filtration. The discontinuous buffer system described by Laemmli (1970) was used for monitoring protein purification. Analysis of cross-linked proteins was performed on the continuous system described by Davies and Stark (1970). All gels were stained with Coomassie Brilliant Blue. Analytical gel filtrations were carried out on a Synchropak GPC 300 HPLC column (0.46 × 25 cm) at a flow rate of 0.3 mL/min in buffer B containing 0.1 M KCl. The gel filtration column was calibrated by monitoring the elution of molecular weight standards (Pierce) by absorbance at 260 nm. Elution of purified 5,10-methylene-THF dehydrogenase from the column was monitored by enzyme activity assay and absorbance at 260 nm which corresponded to the activity peak.

Initial Velocity Kinetic Studies. The conversion of 5,10-methylene-THF to 5,10-methenyl-THF was measured in the same buffer as described for the enzyme assay. Initial velocity measurements were performed in tubes containing 0.4 μg of purified protein, and reactions were initiated by the addition of 5,10-methylene-THF. The concentration of NAD^+ was varied from 0.375 to 6 mM, and 5,10-methylene-THF was varied from 0.001 to 0.2 mM. The equilibrium concentration of 5,10-methylene-THF was calculated by solving the simultaneous equations for the equilibria between THF, formaldehyde, and 2-mercaptoethanol (Kallen & Jencks, 1966). Experimental values are from duplicate assays, and very similar results were obtained on two preparations of purified enzyme. The Michaelis–Menten parameters V_{max} and K_m were determined by the nonlinear regression computer program SEQUEN (Cleland, 1979). This program directly fits the data to the rate equation of an intersecting initial velocity pattern:

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_b + AB}$$

Subcellular Fractionation. Mitochondria were prepared by modification of a method described by McKee and Poyton (1984) and purified by sucrose gradient centrifugation. Strain KSY9 was grown in a semisynthetic medium consisting of 3 g of yeast extract, 0.8 g of $(NH_4)_2SO_4$, 10 g of galactose, 0.7 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of NaCl, 1.0 g of KH_2PO_4, 0.4 g of $CaCl_2$, and 5 mg of $FeCl_3 \cdot 6H_2O$, in 1 L. Cells were grown to a density of OD_{600} = 1.0 and then harvested by centrifugation, washed with dH_2O, and resuspended in 0.1 M Tris-HCl (pH 9.0) and 1 mM DTT at 0.2 g wet weight/mL. After 30 min at 30 °C with gentle shaking, cells were washed twice and resuspended in 1.35 M sorbitol and 1 mM EDTA, pH 7.4, at 0.2 g wet weight/mL. This solution was made 2% in β-glucuronidase and incubated at 30 °C with gentle shaking. Conversion to spheroplasts was monitored by measuring turbidity at 600 nm after removing an aliquot of cells and suspending in 0.1% SDS. Greater than 90% of the cells were converted to spheroplasts after 30 min. The spheroplasts were harvested by centrifugation, washed with 1 M sorbitol, and resuspended at 0.2 g/mL in 0.6 M mannitol and 1 mM EDTA, pH 6.7. The remaining steps were performed at 4 °C. Cells were homogenized in a Teflon homogenizer (10 strokes) and centrifuged at 1900*g* for 5 min, and the resulting supernatant fluid was centrifuged at 13000*g* for 10 min to pellet mitochondria. The pellet was rehomogenized in mannitol buffer, and the low-speed and high-speed spins were repeated. The final pellet was resuspended in 2.5 mL of mannitol/EDTA and layered onto a 32-mL continuous sucrose gradient from 1 to 2 M sucrose (Rickwood et al., 1987). After centrifugation at 80000*g* for 135 min, a distinct brownish band was visualized midway down the gradient and removed with a Pasteur pipet. Mitochondria were recovered after dilution with an equal volume of dH_2O and centrifugation at 20000*g* for 15 min. Unfractionated (washed spheroplasts), cytosol (13000*g* supernatant), and mitochondrial extracts were prepared by dilution with 3 volumes of buffer A and sonication at maximum output on a Vibra-Cell (Danbury, CT) Model VC40 for 30 s. The sonicated extracts were centrifuged at 20000*g* for 30 min, and the supernatant fractions were analyzed for enzyme activities.

