Evidence for Overlapping Active Sites in a Multifunctional Enzyme: Immunochemical and Chemical Modification Studies on C_1-Tetrahydrofolate Synthase from *Saccharomyces cerevisiae*†

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Received December 13, 1984

ABSTRACT: The relationship of the active sites which catalyze the three reactions in the trifunctional enzyme C_1-tetrahydrofolate synthase (C_1-THF synthase) from *Saccharomyces cerevisiae* has been examined with immunochemical and chemical modification techniques. Immunotitration of the enzyme with a polyclonal antiserum resulted in identical inhibition curves for the dehydrogenase and cyclohydrolase activities which were distinctly different from the inhibition curve for the synthetase activity. During chemical modification with diethyl pyrocarbonate (DEPC), the three activities were inactivated at significantly different rates, indicating that at least three distinct essential residues are involved in the reaction with DEPC. The pH dependence of the reaction with DEPC was consistent with the modification of histidyl residues. Treatment of C_1-THF synthase with *N*-ethylmaleimide (NEM) resulted in significant inactivation of only the dehydrogenase and cyclohydrolase activities, with the cyclohydrolase at least an order of magnitude more sensitive than the dehydrogenase. Inactivation of cyclohydrolase was biphasic at NEM concentrations above 0.1 mM, suggesting two essential cysteinyl residues were being modified. $NADP^+$, a dehydrogenase substrate, protected both dehydrogenase and cyclohydrolase activities, but not synthetase activity, against inactivation by either reagent. Synthetase substrates had no protective ability. Pteroylpolyglutamates and *p*-aminobenzoic acid polyglutamates exhibited some protection of all three activities. The *p*-aminobenzoic acid polyglutamate series showed progressive protection with increasing chain length. These results are consistent with an overlapping site for the dehydrogenase and cyclohydrolase reactions, independent from the synthetase active site. Possible active-site configurations and the role of the polyglutamate tail in substrate binding are discussed.

Folate coenzyme mediated one-carbon metabolism plays an essential role in several major cellular processes including nucleic acid biosynthesis, amino acid biosynthesis and conversions, and vitamin metabolism (Rabinowitz, 1960; Blakley, 1969). The variety of pathways which utilize these one-carbon units is dependent upon the ability of the organism to vary the oxidation state of the carbon unit attached to the coenzyme, tetrahydrofolate (THF).[1] In eukaryotes, N^{10}-formyltetrahydrofolate synthetase (EC 6.3.4.3), N^5,N^{10}-methenyltetrahydrofolate cyclohydrolase (EC 3.5.4.9), and N^5,N^{10}-methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5) activities 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). These three reactions are summarized in Figure 1. The enzyme from the yeast *Saccharomyces cerevisiae*, termed C_1-THF synthase, is fairly representative of the other eukaryotic enzymes, existing as a homodimer of M_r 104 000 subunits (Paukert et al., 1977). In prokaryotes, these activites are catalyzed by separate monofunctional enzymes, with the known exceptions of *Escherichia coli* and *Clostridium thermoaceticum* in which the cyclohydrolase and dehydrogenase activities are catalyzed by bifunctional proteins [for a review, see MacKenzie (1984)].

Several lines of evidence, including kinetic, structural, and chemical modification studies, suggest that the dehydrogenase and cyclohydrolase activities of the trifunctional enzyme are catalyzed at closely interacting sites. The synthetase activity can be separated from the other two activities upon proteolysis in the yeast enzyme (Paukert et al., 1977) and porcine enzyme (Tan & MacKenzie, 1977). In both cases, synthetase activity is associated with a large proteolytic fragment (M_r 60 000–80 000) and dehydrogenase and cyclohydrolase activities with a small fragment (M_r 30 000). Schirch (1978) demonstrated 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 in the enzyme from rabbit liver (Schirch, 1978), pig liver (Cohen & MacKenzie, 1978), or chicken liver (Wasserman et al., 1983). Chemical modification studies with the mammalian enzymes have also led workers to suggest a common active site for the dehydrogenase/cyclohydrolase activities (Schirch et al., 1979; Smith & MacKenzie, 1983).

Strong genetic evidence exists for an intimate dehydrogenase/cyclohydrolase relationship in the trifunctional

†Supported by Grant AM2109-26 from the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases of the U.S. Public Health Service and Postdoctoral Fellowships PF-2202 from the American Cancer Society and GM08889 from the National Institutes of Health. A preliminary report of this work was presented at the Second Workshop on Folyl and Antifolyl Polyglutamates, Airlie, VA, 1984.

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[1] Abbreviations: THF, tetrahydrofolate; DEPC, diethyl pyrocarbonate; NEM, *N*-ethylmaleimide; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; $PteGlu_n$, pteroylpolyglutamate with *n* glutamyl residues; $pAbaGlu_n$, *p*-aminobenzoic acid polyglutamate with *n* glutamyl residues; H_4, tetrahydro; PBS-BSA, phosphate-buffered saline containing 1 mg/mL bovine serum albumin.

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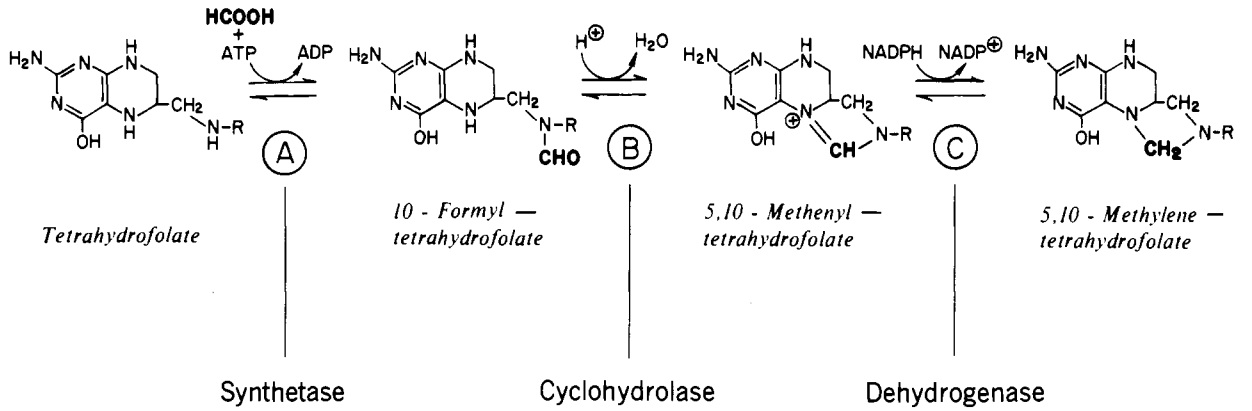

FIGURE 1: Reactions catalyzed by C_1-THF synthase from *Saccharomyces cerevisiae*. One-carbon units are in boldface type.

enzyme of yeast. McKenzie & Jones (1977) analyzed crude extracts from several point mutants that are deficient in one or more of the three activities of C_1-THF synthase. Five of the six mutants lacking dehydrogenase activity also lack cyclohydrolase activity. Synthetase activity is normal in this group. One of the six point mutants was reported to have nearly normal levels of cyclohydrolase; however, this mutant enzyme has been purified in our laboratory and appears to be somewhat deficient in cyclohydrolase activity as well as dehydrogenase activity (Kapor, 1982).

