Active-Site Mapping and Site-Specific Mutagenesis of Glycinamide Ribonucleotide Transformylase from *Escherichia coli*†

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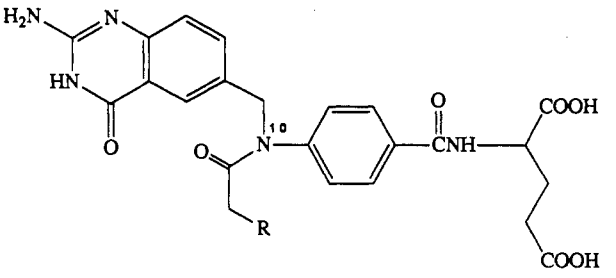
ABSTRACT: The affinity reagent N^{10}-(bromoacetyl)-5,8-dideazafolate has previously been shown to inactivate glycinamide ribonucleotide transformylase (EC 2.1.2.2) from *Escherichia coli* in an active-site-directed manner with a 1:1 stoichiometry [Inglese et al. (1990) *Biochemistry 29*, 1436–1443]. After a series of mild proteolytic digestions, the dideazafolate label was localized to an active-site peptide attached by an ester linkage to the highly conserved residue Asp 144. Subsequent site-specific mutagenesis of Asp 144 to Asn 144 resulted in a catalytically inactive enzyme that retained the ability to bind substrates and inhibitors. The Asn 144 mutant could be further labeled with the affinity reagent in an active-site-directed stoichiometric fashion; however, the site of modification in this case was His 119. These results imply that Asp 144 may function as a general base within the catalytic center of the transformylase and is in close proximity to His 119 in the folded protein.

Glycinamide ribonucleotide transformylase (GAR TFase)[1] catalyzes the first of two reduced folate formyl transfers in the synthesis of purines de novo (Warren & Buchanan, 1957; Scheme I) and is an enzyme of fundamental significance in the anabolism of all organisms. Because of its association with DNA synthesis, this enzyme has become the target of antineoplastic agents (Chabner et al., 1986). To date little is known about GAR TFase at the molecular level compared to other medicinally important and related enzymes such as dihydrofolate reductase (Blakley, 1984) and thymidylate synthase (Santi & Danenberg, 1984; Hardy et al., 1987).

Much of what is known about GAR TFase comes from studies on the avian enzyme, which were primarily aimed at its purification (Caperelli et al., 1980; Young et al., 1984) and interaction with other folate-processing enzymes (Young et al., 1985). Recent work on the avian enzyme in collaboration with Stubbe, Henikoff, and Patterson (Daubner et al., 1985) illustrated the multifunctional nature of the enzyme. Of particular significance to the work presented in this paper was the development of an affinity reagent for GAR TFase based on the N^{10}-formyl-5,8-dideazafolate cofactor analogue (Chart I; Smith et al., 1981; Daubner et al., 1986). The irreversible N^{10}-(bromoacetyl)-DDF inhibitor has been shown to inactivate *Escherichia coli* GAR TFase in an active-site-directed manner with a stoichiometry of 1 label per enzyme monomer (Inglese et al., 1990).

Data on GAR TFase at the level of the gene has been obtained for four species of the enzyme. A comparison of the primary protein sequences of the four species has been made in Figure 1. These sequences were obtained from *E. coli* (Smith & Daum, 1987), *Bacillus subtilis* (Ebbole & Zalkin, 1987), and *Drosophila melanogaster* and *Saccharomyces cerevisiae* (Henikoff, 1986) by translation of the respective cDNA clones. Smith has noted regions of homology within these species. Through affinity labeling we have identified Asp 144, which falls within one of these conserved regions,

Chart I: Chemical Structures of N^{10}-Substituted DDF Cofactors



R = Br, N-10-(bromoacetyl)-DDF

R = OH, N-10-(hydroxyacetyl)-DDF

as an active-site residue for *E. coli* GAR TFase, and we have generated a site-specific mutation at this residue (D144N) that results in an inactive protein retaining the ability to bind substrates. In addition, we have employed this mutant and the affinity reagent to identify another amino acid residue at the active site, His 119. This represents a unique method for mapping the active site of an enzyme by using site-specific mutagenesis and classic affinity-labeling techniques that is complementary to traditional active- and binding-site mapping employing a set of different affinity reagents (Plapp, 1982, and references therein).

Experimental Procedures

Materials

Spectroscopic grade trifluoroacetic acid, PTH amino acids, and amino acid standards were purchased from Pierce Chemical Co. Trypsin–TPCK was from Worthington Enzymes. Endoproteinase Arg C, *Staphylococcus aureus* V8

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[1] Abbreviations: β-TGDDF, β-thioglycinamide dideazafolate; DDF, 5,8-dideazafolate; DHFR, dihydrofolate reductase; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FPLC, fast protein liquid chromatography; GAR TFase, glycinamide ribonucleotide transformylase; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PTC, phenylthiocarbamoyl; PTH, phenylthiohydantoin; QAE, quaternary aminoethyl; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid.

