Purification and Characterization of Selenomethionyl Thymidylate Synthase from *Escherichia coli*: Comparison with the Wild-Type Enzyme†

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Received May 6, 1991; Revised Manuscript Received July 12, 1991

ABSTRACT: Replacement of methionine (Met) residues by selenomethionine (SeMet) was recently shown to facilitate the crystallographic analysis of protein structure through the application of multiwavelength anomalous diffraction techniques [Yang et al. (1990) *Science* (*Washington, D.C.*) *249*, 1398–1405]. The availability of SeMet-containing proteins provides an excellent opportunity to evaluate the effects of the complete replacement of Met by SeMet. We chose to compare the properties of selenomethionyl thymidylate synthase isolated from *Escherichia coli* DL41 (a methionine auxotroph) and wild-type (wt) enzyme obtained from *E. coli* Rue10. An improved purification procedure for thymidylate synthase was developed which permitted the isolation of 25 mg of pure protein from 2 g of *E. coli* in 90% yield in no more than 8 h. The pure wt and SeMet enzymes exhibited specific activities 40% higher than published values. Thermal stability studies at 30 °C in degassed buffer showed that the SeMet enzyme ($t_{1/2}$ 67 h) was 8-fold less stable than wt enzyme ($t_{1/2}$ 557 h). The half-lives for the latter enzymes in nondegassed buffers at 30 °C were decreased by 2-fold, thus indicating the sensitivity of the enzyme to dissolved oxygen. Both enzymes exhibited essentially the same kinetic and binding properties, including $K_{m(dUMP)}$ (1.2×10^{-6} M), specificity constant (1.6×10^{6} s^{-1} M^{-1}), and K_d for 5-fluorodeoxyuridylate binding (1.2 nM) in covalent inhibitory ternary complexes. In addition, X-ray crystallographic analysis by difference Fourier synthesis showed there was no significant difference in conformation between the SeMet enzyme and the wt enzyme.

Recently, it was demonstrated that the selenium atom of selenium-substituted biotin associated with streptavidin provided sufficiently strong anomalous scatter to facilitate the solution of the crystal structure of the resulting protein complex through the utilization of multiwavelength anomalous diffraction (MAD) coupled with synchrotron radiation and improved algebraic methods of MAD data analysis without the necessity of phase information from other sources (Hendrickson et al., 1985, 1989). In an effort to exploit this novel technology to its fullest extent, Hendrickson and co-workers sought to introduce the required selenium atoms into specific sites in proteins via protein biosynthesis. This was accomplished by culturing a methionine auxotroph (DL41) of *Escherichia coli* in a defined medium containing the selenium analogue of methionine, selenomethionine (SeMet),[1] as the sole source of the latter amino acid, thus presumably affording proteins in which SeMet completely replaced the usual Met residues. The enormous potential of this innovative approach is readily demonstrated by the fact that the solutions of the structures of three additional proteins by these techniques have recently been announced (Hendrickson et al., 1990; Graves et al., 1990; Yang et al., 1990).

Selenomethionine-containing proteins offer additional unique investigational potential since the ^{77}Se isotope exhibits properties which make it an attractive probe via nuclear magnetic resonance spectroscopy (NMR) (Dunlap & Odom, 1988). While SeMet proteins offer much in terms of X-ray crystallography and NMR studies, they also provide an almost unprecedented opportunity to compare the properties of a native protein with those of a protein which contains the complete replacement of one of the common amino acids found in proteins with an "unusual" amino acid residue, in this case SeMet, which exhibits subtle, but important differences from Met (Frank et al., 1985). In fact, for the integrity of the X-ray and NMR results, it is crucial to characterize the properties of SeMet proteins fully and to compare them with the properties of their naturally occurring Met-containing counterparts. For this reason, we have purified, characterized, and crystallized *E. coli* selenomethionyl thymidylate synthase and compared its properties and structure to those of the wt enzyme.

Thymidylate synthase catalyzes the reductive methylation of dUMP to dTMP by utilizing the coenzyme 5,10-methylenetetrahydrofolate as the reductant and carbon source. The *E. coli* enzyme exists as a dimer (MW 60 882), composed of identical subunits of 264 residues, containing one active site per monomer (Belfort et al., 1983). There are seven methionine residues in each subunit (1 Met per 45 residues). No methionines are involved in forming the active site or the subunit interface. Only one methionine, residue 141, is strictly conserved evolutionarily. It is the methionine residue nearest the active site with its C_α carbon 11.8 Å from the C_α carbon of the active-site cysteine residue. Its side chain appears to

‡This research was supported by NIH Grant CA 15645 (R.B.D.) from the National Cancer Institute.

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[1] Abbreviations: dUMP, 2′-deoxyuridine 5′-monophosphate; FdUMP, 5-fluoro-2′-deoxyuridine 5′-monophosphate; SeMet, selenomethionine, Met, methionine; TS, thymidylate synthase; CH_2H_4 folate, 5,10-methylenetetrahydrofolate; MES, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; TCA, trichloroacetic acid; BSA, bovine serum albumin; NMR, nuclear magnetic resonance; CFE, cell-free extract; PTH, phenylthiohydantoin; PDDF, 10-propargyl-5,8-dideazafolate.

0006-2960/91/0430-11073$02.50/0

be involved in intramolecular packing interactions.

