FORMYL-METHENYL-METHYLENETETRAHYDROFOLATE SYNTHETASE(COMBINED):* Correlation of Enzymic Activities with Limited Proteolytic Degradation of the Protein from Yeast

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SUMMARY

A protein purified from extracts of *Saccharomyces cerevisiae* contains formyltetrahydrofolate synthetase (51 units/mg), methenyltetrahydrofolate cyclohydrolase (4 units/mg), and methylenetetrahydrofolate dehydrogenase (3 units/mg) activities. The protein is a dimer of identical subunits of M_r = 104,000. A formyltetrahydrofolate synthetase devoid of the other activities, a dimer of subunits of M_r = 76,000 has also been isolated. Exposure of the trifunctional protein to trypsin produces coordinate decay of the dehydrogenase and cyclohydrolase activities and conversion of the 104,000 dalton subunit to one electrophoretically identical to that of the monofunctional synthetase.

Multifunctional proteins involved in the formation and interconversion of one-carbon adducts of tetrahydrofolate (THF)‡ have recently been isolated from mammalian sources (1-4). A protein from ovine liver (1), a dimer of identical subunits, catalyzes the sequential reactions specified by the three enzymes formyl-THF synthetase (EC 6.3.4.3), methenyl-THF cyclohydrolase (EC 3.5.4.9), and methylene-THF dehydrogenase (EC 1.5.1.5). Biochemical and genetic characterization (5) of yeasts (*Saccharomyces cerevisiae*) mutated at the *ade-3* locus, that are partially or completely deficient in these three enzymes, suggests that the enzymes are coded by contiguous regions within the locus and

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*The trivial name for this protein, which contains multiple catalytic activities in a single polypeptide species, is the same proposed for the homologous protein isolated from sheep liver (1).

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‡Abbreviations: dansyl-, 1-dimethylaminonapthalene-5-sulfonyl-; DS, sodium dodecylsulfate; 2-met, 2-mercaptoethanol; PMSF, phenylmethanesulfonylfluoride; THF, tetrahydrofolate.

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are physically associated. We describe here the isolation from yeast of a protein containing these three activities and its structural similarity to the trifunctional mammalian proteins. Additionally, experiments are described which correlate the loss of enzymic activity with the reduction of subunit size by limited proteolytic digestion.

MATERIALS AND METHODS

Chromatographic materials and folate substrates were prepared as previously described (1). Trypsin (TPCK-treated) was purchased from Worthington Biochemicals, soybean trypsin inhibitor from Sigma Chemical Co.

Enzyme assays were performed as previously described (1) except that 1 *M* Tris·Cl, pH 7.6, was substituted for the maleate buffer and ammonium chloride for the potassium chloride in the formyl-THF synthetase assay. Enzyme was diluted in 25 *mM* Tris·Cl, pH 7.5/10 *mM* KCl/10 *mM* 2-met prior to all assays.

DS gel electrophoresis. The slab format of Studier (6) was used with the discontinuous buffer system of Laemmli (7). Gels contained 10% acrylamide cross-linked at a ratio of 38:1. Samples were denatured by heating at 100° for 2 min in 2% DS/2% 2-met. Gels were stained with Coomassie blue in isopropanol/acetic acid (8) and were scanned at 600 nm for quantitation of protein (RFT scanning densitometer, Transidyne Corp.).

Cell growth and storage. Cultures of *Saccharomyces cerevisiae*, strain M16-14C (a haploid strain of genotype *a ser-1, leu-1*), obtained from E. W. Jones (Carnegie-Mellon University, Pittsburgh, Pa.) were routinely grown aerobically at 30° in YEPD medium composed of 1% yeast extract, 2% peptone, and 2% glucose. Cells were also grown on synthetic medium (yeast nitrogen base (9)) supplemented with glucose (20 g/liter), uracil (10 mg/liter), and a mixture of 11 amino acids (Leu, Ser, Ile, Glu, Try, Tyr, His, Arg, Thr, Met, and Lys) (L-isomer each at 10 mg/liter). Cells were harvested at late log phase and were stored at -70° for up to 4 months without loss of activity.

Enzyme purification.

Extract. Cells (800 g) were thawed and suspended in 0.1 *M* Tris·sulfate/0.1 *M* KCl/10 *mM* 2-met, pH 7.5, to a density of 30 to 40 g/100 ml. The suspension was passed through a Manton-Gaulin homogenizer for 6 to 8 passes at a pressure of 8,000 psi. The suspension was maintained at 10 to 30° during homogenization. All subsequent steps were performed at 0 to 4°. The extract was centrifuged at 9,500 rpm (13,500 x *g*) in a Sorvall RC-2 centrifuge (GSA rotor) for 45 min and was decanted through cheesecloth.