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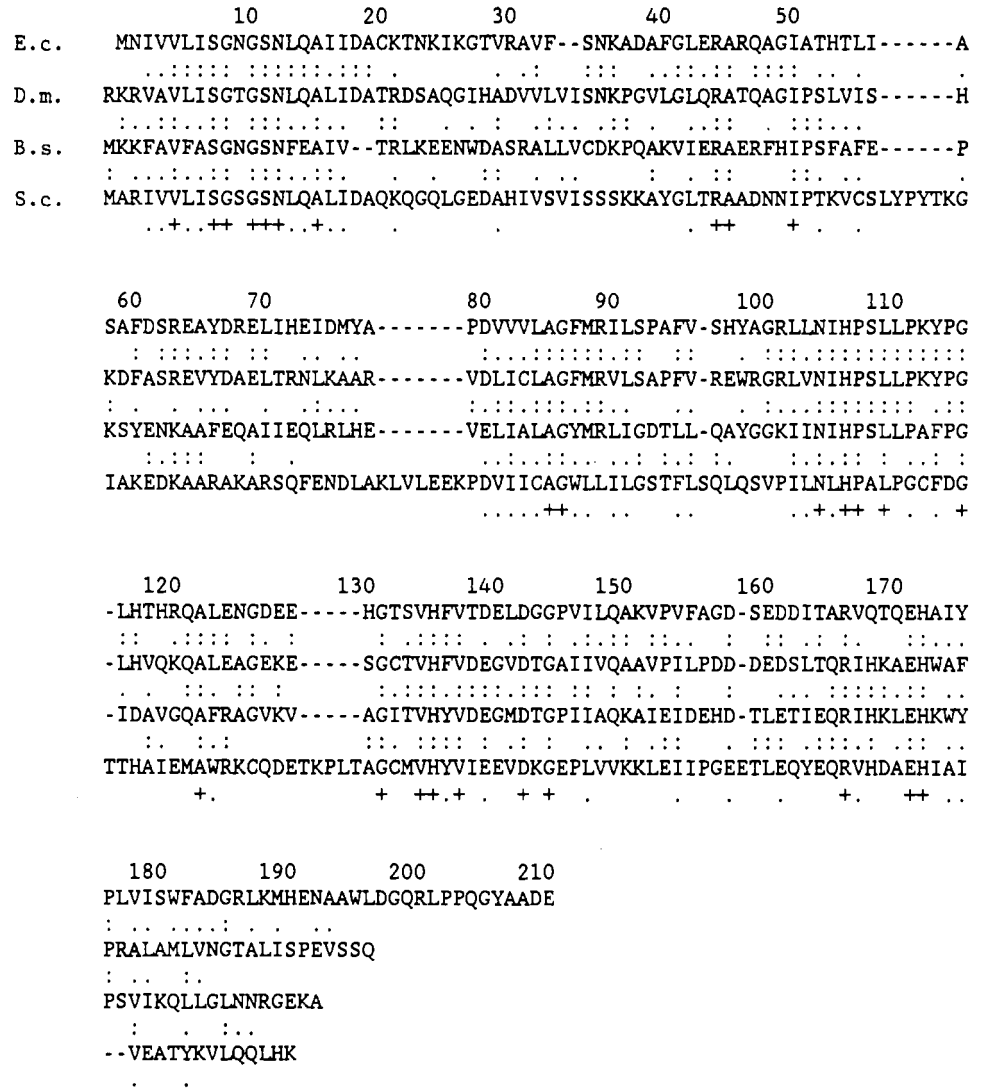
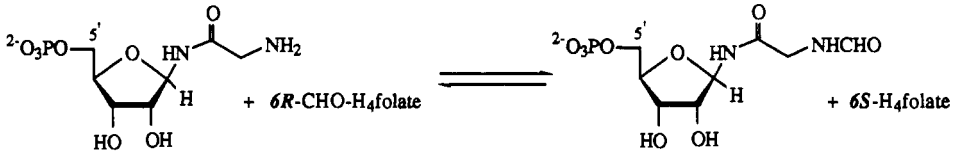


FIGURE 1: Amino acid alignment of GAR TFases. The *E. coli* (E.c.) sequence was taken from Smith and Daum (1987), the *Saccharomyces* (S.c.) and *Drosophila* (D.m.) sequences were from Henikoff (1986), and the *B. subtilis* (B.s.) sequence was from Ebbole and Zalkin (1987). The E.c., B.s., and S.c. sequences begin with residue 1, and the D.m. sequence begins with residue 1153 of the trifunctional GART polypeptide. Alignment was carried out with the algorithm of Wilber and Lipman (1983). The code used is as follows: between adjacent sequences a · implies a conserved residue while a : implies identical residues. For comparison of the entire four sequences, a + indicates invariant residues throughout four species while a · indicates conserved residues according to the system of Amuro et al. (1985).

Scheme I: GAR TFase Reaction



protease, and carboxypeptidase P were obtained from Boehringer Mannheim Biochemicals. Porcine pancreatic pepsin (1:10000) was purchased from Sigma. HPLC-grade water and acetonitrile were purchased from the Baker Chemical Co. Sequencer reagents were from Applied Biosystems, Foster City, CA. All other reagents were of the highest possible quality and were used as obtained unless otherwise specified. Spectrophotometric assays were performed on either a Gilford Model 252 or a Cary 219 spectrophotometer. UV–visible spectra were recorded on a Perkin-Elmer Lambda Array 3840 UV/vis spectrophotometer. HPLC was performed on a Waters 600E system with detection by a Waters 990 photodiode array detector controlled by a NEC PowerMate 2 PC.

Methods

Protein Purification and Concentration Determination. Wild-type GAR TFase was prepared as described by Inglese et al. (1990). Homogeneous GAR TFase was quantitated by using the extinction coefficient measured at 280 nm of 22.1 mM^{-1} cm^{-1} (pH 7.5). The D144N GAR TFase was purified by the same protocol used for wild type except SDS–PAGE was used to locate the protein in each purification step.