Materials and Methods

Materials. Seleno-DL-methionine (SeMet), the 20 common L-amino acids, 2′-deoxyuridine 5′-monophosphate (dUMP), and 5-fluoro-2′-deoxyuridine 5′-monophosphate (FdUMP) were obtained from Sigma as were ampicillin (sodium salt), 2-mercaptoethanol, uridine, cytidine, and adenosine. Radiolabeled [6-^{3}H]FdUMP and [5-^{3}H]dUMP were from Moravek Biochemicals. Tris(hydroxymethyl)aminomethane (Tris) and potassium chloride were products of Research Organics. Guanosine was purchased from Chemical Dynamics. PhastGel gradient gel 8–25%, PhastGel Blue R, PhastGel native buffer strips, and Q-Sepharose fast flow were purchased from Pharmacia. (±)-Tetrahydrofolate (H_4folate) was prepared via catalytic hydrogenation of folic acid (Hatefi et al., 1960) and was converted to CH_2H_4folate by the addition of a 25-fold molar excess of formaldehyde in 125 mM $NaHCO_3$, pH 7.0, containing 300 mM 2-mercaptoethanol to form a stock cofactor solution (1 mM CH_2H_4folate). Folic acid was purchased from Calbiochem.

Cell Line and Culture Conditions. E. coli DL41 (ΔMet), an *E. coli* K-12 derivative with a lesion in the *metA* gene, was developed by Dr. David LeMaster (Hendrickson et al., 1990) and provided to us by Professor Wayne Hendrickson. A thymidylate synthase expression vector with a constitutive promoter (amp^r) (henceforth referred to as pRue10) was supplied to us in *E. coli* Rue10 cells by Agouron Pharmaceuticals. This plasmid was extracted from *E. coli* Rue10 cells, purified, and transformed into *E. coli* DL41 by standard protocols to form *E. coli* DL41 (pRue10) (Maniatis et al., 1982). Cells were grown to stationary phase in the following media (amounts per liter): 0.5 g of each of the 20 common L-amino acids, adenosine (0.5 g), guanosine (0.6 g), uridine (0.5 g), cytidine (0.5 g), sodium acetate (1.5 g), ammonium chloride (1.0 g), sodium hydroxide (1.0 g), and dibasic potassium phosphate (10.0 g). After the above solution was autoclaved, a 40-mL solution containing 15.0 g of glucose and 0.1 g of $MgSO_4$ was passed through a 0.22-μm sterile filter and added to the growth culture (pH 7.0), which was then taken to a final concentration of 50 μg/mL ampicillin and 50 μg/mL L-Met or L-SeMet (100 μg/mL DL-SeMet) using sterile filtered stock solutions. Inocula were made by growing cells to stationary phase at 37 °C in 10 mL of this solution. The inoculum was subsequently added to 1 L of growth medium, and the cells were grown to stationary phase in an environmental shaker at 37 °C. In this defined medium containing SeMet, *E. coli* DL41 (pRue10) grows with a generation time of about 1 h at 37 °C in a shaker bath to a maximal OD_{600} of approximately 4.0, yielding 6.0 g of wet cell paste at harvest.

Preparation of Cell-Free Extract and Q-Sepharose Chromatography of Selenomethionyl Thymidylate Synthase. E. coli DL41 (pRue10) cells (2.0 g) grown in the presence of SeMet were broken by two cycles of freeze/thaw, followed by 10 min of sonication (Heat Systems-Ultrasonics cell disruptor W-220F) in 10 mL of degassed 50 mM Tris buffer, pH 7.8, containing 10 mM 2-mercaptoethanol and 1 mM EDTA, and then centrifuged at 10000*g* for 30 min. The latter step and all subsequent steps were performed at 5 °C. All buffers were degassed by bubbling with He gas for 1 h. After the first 2 min of sonication, the pH dropped to 5.5 and was quickly adjusted to pH 7.0 to avoid loss of enzyme activity. All chromatography was performed with a Pharmacia FPLC system equipped with an LCC 500 liquid chromatography controller. The cell-free extract was applied to a 2.4 cm × 7.3 cm (33-mL) Q-Sepharose column which had been equilibrated with 10 bed volumes of degassed QA buffer. The chromatographic buffers were QA (50 mM Tris, 10 mM 2-mercaptoethanol, 1 mM EDTA, pH 7.2) and QB (50 mM Tris, 1 M KCl, 10 mM 2-mercaptoethanol, 1 mM EDTA, pH 7.2). After elution of unbound proteins with QA buffer (2.0 mL/min), a 400-mL gradient from 20% to 30% QB was initiated at a flow rate of 1.5 mL/min. The fractions (8 mL) were monitored for thymidylate synthase activity by the spectrophotometric assay and protein (A_{280}) and were placed on ice as they were collected. Fractions containing TS activity were pooled and placed in dialysis against degassed QA buffer in a sealed container. Purity was assessed by native polyacrylamide gel electrophoresis (PAGE). Purified thymidylate synthase was activated by dialysis at 5 °C against degassed activation buffer (50 mM Tris, 100 mM KCl, 10 mM 2-mercaptoethanol, 1 mM EDTA, pH 7.0) for 24 h. The dialysis buffer was replaced every 24 h.

Purification of wt E. coli Thymidylate Synthase. E. coli Rue10 cells were grown in defined media containing L-Met and harvested 16 h after they reached stationary phase. Cell-free extract was prepared as described above for the selenomethionine enzyme and subjected to Q-Sepharose chromatography. Thymidylate synthase was eluted from Q-Sepharose (fast flow) as above, and fractions exhibiting activity were pooled. The pool was then made 1 M in ammonium sulfate and applied to a 1 cm × 7.2 cm (5.7-mL) phenyl-Sepharose (Pharmacia) column and immediately eluted with a step gradient from 100% PSA (1 M ammonium sulfate, 50 mM Tris, 10 mM 2-mercaptoethanol, 1 mM EDTA, pH 7.2) to 40% PSB (50 mM Tris, 10 mM 2-mercaptoethanol, 1 mM EDTA, pH 7.2) with a flow rate of 1.5 mL/min. Thymidylate synthase containing fractions were determined by the spectrophotometric assay, pooled, and dialyzed against PSB. Purity was established by native PAGE.

