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Site-Directed Mutagenesis of Yeast C_1-Tetrahydrofolate Synthase: Analysis of an Overlapping Active Site in a Multifunctional Enzyme†

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Received September 20, 1988; Revised Manuscript Received November 2, 1988

ABSTRACT: C_1-tetrahydrofolate (THF) synthase is a trifunctional protein possessing the activities 10-formyl-THF synthetase, 5,10-methenyl-THF cyclohydrolase, and 5,10-methylene-THF dehydrogenase. The current model divides this protein into two functionally independent domains with dehydrogenase/cyclohydrolase activities sharing an overlapping active site on the N-terminal domain and synthetase activity associated with the C-terminal domain. Previous chemical modification studies on C_1-THF synthase from the yeast *Saccharomyces cerevisiae* indicated at least two cysteinyl residues involved in the dehydrogenase/cyclohydrolase reactions [Appling, D. R., & Rabinowitz, J. C. (1985) *Biochemistry 24*, 3540–3547]. In the present work, site-directed mutagenesis of the *S. cerevisiae* ADE3 gene, which encodes C_1-THF synthase, was used to individually change each cysteine contained within the dehydrogenase/cyclohydrolase domain (Cys-11, Cys-144, and Cys-257) to serine. The resulting proteins were overexpressed in yeast and purified for kinetic analysis. Site-specific mutations in the dehydrogenase/cyclohydrolase domain did not affect synthetase activity, consistent with the proposed domain structure. The C144S and C257S mutations result in 7- and 2-fold increases, respectively, in the dehydrogenase K_m for $NADP^+$. C144S lowers the dehydrogenase maximal velocity roughly 50% while C257S has a maximal velocity similar to that of the wild type. Cyclohydrolase catalytic activity is reduced 20-fold by the C144S mutation but increased 2-fold by the C257S mutation. Conversion of Cys-11 to serine has a negligible effect on dehydrogenase/cyclohydrolase activity. A double mutant, C144S/C257S, results in catalytic properties roughly multiplicative of the individual mutations. In addition, the 5′ end of ADE3 mRNA was mapped in yeast grown under conditions which repress or derepress C_1-THF synthase transcription. Four major transcription initiation sites are observed in both cases (−27, −30, −38, −42); however, the relative frequency of initiation at these sites differs between the repressed and derepressed states.

In eukaryotes, 10-formyltetrahydrofolate synthetase (EC 6.3.4.3), 5,10-methenyltetrahydrofolate cyclohydrolase (EC 3.5.4.9), and 5,10-methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5) are present on one polypeptide in the form of a trifunctional enzyme (Paukert et al., 1976, 1977; Tan et al., 1977; Caperelli et al., 1978; Schirch, 1978; de Mata & Rabinowitz, 1980). This enzyme, termed C_1-tetrahydrofolate synthase, is responsible for the interconversion of the one-carbon unit attached to the coenzyme THF.[1] The enzyme from the yeast *Saccharomyces cerevisiae* is similar to the other known eukaryotic C_1-THF synthases, existing as a homodimer of M_r = 102 000 (Paukert et al., 1977; Staben & Rabinowitz, 1986).

Several lines of evidence suggest that the eukaryotic enzyme is composed of two functional domains. Proteolysis of the yeast enzyme (Paukert et al., 1977), the porcine enzyme (Tan & MacKenzie, 1977), or the rabbit enzyme (Villar et al., 1985) results in separation of synthetase activity from the other two activities. In each case, synthetase activity is associated with a large proteolytic fragment (subunit M_r = 60 000–80 000), and dehydrogenase and cyclohydrolase activities are associated with a small fragment (subunit M_r = 30 000). Schirch (1978) demonstrated coordinate protection by $NADP^+$ of the rabbit liver dehydrogenase/cyclohydrolase activities against heat inactivation. In addition, 5,10-methenyl-THF, a product of the dehydrogenase reaction, does not accumulate in the coupled dehydrogenase/cyclohydrolase reaction (Schirch, 1978; Cohen & Mackenzie, 1978; Wasserman et al., 1983). Chemical modification studies with the trifunctional enzyme (Schirch et al., 1979; Smith & Mackenzie, 1978, 1985; Appling & Rabinowitz, 1985a) led to the suggestion of a common active site for the dehydrogenase/cyclohydrolase activities. Experiments with the yeast enzyme (Appling & Rabinowitz, 1985a)

† This work was supported in part by Grant DK36913 to D.R.A. from the National Institutes of Health.

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[1] Abbreviations: THF, tetrahydrofolate; NEM, *N*-ethylmaleimide; Tris, tris(hydroxymethyl)aminomethane; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; bp, base pairs; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride.

0006-2960/89/0428-2099$01.50/0

are consistent with an overlapping active site and indicate the existence of at least two critical histidyl residues and at least two critical cysteinyl residues at the dehydrogenase/cyclohydrolase active site.

The nucleotide sequence of the ADE3 gene of *S. cerevisiae*, which codes for C_1-THF synthase, has been determined (Staben & Rabinowitz, 1986). The two functional domains of the enzyme have been tentatively mapped onto the nucleotide sequence by correlation with the genetic map for the ADE3 locus (McKenzie & Jones, 1977) and with the proteolysis data. In both the yeast enzyme (McKenzie & Jones, 1977) and the pig liver enzyme (Tan & MacKenzie, 1979), the dehydrogenase/cyclohydrolase domain is N-terminal to the synthetase domain. The junction of the two domains is probably located at or around amino acids 306–321, a stretch that contains six proline residues and several potential protease digestion sites. The putative dehydrogenase/cyclohydrolase domain, encompassing the first 300 or so amino acids, contains 11 histidines, but only 3 cysteines, located at positions 11, 144, and 257 (Staben & Rabinowitz, 1986) (Figure 1). In the present work, we have utilized oligonucleotide-directed mutagenesis to investigate the involvement of these three cysteinyl residues in the overlapping dehydrogenase/cyclohydrolase site. The single amino acid substitutions C11S, C144S, and C257S and the double substitution C144S/C257S were made. The resulting proteins were overexpressed in an *ade3*$^-$ strain of *S. cerevisiae* and purified by a single-step method. Kinetic analysis of these site-specific mutants is reported in this paper. In addition, the 5′ end of the ADE3 transcript has been mapped in yeast grown under conditions which repress or derepress C_1-THF synthase.