5,8-Dideazafolate and N^{10}-Substituted Analogues. 5,8-Dideazafolate (DDF), N^{10}-formyl-DDF, and N^{10}-(bromoacetyl)-DDF were prepared according to the methods described and referred to in Inglese et al. (1990). N^{10}-(Hydroxyacetyl)-DDF was prepared through hydrolysis of N^{10}-(bromoacetyl)-DDF at pH 12. One milliliter of 40 μM N^{10}-(bromoacetyl)-DDF in water was taken to approximately pH 12 with several microliters of 1 N NaOH. The solution was kept at 37 °C for 1 h and then buffered with 10 μL of 2 M Tris, pH 7.5. Alternatively, 3 μL of 7.6 mM inactivator

was added to 10 μL of 100 mM K_2CO_3, pH 11.4, and the solution was incubated for several hours at 30 °C. The major product of this hydrolysis is N^{10}-(hydroxyacetyl)-DDF as determined by HPLC. The UV and ^1H NMR spectra of this material were identical with those of the bromoacetyl compound (Daubner et al., 1986) with one exception. In the ^1H NMR spectrum (DMSO-d_6) the CH_2OH resonance (δ 4.15) falls downfield from the corresponding protons in the bromoacetyl group (i.e., CH_2Br δ 3.93) as expected. Also, the hydroxy compound gives a negative result in the active bromide test described by Daubner et al. (1986).

Nondenaturing PAGE. The procedure is a modification of that originally described by Bryan (1977) and Davis (1964) or as outlined in Sigma Technical Bulletin No. MKR-137. Protein was electrophoresed on 9% polyacrylamide mini slab gels at 4 °C for 6 h. Samples (approximately 4 μg each) were dissolved in 5 μL of sample buffer prepared from 1 mL of 50 mM Tris, pH 6.7, 1 mL of glycerol, and 1 mL of water containing 0.25 mg of bromophenol blue.

Enzyme Assays. Assays were performed with the N^{10}-formyl-DDF cofactor as described by Inglese et al. (1990).

Fluorescence Titrations. Dissociation constants (K_D's) were determined by measuring the emission enhancement at 395 nm (excitation 275 nm) of the inhibitor upon addition of either wild-type or D144N GAR TFase. The procedure is described in Inglese et al. (1989).

Wild-Type GAR TFase Inactivations. Inactivations of GAR TFase were carried out according to the general protocol described by Inglese et al. (1990). A typical inactivation was accomplished by incubating 20–60 μM solutions of GAR TFase in 50 mM Hepes buffer, pH 7.5, at 26 °C with 1.5–2 equiv of N^{10}-(bromoacetyl)-DDF. After inactivations were complete (determined by assaying an aliquot for activity), the enzyme solutions were dialyzed into the appropriate buffer for proteolytic or chemical digestion and to remove the excess unreacted N^{10}-(bromoacetyl)-DDF.

Mutant (D144N) GAR TFase Inactivations. In this case 20–60 μM mutant enzyme was used, with the concentrations of N^{10}-(bromoacetyl)-DDF as described under Results. Incubations, however, were done at 37 °C for 8 h, and the extent of labeling was determined by using HPLC (condition C, vide infra), by comparison of the 254/280-nm absorbance ratio to that of inactivated wild-type GAR TFase (ratio is ≈1.44 for 1 label per enzyme monomer).

Stability of Wild-Type GAR TFase–Inactivator Adduct. Aliquots (50 μL in 50 mM potassium phosphate buffer, pH 7) of wild-type GAR TFase were added to 500 μL of each of the following buffers: 50 mM potassium phosphate, pH 2, 7, and 11, and 50 mM potassium phosphate, pH 7, containing either 20 mM DTT or 20 mM hydroxylamine. The samples were incubated at 100 °C for 2 min and then incubated at 65 °C for 3 h, after which time the entire sample was injected onto the HPLC column (condition A, vide infra). The stability of the adduct was determined on the basis of the amount of label released as DDF or N^{10}-(hydroxyacetyl)-DDF.

Cyanogen Bromide Cleavage. Dried, salt-free protein samples [prepared by being injected onto the HPLC column (condition A), collected, and speed-vacuum-dried] were dissolved in 70% formic acid (purged with argon gas) to give a concentration of 20–40 μM GAR TFase. Crystalline CNBr (Pierce Chemical Co., Rockford, IL) was dissolved in a solution of 70% formic acid (usually 20 mg of CNBr/mL of formic acid). Enough of the CNBr–formic acid solution was added to the protein–formic acid solution to give a ≈50-fold excess of CNBr over protein (w/w). The reaction was allowed to proceed in a 1.5-mL Eppendorf tube (argon blanket) with stirring at ambient temperature in the dark. After 24 h, the reaction mixture was evaporated to dryness with a stream of argon or diluted with 4 volumes of water and injected onto the HPLC column (condition A).

S. aureus V8 Protease Digestion of Labeled GAR TFase. Inactivated enzyme (vide supra), 40 μM, was dialyzed versus 50 mM NaH_2PO_4 buffer, pH 4.0, at 4 °C for 12 h. The slightly turbid solution was treated with an aliquot of *S. aureus* V8 protease (0.5 mg/mL of 50 mM NaH_2PO_4 buffer, pH 4.0) and allowed to incubate at 35 °C with stirring for 12 h. The ratio of inactivated enzyme to V8 protease was 100:1 (w/w). After 12 h, another aliquot (100:1 w/w) of protease was added and the incubation was continued for an additional 12 h. Typically experiments were carried out on 4–12 μmol of material. The reactions were quenched by quick freezing at −85 °C or by direct injection onto the HPLC column (condition A).

Endoproteinase Arg C Digestion of Labeled GAR TFase. Endoproteinase Arg C from submaxillary glands of mice was dissolved in water to give a solution of 0.5 mg of lyophilizate/mL. To 25 μL of a 40 μM solution of inactivated enzyme (vide supra) in 50 mM Tris buffer, pH 7.5, was added 1 μL of the above Arg C solution. This sample was incubated at 35 °C for 18 h and then injected directly onto the HPLC column (condition A).