Table I: Purification of NAD^+-Dependent 5,10-Methylene-THF Dehydrogenase from Strain KSY8

step	protein (mg)	units (μmol/min)	specific activity (units/mg)	purification (*x*-fold)	yield (%)
crude extract[a]	2056	56	0.027	1	100
pH 5.2 supernatant	1004	56	0.056	2	100
TSK-DEAE	291	47	0.162	6	84
Orange A	13	12	0.932	35	21
phenyl-Sepharose	2.2	6.3	2.25	83	11
AX500 HPLC	0.95	5.1	5.38	200	9

[a] Prepared from 40 g of cells.

RESULTS

Enzyme Purification. An NAD^+-dependent 5,10-methylene-THF dehydrogenase was purified from *S. cerevisiae*. Cell extracts were prepared by disruption with glass beads since this method allowed for a greater recovery of enzyme activity than disruption by passage through a French pressure cell. The protein was purified from strain KSY8, which contains neither the cytoplasmic C_1-THF synthase, due to an extensive deletion of the encoding *ADE3* locus, nor the mitochondrial isozyme of C_1-THF synthase, due to disruption of the encoding *MIS1* locus (Shannon & Rabinowitz, 1988). This strain was chosen to avoid any possible contamination with either of the trifunctional C_1-THF synthase proteins. However, crude extracts from all *S. cerevisiae* strains tested (including *ADE3*$^+$ and *MIS1*$^+$ strains) exhibited the NAD^+-dependent 5,10-methylene-THF dehydrogenase activity at approximately the same specific activity (data not shown). The protein was purified from a crude extract in five steps by pH treatment and application to DEAE, Orange A, phenyl-Sepharose, and anion-exchange columns. Elution profiles of enzyme activity from these chromatography columns demonstrated a single peak of activity at each step. As summarized in Table I, the enzyme was purified 200-fold with a 9% yield. Figure 1 shows SDS–PAGE of extracts after purification steps, and a single band is observed after the final column. Analysis of the peak activity fractions eluting from the AX500 HPLC

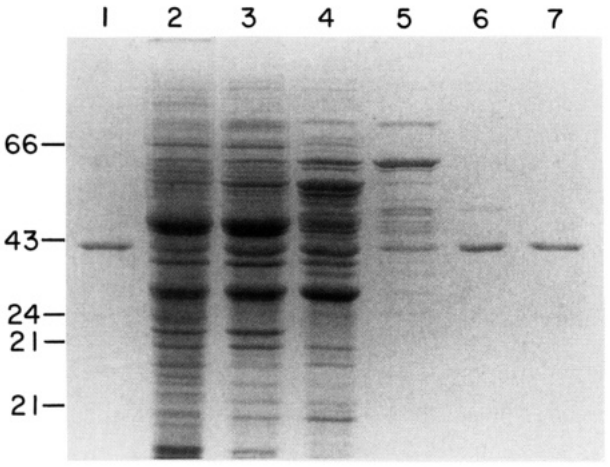

FIGURE 1: Purification of NAD^+-dependent 5,10-methylene-THF dehydrogenase analyzed on a 12.5% SDS–polyacrylamide gel. Lanes 1 and 7, purified protein after HPLC (1 μg); lane 2, crude extract (20 μg); lane 3, pH 5.2 supernatant (20 μg); lane 4, DEAE pool (20 μg); lane 5, Orange A pool (2 μg); lane 6, phenyl-Sepharose pool (1 μg). Reference markers are bovine serum albumin (66 000), ovalbumin (43 000), trypsinogen (24 000), trypsin inhibitor protein (21 500), and α-lactalbumin (14 200).

column by SDS–PAGE demonstrated correlation of activity with the intensity of a single band. The enzyme activity is stable for several weeks after storage in the final elution buffer at 4 °C.