Gel Electrophoretic Procedures. The purity of column fractions was assessed following electrophoresis of native and inhibitory ternary complexes of 20-fold concentrates of each column fraction exhibiting TS activity. Concentration of fraction aliquots was carried out with Centricon microconcentrators (Centricon-30, Amicon Corp.). For native TS lanes, 1 μL of thymidylate synthase was mixed with 2 μL of 0.1% bromophenol blue. Inhibitory ternary complexes were prepared for electrophoresis by incubating 1 μL of thymidylate synthase with 1 μL of 2.5 mM FdUMP and 1 μL of 1 mM CH_2H_4folate for 5 min. Samples were then subjected to electrophoresis on Pharmacia PhastGel gradient gel 8–25% utilizing PhastGel native buffer strips with a Phast electrophoresis system.

Protein Assays. The protein concentrations of column fractions and column pools were estimated by A_{280} values and further determined by the Bio-Rad dye binding microassay using bovine serum albumin as the standard (Bradford, 1976). Concentrations of homogeneous thymidylate synthase were measured using the extinction coefficient of 128000 M^{-1} cm^{-1} at 278 nm for the *E. coli* enzyme.

Amino Acid Composition and Sequence Analysis. Protein samples were hydrolyzed under nitrogen with 6 N HCl for 24 h at 110 °C. Hydrolysates were dried under vacuum at 25 °C, redissolved in sodium citrate buffer (Beckman), pH 2.20, and analyzed on a Beckman system 6300 analyzer using ninhydrin as the reagent. A Beckman system gold integrator was used for quantitation and data analysis. Automated Edman degradation was performed with an Applied Biosystems 477A pulse-liquid sequenator. The phenylthiohydantoin

(PTH) derivatives were analyzed with an Applied Biosystems PTH Model 120A analyzer.

Selenium Analysis. The determination of the selenium content of 900 nM SeMet-TS samples was made fluorometrically, on the basis of the fluorescence of 4,5-benzopiazselenole derivatives, with 900 nM wt-TS samples serving as controls, by Dr. P. D. Whanger and co-workers of Oregon State University by methods described elsewhere (Brown & Watkinson, 1977; Butler et al., 1990).

Spectrophotometric Assay. Enzyme activity was determined by a modification of the method of Wahba and Friedkin (1961) by monitoring the increase in absorbance at 340 nm due to the formation of dihydrofolate for 30 s after the addition of the enzyme to the substrate premix (Dunlap et al., 1971). Typically, 100 μL of a 2.0 μM thymidylate synthase stock solution was added to a 900-μL premix solution consisting of 120 mM Tris, 60 mM MES, and 60 mM acetic acid [Morrison buffer (Ellis & Morrison, 1982)], pH 7.0, containing 111 μM CH_2H_4folate, 33 mM 2-mercaptoethanol (both derived from 100 μL of stock cofactor solution), and 111 μM dUMP. All spectrophotometric assays were carried out at 30 °C. Data were collected with a Hewlett-Packard 8450A UV/vis spectrophotometer and processed with an on-line computer.

Tritium Release Assay. Thymidylate synthase activity constants V_{max}, K_m, and k_{cat} of both wt-TS and SeMet-TS were measured by the tritium release assay (Roberts, 1966). Typically, reaction mixtures contained 100 μM CH_2H_4folate and 1–50 μM [5-^{3}H]dUMP in Morrison buffer and about 20 nM thymidylate synthase in a total volume of 300 μL. Reactions were initiated at 37 °C by the addition of enzyme. Seven 35-μL aliquots of the reaction mixture were withdrawn over the course of 1 min, and the reaction was quenched by delivery into Eppendorf tubes containing 25 μL of 25% trichloroacetic acid (TCA). One milliliter of a 2.5% (w/v) acid-washed charcoal suspension in water was then added to each tube, and the tubes were vortexed, allowed to stand for 10 min, and centrifuged at 14000 rpm for 10 min. A Beckman LS-7500 liquid scintillation counter equipped with a data reduction package was then used to determine the counts in 700 μL of the supernatant. The rate was calculated by converting the number of counts into nanomoles of [^{3}H]H_2O produced per minute. One unit of activity was defined as the amount of enzyme which produced 1 μmol of product/min.

Trichloroacetic Acid Precipitation Assay. The trichloroacetic acid (TCA) assay was employed to determine maximum covalent FdUMP/TS binding ratios along with apparent K_d's for the inhibitory ternary complex formed from enzyme, FdUMP, and CH_2H_4folate (Cisneros & Dunlap, 1990). The 0.5-mL reaction mixture, containing 0.1–100 nM [6-^{3}H]-FdUMP (24 Ci/mmol), 1 nM TS, 50 μM CH_2H_4folate, and Morrison buffer, pH 7.0, was incubated at 37 °C for 10 min, followed by quenching with the addition of 125 μL of 50% TCA. This solution was then centrifuged at 14000 rpm for 2 min, decanted, washed four times with 10% TCA, and dissolved in an ethanolic wash (0.2 N NaOH in 50% ethanol) and transferred to scintillation vials for subsequent counting on a Beckman LS 7500 liquid scintillation counter. The covalent binding ratio was defined as nanomoles of FdUMP bound per nanomole of thymidylate synthase.

Effect of Magnesium Ions on Activity. Maximum specific activity (expressed as micromoles per minute per milligram of protein) was determined for both wt-TS and SeMet-TS by utilizing the spectrophotometric and tritium release assays. Typically, concentrations of Mg^{2+}, ranging from 2 to 100 mM Mg^{2+}, in the form of $MgCl_2$, were present in the reaction premixes described previously.