Pepsin Digestion of the C-Terminal DDF-Labeled Peptide. Further digestion of the purified *S. aureus* V8 protease peptides was accomplished with porcine pancreatic pepsin (1:10000). The peptide (0.1–2 nmol) was taken up in 10 μL of 70% formic acid and added slowly to a stirred solution of 110 μL of 1 mM HCl containing 15 μL of a 0.1 mg/mL solution of pepsin. The digestion was performed in a 1.5-mL Eppendorf tube at 22 °C for 65 min, after which time the sample was injected onto the HPLC column (condition A).

Carboxypeptidase P Digestion of Pepsin-Generated DDF-Labeled Peptide. Carboxypeptidase P from *Penicillium janthinellum* was dissolved in 50 mM K_2HPO_4 buffer, pH 4.0, to a concentration of 0.5 mg/mL. Approximately 1 nmol of peptide was dissolved in 10 μL of 30% CH_3CN/70% H_2O/0.1% TFA, to which 150 μL of 1 mM HCl and 40 μL of the carboxypeptidase P solution were added. The reaction solution was stirred at 25 °C for 20 h in a 1.5-mL Eppendorf tube and injected onto the HPLC column (condition B).

S. aureus V8 Protease Digestion of the Pepsin-Generated DDF-Labeled Peptide. Dried peptide (≈1–0.5 nmol in an Eppendorf tube) was taken up in 1 μL of 70% formic acid, to which was added 100 μL of 50 mM acetate buffer (pH 8.0) or 50 mM phosphate buffer (pH 7.0). The resultant solutions had pH's below 2 and were brought to a pH between 3.5 and 4.5 with 5–10 μL of 1 M NaOH (pH was measured with colorpHast pH indicator strips). To this solution was added 10 μL of the V8 protease solution described above. Reaction times were from 24 to 48 h at 22 °C, after which time the solution was injected onto the HPLC column (condition B).

Trypsin Digestion of Labeled D144N GAR TFase. To 1 mL of 33 μM labeled D144N GAR TFase in 50 mM Hepes, pH 7.5, and 0.5 mM EDTA buffer was added 20 μL of a trypsin–TPCK solution (added as a 0.5 mg/mL solution in 0.1 N HCl). The reaction mixture was allowed to incubate at 22 °C for 24 h, after which time the solution was injected onto the HPLC column (condition C).

Peptide Isolation and Purification.[2] Protein digests and peptide samples were separated and purified on a Waters C18 μBondapak column (3.9 mm × 30 cm; conditions A and B) or a Waters Delta-pak column C18-100 Å (3.9 mm × 30 mm; condition C). Mobile phases were as follows: solvent A, HPLC-grade H_2O/0.1% TFA; solvent B, HPLC-grade CH_3CN/0.7% TFA; solvents A and B were balanced at 220 nm by the addition of TFA to one of the buffers so its OD at 220 nm matched the other.

The following gradients were used for separations: condition A, isocratic at 15% solvent B for 1 min, followed by a linear gradient to 100% solvent B over 100 min with a flow rate of 0.8 mL/min; condition B, a linear gradient from 0% to 50% solvent B over 100 min, followed by a steeper linear gradient from 50% to 100% solvent B over 50 min, flow rate 0.8 mL/min; condition C, same as condition B except the Delta-pak C18-100 Å column was used for the separation. Peaks submitted for sequencing were rechromatographed under the same condition but the gradient was made shallower where the original peak eluted and only the center portion of the peak was collected.

The effluent was monitored at 220, 254, and 280 nm, and individual peaks or groups of peaks were collected as 0.5–1-mL samples by hand. Depending on the digest, 1–0.1 nmol of material was injected per run, giving absorbance values in the range 1.5–0.1 absorbance units at 220 nm. Samples of interest were taken to dryness on a Savant Speed-vac or frozen at −85 °C.

Amino Acid Analysis and N-Terminal Sequencing. Amino acid derivatizations were performed on an Applied Biosystems Model 420A derivatizer and the PTC derivatives analyzed on a Model 120A analyzer (Applied Biosystems). Automated Edman degradation was carried out on an Applied Biosystems Model 477A pulsed-liquid sequenator. The resulting PTH derivatives were analyzed by using an on-line Applied Biosystems Model 120A microbore HPLC system.

C-Terminal Sequencing. C-terminal sequencing was carried out with carboxypeptidase P. To 0.5 mL of water was added 0.7 mg of carboxypeptidase P lyophilizate (0.175 mg of protein/0.7 mg of lyophilizate) to give a 0.35 mg/mL carboxypeptidase P solution. To 2 μL of a solution of approximately 500 pmol of peptide in 30% CH_3CN/70% H_2O/0.1% TFA were added 96 μL of 1 mM HCl, with stirring, and 2 μL of carboxypeptidase P solution. The reaction was allowed to proceed at 22–23 °C in a 1.5-mL Eppendorf tube. Aliquots removed at 1, 2 and 3 h were acidified with 1 drop of TFA and subjected to amino acid analysis as previously described.

Construction of the D144N GAR TFase Mutant. Strain CGSC 6796 from the Coli Genetic Stock Center was used as the *dut*$^-$ *ung*$^-$ host strain, and strain BSJ72 (obtained from Tom St. John) was used as the *dut*$^+$ *ung*$^+$ recovery strain.