Characterization of Enzyme Activity. An absorption spectrum of the product formed from incubation of 5,10-methylene-THF, NAD^+, and enzyme is identical with that of authentic 5,10-methenyl-THF (Temple & Montgomery, 1984) when the spectra are taken in 0.25 N HCl, exhibiting a maximum at 350 nm. In order to obtain a spectrum on the product directly, we carried out the reaction at pH 7.0 in the presence of an NAD^+-regenerating system. The product showed an absorbance maximum at 355 nm, identical with that for authentic 5,10-methenyl-THF at neutral pH (Temple & Montgomery, 1984). $NADP^+$ is neither a substrate nor an inhibitor of the purified enzyme when tested at concentrations up to 5 mM. We did not find any divalent cation requirements for the NAD^+-dependent 5,10-methylene-THF dehydrogenase activity, and the activity was unaffected by addition of EDTA. Within the range of pH 6–10, a pH of 8.0 was found to be optimal, with enzyme activity decreasing approximately 2-fold at pH 7.0 or 9.0. Alterations in ionic strength or type of buffer did not have a significant effect. We were unable to detect 5,10-methenyl-THF cyclohydrolase activity associated with the purified protein when providing either 5,10-methenyl-THF or 10-formyl-THF as substrates under several conditions including the addition of Mg^{2+} or NAD^+. Our ability to assay for 5,10-methenyl-THF cyclohydrolase activity in both directions was verified by using purified yeast C_1-THF synthase which contains cyclohydrolase activity.

Initial velocity measurements for 5,10-methylene-THF dehydrogenase were obtained at various concentrations of NAD^+ and methylene-THF. Plots of inverse substrate–velocity data are summarized in Figure 2 and reveal a set of converging lines. The converging linear plots are indicative of a sequential enzyme reaction mechanism (Cleland, 1970). The kinetic parameters with standard errors were obtained by a nonlinear least-squares analysis computer program SEQUEN, which gave a good fit to the rate equation for a sequential initial velocity pattern (see Experimental Procedures) (Cleland, 1979). The calculated K_m values for NAD^+ and (6*R*,*S*)-5,10-methylene-THF are 1.6 ± 0.3 mM and 0.06 ± 0.01 mM, respectively, and the V_{max} is 9.7 ± 0.8 μmol min^{-1} mg^{-1}.

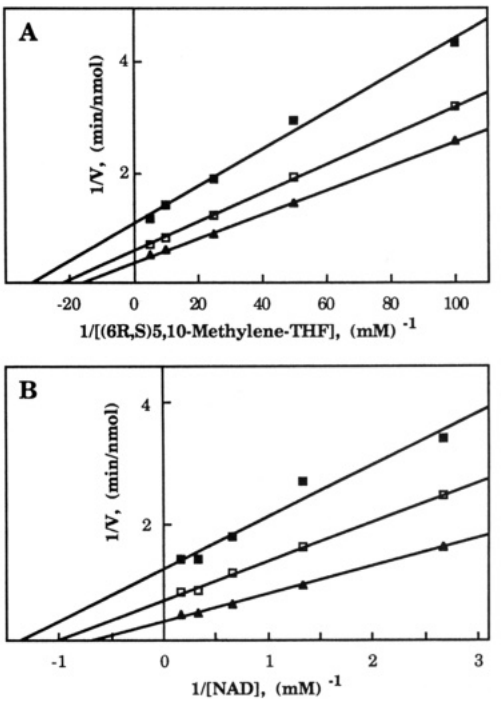

FIGURE 2: Lineweaver–Burk plots of initial velocity data for *S. cerevisiae* 5,10-methylene-THF dehydrogenase. (A) Plot of initial velocity data at 0.75 mM (■), 1.5 mM (□), and 3.0 mM (▲) NAD^+ while (6*R*,*S*)-5,10-methylene-THF concentrations were varied from 0.01 to 0.2 mM. (B) Plot of initial velocity data at 0.02 mM (■), 0.04 mM (□), and 0.2 mM (▲) (6*R*,*S*)-5,10-methylene-THF while NAD^+ concentrations were varied from 0.38 to 6.0 mM.