The ADE3 gene of *S. cerevisiae*, which encodes C_1-THF synthase, has recently been sequenced in our laboratory (C. Staben and J. C. Rabinowitz, unpublished results). Our utltimate goal is to investigate the active-site relationships through the use of site-specific mutagenesis to make single amino acid changes in the enzyme. The present study utilizes immunochemical and chemical modification experiments to examine these active-site relationships in the yeast enzyme. Our results reveal subtle structural differences between the yeast enzyme and the mammalian enzymes and have allowed us to identify potential dehydrogenase/cyclohydrolase active-site residues as targets for in vitro mutagenesis.

Experimental Procedures

Materials

Common reagents were commercial products of the highest grade available. NEM was purchased from Eastman and DEPC from Sigma Chemical Co. Protein assay dye reagent was purchased from Bio-Rad. (6*RS*)-Tetrahydrofolate was prepared by the hydrogenation of folic acid (Sigma Chemical Co.) over platinum oxide (Matheson Coleman and Bell) in neutral aqueous solution (Blakley, 1957) and was purified by chromatography on DEAE-cellulose (Curthoys & Rabinowitz, 1971). The stock solution contained 10 mM (6*RS*)-tetrahydrofolate, 0.2 M Tris-HCl, pH 7.0, and 0.5 M 2-mercaptoethanol. (6*RS*)-5,10-methylene-THF was chemically prepared from formaldehyde and (6*RS*)-tetrahydrofolate at a molar ratio of 1.5:1 (Delk et al., 1976). $PteGlu_n$ and $pABAGlu_n$ were prepared and generously provided by L. D'Ari (D'Ari & Rabinowitz, 1985).

Methods

Antibody Preparation. Antisera to yeast C_1-THF synthase were raised in rabbits by C. Staben in this laboratory. IgG was purified by ammonium sulfate precipitation and DEAE-cellulose chromatography. The protein concentration was 1.12 mg/mL calculated from the absorbance at 280 nm (Livingston, 1974).

Immunoinactivation Experiments. Two hundred microliters of enzyme [3 μg in 25 mM Tris–sulfate (pH 7.5)/10 mM KCl/10 mM 2-mercaptoethanol] and 200 μL of antiserum (serially diluted with PBS-BSA) were incubated at 37 °C for 1 h. The tubes were then placed on ice, and aliquots were assayed for the three activities. Immunoprecipitation was accomplished by further incubation of the tubes on ice overnight. One hundred microliters of 4× IP buffer [0.4 M Tris-HCl (pH 8.0)/0.4 M NaCl/2.0% Nonidet P-40 (Sigma)] and 10 μL of prewashed *Staphylococcus aureus* cells (IgGSORB, 10% suspension, The Enzyme Center) were added, and incubation was continued on ice for 1 h. Precipitates were removed by centrifugation, and aliquots of the supernatant fraction were assayed for the three activities.

Reaction of C_1-THF Synthase with N-Ethylmaleimide. All reactions were done in 50 mM HEPES (potassium salt), pH 6.5, containing 0.1 M KCl at 25 °C. NEM stock solutions were prepared in the same buffer just before use, and the concentration was calculated from the molar extinction coefficient of NEM at 302 nm (ϵ = 620 cm^{-1} M^{-1}) (Means & Feeney, 1971). Enzyme (0.18 mg/mL final concentration) was added from a stock solution which was rendered free of 2-mercaptoethanol by desalting on a PD-10 column (Pharmacia) equilibrated with 50 mM K-HEPES, pH 7.5, containing 0.1 M KCl. Other additions are indicated in the figure legends. Reactions were terminated by removing aliquots at various times and diluting 50-fold into ice-cold 50 mM K-HEPES, pH 7.5, containing 0.1 M KCl and 1 mM cysteine.

Reaction of C_1-THF Synthase with Diethyl Pyrocarbonate. All reactions were carried out in 50 mM potassium phosphate buffer, pH 7.0, at 20 °C. DEPC stock solutions were prepared in absolute ethanol immediately before use. The stock concentration was calculated from the increase in absorbance at 242 nm following reaction of DEPC with an excess of histidine (10 mM) using 3.2 × 10^3 cm^{-1} M^{-1} for the extinction coefficient for *N*-carbethoxyimidazole (Miles, 1977; Carrillo & Vallejos, 1983). The ethanol concentration was 5% or less in all reactions and had no effect on enzyme activity. The final enzyme concentration was 0.18 mg/mL. All other additions are described in the figure legends. Reactions were terminated by dilution of aliquots into ice-cold 25 mM K-HEPES, pH 7.5, containing 10 mM KCl, 10 mM 2-mercaptoethanol, and 1 mM histidine.

Protection Experiments. Enzyme was preincubated for a short time at the appropriate temperature with each substrate or analogue before addition of reagent. Each was tested for its ability to react with or to inactivate the reagent before use in these protection experiments. Formate was found to inactivate DEPC and thus was not used in those experiments.

Assays. Purification of C_1-THF synthase (Staben & Rabinowitz, 1983) and assay of 10-formyl-THF synthetase and 5,10-methylene-THF dehydrogenase (Appling & Rabinowitz,

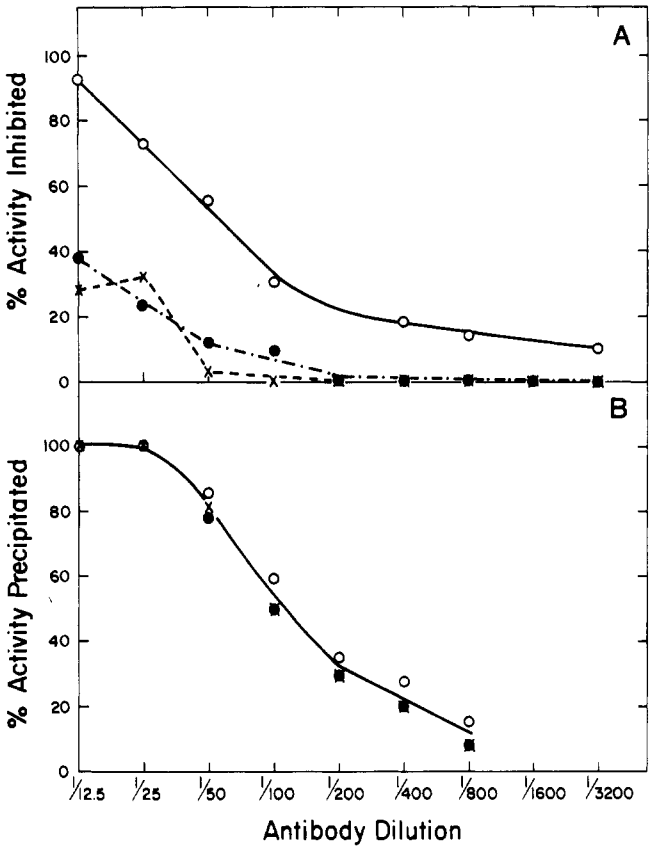

FIGURE 2: Immunotitration of yeast C_1-THF synthase with a polyclonal antiserum. (A) Percent of activity inhibited after 1-h incubation with various dilutions of antiserum. (B) Percent of activity immunoprecipitated after addition of *Staphylococcus aureus* cells and centrifugation: (O) synthetase; (●) dehydrogenase; (×) cyclohydrolase.