The goal of the site-specific mutagenesis was to change the active-site Asp 144 codon (GAC) into an Asn codon (AAC). Accordingly, a 20mer mutagenic oligonucleotide (5′-GGGCCACCGT*T*CAGTTCATC-3′) was synthesized on a Milligen/Biosearch Cyclone, purified, and phosphorylated with polynucleotide kinase (Maniatis et al., 1982). The site-specific mutagenesis was carried out essentially as described by Ner et al. (1988). The *Eco*RI–*Sal*I DNA fragment from the expression vector pJS167 containing the GAR TFase gene was subcloned into the phagemid Bluescript M13 SK$^+$ (Stratagene, Inc.) via the *Eco*RI–*Sal*I sites. After verification of its structure, the plasmid was transformed into the *dut*$^-$ *ung*$^-$ strain CGSC 6796 by selection for ampicillin resistance. Uracil-containing single-stranded DNA was produced by superinfection with the helper phage M13K07 (Vieira & Messing, 1987). The mutagenic oligonucleotide was annealed, elongated, and ligated as described (Ner et al., 1988), and the resulting mutagenesis mixture was transformed into strain BSJ72 with selection for ampicillin resistance. Sequencing DNA was prepared from random colonies and screened by Sanger dideoxy sequencing (Sanger et al., 1977). The sequencing primer (5′-CTGGGTTTGCACGCGGGCGG-3′) was selected to anneal approximately 100 nucleotides away from the desired mutation, and the mutagenized GAR TFase gene was transferred into the expression vector pJS88 via the *Eco*RI–*Sal*I restriction sites. A representative plasmid containing the Asn 144 mutation was selected, designated pJS212, and employed for the production of the D144N GAR TFase for subsequent characterization.

The *Eco*RI–*Sal*I restriction fragment containing the Asn 144 mutation was resequenced in its entirety by using the oligonucleotides 5′-CTGGGTTTGCACGCGGGCGG-3′, 5′-GCCAGCACGACCACATCGGG-3′, 5′-CAGACGACCATCGGCAAACC-3′, and 5′-GTTTTCCCAGTCACGAC-3′ (universal) as sequencing primers. The construct had only the desired mutation.

Results and Discussion

Active-Site Mapping

Properties of Affinity-Labeled GAR TFase. As we previously reported (Inglese et al., 1990), *E. coli* GAR TFase is inactivated with 1 equiv of N^{10}-(bromoacetyl)-DDF. In order to determine a viable route toward isolation of an active-site residue, the stability of the enzyme–inactivator (E∼I) adduct was examined under various conditions of pH and susceptibility to nucleophiles. This analysis indicated that the adduct is labile at basic pH and to nucleophiles but is rather stable at acidic pH (50 mM phosphate buffer, pH 2). In all cases the label was released as N^{10}-(hydroxyacetyl)-DDF (Chart I) as determined by HPLC. These results suggest the nature of the covalent linkage between enzyme and cofactor is that of an ester but do not rule out the less likely possibility of a reversible alkylation followed by hydrolysis of an intermediate species to N^{10}-(hydroxyacetyl)-DDF.

If the E∼I adduct is subjected to reverse-phase HPLC (condition A), it elutes as a single peak having the UV spectrum shown in Figure 2b. The HPLC profile of native GAR TFase (Figure 2a) shows that it possesses retention time similar to that of the labeled enzyme, indicating that the label has little effect on the elutropic properties of the enzyme. The UV spectrum of the labeled enzyme has a maximum, due to bound cofactor, that appears at 254 nm. In subsequent experiments the 254/280-nm absorbance ratio was used during HPLC elutions of E∼I digests to identify peptides carrying the cofactor. Any peak with a 254/280-nm absorbance >1 indicated the presence of label.

Cyanogen Bromide Digestion of E∼I. GAR TFase contains four methionine residues, the first Met being the N-terminal amino acid of the protein. Cleavage of E∼I with CNBr results in three reverse-phase-HPLC-separable peptides. The DDF chromophore is associated with a single eluting peak (CB III) at 95–100 min. No label was hydrolyzed under these

[2] Peptides obtained from chemical and proteolytic digests are designated by the chemical or protease used to generate the peptide and the position at which the peptide elutes from the column relative to other peptides within that separation; e.g., CB I indicates the first peptide eluting from the separation of a cyanogen bromide digest. The proteases are abbreviated as follows: V8, *S. aureus* V8 protease (also known as endoproteinase Glu C); Arg C, endoproteinase Arg C; and P, pepsin.

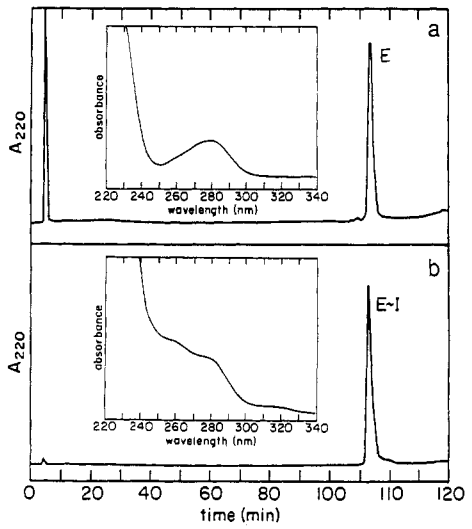


FIGURE 2: HPLC profiles and absorbance spectra of native and inactivated GAR TFase. (a) Reverse-phase HPLC (condition A) profile of *E. coli* GAR TFase. (Inset) UV spectrum of GAR TFase (E). (b) Reverse-phase HPLC (condition A) profile of *E. coli* GAR TFase inactivated with N^{10}-(bromoacetyl)-DDF. (Inset) UV spectrum of E~I adduct.