Experimental Procedures

Materials. Common reagents were commercial products of the highest grade available. Culture media were obtained from Difco (Detroit, MI). [^{35}S]-α-Thio-dATP and [γ-^{32}P]-ATP were purchased from Amersham (Arlington Heights, IL) and ICN (Costa Mesa, CA), respectively. Other radiochemicals were obtained from NEN (Boston, MA). Restriction enzymes were obtained from BRL (Gaithersburg, MD) or New England Biolabs (Beverly, MA). Oligonucleotides were synthesized on an Applied Biosystems Model 381A DNA synthesizer, deprotected, and purified by thin-layer chromatography (Alvardo-Urbana et al., 1981). T7 RNA polymerase was prepared as described (Grodberg & Dunn, 1988) from an overproducing strain of *Escherichia coli*, obtained from J. Dunn (Brookhaven National Laboratory). (6*R*,*S*)-Tetrahydrofolate was prepared by the hydrogenation of folic acid over platinum oxide (Blakley, 1957) and purified by chromatography on DEAE-cellulose (Curthoys & Rabinowitz, 1971). (6*R*)-5,10-Methenyl-THF was synthesized enzymatically (Curthoys & Rabinowitz, 1971).

Strains and Growth Conditions. Haploid strains of *Saccharomyces cerevisiae* were used in all studies. M1614C (**a** *ser1 leu1*) and *ade3*-5281 (α *ser1 ura1 ade3*-130) were obtained from E. Jones (Carnegie Mellon University, Pittsburgh, PA). *ade3*-5281 has a large deletion at the ADE3 locus. DAY3 (**a** *ura3*-52 *ade3*-130 *trp1 leu2 his3 his4 ser1*-171) was constructed by crossing *ade3*-5281 with JRY478 (**a** *his3 his4 trp1 leu2 ura3*-52 *sir3*^{ts}) (Sherman et al., 1986). JRY478 was a gift from J. Rine (University of California, Berkeley). Rich media (YPD) contained 1% yeast extract, 2% bactopeptone, and 2% dextrose. Minimal media (YMD) consisted of 0.7% yeast nitrogen base and 2% dextrose and was supplemented with the following nutrients as needed (final concentration in mg/L); serine (375), leucine (30), hisitidine (20), tryptophan (20), uracil (20), adeninine (20), glycine (20), and formate (1000).

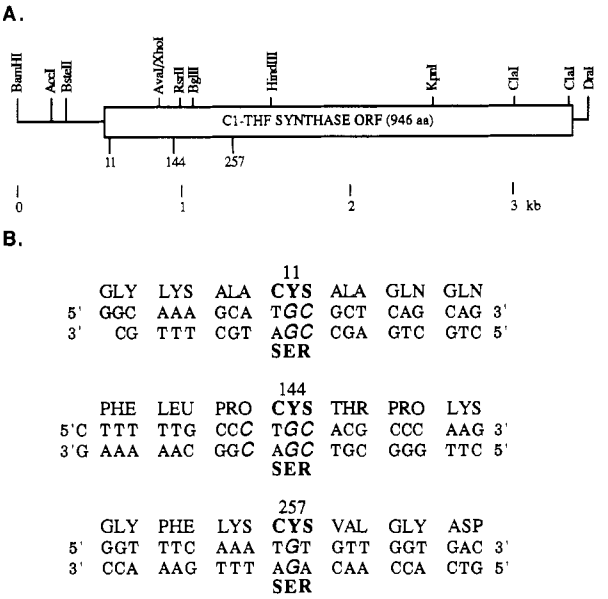

FIGURE 1: (A) A portion of the ADE3 locus showing the C_1-THF synthase open reading frame (ORF) and the 5′ and 3′ noncoding regions (thin lines). Restriction endonuclease sites referred to in the text are identified. Locations of the three cysteine codons are shown below the map. (B) Amino acid and nucleotide sequences surrounding the three cysteine-to-serine mutations are shown. The upper nucleotide strand (5′ → 3′) is the sequence of the mp18/HIND template (see Experimental Procedures). The lower nucleotide strand (3′ → 5′) is the mutagenic oligonucleotide. The mismatches are indicated in large italics.

In Vitro Mutagenesis. Figure 1A shows the 2838 base pair open reading frame for C_1-THF synthase and the restriction sites used in constructing site-specific mutants. The mutagenic oligonucleotides for conversion of cysteine codons to serine are seen in Figure 1B. Oligonucleotide-directed mutagenesis was carried out according to Nisbet and Beilharz (1985). The single-stranded template used for all the experiments reported here is referred to as mp18/HIND (refer to Figure 2 for an outline of constructs). It is comprised of the 5′-terminal *Bam*HI–*Hin*dIII restriction fragment, containing the first 1508 nucleotides of the ADE3 gene, ligated into the multiple cloning site of M13mp18 (Norrander et al., 1983). Following transformation (Hanahan, 1985) of *E. coli* TG-1 (Carter et al., 1985), mutants were detected by hybridization of plaque lifts with mutant oligonucleotide and washing at increasing stringency (Zoller & Smith, 1983). Putative mutants were plaque purified and sequenced. The mutagenized region was removed from the M13mp18 vector by in vitro primer extension and restriction digestion as follows (refer to Figure 1). *Bst*EII and *Xho*I were used for the C11S mutation, and the resulting 611-bp fragment was purified and ligated into pYC1Syn previously cut with the same enzymes to remove the wild-type fragment. [pYC1Syn was derived from YEpADE3 (Staben & Rabinowitz, 1986) by transfer of the 3194-bp *Acc*I–*Dra*I fragment into pUC13.1 (Figure 2). pUC13.1 is pUC13 (Messing, 1983) in which the *Hin*dIII site was removed by digestion with *Hin*dIII, filling in the recessed ends with the Klenow fragment of *E. coli* DNA polymerase (BRL), and ligation of the resulting blunt ends. This was done to facilitate reconstruction of some of the mutant genes.] For the C144S mutation, the *Xho*I and *Bgl*II sites were used. The resulting 172-bp fragment was used to reconstruct pYC1Syn as above. For the C257S mutant, the primer-extended vector was digested with *Hin*dIII and *Bgl*II, and the resulting 489-bp