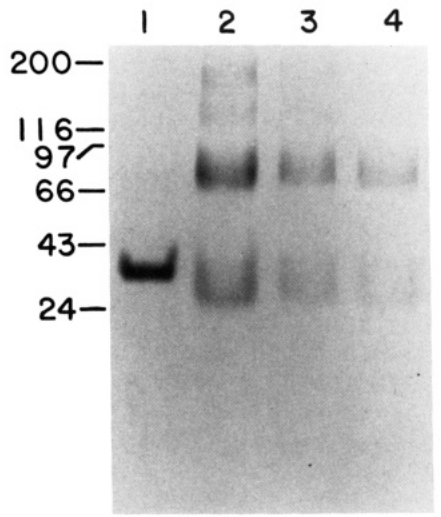

FIGURE 3: Cross-linking of purified 5,10-methylene-THF dehydrogenase with dimethyl suberimidate. Purified protein was incubated at final concentrations of 0.2 mg/mL (lane 2), 0.1 mg/mL (lane 3), and 0.05 mg/mL (lane 4) with 2.5 mg/mL dimethyl suberimidate in a buffer containing 0.02 M KCl and 0.2 M triethanolamine (pH 8.5). Reactions were incubated at room temperature for 3 h in a total volume of 50 μL. Twenty-microliter aliquots of the reaction mixtures were analyzed on 7% SDS–polyacrylamide gels as described by Davies and Stark (1970). Lane 1 contains 2 μg of untreated protein, and reference markers are myosin (200 000), β-galactosidase (116 000), phosphorylase *b* (97 000), bovine serum albumin (66 000), ovalbumin (43 000), and trypsinogen (24 000).

Physical Properties. Analysis of the purified protein by discontinuous SDS–PAGE reveals a single band migrating below the ovalbumin molecular weight marker (Figure 1). A plot of electrophoretic mobility versus the log molecular weight of marker proteins yields a line which was used to calculate

Table II: Subcellular Fractionation of Strain KSY9

fraction[a]	ratio for 10-formyl-THF synthetase	fumarase	5,10-methylene-THF dehydrogenase[b]
(mitochondrial activity)/(unfractionated activity)	0.03	7.87	0.08
(cytoplasmic activity)/(unfractionated activity)	1.31	0.39	1.48

[a] Values shown represent the ratio of enzyme specific activity (units/mg) between mitochondrial or cytoplasmic fractions and the unfractionated extract. [b] Activity is NAD+-dependent 5,10-methylene-THF dehydrogenase.

a molecular weight of 38K for the methylene-THF dehydrogenase protein. The molecular weight of the native protein was estimated to be 64K by gel filtration on a GPC-300 column. An additional experiment to investigate the subunit arrangement of the NAD+-dependent methylene-THF dehydrogenase is shown in Figure 3. The purified protein was incubated with the chemical cross-linker dimethyl suberimidate and analyzed by a continuous SDS–PAGE system described by Davies and Stark (1970). Cross-linking results in the appearance of an additional species with an electrophoretic mobility corresponding to twice the size of the monomer molecular weight, suggesting a homodimer arrangement for the native protein. It is interesting to note that the electrophoretic mobility of untreated protein in the continuous system (Figure 3, lane 1) predicts a subunit molecular weight of 33K for methylene-THF dehydrogenase. The lower molecular weight species observed in lanes containing protein treated with cross-linking agent (Figure 3, lanes 2–4) migrates more diffusely and slightly faster than untreated monomer. We believe this increase in electrophoretic mobility is due to *intra*molecular cross-linking of dehydrogenase monomer, resulting in a more compact molecule. In any event, the cross-linking results and the gel filtration results are consistent with the native enzyme existing as a homodimer with an approximate subunit molecular weight of 33K–38K.