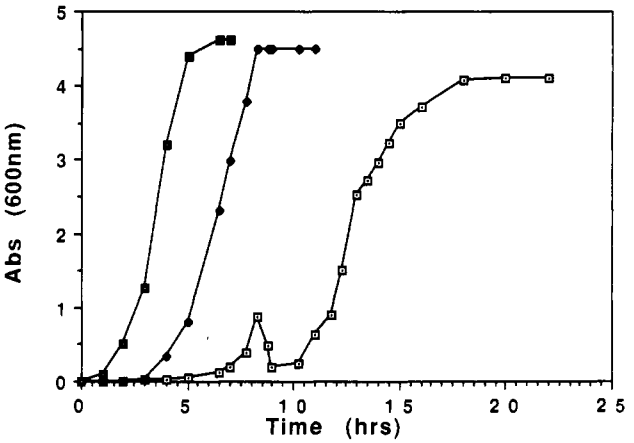

FIGURE 1: Growth curves for (■) *E. coli* Rue10 (pRue10) on minimal LB media, (◆) *E. coli* DL41 (pRue10) on defined L-Met-containing media, and (⊡) *E. coli* DL41 (pRue10) on defined SeMet-containing media.

Effects of Temperature, pH, and Urea on TS Stability. The stability (expressed as percent activity remaining) of both wt- and SeMet-TS was evaluated under a variety of conditions since the kinetic and binding properties of both enzymes were being elucidated. Stability studies to examine thermal inactivation of both enzyme forms were carried out by incubating the enzyme in Morrison buffer at 30 and 37 °C, removing aliquots vs time, and determining the activity with the spectrophotometric assay at 30 °C. The dependence of activity on pH was measured with the spectrophotometric assay by incubating the enzyme in Morrison buffers at pH's 5.5, 7.0, and 8.5, withdrawing aliquots vs time, and determining the activity at 30 °C.

Crystallography. SeMet-TS was crystallized under conditions very similar to those for the wt enzyme (Matthews et al., 1990). The ternary complex was formed by adding 4-fold molar excesses of FdUMP and PDDF to a 20 mg/mL solution of protein in crystal buffer (10 mM Tris, pH 7.2, 18 mM 2-mercaptoethanol). To this solution is added enough of a 4 M Na,K-phosphate solution to give a final concentration of 0.4 M Na,K-phosphate. Ten-microliter aliquots of this solution were set up at 4 °C as hanging drops and allowed to equilibrate over reservoirs containing 0.1–1.4 M Na,K-phosphate, 0.05 M Tris, pH 7.5, and 4 mM 2-mercaptoethanol. Diffraction analysis of the SeMet-TS crystals showed that they were perfectly isomorphous with the wt crystals.

Diffraction data complete to 2.8 Å were collected at 4 °C from a single crystal using a Rigaku AFC-6 diffractometer equipped with a dual chamber area detector system of the Xuong–Homlin design (San Diego Multiwire Systems). The scaling R for the SeMet-TS data versus the wt data was 15.1%. Difference Fourier maps were calculated using coefficients $(F_{SeMet} - F_{wt})^{e i\alpha_{wt}}$. F_{SeMet} and F_{wt} refer to observed structure factor amplitudes for ternary complexes with FdUMP and PDDF for the SeMet-TS and wt-TS, respectively. α_{wt} is a calculated phase for the wt TS–FdUMP–PDDF complex based on the refined model for that structure. The maps were displayed and interpreted on an Evans and Sutherland PS 330 graphics display system using the program FRODO.

Results

Culture. The growth of *E. coli* DL41 (pRue10) in L-Met-containing medium differed markedly from that of the bacteria in DL-SeMet-containing media (Figure 1). When *E. coli* DL41 was grown on media in which DL-SeMet replaced

Table I: *E. coli* Thymidylate Synthase Purification Table

purification step	total act. (units)	total protein (mg)	specific act. (units/mg)	vol (mL)	*x*-fold purification	recovery (%)
SeMet-TS						
(1) CFE	212	625	0.34	10	1	100
(2) Q-Sepharose	203	27	7.50	72	22	96
wt-TS						
(1) CFE	210	600	0.35	10	1	100
(2) Q-Sepharose	202	32	6.30	80	18	95
(3) phenyl-Sepharose	192	26	7.40	32	21	90

Met, both a very extended lag phase (10 h) and a repeatedly observed rise and fall in cell density during late lag phase (7–9 h) were characteristics of the growth curves. Identical cell growth characteristics were observed when L-SeMet was substituted for DL-SeMet. The deviation in late lag phase found with SeMet-containing media was not observed when either *E. coli* DL41 (pRue10) or *E. coli* Rue10 was cultured in L-Met-containing media. The growth of *E. coli* Rue10 (pRue10) cells was faster than the *E. coli* DL41 (pRue10) cells grown on L-Met.

Control experiments in which methionine was omitted from the defined media resulted in no growth, thus confirming methionine auxotrophy. Although similar cell density at stationary phase was observed with *E. coli* DL41 (pRue10) on L-Met-containing medium in the presence of ampicillin, no wt-TS overexpression was ever observed in these cells. The vector pRue10, which confers ampicillin resistance to the cell line and which carries the TS gene, was purified from *E. coli* DL41 (pRue10). Alkaline lysis of *E. coli* DL41 (pRue10) followed by restriction digestion, purification, and separation on agarose gels by standard molecular biological protocols (Maniatis, 1982) verified the inclusion of the plasmid pRue10 in the methionine auxotrophic host. This observation, along with the inability of *E. coli* DL41 to proliferate on ampicillin-containing media, verified the presence of the TS-gene-containing vector.