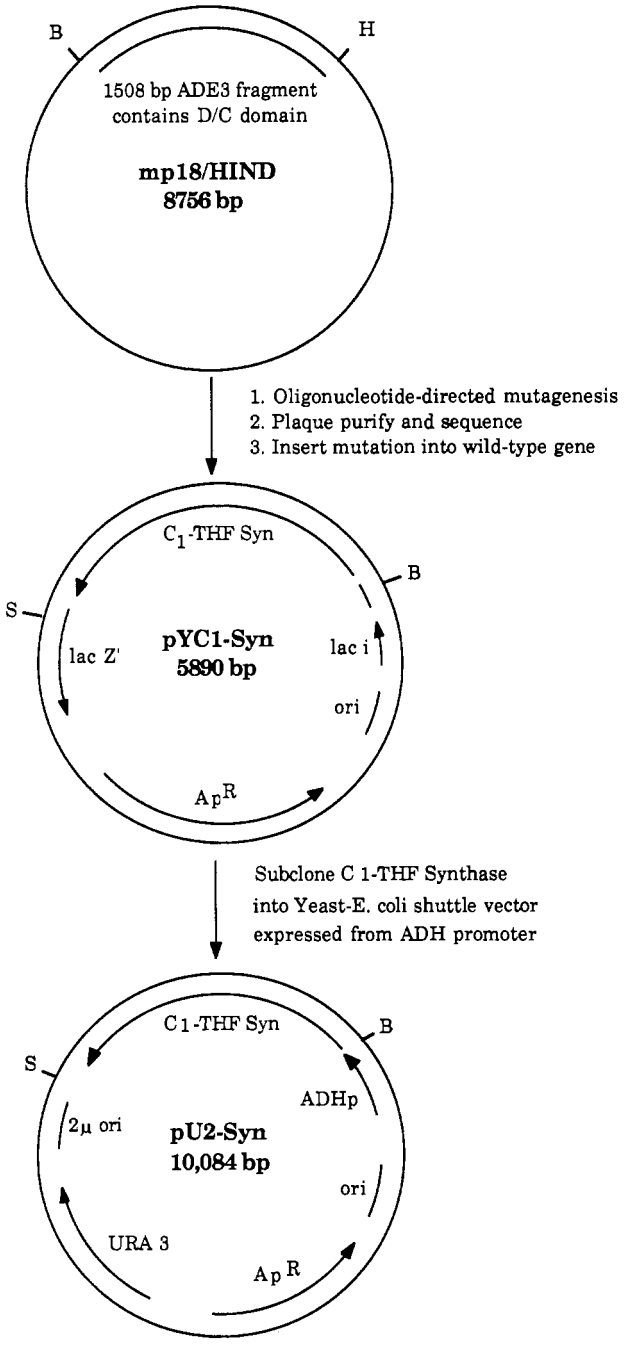

FIGURE 2: Restriction sites are indicated as follows: B = *Bam*HI, H = *Hin*dIII, and S = *Sst*I. The single-stranded mp18/HIND contains the *Bam*HI–*Hin*dIII fragment of the ADE3 gene encoding the dehydrogenase/cyclohydrolase (D/C) domain. The *Acc*I–*Dra*I fragment from YEpADE3 (see text and Figure 1A) was blunt ended and ligated into the *Sma*I site contained within the multiple cloning region of pUC13.1 (see text) to produce pYC1Syn. The *Bam*HI and *Sst*I sites of pYC1Syn are derived from the pUC13.1 multiple cloning site. The *Bam*HI–*Sst*I fragment from pYC1Syn, containing the entire C_1-THF synthase ORF, was then transferred into pVT-102U to produce pU2-Syn (see text). Other abbreviations are as follows: ori, *E. coli* origin of replication; Ap^R, ampicillin resistance (β-lactamase gene); 2μ ori, yeast 2μ origin of replication; ADHp, yeast alcohol dehydrogenase gene promoter.

fragment was isolated and ligated into pYC1Syn previously cut at the same sites. Before the mutagenized fragments were removed from mp18/HIND, the entire fragment was sequenced to ensure that no secondary mutations had occurred. Dideoxy sequencing was performed with [^{35}S]-α-thio-dATP (Biggin et al., 1983) and either Sequenase (USB, Cleveland, OH) or the Klenow fragment of *E. coli* DNA polymerase. The mutant pYC1Syn constructs were used to transform *E. coli* SCS1 or XL1-Blue (Stratagene, La Jolla, CA) to ampicillin resistance. The double mutant, C144S/C257S, was constructed by replacing the 802-bp *Bam*HI–*Rsr*II fragment of C257S in pU2-Syn (Figure 2) with the same *Bam*HI–*Rsr*II fragment from C144S.

Expression and Purification of Mutant Enzymes. The yeast shuttle vector pVT-102U (Vernet et al., 1987) was a gift from T. Vernet and was utilized for overexpression of the wild-type and mutant proteins. The 3209-bp *Bam*HI–*Sst*I fragment from pYC1Syn was inserted into the multiple cloning site of pVT-102U to yield pU2-Syn (see Figure 2). The resulting construct was used to transform yeast.

The *ade3*$^-$ strain of *S. cerevisiae*, DAY3, was transformed to uracil prototrophy with pU2-Syn by a LiCl method (Ito et al., 1983). Transformants were streaked onto YMD plates plus essential nutrients. To ensure that the proper construct had been introduced into DAY3, yeast plasmid DNA was isolated as described (Burke et al., 1983) except that Geneclean (Bio 101, La Jolla, CA) was used to recover plasmid DNA instead of ethanol precipitation. Restriction analysis (the C11S mutation removes an *Sph*I site while the C144S produces a new *Sal*I site) and DNA sequencing confirmed the proper constructs of pU2-Syn in DAY3 transformants. The transformed strains express C_1-THF synthase approximately 40-fold over wild-type *S. cerevisiae*.