Subcellular Localization of NAD+-Dependent 5,10-Methylene-THF Dehydrogenase. We investigated the subcellular location of yeast NAD+-dependent 5,10-methylene-THF dehydrogenase by differential centrifugation. *S. cerevisiae* strain KSY9, which contains a disruption of the *MIS1* gene encoding mitochondrial C_1-THF synthase and is wild type at the *ADE3* locus encoding cytoplasmic C_1-THF synthase, was chosen for this study so that cytoplasmic C_1-THF synthase could be used as a cytoplasmic marker activity. Cells were converted to spheroplasts, gently lysed, and fractionated by differential centrifugation. Mitochondria were further purified by sucrose gradient centrifugation. The results of this fractionation are shown in Table II. Analyses of the marker activities demonstrate the mitochondrial fraction obtained after sucrose gradient purification was highly enriched in fumarase activity (a mitochondrial matrix enzyme) while containing only trace amounts of cytoplasmic marker activity (10-formyl-THF synthetase activity contained on C_1-THF synthase). The very low amount of NAD+-dependent 5,10-methylene-THF dehydrogenase activity associated with the mitochondrial fraction indicates this activity is not localized to mitochondria. The cytoplasmic fraction was initially obtained by collecting the 20000*g* supernatant from lysed spheroplasts. This supernatant extract was then centrifuged at 80000*g*, and both pellet and supernatant fluid were assayed for NAD+-dependent 5,10-methylene-THF dehydrogenase. Greater than 95% of the methylene-THF dehydrogenase activity remained associated with the supernatant fraction (data not shown). These results support the localization of the NAD+-dependent 5,10-methylene-THF dehydrogenase to the cytosol.

Discussion

This enzyme represents the first reported example of a monofunctional 5,10-methylene-THF dehydrogenase in eukaryotes. The size, subunit structure, and coenzyme specificity of the yeast enzyme are similar to that of the NAD+-dependent 5,10-methylene-THF dehydrogenase from mammalian tumor cells (Mejia et al., 1986). However, the enzyme we have purified from yeast differs from the mammalian enzyme in several respects. First, the yeast enzyme is monofunctional. We could detect no 5,10-methenyl-THF cyclohydrolase activity in the purified enzyme, using either 5,10-methenyl-THF or 10-formyl-THF as substrate. Second, although both dehydrogenases are specific for NAD+, the mammalian enzyme requires Mg^{2+} for maximal activity (Mejia et al., 1986). The yeast enzyme exhibited no Mg^{2+} dependence and was not inhibited by EDTA. Thus, the monofunctional 5,10-methylene-THF dehydrogenase from yeast appears most similar to the NAD+-dependent enzymes found in the bacteria *Clostridium formicoaceticum* (Moore et al., 1974) and *Acetobacterium woodii* (Ragsdale & Ljungdahl, 1984). All of the prokaryotic 5,10-methylene-THF dehydrogenases characterized to date, whether monofunctional or multifunctional, exhibit a similar subunit molecular weight (30K–35K). Even in the trifunctional C_1-THF synthases, the dehydrogenase/cyclohydrolase domain is approximately 30K (Paukert et al., 1977; Tan et al., 1977; Villar et al., 1985). Nucleotide sequences are available for five dehydrogenase/cyclohydrolase domains, including C_1-THF synthases from yeast cytoplasm (Staben & Rabinowitz, 1986), yeast mitochondria (Shannon & Rabinowitz, 1988), human (Hum et al., 1988), and rat (Thigpen et al., 1990) and the bifunctional NAD+-dependent enzyme from tumor cells (Belanger & MacKenzie, 1989). Comparison of the predicted amino acid sequences of these enzymes reveals significant homologies, ranging from 40% to 90% identity, including one 15-residue stretch of perfect matches across all five sequences (Thigpen et al., 1990). Clearly these represent an evolutionarily related gene family. The relationship of the yeast monofunctional dehydrogenase to this family is not known. We were unable to detect any cross-reaction on immunoblots of this enzyme with antibodies against the yeast cytoplasmic C_1-THF synthase (unpublished results). In an effort to obtain sequence information, experiments are now in progress to isolate the yeast gene encoding the monofunctional 5,10-methylene-THF dehydrogenase.