Protamine sulfate fractionation. A solution of protamine sulfate (Elanco Products Div., Eli Lilly & Co.), (2%, w/v in H_2O, neutralized with Tris base) was added dropwise to the extract to a final concentration of 0.3%. After stirring for 45 min, the solution was centrifuged as above. The supernatant was collected.

Ammonium sulfate fractionation. The preparation was fractionated with ammonium sulfate between 42% and 62% of saturation as previously described (1) except that 24.3 g/100 ml was added initially. There was no heat step. The 62% pellet was dissolved in 200 ml (total) of column buffer (25 *mM* Tris·sulfate/10 *mM* KCl/10 *mM* 2-met, pH 7.5). A solution of PMSF (40 *mM* in 95% ethanol) was added gradually to a final concentration of 1 *mM*.

Agarose chromatography. The preparation was chromatographed in column buffer on a column of Bio-Gel A-0.5m (Bio-Rad Labs) 5 x 160 cm. Fractions containing formyl-THF synthetase activity were pooled and were treated with PMSF as above.

DEAE-cellulose chromatography. The sample, at a conductivity of 1.25 mMho (at 5°) and protein concentration of 16 mg/ml, was applied to a column of

DEAE-cellulose (5 x 40 cm) in column buffer. The column was developed with a linear gradient of 0 to 0.3 *M* KCl in 4.0 liters of buffer. Fractions containing synthetase activity were pooled and treated with PMSF as above. Phosphoryl-cellulose chromatography, 1. The sample was diluted to a conductivity (at 0^0) of less than 4 mMho and applied to a column of phosphoryl-cellulose (3.4 x 28 cm) in column buffer. The column was developed with a linear gradient of 0 to 0.3 *M* KCl in 2.0 liters of buffer. Fractions containing the synthetase activity were pooled and treated with PMSF as above. Phosphoryl-cellulose chromatography, 2. The sample was diluted as above and applied to a column of phosphoryl-cellulose (2.0 x 17 cm). The column was washed sequentially with 100 ml each of buffers containing 100, 125, and 10 *mM* (total) KCl. In the preparation in Table 1, elution was batch-wise with buffer containing 12 *mM* Na_2ATP. More homogeneous preparations have been obtained by elution with a gradient of 0 to 15 *mM* $Na_2ATP/MgCl_2$ in 25 *mM* Tris·sulfate, pH 7.5/10 *mM* 2-met.

RESULTS AND DISCUSSION

Enzyme Purification. A modification of the method used for the purification of the trifunctional protein from sheep liver (1) has been applied to extracts of yeast to yield a 950-fold purification with 21% recovery. The progress of one large-scale preparation is summarized in Table 1. The product is essentially homogeneous by the criterion of DS gel electrophoresis (see below) and contains the three enzyme activities at ratios comparable to those in crude extracts. The ratios of the dehydrogenase and of the cyclohydrolase to the synthetase are somewhat lower than those for the sheep protein (0.2 and 0.5, respectively) (1) or for the porcine protein (0.5 and 1.6 respectively) (3).

Native Molecular Weight. The molecular weight of the protein has been estimated by molecular-exclusion chromatography on a column of Sephadex G-150 in 25 *mM* Tris·sulfate, pH 7.5/0.1 *M* KCl/10 *mM* 2-met. The elution position of the formyl-THF synthetase activity corresponds to a molecular weight of 201,000.

Subunit Molecular Weight and Number. The molecular weight of the only major component in highly purified preparations has been estimated from its relative mobility in DS gel electrophoresis (10). The subunit molecular weight from four determinations is 104,500 ± 900.