1985) and of 5,10-methenyl-THF cyclohydrolase (de Mata & Rabinowitz, 1980) have been described. Protein concentration was estimated by the dye binding assay of Bradford (1976) with BSA as a standard.

Results

Immunotitration of C_1-THF Synthase. Immunotitration of the three activities of C_1-THF synthase was accomplished by incubation of the enzyme with various dilutions of a polyclonal antiserum prepared against the purified protein. Assay of the residual activities revealed similar inactivation curves for dehydrogenase and cyclohydrolase activities, but a distinctly different curve for synthetase activity (Figure 2A). At a dilution of antiserum which resulted in inactivation of 95% of synthetase activity, only 30–40% of the dehydrogenase and cyclohydrolase activities were inhibited. When the supernatant fractions were assayed following precipitation of the immune complexes (Figure 2B), the three activities exhibited identical curves, as would be expected for a multifunctional enzyme. Note that the 1/25 dilution of antiserum was able to complex with and direct the precipitation of 100% of the enzyme. These results are consistent with a structure in which the dehydrogenase and cyclohydrolase active sites are recognized by the same subpopulation of antibodies, different from the set of antibodies which interact with the synthetase active site.

Inactivation of C_1-THF Synthase by Diethyl Pyrocarbonate. All three activities of C_1-THF synthase showed some degree of inactivation when the enzyme was treated with DEPC. Cyclohydrolase and dehydrogenase inactivations were performed with DEPC concentrations ranging from 5 to 20 μM whereas synthetase inactivation required concentrations ranging from 500 to 1250 μM. Semilogarithmic plots of

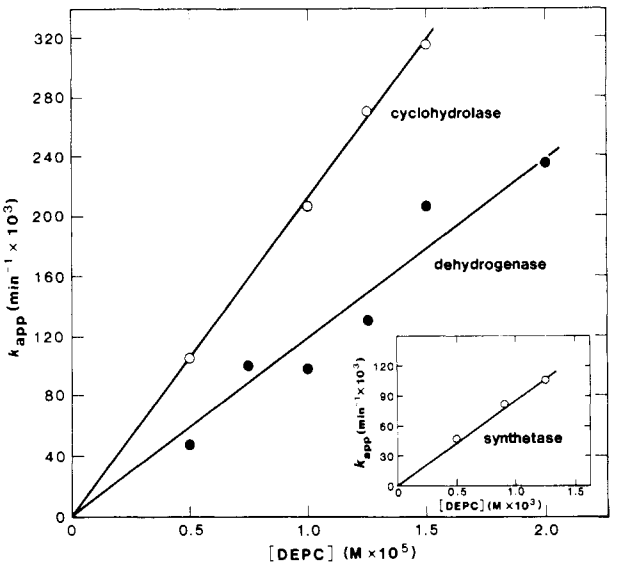

FIGURE 3: Effect of DEPC concentration on the apparent first-order rate constant for inactivation. Apparent first-order rate constants for cyclohydrolase (O), dehydrogenase (●), and synthetase (inset) are plotted vs. reagent concentration. *Second*-order rate constants were calculated from the slopes of the resulting lines.

residual activity vs. time were linear, indicating that the inactivation was first order with respect to enzyme concentration for each activity. The apparent first-order rate constants (K_{app}) were calculated from the relationship $\ln (E/E_0) = -K_{app}t$, where E is the residual activity and E_0 is the initial activity (Colman, 1969). The rate of inactivation was a function of DEPC concentration, but the three activities showed different sensitivities to the reagent. Second-order rate constants (k_2) were calculated from plots of the apparent first-order rate constants vs. reagent concentration (Figure 3). Dehydrogenase and cyclohydrolase activities (k_2 = 12.4 and 21.2 mM^{-1} min^{-1}, respectively) were at least 2 orders of magnitude more sensitive to DEPC than synthetase activity (k_2 = 86 M^{-1} min^{-1}). The significant difference in slope for dehydrogenase and cyclohydrolase inactivation indicates different residues are involved in the reaction with DEPC. A double-logarithmic plot of K_{app} vs. reagent concentration (Levy et al., 1963) yields reaction orders of 0.92, 1.01, and 1.16 with respect to inhibitor for synthetase, cyclohydrolase, and dehydrogenase activities, respectively.

Reaction of DEPC with proteins at pH 7.0 is relatively specific for histidyl residues; however, reaction with other nucleophilic groups has been observed at much lower rates (Melchior & Fahrney, 1970; Wells, 1973; Burstein et al., 1974). The pH dependence of inactivation of dehydrogenase and cyclohydrolase activities with DEPC was examined. The rate of inactivation should depend on the degree of protonation of the reacting residue if the unprotonated residue is modified at a substantially faster rate than the protonated one. K_{app} at different pHs was plotted against [H$^+$] according to the equation of Cousineau & Meighen (1976):

$$1/K_{app} = 1/k_2 + [H^+]/(k_2K_a)$$

where K_a is the apparent acidic dissociation constant and k_2 is the second-order rate constant of formation of the covalent bond for the totally unprotonated form of the enzyme. From the slopes and intercepts (Figure 4), the apparent pK_a's of the inactivation of dehydrogenase and cyclohydrolase were calculated to be 6.4 and 6.6, respectively. These values are within experimental error of each other and are consistent with the modification of histidyl residues. Curvature downward at

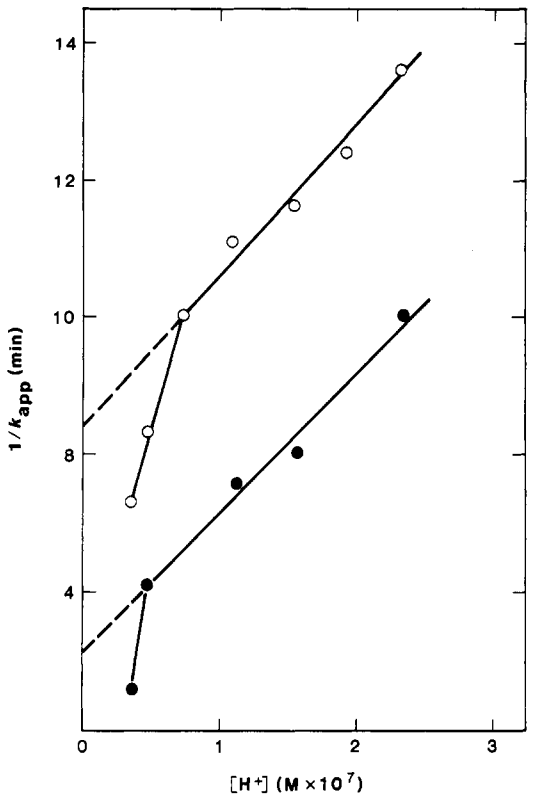

FIGURE 4: pH dependence of inactivation of cyclohydrolase (●) and dehydrogenase (○) by DEPC. All reactions were conducted in 50 mM phosphate buffer adjusted to the desired pH with KOH. The apparent first-order rate constants of inactivation for each pH were plotted according to Cousineau & Meighen (1976).