The initial velocity measurements obtained by varying the concentrations of NAD+ and 5,10-methylene-THF indicate a sequential enzyme reaction mechanism for the yeast NAD+-dependent 5,10-methylene-THF dehydrogenase. The only other report on an NAD+-dependent methylene dehydrogenase reaction mechanism is for the enzyme from *A. woodii* and indicates a substituted (ping-pong) model (Ragsdale & Ljungdahl, 1984). The reason for different mechanisms between the *A. woodii* and yeast enzymes is not known. Kinetic parameters for the yeast enzyme were obtained by fitting the initial velocity data to the rate equation for bireactant sequential reactions. The Michaelis constants for NAD+ and 5,10-methylene-THF obtained by this method are similar to K_m values for NAD+-dependent methylene dehydrogenases from *A. woodii* (Ragsdale & Ljungdahl, 1984) and *C. formicoaceticum* (Moore et al., 1974). However, the specific activities of the purified prokaryotic methylene dehydrogenases are approximately 100-fold greater than we observed for the yeast protein.

The metabolic role of the monofunctional enzyme in yeast

is at present undefined. However, the unique properties of the enzyme provide several clues. Subcellular fractionation results indicate that the enzyme is confined to the cytoplasm of yeast cells. Thus, we can assume the enzyme is involved in the oxidation or reduction of the one-carbon unit for some cytoplasmic folate-dependent process, such as de novo purine synthesis. The cofactor specificity provides an important clue as to the predominant direction of flux through this enzyme in vivo. Serine, generated in the cytoplasm, is believed to be the major one-carbon donor in most cells (Schirch, 1984). Cytoplasmic synthesis of 10-formyl-THF utilizing the 3-carbon of serine would require oxidation of 5,10-methylene-THF to 5,10-methenyl-THF via 5,10-methylene-THF dehydrogenase. A question arises as to the role of the $NADP^+$-dependent dehydrogenase activity of the cytoplasmic C_1-THF synthase in this process (Barlowe & Appling, 1988). Most cytoplasmic $NADP^+$-linked dehydrogenases operate in the opposite direction (i.e., oxidation of NADPH) (Saier, 1987). On the other hand, the NAD^+/NADH redox ratio typically observed in the cytoplasm of eukaryotic cells (Sies, 1982) would favor the oxidation of 5,10-methylene-THF by a NAD^+-linked dehydrogenase such as the one described here. The level of NAD^+-linked activity in rapidly growing yeast is certainly adequate to support this pathway: the specific activities of the NAD^+- and $NADP^+$-dependent 5,10-methylene-THF dehydrogenases are virtually identical in crude extracts from wild-type yeast (27 vs 29 milliunits/mg, respectively). Further support for a role for this enzyme in the oxidation of one-carbon units is the ability of yeast expressing an inactive cytoplasmic C_1-THF synthase to produce 10-formyl-THF at sufficient levels to support normal rates of purine synthesis (Barlowe and Appling, submitted for publication). It is not known whether an additional 5,10-methenyl-THF cyclohydrolase is also present in the cytoplasm of yeast to catalyze the conversion of 5,10-methenyl-THF to 10-formyl-THF.

The discovery and characterization of this novel NAD^+-dependent 5,10-methylene-THF dehydrogenase from yeast shed new light on our understanding of folate-mediated one-carbon metabolism in eukaryotes. Isolation of the gene will allow us to apply powerful genetic and biochemical methods to determine the role of this enzyme and its relationship to the other folate-dependent interconverting enzymes in yeast.

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