The protein was subjected to amino-terminal sequence analysis by the Edman degradation/dansylation protocol of Weiner et al. (11). Labelling after zero, one, and two cycles of degradation produced the dansyl derivatives of

TABLE 1

Purification of Three Tetrahydrofolate Enzymes

Purification step	Total protein	Formyl-THF Synthetase		Specific activity ratios	
		Total	Specific activity	Dehydrogenase / Synthetase	Cyclohydrolase / Synthetase
	grams	units	units/mg		
1. Homogenate	78.1	4,280	0.054	0.143	0.078
2. Protamine sulfate 0.3% supernatant	38.7	3,950	0.101	0.138	0.118
3. Ammonium sulfate 42% supernatant	36.1	3,280	0.091	0.086	0.133
4. Ammonium sulfate 42-62%	23.9	3,340	0.139	0.077	0.040
5. Bio-Gel A-0.5m	10.8	2,520	0.232	0.063	0.017
6. DEAE-cellulose	0.947	1,570	1.66	0.057	0.045
7. Phosphoryl-cellulose 1	0.0312	1,290	41.2	0.068	0.058
8. Phosphoryl-cellulose 2	0.0174	895	51.3	0.056	0.083

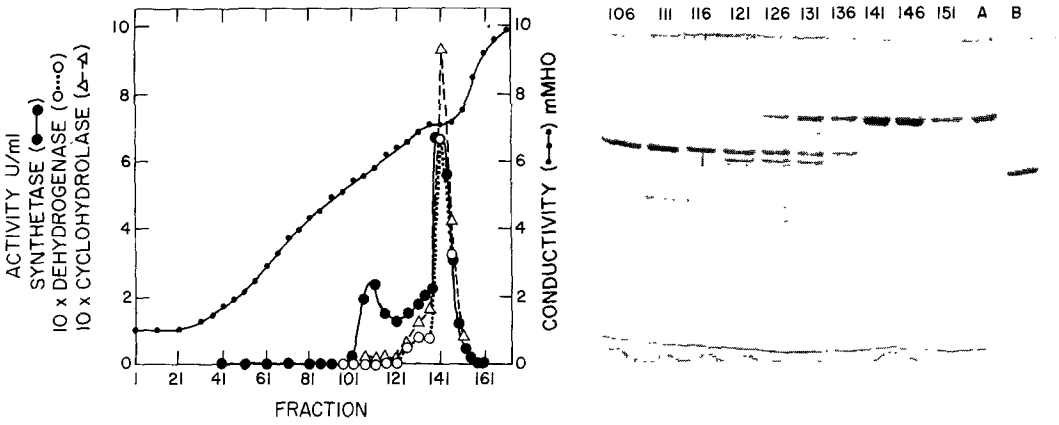

Figure 1. Separation of formyl-THF synthetases on phosphoryl-cellulose. Left. Elution profile of enzymes from phosphoryl-cellulose developed with a gradient of potassium chloride as described in "Methods." Right. DS gel electrophoresis of column fractions. Aliquots of the indicated fractions containing 0.08 units of synthetase activity were analyzed as described in "Methods." Reference proteins are A. trifunctional protein from sheep liver (M_r = 108,500) 0.8 µg and B. formyl-THF synthetase from *Clostridium acidi-urici* (M_r = 60,000), 0.8 µg.

alanine, glycine, and proline, respectively. There was no evidence of amino-terminal heterogeneity.

Isolation of Two Formyl-THF Synthetases. An extract of yeast grown on purine-deficient synthetic medium was processed through the DEAE-cellulose chromatography step as described above. PMSF was not added at any time, and no anomalous or heterogeneous behavior was noted in the early steps. When the DEAE-cellulose pool was chromatographed on phosphoryl-cellulose, as shown in Figure 1, a small peak containing about 25% of the total activity eluted prior to the main activity peak. The minor peak was devoid of dehydrogenase and cyclohydrolase activities, while the major peak contained them in approximately the expected ratios.

Analysis of the column fractions by DS gel electrophoresis (Fig. 1,right) shows them to contain two major polypeptide species and numerous minor ones. The intensity of a band having a mobility corresponding to a M_r = 76,000 paral-