Table I: Protection of C_1-THF Synthase Activities against DEPC Inactivation[a]

	K_{app} ($\times 10^3$ min^{-1})[b]		
analogue[c]	synthetase	dehydrogenase	cyclohydrolase
none	79.4 ± 7.7[d]	89.6 ± 22.1	119.3 ± 56.1
$PteGlu_1$	79.9 ± 3.6	7.5 ± 0.2[e]	7.4 ± 0.0[e]
$PteGlu_2$	69.8 ± 11.2	9.2 ± 1.5[e]	7.9 ± 7.9[e]
$PteGlu_3$	78.8 ± 3.5	7.1 ± 0.7[e]	7.9 ± 5.7[e]
$PteGlu_4$	57.9 ± 7.6[e]	6.0 ± 3.5[e]	8.0 ± 0.1[e]
$PteGlu_5$	62.6 ± 8.3[e]	7.7 ± 0.8[e]	7.5 ± 2.5[e]
$pAbaGlu_2$	58.3 ± 3.5[e]	32.0 ± 4.0[e]	39.7 ± 5.0
$pAbaGlu_3$	44.6 ± 10.3[e]	17.4 ± 3.5[e]	19.5 ± 5.6[e]
$pAbaGlu_4$	66.0 ± 10.9[e]	9.0 ± 3.0[e]	16.1 ± 4.1[e]
γ-diGlu	69.1 ± 10.0	56.8 ± 4.0	49.5 ± 1.5
heparin	94.7 ± 9.0[e]	71.3 ± 1.0	80.7 ± 10.9

[a] DEPC was added to a final concentration of 1.0 mM for synthetase inactivations and 7.5 μM for dehydrogenase and cyclohydrolase inactivations in order to achieve similar rates of inactivation in the absence of analogue. Reactions were carried out at 20 °C and stopped after 1 and 10 min by dilution with ice-cold buffer containing 1 mM histidine as described under Experimental Procedures. [b] K_{app} was calculated from the relationship $\ln (E_1/E_2) = K_{app}(T_2 - T_1)$ (Burstein et al., 1974). [c] Concentration of all analogues was 2.0 mM except heparin, which was 100 μg/mL. [d] Values represent the mean ± standard deviation of at least two determinations. [e] Different from no analogue ($p < 0.05$).

alkaline pH presumably reflects the modification of other essential residues.

Protection of C_1-THF Synthase Activities against DEPC Inactivation by Substrates and Substrate Analogues. The effects of saturating amounts of substrates on the inhibition of C_1-THF synthase by DEPC are shown in Figure 5. MgATP, a substrate for the synthetase, had no effect on the inactivation of any of the three activities. $NADP^+$, a substrate of only the dehydrogenase, showed near-complete protection

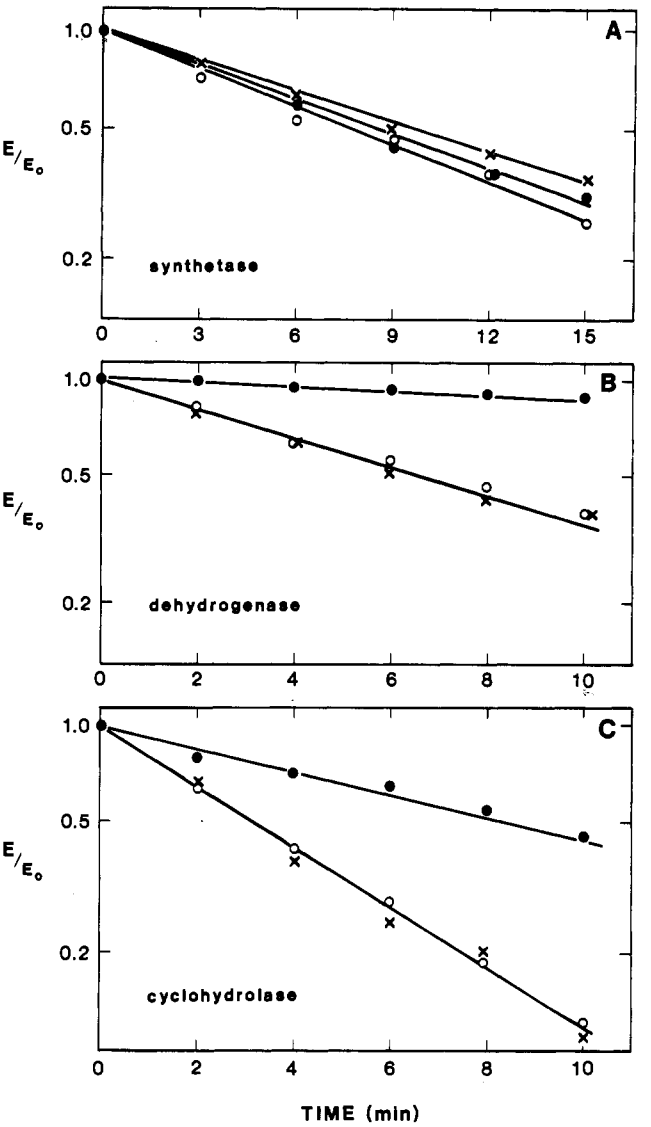

FIGURE 5: Kinetics of inactivation of C_1-THF synthase by DEPC in the presence and absence of substrates. Enzyme (0.18 mg/mL) was incubated in the absence of substrate (○) or in the presence of 0.5 mM $NADP^+$ (●) or 5 mM MgATP (×). DEPC concentration was 0.9 mM for synthetase inactivations (A) and 10 μM for dehydrogenase (B) and cyclohydrolase (C) inactivations.

of dehydrogenase activity and significant protection of cyclohydrolase activity but no protection of synthetase activity. These results are consistent with an overlapping active site for the dehydrogenase/cyclohydrolase activities, distinct from the synthetase active site.