C_1-THF synthase activity determinations and purification of overexpressed protein were performed essentially as described previously (Staben et al., 1987). Transformed yeast were grown in 3 L of minimal media to an OD_{600} of about 2 and harvested by centrifugation. After being washed with deionized water, cells were resuspended in 2 volumes of buffer A [25 mM Tris–SO_4 (pH 7.5), 10 mM KCl, 10 mM 2-mercaptoethanol, 1 mM PMSF] and passed three times through a French pressure cell at 4 °C. This lysate was centrifuged at 25000*g* for 1 h, and the resulting supernatant fluid was filtered through cheese cloth and applied to a 2.5 × 20 cm heparin–agarose column (Davison et al., 1979) equilibrated with buffer A. The column was washed with 400 mL of buffer A followed by 400 mL of buffer A containing 0.1 M KCl. C_1-THF synthase was eluted with a 1 L gradient from 0.1 to 0.8 M KCl in buffer A, and fractions were monitored for 10-formyl-THF synthetase activity. Peak activity fractions were analyzed by 10% SDS–PAGE (Laemmli, 1970), after staining with Coomassie R250. Fractions containing purified protein were pooled and concentrated by dialysis against 50% glycerol (v/v) in buffer A and stored at −20 °C. Typically, 8–12 mg of purified protein was obtained from 10 g wet weight of DAY3 transformed with pU2-Syn.

Enzymatic Assays. 10-Formyl-THF synthetase was assayed under saturating conditions as previously described (Appling & Rabinowitz, 1985b). Initial velocity measurements of 5,10-methylene-THF dehydrogenase were determined by a modified end-point assay (Scrimgeour & Huennekens, 1963). Assays contained in a total volume of 1 mL 50 mM HEPES (pH 7.5) and 100 mM KCl. The dependence of initial velocity on $NADP^+$ concentration was made with 0.5 mM (6*R*,*S*)-THF, 0.8 mM formaldehyde, 20 mM 2-mercaptoethanol, and varying amounts of $NADP^+$. The dependence of initial velocity on 5,10-methylene-THF concentration was determined at 0.6 mM $NADP^+$, 10 mM 2-mercaptoethanol, 0.8 mM formaldehyde, and varying amounts of (6*R*,*S*)-THF. Reactions were preincubated at 37 °C for 4 min to allow for equilibration of the condensation reaction of THF with formaldehyde to produce 5,10-methylene-THF. The assays were

initiated by the addition of enzyme at 37 °C and terminated after 1 min by the addition of 0.25 mL of a solution containing 1.25 M HCl and 0.075 M 2-mercaptoethanol. Enzymatic activity was calculated from the extinction coefficient at 350 nm of 24 900 M^{-1} for 5,10-methenyl-THF. 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). The Michaelis–Menten parameters V_{max} and K_m were determined by a nonlinear regression computer program (Duggleby, 1981). The initial velocity measurements of 5,10-methenyl-THF cyclohydrolase activity were obtained by following the decrease in absorbance at 355 nm as described elsewhere (de Mata & Rabinowitz, 1980) except that a final concentration of 50 mM 2-mercaptoethanol was used. Protein concentrations were estimated according to the dye-binding assay of Bradford (1976) with BSA as a standard. Absorbance readings were performed on a Perkin-Elmer Model Lambda 3A spectrophotometer.

Primer Extensions. RNA was isolated from yeast as previously described (Appling & Rabinowitz, 1985b). Poly(A+) RNA was prepared according to Aviv and Leder (1972). A synthetic oligonucleotide [5′-CCAACACTTGACCAGC-CAT-3′; designed to hybridize to the first 19 nucleotides of the large open reading frame predicted from the sequence of the ADE3 gene (Staben & Rabinowitz, 1986)] was phosphorylated at its 5′ end with [γ-^{32}P]ATP and T4 polynucleotide kinase (BRL) and separated from unincorporated label by chromatography on DEAE-cellulose. Ten micrograms of poly(A+) RNA and 0.1 pmol of the labeled oligonucleotide primer were dried in a Speed-Vac and redissolved in 10 μL of solution containing 50 mM Tris-HCl (pH 7.5), 150 mM KCl, and 0.1 mM EDTA. Annealing was carried out by incubation at 90 °C for 10 min followed by 2 h at 37 °C. Upon transfer to 37 °C, 1 μL of a solution containing 30 units of RNasin (Promega Biotec) and 100 mM DTT was added. Following annealing, 10 μL of a solution containing 50 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 10 mM DTT, 1 mM each of dATP, dCTP, dGTP, and dTTP, and 100 units of Moloney-Murine leukemia virus reverse transcriptase (BRL) was added to the primer-template solution. Reaction proceeded at 37 °C and was stopped after 30 min by addition of 1 μL of 0.5 M EDTA. The sample was extracted with phenol/chloroform and precipitated with ethanol. The pellet was dissolved in 50 μL of 0.2 M NaCl and reprecipitated with 125 μL of 95% ethanol. After being rinsed with 70% ethanol, the pellet was dissolved in 10 μL of loading dye (99% formamide, 0.03% each bromphenol blue and xylene cyanol, and 10 mM EDTA). Samples were heated to 95 °C for 3 min before electrophoresis on an 8% polyacrylamide sequencing gel (Maxam & Gilbert, 1980). Dideoxy sequencing reactions were carried out on the mp18/HIND template with the same oligonucleotide as a primer.

S1 Nuclease Mapping. Mapping of the 5′ end of the transcripts was performed as described by Quarless and Heinrich (1986). Single-stranded [^{32}P]cRNA probe was prepared from pTZ19R/AvaI by in vitro transcription with T7 RNA polymerase. Transcription reactions were carried out essentially according to protocols provided by Promega Biotec. pTZ19R/AvaI was derived from pYC1Syn by subcloning the 847-bp *Bam*HI–*Ava*I fragment into the *Sma*I site of pTZ19R (Pharmacia). The orientation is such that transcription by T7 RNA polymerase will produce a complementary RNA. pTZ19R/AvaI was linearized with *Bst*EII before being transcribed to yield a cRNA of 611 nucleotides.