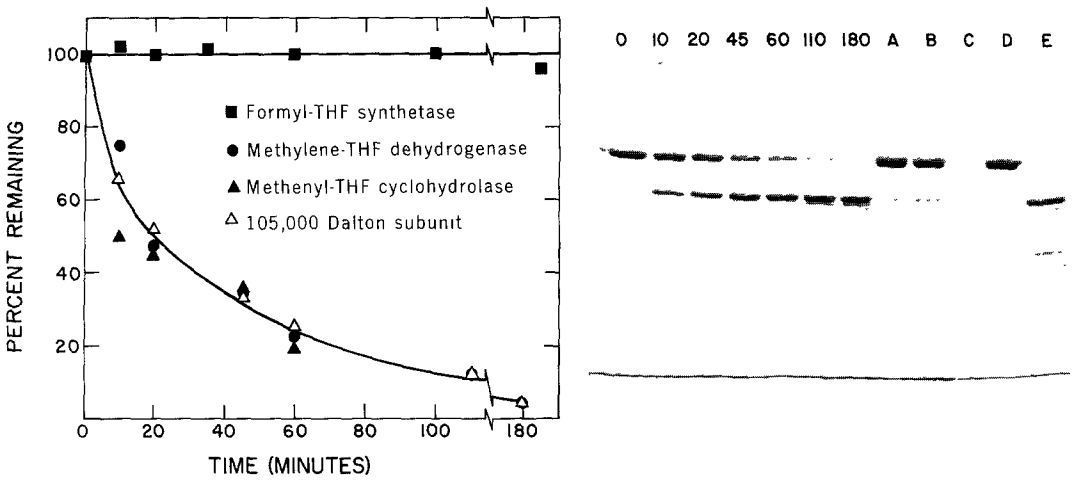

Figure 2. Tryptic degradation of the trifunctional protein. Protein was incubated at 2.2 μ*M* (in subunits) with 0.27 μ*M* trypsin in 22 *mM* Tris/10 *mM* Na_2ATP/10 *mM* $MgCl_2$/9 *mM* 2-met at 37° for periods of up to 3 hours. Aliquots were diluted in 3 x volume of buffer at 0° containing a 10 x molar excess of trypsin inhibitor. Control reactions contained trypsin inhibitor during the incubation period. Left. Change in enzyme activities and in remainder of the large subunit during the course of trypsin treatment. Right. Change in protein composition during the course of trypsin treatment. Samples containing 0.08 units of formyl-THF synthetase activity were analyzed by DS gel electrophoresis as described in "Methods." A. Control treated zero min. B. Control treated 180 min. C. Blank. D. Sample not exposed to trypsin. E. Sample from the first peak of the column profile in Figure 1 Left.

lels the synthetase activity of the minor peak and a band of M_r = 105,000 parallels the three enzyme activities of the major peak.

The major peak fractions were pooled and chromatographed on phosphoryl-cellulose developed with a gradient of MgATP (see "Methods"). A symmetric peak containing 75% of the applied activity was obtained. This pool (Fig. 2, right, slot D) was nearly devoid of contaminant protein and contained only a trace of the 76,000 dalton species. It contained dehydrogenase and cyclohydrolase activities at 7.2% and 14.3%, respectively, of the synthetase.

The molecular weight of the native monofunctional formyl-THF synthetase was estimated by molecular-exclusion chromatography as described above. Its elution position corresponds to a M_r = 145,000. Thus, both proteins have native molecular weights consistent with homodimeric structure, the trifunctional

protein having a subunit M_r = 104,000, the monofunctional one having a subunit M_r = 76,000.

Tryptic Degradation of the Trifunctional Protein. It was hypothesized that the monofunctional synthetase was produced by the action of a protease on the trifunctional protein, either *in vivo* or during isolation. In an attempt to reproduce the action of the putative protease, the purified trifunctional protein was subjected to limited proteolysis *in vitro*. After incubation with trypsin for various periods of time, aliquots were assayed for the three enzymic activities and were examined by DS gel electrophoresis for changes in protein composition. As illustrated in Fig. 2,left, there was no change in formyl-THF synthetase activity, but the other two activities declined coordinately. The fraction of total protein remaining as the large subunit decreased coordinately with the two enzymic activities, with the appearance of a smaller subunit having a mobility comparable to that of the monofunctional synthetase described above (Fig. 2,right, slot E). The sum of the intensities of the two bands remains approximately constant and there is no appearance of species of intermediate mobility. In control incubations, there was no significant degree or direction of change of any of the activities or of the protein composition (Fig. 2,right, slots A and B).

The proteolytic processing of the dimeric, trifunctional protein by a discrete and uniform removal of 27% of the mass of each subunit to produce a dimeric monofunctional synthetase is quite similar to the processing of other multifunctional proteins (containing multiple catalytic functions with a single type of polypeptide chain) (12). Some of these, most notably the aspartokinase I:homoserine dehydrogenase I (13) and the anthranilate synthetase component II (14) of the enteric bacteria, have been proposed on genetic and structural evidence to be comprised of discrete domains produced by the folding of sections of the polypeptide chain, to which are localized their constituent functions.

The existence of this trifunctional protein involved in folate-mediated

metabolism of one-carbon units clarifies the nature of the lesion produced by mutation at the *ade-3* locus in yeast. The pleiotropic effects and complex intragenic complementation patterns of fungal gene clusters are seen with increasing frequency to be due to the production of single polypeptide species having multiple catalytic functions (15-18).

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