The inability of the latter expression system (DL41 containing the Rue10 TS expression vector) to produce wt enzyme with L-methionine mandated the use of *E. coli* Rue10 (pRue10) cells for the isolation of large quantities of wt *E. coli* TS. When *E. coli* DL41 (pRue10) was grown on SeMet-containing media, TS levels approaching 6% of total protein were found (Table I). Both *E. coli* DL41 (pRue10) (SeMet-containing defined media) and *E. coli* Rue10 (L-Met-containing defined media) exhibited essentially the same degree of TS overexpression. The possibility of L-Met being present in the SeMet-containing defined media for *E. coli* DL41 (pRue10) was substantially diminished by utilizing inocula containing less than 0.5 ng/mL L-Met contamination. Furthermore, the amino acid composition of the media was determined before inoculation and after cell harvest (supernatant) with no Met being detected in either instance. A small amount (0.5 nmol/mL) of homoserine, which would result if selenomethionine was utilized as a methyl donor, was detected in the media supernatant and CFE after cell harvest.

Purification of SeMet-TS and wt-TS. Facile procedures requiring 8 h or less were developed for the isolation of substantial quantities (>25 mg) of homogeneous SeMet-TS and wt-TS from *E. coli* with recoveries of no less than 90% (Table I). First, subjecting the cells to the combination of two cycles of freeze/thaw and 10-min sonication (2-min bursts at full power followed by cooling to 4 °C) was found to yield both maximum cell breakage and optimum enzyme levels. The key to the rapid isolation of highly purified enzyme was the Q-Sepharose step performed on a Pharmacia fast protein liquid chromatography instrument (Figure 2). This procedure can be performed in 3 h and led to an 18–22-fold increase in specific activity and at least 95% recovery of enzyme activity. When the procedure was started with 2 g of *E. coli* DL41 (pRue10) grown on SeMet-containing media, the latter purification scheme provided greater than 25 mg of SeMet-TS which was pure as reflected by gel electrophoresis of the native enzyme or its inhibitory ternary complexes (Aull et al., 1974). An additional step, phenyl-Sepharose (Pharmacia) chromatography, was necessary to achieve homogeneous enzyme when the procedure was started with *E. coli* Rue10 (wt enzyme) or with 6 g or greater of *E. coli* DL41 (pRue10) containing SeMet. The latter step provided relatively concentrated enzyme solutions (about 0.8 mg/mL) with a very high recovery (95%) of activity in no more than 2 h (Table I). Previous purification protocols (Maley & Maley, 1988; Dev et al., 1988) required as many as three to five steps and multiple days to afford the pure enzyme. Maley and Maley (1988) reported a specific activity of 5.47 units/mg at 30 °C, determined spectrophotometrically, after the fifth and final step of the purification. By comparison, the homogeneous TS resulting from our 8-h procedure exhibited a specific activity of 7.5 units/mg at 30 °C; however, when nondegassed buffers were employed during sonication and purification, the specific activity dropped to 3.6 units/mg for SeMet-TS and 4.5 units/mg for wt-TS.

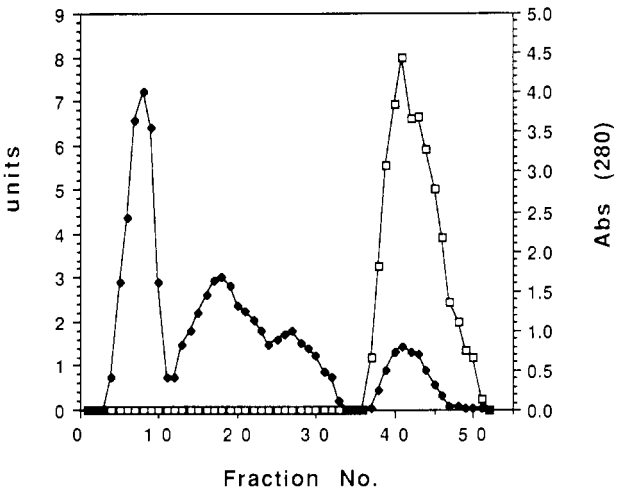

FIGURE 2: Purification of SeMet-TS with Q-Sepharose (fast flow) chromatography: (□) units of TS detected spectrophotometrically; (◆) total protein per fraction as determined by A_{280}. One unit of enzyme is defined to be the amount of TS which produced 1 μmol of product/min.

Elemental Selenium, Amino Acid Composition, and N-Terminal Sequence Analysis. Elemental selenium analysis of 1 mL of 900 nM SeMet-TS and wt-TS by fluorometric techniques yielded 1003 ng/mL Se and 1.300 ng/mL Se, respectively (Brown & Watkinson, 1977; Butler et al., 1990). The detection limit of the fluorometric technique employed was 0.044 ng/g. The former value corresponds to 14.06 mol

Table II: Effects of Temperature and pH on TS Stability

	half-life (min)[a]			
	wt-TS		SeMet-Ts	
	degassed	nondegassed	degassed	nondegassed
temp (°C)[b]				
30	33400	16000	4050	2005
37	346	230	122	65
pH[c]				
5.5	6	5	0.5	
7.0	346	230	122	65
8.5	75	35	20	5

[a] Activity determinations were by the spectrophotometric assay. [b] pH was held constant at 7.0. [c] Temperature was held constant at 37 °C.

of Se/mol of SeMet-TS, which is consistent with the presence of 14 SeMet per TS, since sequence analysis and amino acid composition of *E. coli* wt-TS indicated the presence of 14 Met residues (Belfort et al., 1983). Sequence analysis of the first 40 residues of the amino-terminal region of the wt-TS confirmed the published amino acid sequence and the presence of Met at positions 1, 8, and 34 (Belfort et al., 1983) (data not shown). When SeMet-TS was subjected to sequence analysis, no stable Edman degradation product was present after cycles 1, 8, or 34, but the remainder of the sequence analyzed was identical to that for wt-TS (data not shown). These results demonstrated that authentic Met did not exist at these positions in SeMet-TS, but also supported the presence of SeMet at these sites, since the PTH derivative of SeMet was found to be unstable. Amino acid composition data showed the expected 14 Met residues per dimer in wt-TS and the absence of Met in SeMet-TS. The sequence results, amino acid composition data, and elemental selenium analysis, when considered together, provide strong evidence for the complete replacement of Met residues in wt-TS by 14 SeMet residues in SeMet-TS.