The general structure of folate coenzymes consists of a pteridine moiety linked to *p*-aminobenzoic acid through a one-carbon bridge. The major cellular forms of the coenzymes contain poly(γ-glutamate) tails conjugated to the pAba moiety (Binkley et al., 1944; Pfiffner et al., 1945, 1946; Bird et al., 1945). It has been suggested (MacKenzie & Baugh, 1980; Ross et al., 1984) that the polyglutamyl moiety facilitates binding to active sites and may act as a swinging arm to move the folate substrate from one active site to another in a multifunctional enzyme. Table I shows the results of protection experiments with various polyglutamyl substrate analogues. (The natural substrates for C_1-THF synthase are reduced folates; the analogues described below are unreduced.) $PteGlu_n$ ($n = 1$–5) and $pAbaGlu_n$ ($n = 2$–4) were tested for their ability to protect the three activities against inactivation by DEPC. All of the $PteGlu_n$ derivatives showed significant

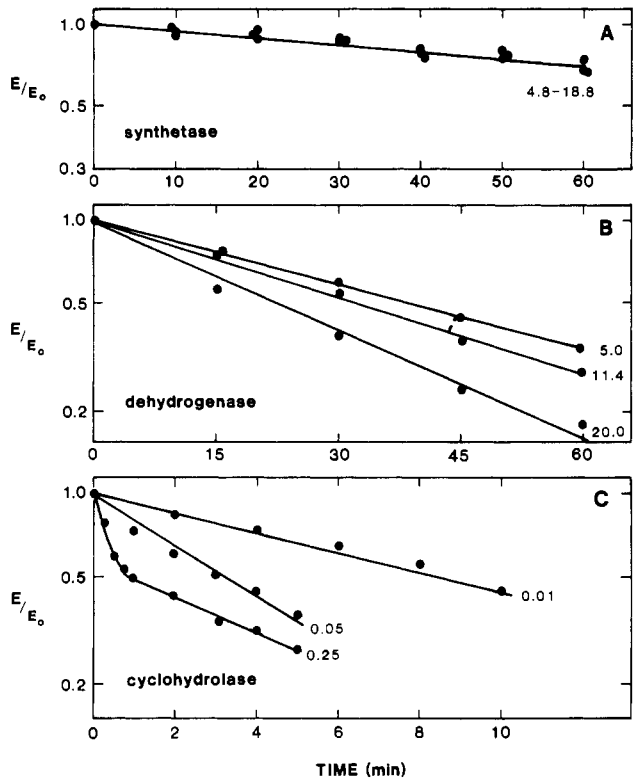

FIGURE 6: Kinetics of inactivation of C_1-THF synthase by NEM. Enzyme (0.18 mg/mL) was incubated with different concentrations of reagent and aliquots were withdrawn, diluted, and assayed for each activity as described under Experimental Procedures. The numbers on the slopes indicate NEM concentration in millimolar. Note the different time scale for the cyclohydrolase inactivations (C).

protection of dehydrogenase and cyclohydrolase activities, with K_{app} values approximately 10-fold lower than in the absence of analogue. Increasing the number of glutamyl residues had little additional protective effect, except for synthetase activity. In general, however, synthetase activity was protected very little, and only by the analogues with four or five glutamyl residues. The pAbaGlu$_n$ series also showed significant protection of dehydrogenase and cyclohydrolase activities. In this case, additional glutamyl residues had a progressive effect on lowering the K_{app} of inactivation. Again, protection of synthetase activity was not as dramatic. The ability of analogues which lack a pteridine moiety (pAbaGlu$_n$) to protect the enzyme as well as the progressive nature of the protection suggests a major role for the polyglutamyl moiety in substrate binding, at least at the dehydrogenase/cyclohydrolase site. γ-Diglutamyl peptide and heparin were tested for their protective ability. [Yeast C_1-THF synthase has a demonstrated affinity for heparin–agarose which has been used to purify the enzyme (Staben & Rabinowitz, 1983). Heparin might be considered an analogue of poly(γ-glutamate) due to its repeated glucuronic acid residues.] However, neither heparin nor γ-diglutamyl peptide had a significant protective effect on any of the three activities. (Heparin actually slightly stimulated the inactivation of synthetase activity.)

Inactivation of C_1-THF Synthase by N-Ethylmaleimide. NEM is a highly specific reagent for protein sulfhydryl groups below pH 7 (Means & Feeney, 1971). Treatment of C_1-THF synthase with this sulfhydryl reagent at pH 6.5 resulted in significant inactivation of only the dehydrogenase and cyclohydrolase activities. Dehydrogenase activity (Figure 6B) was inhibited with apparent first-order rate constants ranging from 18.5×10^{-3} min^{-1} at 5.0 mM NEM to 28.5×10^{-3} min^{-1} at 20 mM NEM. Cyclohydrolase activity (Figure 6C) was at least an order of magnitude more sensitive than dehydrogenase activity, with apparent first-order rate constants of 78.6×10^{-3} and 195×10^{-3} min^{-1} at NEM concentrations of 0.01 and 0.05 mM, respectively. At NEM concentrations above 0.1 mM, inactivation of cyclohydrolase was clearly biphasic, suggesting a second cysteinyl residue was being modified at these higher concentrations. Two observations suggest that reaction of NEM with dehydrogenase and cyclohydrolase active-site sulfhydryl groups is not a simple bimolecular reaction. First, the rates of inactivation are not linearly dependent on reagent concentration as for the DEPC reactions, and second, double-logarithmic plots of K_{app} vs. reagent concentration yield reaction orders with respect to inhibitor of much less than 1. It is possible that NEM first forms a Michaelis complex at the active site and then reacts with the sulfhydryl group. In any event, the vastly different rates of inactivation of dehydrogenase and cyclohydrolase activities indicate that different cysteinyl residues are involved in each case.

Synthetase activity (Figure 6A) was quite resistant to the reagent, exhibiting only slight inactivation after 60 min with NEM concentrations up to 18.8 mM [$K_{app} = (4.6–7.2) \times 10^{-3}$ min^{-1}]. Below 4.8 mM NEM, residual synthetase activity at 60 min was indistinguishable from that of C_1-THF synthase incubated in the absence of reagent. Furthermore, addition of substrates of the synthetase reaction had no effect on the slight inactivation observed (data not shown). The lack of NEM concentration dependence on synthetase inactivation and the inability to protect with substrates suggest that this inactivation is not mechanism based.

Protection of Cyclohydrolase/Dehydrogenase Activities against Inactivation by NEM by Substrates. As was found with the DEPC experiments, NADP$^+$ protected both the dehydrogenase and cyclohydrolase activities against inactivation by NEM. Under the conditions used, the extent of protection by 0.5 mM NADP$^+$ was approximately 2-fold for both activities. NADP$^+$ decreased the K_{app} values for dehydrogenase inactivation from 22.0×10^{-3} to 10.3×10^{-3} min^{-1} and for cyclohydrolase inactivation from 78.6×10^{-3} to 41.3×10^{-3} min^{-1}. Saturating levels of MgATP and formate, substrates of the synthetase reaction, did not protect the dehydrogenase/cyclohydrolase activities. In fact, MgATP appeared to increase the rate of inactivation of dehydrogenase activity ($K_{app} = 27.7 \times 10^{-3}$ min^{-1}). The reason for this apparent potentiation is not known.