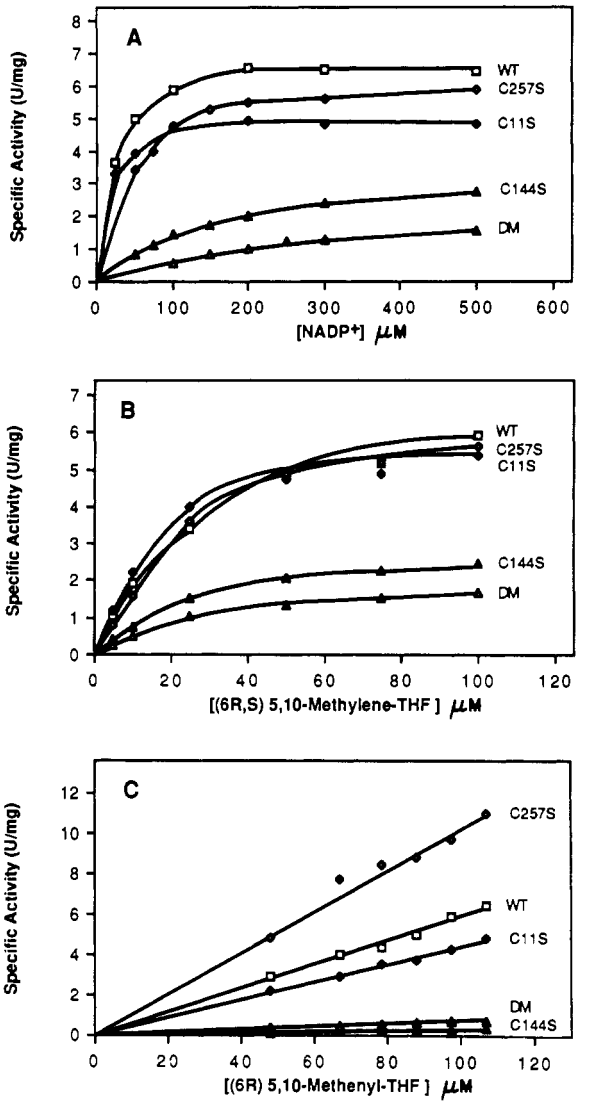

FIGURE 3: Dependence of dehydrogenase enzyme activity on $NADP^+$ concentration (A) and (6*R*,*S*)-5,10-methylene-THF concentration (B) for wild-type and mutant C_1-THF synthases. (C) Dependence of cyclohydrolase activity on (6*R*)-5,10-methenyl-THF concentration. The abbreviation DM represents the double mutant C144S/C257S. Specific activity is expressed as μmol·min^{-1}·(mg of purified protein)$^{-1}$.

The labeled probe was gel purified before use as described (Quarless & Heinrich, 1986). Samples were analyzed on a 5% sequencing gel with a sequencing ladder for markers.

Results

The pU2-Syn construct allowed for overexpression and purification of wild-type C_1-THF synthase and four site-specific mutants in this trifunctional enzyme. Figure 3 shows the dependence of specific activity on substrate concentration for these enzymes, and kinetic constants calculated from these data are listed in Table I. Substitution of cysteine residues in the dehydrogenase/cyclohydrolase portion of C_1-THF synthase has varying effects on the enzymatic properties of 5,10-methenyl-THF cyclohydrolase and 5,10-methylene-THF dehydrogenase. However, none of the substitutions significantly affects the 10-formyl-THF synthetase activity (data not shown), which is catalyzed by a functionally independent domain.

Effect of Mutations on the Catalytic Properties of 5,10-Methylene-THF Dehydrogenase. Mutant C11S has properties very similar to those observed for the wild-type enzyme, with only slight effects on K_m and V_{max}. Replacement of Cys-144

Table I: Kinetic Constants[a] for Wild-Type and Mutant C_1-THF Synthase

	dehydrogenase						cyclohydrolase, 5,10-methenyl-THF,
	NADP$^+$			5,10-methylene-THF			
enzyme	K_m	V_{max}	k_{cat}/K_m	K_m	V_{max}	k_{cat}/K_m	V_{100}[b]
wild type	23 ± 2	7.2 ± 0.1	1060	31 ± 1	7.6 ± 0.2	834	5.9
C11S	14 ± 1	5.2 ± 0.1	1260	22 ± 2	6.7 ± 0.3	1035	4.4
C144S	172 ± 6	3.7 ± 0.1	73	36 ± 2	3.4 ± 0.1	321	0.3
C257S	46 ± 3	6.6 ± 0.1	488	45 ± 4	8.6 ± 0.5	650	10.3
C144S/C257S	397 ± 42	3.0 ± 0.2	26	42 ± 2	2.4 ± 0.1	194	0.7

[a] K_m, V_{max}, and k_{cat}/K_m are expressed in μM, μmol·min^{-1}·mg^{-1}, and s^{-1}·mM^{-1}, respectively. k_{cat} is calculated by use of a M_r of 204 000 for C_1-THF synthase (Staben & Rabinowitz, 1986). Kinetic constants and standard deviations were determined by a nonlinear regression computer program previously described by Duggleby (1981). [b] V_{100} = initial velocity in μmol·min^{-1}·mg^{-1} at 100 μM 5,10-methenyl-THF.

with serine has the most marked effect of the single amino acid changes. An increase in K_m for NADP$^+$ of approximately 7-fold is observed while V_{max} is reduced roughly 50% for this mutant. The K_m for 5,10-methylene-THF is not significantly different when C144S is compared to the wild-type, and in fact, the dehydrogenase K_m for this substrate is largely unchanged for all mutants that were generated. Substitution of Cys-257 with serine increases the K_m for NADP$^+$ by about 2-fold, while having a small effect on the V_{max} of this enzyme. The double mutant, C144S/C257S, has a K_m of about 0.4 mM, a 17-fold increase over that of the wild-type, and also exhibits a 60% decrease in V_{max}.