Effects of Degassed Buffer, Temperature, and pH on TS Stability. The pronounced sensitivity of both the wt and SeMet enzymes to dissolved oxygen, even in buffers containing 10 mM 2-mercaptoethanol, was initially observed during purification and storage, wherein TS isolated in nondegassed buffers exhibited values for specific activity and covalent inhibitory ternary complex stoichiometry which were 30%–50% less than those found when the enzymes were purified and dialyzed using degassed buffers. For example, when dialyzed at 5 °C for 48 h in nondegassed buffer followed by incubation at 30 °C in nondegassed buffers, the SeMet and wt enzymes yielded $t_{1/2}$ values some 200-fold smaller than those obtained for the enzymes in degassed buffers (data not shown). However, when the enzymes were dialyzed in degassed buffer prior to incubation in buffer containing dissolved oxygen, only a 50% reduction in half-life was observed (Table II). These results suggested that the dramatic decrease in stability when the enzyme was incubated in nondegassed buffers was probably due to the oxidation of important sulfhydryl groups in the enzyme and thus mandated the use of degassed buffers in the purification process and further studies. TS activity could not be restored by dialysis in buffers containing fresh 2-mercaptoethanol. When both the SeMet and wild-type enzymes (0.2 μM) (purified in the presence of degassed buffers) were incubated in nondegassed buffers containing 50 μM dUMP, they exhibited half-lives which were similar to those presented in Table II for enzyme incubated in degassed buffers, but in the absence of dUMP, at 30 °C. These results could be interpreted to indicate that dUMP binding provided substrate protection against oxidation to sulfhydryl groups at or near the active sites of the enzyme.

When thermal stability was evaluated by incubation at 30 °C in degassed buffers, the wt enzyme ($t_{1/2}$ 557 h) was found to be some 8 times more stable than the SeMet enzyme ($t_{1/2}$ 67 h) (Table II). When nondegassed buffers were used, the wt enzyme was 8 times more stable than the SeMet enzyme at 30 °C. The apparent destabilizing effect of SeMet incorporation on enzyme activity was also observed when incubations were carried out at 37 °C (Table II). When Huber and Criddle (1967) performed heat inactivation studies of *E. coli* β-galactosidase in which some 80 of the 150 methionine residues were replaced by SeMet, they found that the SeMet-containing enzyme was slightly less stable than the wt β-galactosidase at incubation temperatures above 50 °C. Presently, it is unclear whether or not the decreased stability of the SeMet enzymes studied by us and Huber and Criddle (1967) can be attributed to a particular difference in the chemical or physical properties of selenium when compared to those of sulfur.

When the enzymes were incubated at pH 8.5 and 5.5 at 37 °C and assayed at 30 °C, the half-lives of the wt enzyme were decreased by 8- and 57-fold, respectively, when compared to that of enzyme incubated at pH 7.0 (Table II). In related studies, the SeMet enzyme exhibited a 6-fold decrease in $t_{1/2}$ at pH 8.5 when compared to that of enzyme at pH 7.0 while the SeMet enzyme had a $t_{1/2}$ estimated at 0.5 min at pH 5.5. Just as with the thermal stability studies, the results of the pH experiments indicate that the SeMet enzyme is severalfold less stable than the wt enzyme (Table II). However, when the loss of activity due to urea-dependent denaturation was followed, both enzymes exhibited the same results, with total inactivation occurring at 3 M urea following a 5-min incubation at 0 °C (data not shown).

Steady-State Kinetic Analysis. Kinetic data for both wt-TS and SeMet-TS, which were determined by the spectrophotometric and tritium release assays, indicated that the replacement of sulfur by selenium in the methionine residues has little or no effect on these properties of the enzyme. Both enzymes exhibited specific activities of 7.5 units/mg in Morrison buffer, pH 7.0, at 30 °C, when assayed spectrophotometrically. In comparison, Maley and Maley (1988) reported a value of 5.47 units/mg under similar conditions utilizing the same assay. The nearly 40% increase in specific activity reported herein is probably due to the streamlined purification protocol and the use of degassed buffers in all experimental procedures. The values cited above were measured in buffers containing 75 mM Mg^{2+}. TS activity, which exhibited a 3–4-fold increase with increasing Mg^{2+} concentrations, attained an optimum value at about 30 mM Mg^{2+}. This behavior was found for both SeMet-TS and wt-TS and is consistent with the results of other groups (Wahba & Friedkin, 1961; Lomax & Greenberg, 1967; Maley & Maley, 1988) working with *E. coli* TS.