Table I: Summary of Amino Acid Sequence Analyses of Peptides Isolated from *E. coli* GAR TFase[a]

peptide–DDF adduct[b]	HPLC peak	N-terminal sequence[c]
	N-term	MNIV...
no	CB I	HENAAWLDGQRLPPQGYAAD
no	CB II	YAPDVVVLAGF
yes	CB III(1)[d]	RILSPAFVSHYAGRLLNIHPSLLP...
	CB III(2)[d]	NIVVLISGNGSNLQAIIDACKTN...
yes	V8 I	NGDEEHGTSVHFVTDELDGGPVILQAKVP...
no	V8 II	MNIVV...
yes	Arg I	QALEN...
yes	P II	NGDEEHGTSVHFVTDELDGGPVI*L*[e]
no	PIV	QAKVPVFAG
no	V8′ I	NGDEEHGTSVHFVTDE
yes	V8′ II	LDGGPVI
yes	V8′ III	LDGGPVIl

[a] See text for additional information as to the source of these peptides. [b] Determined by the 254/280-nm ratio for each HPLC peak. [c] Determined by using an automated pulsed-liquid ABI 477A sequencer. [d] Represents a single HPLC peak with the sequences being obtained from a single sequencing run. [e] This sequence is an approximately equal mixture of two peptides differing in their C-termini, one ending in Ile 149 and one ending in Leu 150. Sequences ending with ... represent programmed stops while sequences without indicate a significant drop in yield after that amino acid.

conditions. On the basis of the Met content of the enzyme, excluding the N-terminal Met, one would expect four unique peptides from complete CNBr cleavage. N-Terminal Edman degradation of the peptides isolated from the HPLC separation of the CNBr peptides revealed (Table I) that the affinity-labeled peptide is actually a mixture of two peptides comprising residues 2–77 and 90–191. Thus, the DDF label is attached to either one or both of these peptide fragments. Attempts to resolve these two peptides were unsuccessful. Label is, therefore, not associated with the two other CNBr produced peptide samples (CB II, CB I) that comprise residues 78–89, a highly conserved but also very hydrophobic region, or residues 192–212, the C-terminal nonconserved region of GAR TFase.

Trypsin Digestion of E~I. Digestion of E~I with trypsin results in greater than 95% release of the label as N^{10}-(hydroxyacetyl)-DDF under a variety of conditions studied (data not shown). This result is consistent with the probability that the covalent linkage between enzyme and affinity lable is an ester. The potent esterolytic activity of trypsin (Keil, 1971) and the pH at which the reactions were run give this particular ester linkage little chance to survive. This prompted the use of other proteases with lower pH optima and less vigorous esterase activities.

S. aureus V8 Protease Digestion of E~I. V8 protease has an acid optimum, cleaving specifically at the carboxyl side of aspartic and glutamic acids (Houmard & Drapeau, 1972). Treatment of E~I with this protease in phosphate buffer at pH 4 and subsequent injection onto a reverse-phase HPLC column indicated that E~I was cleaved at a single residue to generate two almost equal-sized peptide fragments, V8 I and V8 II. From the 254-nm absorbance, the DDF label was associated with peptide V8 I. It is also important to note that no N^{10}-(hydroxyacetyl)-DDF was liberated during this digestion. The results of N-terminal Edman degradation of V8 I and V8 II are shown in Table I. These data restrict the V8 proteolytic cut to the following area:

$$...^{124}\text{Ala-Leu-Glu}\downarrow\text{-Asn-Gly}^{128}...$$

The V8 I sequence begins with residue N127, which is preceded by a glutamic acid in the primary sequence. Taken with the CNBr cleavage data, this positions the label somewhere between residues N127 and M191. The N-terminal region of this peptide (residues 133–149) contains a stretch of conserved amino acids, as shown in Figure 1.

Endoproteinase Arg C, which is specific for the carboxyl terminus of arginine residues (Levy et al., 1970), also cleaves at only a single arginine in the E~I adduct. The HPLC chromatogram of this proteolytic experiment shows two peptide fragments similar to those observed for V8 protease. The label containing peptide, Arg I, was subjected to N-terminal sequencing (see Table I), from which the point of attack by Arg C was determined to be

$$...^{120}\text{Thr-His-Arg}\downarrow\text{-Gln-Ala}^{124}...$$

This arginine residue is within four amino acids of E126 and indicates that when the inactivator is bound to GAR TFase, a region encompassing these amino acids (residues 122–126) is exposed and accessible to these proteases (V8 and Arg C).

Subcleavage of V8 I Labeled Peptide with Pepsin. To further narrow down the location of the modified amino acid, the V8 I peptide was subcleaved with porcine pancreatic pepsin. This enzyme was chosen because of its acidic optimum (pH 2). We have found that the depsipeptide is more stable in acidic medium than in neutral or basic media, especially in the presence of proteolytic enzymes. An undesirable property of pepsin is its documented lack of specificity (Fruton, 1971), cutting primarily at hydrophobic residues, and lack of reproducibility (Allen, 1986). This, however, did not create a problem, and in fact the pepsin cleavage of V8 I was highly reproducible and did not result in any detectable N^{10}-(hydroxyacetyl)-DDF formation.

The HPLC profile for the reaction of V8 I with pepsin is shown in Figure 3. The N-terminal sequence of the entire P II fragment (peptide with DDF chromophore) is shown in Table I. Since the covalent linkage of the peptide–DDF adduct is base sensitive, one could not expect the linkage to survive the Edman degradation, since there is an aqueous diisopropylethylamine treatment step in each cycle. On occasion, however, a conspicuously low yield of D144 was observed during sequencing of the V8 I and P II peptides. These observations are insufficient to make any judgments concerning the possibility of D144 as the modified residue but are consistent with an ester linkage being the covalent bond and with the data presented below.

The C-terminus of P II was confirmed by C-terminal sequencing with carboxypeptidase P (Yokoyama et al., 1975).