Effect of Mutations on the Catalytic Properties of 5,10-Methenyl-THF Cyclohydrolase. The cyclohydrolase from *S. cerevisiae* has a reported K_m for (6*R*)-5,10-methenyl-THF of 0.5 mM (de Mata & Rabinowitz, 1980). In these experiments, cyclohydrolase activity could not be determined at or near saturating conditions due to the high absorbance of substrate; therefore, accurate values for K_m and V_{max} could not be calculated. However, the specific activity plots clearly reveal dramatic effects on cyclohydrolase activity due to the site-specific substitutions (Figure 3C). C144S shows a decreased specific activity of 20-fold at 0.1 mM (6*R*)-5,10-methenyl-THF compared to the wild-type enzyme. In contrast to C144S, a doubling of specific activity is demonstrated by the C257S mutant. Substitution of both Cys-144 and Cys-257 results in a specific activity plot similar to that of the single Cys-144 replacement but with a slightly higher activity than that of C144S at a substrate concentration of 0.1 mM.

Mapping the 5′ End of the ADE3 Transcript. Figure 4 shows an autoradiogram of the products resulting from primer extension of poly(A+) RNA isolated from M1614C grown under repressing (YPD) or derepressing (glyine + formate) conditions and from a deletion strain (3-5281). The ADE3 transcripts were 2–3-fold more abundant in RNA from the derepressed strain than from the repressed strain, consistent with results obtained previously from an in vitro translation/immunoprecipitation assay for ADE3 mRNA (Appling & Rabinowitz, 1985b). The same three to four major start sites are seen for M1614C from the two growth conditions. However, the relative intensities of the bands differ between repressing and derepressing conditions. Nearly half (44%) of the ADE3 transcripts detected in RNA from yeast grown in the rich medium (YPD) start at a position 30 nucleotides upstream from the AUG codon. Three other start sites occur with approximately equal frequency at −42 (14%), −38 (19%), and −27 (23%). Transcripts from derepressed yeast start with approximately equal frequency at −42 (29%), −38 (33%), and −30 (27%), with initiation at −27 observed in only 11% of the transcripts. RNA from 3-5281 yields no primer extension products, as expected in this strain carrying a complete deletion of the ADE3 locus. These start sites were confirmed by S1 mapping (data not shown).

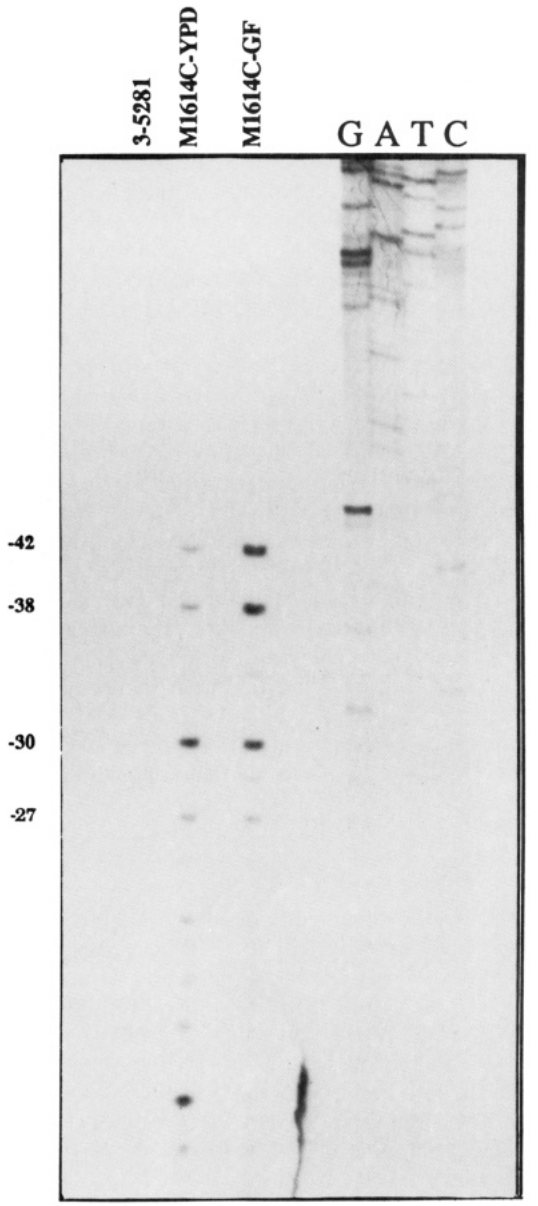

FIGURE 4: Primer extension of ADE3 mRNA. Polyadenylated RNA from yeast strain 3-5281 (ADE3 deletion) or M1614C (wild type) grown in rich medium (YPD) or glycine + formate (GF) was primer extended with a synthetic oligonucleotide complimentary to nucleotides +1 to +19 (see Figure 5) as described under Experimental Procedures. A sequencing ladder, generated from mp18/HIND with the same oligonucleotide primer, provided markers. Samples were electrophoresed on an 8% polyacrylamide sequencing gel and autoradiographed. The major start sites are indicated on the left.

Discussion

Several reports on kinetic, structural, and genetic properties of C_1-THF synthase indicate an intimate relationship between

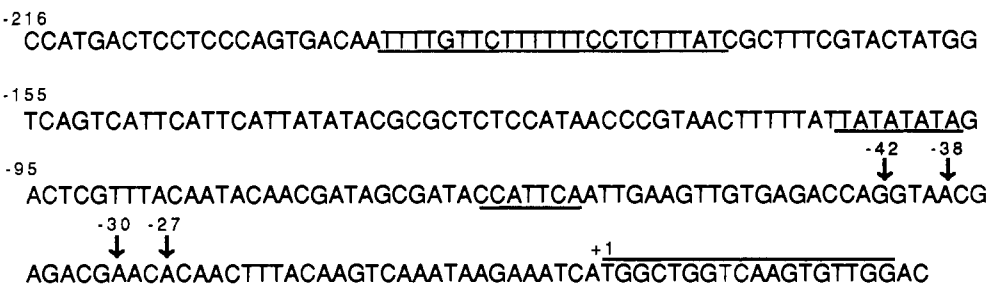

FIGURE 5: Initiation sites of the yeast ADE3 transcript. Sequence at the 5′ end of the ADE3 locus showing the major start sites (arrows) identified by extension of an oligonucleotide primer complimentary to the sequence *overlined* (+19 → +1). Potential promoter elements, as discussed in the text, are *underlined*.

dehydrogenase and cyclohydrolase activities. Chemical modification of yeast C_1-THF synthase with the sulfhydryl modifying agent NEM revealed at least two critical cysteinyl residues for dehydrogenase/cyclohydrolase activities, whereas synthetase activity is quite resistant to NEM (Appling & Rabinowitz, 1985a). On the basis of these results, we made single amino acid substitutions at each cysteine residue in the dehydrogenase/cyclohydrolase domain to probe the function of these residues in enzyme catalysis. Indeed, the results of this study indicate Cys-144 and Cys-257 are important residues as site-specific substitution of either of these amino acids affects both dehydrogenase and cyclohydrolase activities.