The tritium release assay (37 °C) yielded a value of 1.3 μM for the $K_{m(dUMP)}$ of both SeMet- and wt-TS. SeMet- and wt-TS both exhibited identical k_{cat} values of 3.6 s^{-1}. In comparison, Maley and Maley (1988) reported a $K_{m(dUMP)}$ of 5 μM for the *E. coli* enzyme while K_m's for dUMP of mouse and *Lactobacillus casei* TS were 2.7 μM (Zhang et al., 1991) and 2.7 μM (Santi et al., 1990), respectively. Using the tritium release assay at 37 °C, Dev et al. (1988, 1989) reported k_{cat} values of 3.2 and 5.94 s^{-1} and $K_{m(dUMP)}$ values of 2.7 and 3.7 μM, respectively, for wt *E. coli* TS. Determinations of k_{cat} and k_{cat}/K_m by Dev et al. (1988) were 3.2 s^{-1} and 1.2×10^6 s^{-1} M^{-1}, respectively, which agree well with our results of 3.6 s^{-1} for k_{cat} and 1.6×10^6 s^{-1} M^{-1} for k_{cat}/K_m. In 1989, Dev et al. reported 1.6×10^6 s^{-1} M^{-1} for wt *E. coli* TS, which was

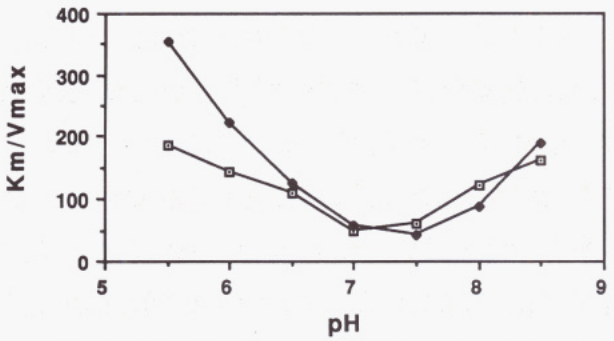

FIGURE 3: Analysis of K_m/V_{max} versus pH for (⊡) wt-TS and (◆) SeMet-TS.

the same value we found for both SeMet- and wt-TS.

pH Effects on K_m and V_{max}. Effects of pH on K_m and V_{max} were analyzed as a function of pH for both the wt and SeMet enzymes by the tritium release assay. This was accomplished by changing the pH of the Morrison buffers in the reaction premixes as described previously. Both enzyme forms exhibited a maximum value for V_{max} between pH 7.0 and pH 7.5 (data not shown). Neither enzyme showed much change in K_m over the pH range 6.0–8.0 (data not shown). Graphical analysis of K_m/V_{max} vs pH yielded very similar parabolic curves for both enzymes (Figure 3), with the most obvious differences appearing at pH 6.0 and 5.5. By comparison, recombinant mouse thymidylate synthase purified from *E. coli* exhibited pH-dependent profiles of K_m, V_{max}, and K_m/V_{max} between pH 5.5 and pH 8.5 (Zhang et al., 1991), which were similar to those of the *E. coli* enzymes.

Covalent Inhibitory Ternary Complex Formation. Covalent binding of the nucleotide inhibitor, FdUMP, to TS in the presence of a large excess of CH_2H_4folate was determined by the trichloroacetic acid precipitation assay, and binding ratios are expressed as moles of FdUMP per mole of enzyme. Maximum binding ratios of 1.7 were determined for both the wt and SeMet enzymes in Morrison buffer, pH 7.0, following purification and storage of the TS in degassed dialysis buffers. However, when the enzymes were purified in degassed buffers and stored in nondegassed dialysis buffers, the covalent FdUMP binding ratios for both enzymes decreased from 1.7 to 1.2 after 48 h. The binding ratio was found to be 1.2 when the enzyme was purified in nondegassed dialysis buffers. When apparent K_d determinations were made at 37 °C, pH 7.0, in Morrison buffer with 1 nM TS, values of 1.2 and 1.1 nM were calculated for the wt and SeMet enzymes, respectively, and are in close agreement with the K_d of 1.6 nM reported by Dev et al. (1988). In comparison, the apparent K_d determined for mouse TS was 1.0 nM (Zhang et al., 1991).

Crystal Structure. The difference Fourier maps (Figure 4) were contoured at various levels in order to more carefully scrutinize them for any differences between the wt and SeMet structures. There were no significant shifts in atomic position detected in either the side chain or the backbone atoms of the methionines, and no other residue positions seemed to be affected. The maps simply showed additional electron density located at the position of the sulfur (selenium) atom of Met (SeMet).

Discussion

Crystal structure analysis by the MAD technique eliminates the necessity of phase information from other sources and thus facilitates protein structure determinations from a single crystal. This methodology, considered together with the facile incorporation of the derivative amino acid necessary for scattering, in this case SeMet, exhibits considerable advantages over traditional heavy-atom derivatization and isomorphous replacement techniques. In addition, each selenium atom present in such selenoproteins could act as a probe via nuclear magnetic resonance spectroscopy if [^{77}Se]SeMet is employed during culture growth. Potentially, this enables one to study the environment of the ^{77}Se nuclei in any selected position of a protein via site-directed mutagenesis. The biosynthetic incorporation of SeMet into proteins enables the characterization of the resulting selenoproteins by structural, spectroscopic, and mechanistic means and a comparison of their properties with those of the native methionine-containing proteins.

Selenomethionine has been incorporated into protein in *E. coli* (Cowie & Cowan, 1957), yeast (Blau, 1961), and plants (Peterson & Butler, 1962) and appears to be well tolerated by such organisms. In addition, it has been shown to share the same metabolic fate as methionine in *E. coli*, including the initiation mechanism involving the N-formylated methionyl-tRNA and its incorporation as the amino-terminal residue in proteins (McConnell & Hoffman, 1972). Early comparisons of the properties of selenomethionyl proteins with their wt counterparts were restricted to systems which lacked complete replacement of Met by SeMet (Huber & Criddle, 1967; Coch & Greene, 1971). In the former case, Huber and Criddle (1967) compared the properties of β-galactosidase isolated from *E. coli* grown on selenate-containing media with those of the enzyme from cells grown on sulfate-containing media. They estimated that nearly 55% of the 150 methionine residues in the enzyme had been replaced by SeMet and found that, while the values for K_m and V_{max} were unaltered, the selenoenzyme seemed to be less stable to heat and urea. Further, Coch and Greene (1971) investigated the biosynthetic incorporation of SeMet by *E. coli*, achieved up to a 75%