Discussion

Evidence has accumulated from several lines of investigation that suggests an intimate relationship for the dehydrogenase and cyclohydrolase active sites in C_1-THF synthase from a number of mammalian sources. In the work presented here, we have examined the nature of this relationship in the trifunctional enzyme from yeast. Results from the chemical modification and protection experiments support the view of an overlapping active site for the dehydrogenase and cyclohydrolase activities. NADP$^+$, a substrate of only the dehydrogenase, protected both dehydrogenase and cyclohydrolase activities against inactivation by either DEPC or NEM. NADP$^+$ was ineffective in protecting the synthetase. Conversely, synthetase substrates did not protect the dehydrogenase/cyclohydrolase activities. These results are in basic agreement with those obtained previously with the mammalian enzymes, but some differences are apparent. Schirch et al. (1979) observed coordinate inhibition of the dehydrogenase and cyclohydrolase activities of rabbit liver C_1-THF synthase upon reaction with the sulfhydryl reagent 5,5′-dithiobis(2-nitrobenzoic acid) whereas these activities

exhibited vastly different sensitivities to sulfhydryl modification of the yeast enzyme. Furthermore, in contrast to the near-complete resistance of the yeast synthetase to NEM, that activity is the most sensitive of the three activities in rabbit liver C_1-THF synthase. Differences were also observed between the yeast enzyme and the pig liver enzyme during the modification of histidines. Smith & MacKenzie (1983) demonstrated protection by folate of the dehydrogenase/cyclohydrolase activities against DEPC inactivation in the porcine enzyme. $NADP^+$ does not protect either activity in the porcine enzyme when used alone, but it does potentiate the protection by folate. Synthetase activity is resistant in that enzyme, at least to concentrations of DEPC below 0.6 mM. In the case of the yeast dehydrogenase/cyclohydrolase activities, $NADP^+$ was quite effective alone in protection. Folate also had significant protective ability, but only for dehydrogenase and cyclohydrolase activities (Table I, $PteGlu_1$). Furthermore, in our experiments, synthetase activity was inhibited by DEPC, although at a much lower rate than that for the dehydrogenase/cyclohydrolase activities. Inactivation of synthetase activity by DEPC is suggestive of a possible catalytic role for histidine in that mechanism. In this regard, photooxidation of formyl-THF synthetase from *Clostridium acidi-urici* results in a rapid loss of activity which correlates with the destruction of histidyl residues (MacKenzie et al., 1972). These differences in the effect of histidine and sulfhydryl modification on the yeast and mammalian enzymes may reflect subtle changes in the surface structure of the enzymes which allow more or less access of the reagents to essential residues.

Our immunoinactivation experiments demonstrate the existence of a close spatial relationship between the dehydrogenase and cyclohydrolase active sites and confirm the existence of two structurally independent domains in yeast C_1-THF synthase. Controlled proteolysis of the enzyme from yeast (Paukert et al., 1977) and pig liver (Tan & MacKenzie, 1977) previously revealed a two-domain structure. These domain sizes are similar to the sizes of the respective monofunctional prokaryotic enzymes (Ljungdahl et al., 1970, 1980; MacKenzie & Rabinowitz, 1971; McGuire & Rabinowitz, 1978; Uyeda & Rabinowitz, 1967; Dev & Harvey, 1978; Clark & Ljungdahl, 1982; Ragsdale & Ljungdahl, 1984), suggesting an evolutionary relationship. In view of the large size of IgG, the fact that the synthetase immunoinactivation curve was distinct from the other two activities indicates a considerable spatial separation of the active sites of the two domains. Although this is only an indirect measure, the distance is potentially great enough to rule out a mechanism suggested by MacKenzie & Baugh (1980, 1983) in which the polyglutamyl moiety acts as a swinging arm to transfer the folate substrate from the dehydrogenase/cyclohydrolase active site to the synthetase active site. In this regard, Wasserman et al. (1983) saw no evidence for channeling between the synthetase and cyclohydrolase sites, using $H_4PteGlu_3$ as a substrate.

The chemical modification experiments indicate the existence of at least two essential histidyl residues and at least two essential cysteinyl residues at the dehydrogenase/cyclohydrolase active site. The difference in the second-order rate constants for inactivation of the dehydrogenase and cyclohydrolase activities by DEPC (Figure 3) indicates that different histidyl residues are involved in the inactivation of each activity. Calculated second-order rate constants for DEPC inactivation of the porcine dehydrogenase and cyclohydrolase activities differ by 20% (Smith & MacKenzie, 1983), suggesting that distinct histidyl residues are involved in the dehydrogenase/cyclohydrolase active site of that enzyme as well. Inactivation of the yeast enzyme by NEM revealed vastly different sensitivities for dehydrogenase and cyclohydrolase (Figure 6). Furthermore, inactivation of cyclohydrolase by 0.1 mM or greater NEM resulted in biphasic curves, suggesting that two cysteinyl residues are involved in the cyclohydrolase reaction. Keeping in mind the coordinate protection afforded by $NADP^+$, these results are consistent with an overlapping dehydrogenase/cyclohydrolase active site in which one histidine and one cysteine are essential for dehydrogenase activity and a different histidine and two cysteines are essential for cyclohydrolase activity. One of the cyclohydrolase cysteinyl residues must be distinct from the essential cysteine of the dehydrogenase, but the second, less reactive one may be involved in both reactions. The dehydrogenase/cyclohydrolase domain contains only three cysteinyl residues (C. Staben and J. C. Rabinowitz, unpublished results). We are now utilizing in vitro directed mutagenesis of the ADE3 gene to identify which of these cysteinyl residues are involved in the dehydrogenase/cyclohydrolase overlapping active site.

The degree of overlap of the dehydrogenase/cyclohydrolase active site remains unknown. Our protection results suggest that the dehydrogenase active-site residues are in close proximity to the cyclohydrolase active-site residues. An alternate interpretation is suggested by kinetic experiments carried out on the chicken liver and rabbit liver enzymes which indicate that coupling of the dehydrogenase/cyclohydrolase reactions affects the binding characteristics of the cyclohydrolase site. 5,10-Methylene-THF and 5,11-methenyltetrahydrohomofolate are both competitive inhibitors against 5,10-methenyl-THF for the cyclohydrolase in the separate activity assay (Schirch, 1978; Caperelli et al., 1981; Wasserman et al., 1983). However, neither of these inhibits cyclohydrolase activity in coupled dehydrogenase/cyclohydrolase reactions. Furthermore, exogenous 5,10-methenyl-THF, the actual substrate in the cyclohydrolase reaction, is not hydrolyzed under coupled assay conditions (Wasserman et al., 1983). These results suggest that once the dehydrogenase/cyclohydrolase couple is running, the cyclohydrolase active site can no longer bind substrates directly from solution, but only those presented to it from the dehydrogenase reaction. It is possible, then, that the two active sites are spatially separate but a conformational change induced by the binding of dehydrogenase substrates might bring the two active sites together to allow the efficient channeling observed.