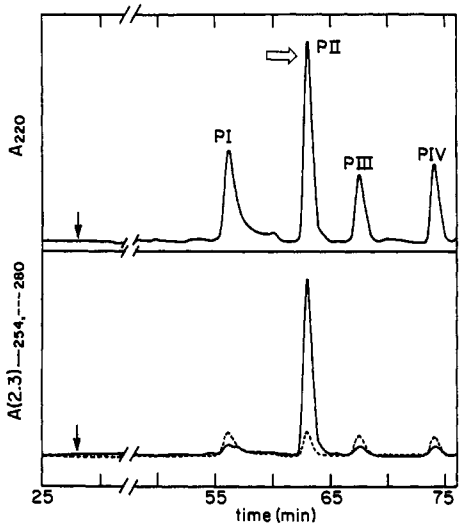


FIGURE 3: Reverse-phase HPLC profile of a pepsin digest of the V8 I labeled peptide. The solid arrow indicates the expected point of elution of N^{10}-(hydroxyacetyl)-DDF, and the open arrow indicates the labeled peptide. Condition A as described under Methods was used for elution of peptides.

Table II: Amino Acid Analysis of DDF–Adduct Obtained from Complete Digestion of P II Peptide with Carboxypeptidase P

material	pre amino acid analysis treatment[a]	amino acids detected (pmol)
N^{10}-(hydroxy-acetyl)-DDF digestion peak	Δ, H^+	glutamic acid
	OH^-	none
	Δ, H^+	aspartic acid (128), glutamic acid (132)
	OH^-	aspartic acid (119)

[a] Δ, H^+: standard vapor-phase acid hydrolysis (165 °C for 45 min in a sealed Teflon bomb with constant-boiling HCl) prior to analysis. OH^-: sample was treated with potassium carbonate (pH 11.5) prior to analysis.

The analysis reveals that P II ends in a Leu or is a mixture of two peptides, one ending with I149 and the other with L150, since both Ile and Leu were observed at the first time point. N-terminal sequence data obtained from P IV (Table I) indicates that this peptide begins with Q151, confirming that P II must end near I149 or L150. Given the known preference of pepsin for aliphatic residues and the above observations, the most likely cleavage points on V8 I to give P II are

...^{147}Pro-Val-Ile↓-Leu↓-Gln-Ala^{152}...

Carboxypeptidase P Digestion of P II Peptide. An ester linkage derived from N^{10}-(bromoacetyl)-DDF and the peptide region N127–L150 would implicate an aspartic or glutamic acid residue in this peptide. There are two glutamic and three aspartic acid residues located within this segment. Total digestion of P II by carboxypeptidase P in acidic medium resulted in the appearance of a DDF derivative that eluted earlier than N^{10}-(hydroxyacetyl)-DDF on the reverse-phase HPLC system. Analysis of this peak by amino acid analysis using the conditions described in Table II suggests that this peak is a 1:1 conjugate of DDF and aspartic acid (Chart II). Because greater then 95% of the DDF label was associated with this adduct, the GAR TFase nucleophile tagged by N^{10}-(bromoacetyl)-DDF must be one or more of the three aspartic acids in the P II peptide (D129, D141, or D144).

Localization of Label to D144. V8 protease was once again recruited in an attempt to further narrow down the position of the label in active-site peptide P II. Treatment of this peptide with V8 protease resulted in a significant release of label from the peptide as N^{10}-(hydroxyacetyl)-DDF in addition

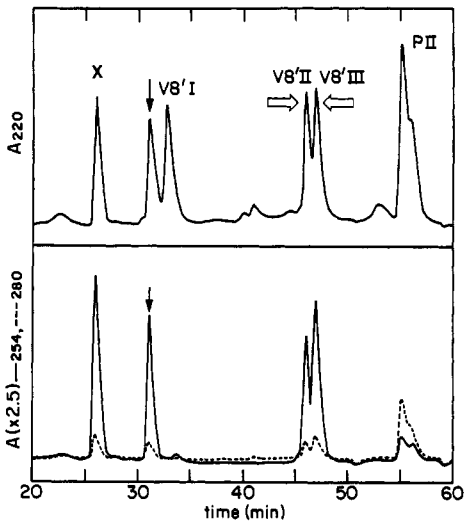
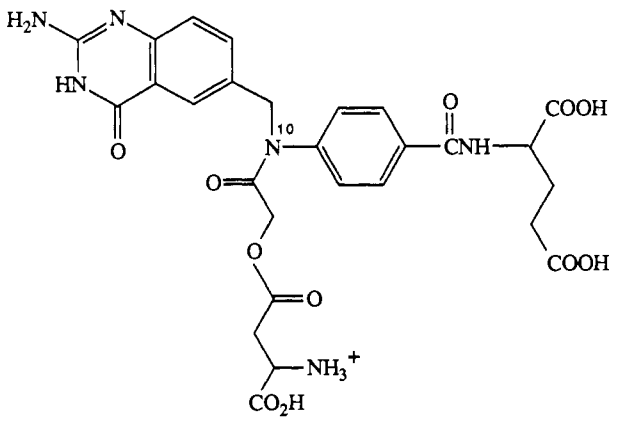


FIGURE 4: Reverse-phase HPLC profile of *S. aureus* V8 protease digest of P II labeled peptide. Condition B was used for elution of the peptides. The solid arrow indicates released N^{10}-(hydroxyacetyl)-DDF from the P II peptides (peak P II). The open arrows indicate labeled peptides. Peak X is a non-peptide-containing DDF derivative, possibly a V8 protease catalyzed transesterification product generated from acetate or formate buffer components and labeled peptide; this peak was observed to be formed to a greater extent in acetate buffer and only in the presence of protease.

Chart II: DDF–Asp Adduct



to the protease clipping P II at a single glutamyl residue (Figure 4):

...^{140}Thr-Asp-Glu↓-Leu-Asp-Gly-Gly^{146}...