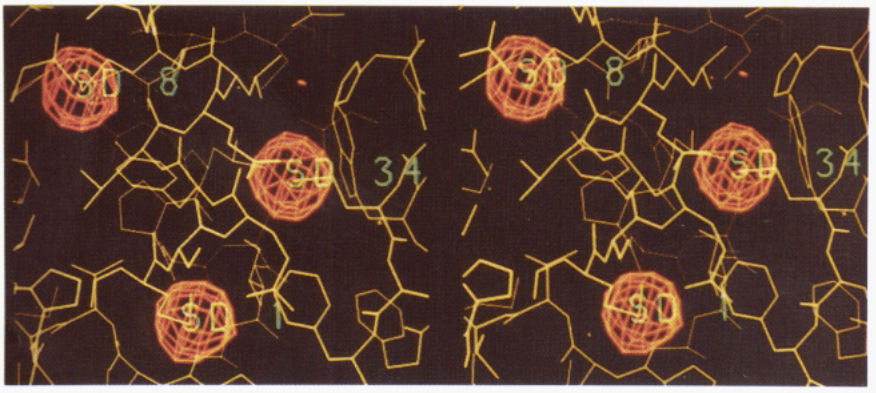

FIGURE 4: Difference Fourier map of SeMet-TS versus wt-TS around residues Met1, Met8, and Met34. The red envelope is the positive density at a 3.5σ contour level. No negative density is observed. The coefficients in this calculation are $(F_{SeMet} - F_{wt})^{\varphi_{wt}}$.

replacement of Met in β-galactosidase by SeMet, and found little change in the properties of the enzyme. In more recent work, Frank et al. (1985) studied a SeMet-containing azurin from a methionine auxotroph of *Pseudomonas aeruginosa* and obtained as much as 88% replacement of the six Met residues by SeMet. These authors found that the SeMet-containing azurin and native azurin had very similar visible, circular dichroic, and electron paramagnetic resonance spectra, with the observed differences allowing insight into the charge-transfer complexes of this protein. Even with the apparent complete replacement of Met by SeMet through the use of *E. coli* DL41 (Hendrickson et al., 1990) and the novel X-ray crystallographic analysis of thioredoxin, interleukin 1α (Graves et al., 1990), and ribonuclease H (Yang et al., 1990), few, if any, data comparing the biochemical properties of the native proteins and their SeMet homologues have been published, and in addition, comparative X-ray crystallographic analysis has not been published to show the structural comparison.

Other attempts of introducing unnatural amino acids into protein via in vivo bacterial protein biosynthesis have met with varied success. For example, the methionine analogue norleucine has been shown to replace methionine residues both at internal positions and at the amino terminus (Bogosian et al., 1989, and references cited therein). However, attempts to substitute selenolysine for lysine (Cini et al., 1981) in *E. coli* proteins resulted in incomplete incorporation, reduced protein synthesis, and inhibition of cellular mechanisms. Additionally, attempts to replace tyrosine or tryptophan completely with their fluorine-labeled derivatives resulted in the lack of cell growth and division. However, when the media contained both the fluoro-substituted amino acid and the natural amino acid, cell growth and division proceeded with the incorporation of fluorinated amino acid at levels up to 60%–75%, thus producing mixtures of only partially substituted protein products (Browne et al., 1970; Kimber et al., 1977, 1978). Finally, automated peptide synthesis has recently been utilized to introduce unusual amino acids into proteins, such as the replacement of the seven cysteines in the copper metallothionein of *Neurospora crassa* with selenocysteine (Tanaka et al., 1988), and chemical modification of the active-site serine in subtilisin to selenocysteine has afforded its conversion to selenosubtilisin (Wu & Hilvert, 1989).

The opportunity to compare the properties of a native protein with those of a protein in which one particular type of amino acid has been completely replaced throughout the protein by another amino acid is rare. In this case, utilizing thymidylate synthase as a model system, we have demonstrated that the complete replacement of L-Met with SeMet failed to alter the typical kinetic and binding properties of the native enzyme. This conclusion is convincingly demonstrated by comparison of the kinetic and binding data discussed in the Results section. Perhaps the latter results could have been anticipated in light of the fact that X-ray crystallographic analysis of TS (Mathews et al., 1990) did not reveal an essential role in binding or catalysis for any of the Met residues. However, while binding and catalytic events remain unperturbed by SeMet replacement, SeMet incorporation produced major differences in the stability of the enzyme as a function of temperature and pH of incubation. In light of the differential effects in normal versus degassed buffers, the instability of the SeMet-TS seems to be an exacerbation of an inherent susceptibility of TS to oxidation. What is unclear is whether this increased susceptibility was a general result of SeMet incorporation or because one or a few of the Met residues play subtle but important roles in the maintenance of the three-dimensional structure of the enzyme. Unfortunately, the crystal structure of SeMet-TS does not reveal a structural basis for a difference in stability between SeMet-TS and wt-TS. It does establish, however, that the stability difference does not arise from a general change in the native protein conformation. This is consistent with the activity data. It also shows that it does not arise from a specific and isolated change in the protein structure that is somehow disconnected from the function of the active sites.

Acknowledgments

We thank Yoshi Ishikawa for the determination of the amino acid composition and N-terminal sequence analysis of wt- and SeMet-TS along with P. D. Whanger for the Se analysis. We are indebted to Brenna P. Modi and Chris Mohr for technical assistance as well as Terry Jones for the PDDF used in the TS crystals. We also thank W. A. Hendrickson for the methionine auxotroph *E. coli* DL41 and J. Horton for helpful discussions.

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