Protection experiments with the polyglutamate derivatives suggest the importance of the polyglutamyl moiety in the binding of substrate in yeast C_1-THF synthase. In the $pAbaGlu_n$ series, a general trend of increased protection with increasing chain length was observed for the dehydrogenase/cyclohydrolase activities. Furthermore, $pAbaGlu_4$, which lacks a pteridine moiety, was about as effective as the $PteGlu_n$ analogues. It should be noted that the predominant forms of folate coenzymes in *S. cerevisiae* contain six to eight glutamyl residues, with the heptaglutamate conjugate the major form (Bassett et al., 1976). In the present study, analogues containing only up to five glutamyl residues were used. Whether the predominant forms of the coenzyme found in vivo are, in fact, the optimal ones for a particular enzyme will have to be determined for each case. The importance of additional γ-glutamyl residues on the folate substrate has been inferred previously from kinetic studies on the trifunctional enzymes from several sources. MacKenzie & Baugh (1980) observed 10- and 40-fold lower K_m values for

the tetrahydropteroylpolyglutamates than for the monoglutamate derivatives with the dehydrogenase and synthetase activities of the porcine enzyme. V_{max} is not changed. In experiments with methylene-$H_4PteGlu_n$ substrates, polyglutamate chain lengths have no effect on the kinetic parameters of the dehydrogenase (Ross et al., 1984). Rabinowitz (1983) reported a 1000-fold decrease in the K_m of $H_4PteGlu_n$ with increasing glutamyl residues up to four for the synthetase of the yeast trifunctional enzyme. This dramatic effect on K_m was not observed for the substrates of the other two activities of the enzyme. Wasserman et al. (1983) demonstrated that increasing the number of glutamyl residues from one to three results in 5- and 16-fold tighter binding constants for the methenyl (cyclohydrolase) and THF (synthetase) substrates, respectively, in the chicken liver enzyme.

The function of the polyglutamyl tail of the folate substrates, beyond increasing binding affinity, is unclear. Wasserman et al. (1983) saw increased channeling in the dehydrogenase/cyclohydrolase-coupled reaction with the triglutamate substrate relative to the monoglutamate derivative. MacKenzie & Baugh (1980), however, found that channeling between the dehydrogenase and cyclohydrolase sites was not affected by polyglutamate chain length in the porcine enzyme. It has also been suggested that the polyglutamate tail plays a role in the regulation of flux through competing pathways of one-carbon metabolism (Baggott & Krumdieck, 1979). Until the polyglutamate specificity of the enzymes in vivo is known, the physiological role(s) of these moieties will remain uncertain.

ACKNOWLEDGMENTS

We thank J. Kirsch and M. Graves for helpful discussions and L. D'Ari for preparation of the polyglutamate analogues.

REFERENCES

Appling, D. R., & Rabinowitz, J. C. (1985) *J. Biol. Chem. 260*, 1248–1256.

Baggott, J. E., & Krumdieck, C. L. (1979) *Biochemistry 18*, 1036–1041.

Bassett, R., Weir, D. G., & Scott, J. M. (1976) *J. Gen. Microbiol. 93*, 169–172.

Binkley, S. B., Bird, O. D., Bloom, E. S., Brown, R. A., Calkins, D. G., Campbell, C. J., Emmett, A. D., & Pfiffner, J. J. (1944) *Science (Washington, D.C.) 100*, 36.

Bird, O. D., Bressler, B., Brown, R. A., Campbell, C. J., & Emmett, A. D. (1945) *J. Biol. Chem. 159*, 631–636.

Blakley, R. L. (1957) *Biochem. J. 65*, 331–342.

Blakley, R. L. (1969) *The Biochemistry of Folic Acid and Related Pteridines*, pp 219–353, North-Holland Publishing Co., Amsterdam.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Burstein, Y., Walsh, K. A., & Neurath, H. (1974) *Biochemistry 13*, 205–210.

Caperelli, C. A., Chettur, G., Lin, L. Y., & Benkovic, S. J. (1978) *Biochem. Biophys. Res. Commun. 82*, 403–410.

Caperelli, C. A., Domanico, P., & Benkovic, S. J. (1981) *J. Med. Chem. 24*, 1086–1088.

Carrillo, N., & Vallejos, R. H. (1983) *Biochemistry 22*, 5889–5897.

Clark, J. E., & Ljungdahl, L. G. (1982) *J. Biol. Chem. 257*, 3833–3836.

Cohen, L., & MacKenzie, R. E. (1978) *Biochim. Biophys. Acta 522*, 311–317.

Colman, R. F. (1969) *Biochemistry 8*, 888–898.

Cousineau, J., & Meighen, E. (1976) *Biochemistry 15*, 4992–5000.

Curthoys, N. P., & Rabinowitz, J. C. (1971) *J. Biol. Chem. 246*, 6942–6952.

D'Ari, L., & Rabinowitz, J. C. (1985) *Methods Enzymol.* (in press).

Delk, A. S., Romeo, J. M., Nagle, D. P., Jr., & Rabinowitz, J. C. (1976) *J. Biol. Chem. 251*, 7649–7656.

de Mata, Z. S., & Rabinowitz, J. C. (1980) *J. Biol. Chem. 255*, 2569–2577.

Dev, I. K., & Harvey, R. J. (1978) *J. Biol. Chem. 253*, 4245–4253.

Foo, S. K., & Shane, B. (1982) *J. Biol. Chem. 257*, 13587–13592.

Kapor, G. S. (1982) Ph.D. Thesis, University of California, Berkeley.

Levy, H. M., Leber, P. D., & Ryan, E. M. (1963) *J. Biol. Chem. 238*, 3654–3659.

Livingston, D. M. (1974) *Methods Enzymol. 34*, 723–731.

Ljungdahl, J., Brewer, J. M., Neece, S. H., & Fairwell, T. (1970) *J. Biol. Chem. 245*, 4791–4797.

Ljungdahl, L. G., O'Brien, W. E., Moore, M. R., & Liu, M.-T. (1980) *Methods Enzymol. 66*, 599–609.

MacKenzie, R. E. (1984) in *Folates and Pterins* (Blakley, R. L., & Benkovic, S. J., Eds.) Vol. 1, pp 255–306, Wiley, New York.

MacKenzie, R. E., & Rabinowitz, J. C. (1971) *J. Biol. Chem. 246*, 3731–3736.

MacKenzie, R. E., & Baugh, C. M. (1980) *Biochim. Biophys. Acta 611*, 187–195.

MacKenzie, R. E., & Baugh, C. M. (1983) in *Folyl and Antifolyl Polyglutamates* (Goldman, I. D., Chabner, B. A., & Bertino, J. R., Eds.) pp 19–34, Plenum Press, New York.

MacKenzie, R. E., D'Ari-Straus, L., & Rabinowitz, J. C. (1972) *Arch. Biochem. Biophys. 150*, 421–427.

McGuire, J. J., & Rabinowitz, J. C. (1978) *J. Biol. Chem. 253*, 1079–1085.

McKenzie, K. Q., & Jones, E. W. (1977) *Genetics 86*, 85–102.

Means, G. E., & Feeney, R. E. (1971) *Chemical Modification of Proteins*, p 111, Holden-Day, San Francisco, CA.