A wide range of kinetic parameters has been reported for C_1-THF synthase activities from different sources. Dehydrogenase K_m values of 100 μM for $NADP^+$ and 38 μM for 5,10-methylene-THF have been reported for the *S. cerevisiae* enzyme (de Mata & Rabinowitz, 1980). We report dehydrogenase values somewhat different from those (23 μM for $NADP^+$ and 31 μM for 5,10-methylene-THF); however, the two determinations were performed under different conditions. Cys-144 appears to be a critical amino acid for both dehydrogenase and cyclohydrolase activity. The rather conservative substitution of this residue with a serine results in a 7-fold increase in dehydrogenase K_m for $NADP^+$ and a 50% decrease in V_{max}. Further, the cyclohydrolase specific activity plot for C144S shows an even more dramatic decrease in this activity. An apparent greater sensitivity of cyclohydrolase to this substitution is consistent with previous data showing that NEM inactivated both dehydrogenase and cyclohydrolase but with cyclohydrolase activity at least an order of magnitude more sensitive to this modifying agent than dehydrogenase (Appling & Rabinowitz, 1985a). The dehydrogenase K_m for 5,10-methylene-THF is not significantly affected by this mutation. Our results imply that Cys-144 is important in binding $NADP^+$ at the active site, and this idea is further supported by the observation that $NADP^+$ protects dehydrogenase and cyclohydrolase activities against sulfhydryl inactivation in the yeast (Appling & Rabinowitz, 1985a) and the rabbit (Schirch et al., 1979) enzymes. Replacement of Cys-257 with serine also affects the catalytic properties of both dehydrogenase and cyclohydrolase. The K_m for $NADP^+$ is doubled while only slight changes are seen in K_m for 5,10-methylene-THF and maximal velocity. Interestingly, this mutation doubles the cyclohydrolase specific activity compared to that of wild type at a 100 μM concentration of 5,10-methenyl-THF. Enhancement in catalytic efficiency of other enzymes as a result of site-specific mutations has been previously reported (Carter et al., 1984; Estell et al., 1986). In studies on the chicken liver enzyme, dehydrogenase/cyclohydrolase activities are efficiently coupled such that substrate from the dehydrogenase is channeled between the cyclohydrolase and dehydrogenase with negligible cyclohydrolase activity toward exogenous 5,10-methenyl-THF (Wasserman et al., 1983). Other investigators have found that $NADP^+$ activates the rabbit cyclohydrolase (Schirch, 1978) or acts as a competitive inhibitor of the porcine activity (Tan et al., 1977). It has also been suggested that a conformational change in the protein allows for this channeling to occur (Appling & Rabinowitz, 1985a). Specific activity measurements in this paper utilized unchanneled 5,10-methenyl-THF; hence, the dramatic decrease in activity of C144S and increase in activity of C257S may reflect changes in the accessibility that this substrate has to the cyclohydrolase catalytic site. Analyzing the effect that these site-specific mutations have on dehydrogenase/cyclohydrolase coupling should contribute to our understanding of this process.

We also constructed C144S/C257S, containing amino acid replacements at both Cys-144 and Cys-257. This enzyme exhibited the lowest catalytic efficiency with an increase in K_m for $NADP^+$ of 17-fold and a maximal velocity only 40% that of the wild type. This K_m for $NADP^+$ is roughly multiplicative of the 7- and 2-fold increases in K_m observed for the individual mutations. The slight increase in cyclohydrolase specific activity of C144S/C257S over C144S may again reflect a combination of inactivation due to C144S and 2-fold stimulation in activity due to C257S.

Our results are consistent with genetic evidence for the intimate nature of the dehydrogenase/cyclohydrolase activities. McKenzie and Jones (1977) analyzed several point mutants of the ADE3 gene deficient in one or more of the three activities of C_1-THF synthase. All mutants which were deficient in dehydrogenase activity also lacked cyclohydrolase activity (McKenzie & Jones, 1977; Kapor, 1982). Similarly, we find that site-specific mutations which effect one activity also alter the other. In contrast to an ADE3 deletion mutant, the point mutants described by McKenzie and Jones did not have an absolute requirement for adenine, but growth was stimulated by addition of this nutrient. The site-specific mutants which we generated can also grow on minimal media lacking adenine (data not shown). However, caution should be taken in comparing these transformed strains, which overexpress C_1-THF synthase from multicopy plasmids, with strains containing a single copy of the gene.

Recently, the gene for mitochondrial C_1-THF synthase from *S. cerevisiae* has been sequenced (Shannon & Rabinowitz, 1988). The predicted amino acid sequence of mitochondrial C_1-THF synthase shares a striking 71% identity with the cytoplasmic enzyme. In addition, Hum et al. (1988) have isolated and sequenced the cDNA for human C_1-THF synthase. The deduced amino acid sequence for the human protein shares a 58% amino acid identity with yeast cytoplasmic C_1-THF synthase. The region around and including Cys-144 is highly conserved between the three proteins (Figure 6A). The amino acid sequence around Cys-257 is also highly conserved al-

A

HU:	F I P **C** T P K G **C** l E L i	(144-156)
ADE3:	F l P **C** T P K G i i E L L	(141-153)
MIS1:	F I P **C** T P K G **C** m k L L	(172-184)

B

HU:	V I D **c** G I N Y V P D d k K p n G r K v V G D V	(233-256)
ADE3:	V I D V G t N Y V a D p S K K S G f K **c** V G D V	(238-261)
MIS1:	V I D V G I N Y V P D i S K K S G q K l V G D V	(261-284)

FIGURE 6: Amino acid sequences of human (HU), yeast cytoplasmic (ADE3), and yeast mitochondrial (MIS1) C_1-THF synthases surrounding cysteine-144 (A) and cysteine-257 (B). Capital letters indicate residues conserved among two or more proteins, and all cysteines are shown in bold face.

though a cysteine is found at that position only in the yeast cytoplasmic C_1-THF synthase (Figure 6B). It is interesting to note that both the human and yeast mitochondrial proteins have a second cysteine five residues away from the conserved Cys-144 while the yeast cytoplasmic protein does not. Cys-11 is not conserved, nor is there appreciable amino acid identity in this region, which may explain the minimal effect mutation of this residue has on dehydrogenase/cyclohydrolase properties.

It is difficult to provide extensive active site information without a three-dimensional structure. However, our results are consistent with the involvement of Cys-144 and Cys-257 in binding and/or positioning of substrate(s) at the active site. The fact that Cys-144 and Cys-257 are not essential for enzyme activity argues against the direct electronic involvement of these residues in catalysis since the chemical reactivities of the oxygen atom in the hydroxyl group of serine and the sulfur atom in the sulfhydryl of cysteine are very different. Also, the possibility exists that these mutations distort the polypeptide structure although this is unlikely since serine and cysteine have a similar spatial arrangement. The facile overexpression and purification of yeast C_1-THF synthase make attempts to crystallize this protein possible, and we hope to obtain x-ray diffraction data on the wild-type and mutant proteins to answer some of these questions and elucidate the mechanistic detail of the trifunctional activities. The identification of cysteinyl residues 144 and 257 as important amino acid residues for dehydrogenase/cyclohydrolase activity contributes to the understanding of this overlapping active site.

Structure of the ADE3 Transcript. Transcription initiation sites at −42, −38, −30, and −27 (+1 defined as the first base in the initiator codon) identified by primer extension are consistent with the existence of consensus sequences for promoter elements within the 5′ noncoding region (see Figure 5). A poly(dA·dT) region between −194 and −172, containing 17 T residues in the coding strand, is similar to sequences which serve as upstream promoter elements for constitutive expression in at least three other yeast genes (Struhl, 1985). If this element is serving the same function in the ADE3 gene, it may control expression from the −30 site, since the frequency of initiation at that site showed little or no change during derepression. The observed initiation sites place the best candidate for the TATA element at −100, as the distance between TATA element and the RNA start site averages 60 bp for yeast genes (Struhl, 1986).

Heterogeneity in the 5′ end of yeast transcripts is common (McIntosh & Haynes, 1986; Zalkin et al., 1984; Zalkin & Yanofsky, 1982), and in a few cases difference in the relative frequencies of initiation from multiple sites is observed under various conditions (Struhl, 1986). The physiological significance, if any, of changes in the frequency of utilization of the ADE3 start sites under repressed versus derepressed conditions is unknown. These primer-extension experiments confirm and extend our previous results on the regulation of expression of the ADE3 gene. Previously, an in vitro translation/immunoprecipitation assay to measure C_1-THF synthase mRNA levels demonstrated a 2–3-fold increase upon switching the yeast to derepressing growth conditions (Appling & Rabinowitz, 1985b). A 2–3-fold increase in ADE3 transcript is also observed with the primer-extension assay. It appears that constitutive expression (YPD medium) occurs primarily from the −30 site, since the absolute amount of initiation at this site is unchanged upon derepression. The increase in amount of ADE3 transcript upon derepression is primarily due to increased initiation at the −42 and −38 sites.

Acknowledgments

We thank Mallory Barcus for her expert technical assistance and Robert MacKenzie for sharing the human C_1-THF synthase sequence prior to its publication.

Registry No. NADP, 53-59-8; [γ-^{32}P]ATP, 2964-07-0; Cys, 52-90-4; THF synthase, 73699-18-0; 5,10-methylene-THF dehydrogenase, 9029-14-5; 5,10-methylene-THF cyclohydrolase, 9027-97-8; synthetic oligonucleotide, 118436-82-1; 5,10-methylenetetrahydrofolic acid, 3432-99-3.

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α-Secondary Isotope Effects in the Lipoxygenase Reaction

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Received June 21, 1988; Revised Manuscript Received October 20, 1988

ABSTRACT: Isotope effects for the oxidation of [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid catalyzed by soybean lipoxygenase and by 5-lipoxygenase were measured. This labeling pattern represents substitution at each of the vinylic hydrogens of the substrate. The observed isotope effect for soybean lipoxygenase was 1.16 ± 0.02 and for 5-lipoxygenase was 1.11 ± 0.05. These isotope effects are inconsistent with any change in hybridization (sp^2 to sp^3) at the vinylic carbons prior to or during the rate-determining step and are concluded to be most consistent with the formation of a carbanion-like intermediate or transition state. In contrast, the oxidation of arachidonic acid by Ce(IV), which is thought to proceed via a cation radical intermediate, exhibited at most a small isotope effect (1.02 ± 0.01). The reduction potential for the cation radical formed from arachidonic acid in this reaction is estimated to be 2.7 V vs NHE by comparison of the rates of oxidation of arachidonic acid and cyclohexene by Ce(IV). This is similar to the potential for the cation radical of 2-butene. No isotope effect (1.00 ± 0.03) was observed in the 5-lipoxygenase reaction for conversion of the initially formed product 5-hydroperoxyeicosatetraenoic acid to the epoxide leukotriene A_4. From this it is concluded that there is little carbon–oxygen bond formation prior to or during the rate-determining step for epoxide formation.

The kinetics and mechanism of soybean lipoxygenase have been extensively studied with the expectation that the results will be generalizable to other enzymes of this class, and the available information has been collected in several recent reviews (Schewe et al., 1986; Kühn et al., 1986; Lands, 1984; Veldink et al., 1984). The enzyme is a monomer with a single iron per active site, which is neither a heme nor an iron–sulfur center. The iron is considered to be the oxidant that oxidizes the 1,4-diene of a polyunsaturated fatty acid to a pentadienyl radical intermediate. The pentadienyl radical is then trapped by oxygen to give a lipid hydroperoxide as the final product. For example, when the substrate is arachidonic acid, as in this study, the product is 15-HPETE.[1] A pentadienyl radical

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0006-2960/89/0428-2106$01.50/0