Melchior, W. B., Jr., & Fahrney, D. (1970) *Biochemistry 9*, 251–258.

Miles, E. W. (1977) *Methods Enzymol. 47*, 431–442.

Paukert, J. L., Straus, L. D., & Rabinowitz, J. C. (1976) *J. Biol. Chem. 251*, 5104–5111.

Paukert, J. L., Williams, G. R., & Rabinowitz, J. C. (1977) *Biochem. Biophys. Res. Commun. 77*, 147–154.

Pfiffner, J. J., Calkins, D. G., O'Dell, B. L., Bloom, E. S., Brown, R. A., Campbell, J. C., & Bird, O. D. (1945) *Science (Washington, D.C.) 102*, 228.

Pfiffner, J. J., Calkins, D. G., Bloom, E. S., & O'Dell, B. L. (1946) *J. Am. Chem. Soc. 68*, 1392.

Rabinowitz, J. C. (1960) *Enzymes, 2nd Ed. 2*, 185–252.

Rabinowitz, J. C. (1983) in *Folyl and Antifolyl Polyglutamates* (Goldman, I. D., Chabner, B. A., & Bertino, J. R., Eds.) pp 75–83, Plenum Press, New York.

Ragsdale, S. W., & Ljungdahl, L. G. (1984) *J. Biol. Chem. 259*, 3499–3503.

Ross, J., Green, J., Baugh, C. M., MacKenzie, R. E., & Matthews, R. G. (1984) *Biochemistry 23*, 1796–1801.

Schirch, L. (1978) *Arch. Biochem. Biophys. 189*, 283–290.

Schirch, L., Mooz, E. D., & Peterson, D. (1979) in *Chemistry and Biology of Pteridines* (Kisliuk, R. L., & Brown, G. M., Eds.) pp 495–500, Elsevier/North-Holland, New York.

Smith, D. D. S., & MacKenzie, R. E. (1983) *Can. J. Biochem. Cell Biol. 61*, 1166–1171.
Staben, C., & Rabinowitz, J. C. (1983) in *Chemistry and Biology of Pteridines* (Blair, J. A., Ed.) pp 381–385, Walter de Gruyter & Co., Berlin.
Tan, L. U. L., & MacKenzie, R. E. (1977) *Biochim. Biophys. Acta 485*, 52–59.
Tan, L. U. L., Drury, E. J., & MacKenzie, R. E. (1977) *J. Biol. Chem. 252*, 1117–1122.
Uyeda, K., & Rabinowitz, J. C. (1967) *J. Biol. Chem. 242*, 4378–4385.
Wasserman, G. F., Benkovic, P. A., Young, M., & Benkovic, S. J. (1983) *Biochemistry 22*, 1005–1013.
Wells, M. A. (1973) *Biochemistry 12*, 1086–1093.

Characterization of an Early Intermediate in the Folding of the α Subunit of Tryptophan Synthase by Hydrogen Exchange Measurement†

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ABSTRACT: The development of the hydrogen bonding network in the early stages of the folding of the α subunit of tryptophan synthase was monitored with a hydrogen exchange technique. The orders of magnitude difference between the rapid conversions of the unfolded forms to two stable intermediates (milliseconds) and the subsequent slow conversions of the intermediates to the native form (>100 s) was used to selectively label with tritium the hydrogen bonds that form in the first 30 s of folding at 0 °C. Rapid removal of the tritiated solvent by gel filtration ensured that hydrogen bonds formed in subsequent folding reactions would be unlabeled. Limited proteolysis and separation of peptides by high-pressure liquid chromatography permitted the determination of the amount of label retained in individual peptides by scintillation counting. Peptides 1–70 and 71–188, which when covalently linked comprise the stable amino domain in the native conformation, retain 91% and 93%, respectively, of the label retained when the protein is allowed to completely refold in tritiated solvent. Peptide 189–268, the marginally stable carboxyl domain, only retains 43% of the label. The striking difference in retention of label confirms the independent folding of these two domains and shows that the kinetic intermediates that appear in the folding of α subunit correspond to structural domains in the native conformation. The near-equality of the labeling of the two peptides comprising the amino domain shows that this domain folds as a single entity and that subdomain folding is unlikely. Refolding into consecutively higher final urea concentrations shows that the amino domain consistently retains a greater fraction of label than does the carboxyl domain. This result demonstrates the greater stability of the amino domain.

The mechanism by which the amino acid sequence of a protein directs the folding to the native functional conformation is not known in detail for any protein. This lack of understanding is due in part to the high cooperativity of the unfolding transition and the concomitant absence of stable intermediates and in part to the rapid rate of folding which precludes the use of high-resolution techniques such as X-ray or nuclear magnetic resonance (NMR)[1] spectroscopy. The proliferation of DNA sequences for structural genes makes clear the desirability of being able to predict the three-dimensional conformation of a protein from its primary sequence.

In an effort to elucidate the mechanism of folding, we have been investigating the effects of single amino acid replacements on the folding and stability of the α subunit of tryptophan synthase from *Escherichia coli*. Over two dozen point mutations have been isolated and identified by Yanofsky and his colleagues (Yanofsky et al., 1967; Yanofsky & Horn, 1972; Murgola & Yanofsky, 1974). The basic premise of our work is that amino acids that play key roles in folding and stability will have observable effects on the kinetic and equilibrium properties of the unfolding reaction in vitro. Obviously, the correct interpretation of such effects depends upon a folding model for the wild-type protein that is as detailed as possible in both kinetic and structural terms.

Previous studies on the folding of the α subunit have proposed that one or more stable intermediates appear during folding (Yutani et al., 1979, 1982; Matthews & Crisanti, 1981; Crisanti & Matthews, 1981; Matthews et al., 1983). The structural interpretation of the principal intermediate is based upon the observation of Higgins et al. (1979) that the protein can be converted to two fragments by limited tryptic digestion. The amino domain consists of residues 1–188 and the carboxyl domain of residues 189–268. The isolated fragments spontaneously complement to form active enzyme. The principal intermediate has been proposed to consist of a folded amino domain and an unfolded carboxyl domain on the basis of both difference UV and circular dichroism spectroscopies. Supporting this hypothesis are the recent results of Miles et al. (1982) on the stabilities of the isolated domains to guanidine hydrochloride unfolding. The amino domain was shown to be substantially more stable than the carboxyl domain. Also,

† This work was supported by U.S. Public Health Service Grant GM 23303. C.R.M. is the recipient of Research Career Development Award K04 AG00153 from the U.S. Public Health Service.

[1] Abbreviations: NMR, nuclear magnetic resonance; $NaDodSO_4$, sodium dodecyl sulfate; HPLC, high-pressure liquid chromatography; $(NH_4)_2SO_4$, ammonium sulfate; DTE, dithioerythritol; Na_2EDTA, disodium ethylenediaminetetraacetate; NaOAc, sodium acetate; $^3H_{rem}$, moles of tritium remaining per mole of protein or peptide; Gdn-HCl, guanidine hydrochloride.

0006-2960/85/0424-3547$01.50/0