Enough label, however, remained (~50%) under the conditions employed to show, as seen in Table I, that the only peptides carrying the DDF chromophore contained D144. This evidence also shows that all the retained label is attached through the β-carboxylate of D144. We cannot, however, unequivocally rule out the attachment of the remainder of the affinity reagent at aspartate sites (D129, 141) in the P II peptide, but they would have to be particularly prone to hydrolysis. A summary of the digests and resultant peptides is shown in Figure 5.

Site-Specific Mutagenesis and Analysis of D144N GAR TFase

In order to confirm the significance of Asp 144, site-directed mutagenesis of this residue to an asparagine was accomplished as described under Experimental Procedures. Overproduction of the mutant GAR TFase was the same as that observed for the wild type from strain TX635 (Inglese et al., 1990). The crude extract harboring the mutant GAR TFase, however, was essentially devoid of transformylase activity when assayed with

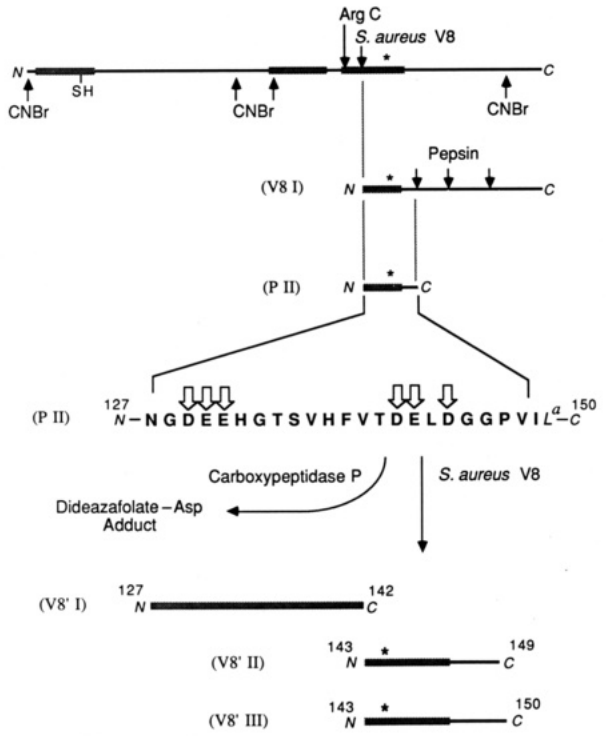


FIGURE 5: Summary of chemical and proteolytic digests used to locate the position of attachment of the affinity label to wild-type GAR TFase. The solid horizontal lines represent the polypeptide backbone, with shaded regions denoting conserved regions. The asterisk represents the label, and the open arrows identify the residues that could form potential ester linkages with the affinity label. The P II peptide obtained from the V8 I peptide was used to establish the amino acid type to which the affinity label was attached and its location on the peptide. Details are given in the text. For footnote *a*, see Table I.

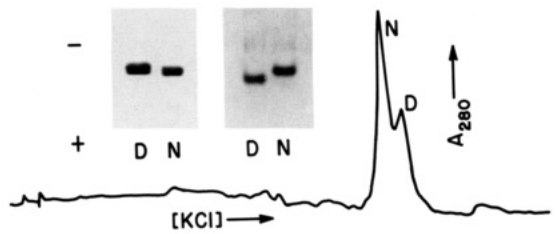


FIGURE 6: Anion-exchange chromatographic and electrophoretic analysis of D144N and wild-type GAR TFase. The trace is a separation of a 3:1 mixture of D144N and wild-type GAR TFase, N and D, respectively, on QAE anion-exchange FPLC with a KCl salt gradient. (Left inset) SDS–PAGE of N and D and (right inset) nondenaturing PAGE of N and D. All procedures were carried out as described under Methods.

either (6*R*)-N^{10}-formyl-5,6,7,8-tetrahydrofolate (D. L. Johnson, personal communication) or N^{10}-formyl-DDF. The protein was purified as previously described with SDS–PAGE used to locate and assay the purity of the enzyme. The mutant GAR TFase behaved the same as the wild type under the purification protocol.

Physical Properties of the D144N GAR TFase. Wild-type and mutant GAR TFase had virtually the same mobility on SDS–PAGE, as would be expected for a single-residue replacement. However, the mutant has one less negative charge than the wild type. This charge decrease is observable in two separate experiments. Under nondenaturing PAGE conditions, wild-type GAR TFase migrates faster toward the anode than does the mutant, and with high-resolution anion-exchange chromatography (i.e., FPLC), mutant GAR TFase elutes before wild type under a KCl gradient (Figure 6). Both results are consistent with a decrease in the net charge of the protein.

Table III: Comparison of Catalytic, Binding, and Fluorescent Properties of Wild-Type and N144 Mutant *E. coli* GAR TFase

species	rel velocity	K_D, β-TGDDF[a] (pM)	rel fluorescence enhancement[b]
D144 (wild type)	1	210 ± 60	1
N144 (mutant)	0.0001[c]	220 ± 80	1.45

[a] β-TGDDF is a potent bisubstrate adduct inhibitor of GAR TFase (Inglese et al., 1989). [b] Describes the relative enhancement in the emission spectrum of β-TGDDF at 395 nm when bound to GAR TFase. [c] This residual activity may be due to contaminating wild type from the chomosomal gene (see text for further discussion).

The N-terminus of the mutant GAR TFase was sequenced and compared to the wild type as an additional verification of protein integrity. Automated Edman degradation indicated that the mutant protein contained the same leading amino acids as wild type: MNIVVL. Additionally, the entire gene was sequenced and found to have the expected sequence.

Binding and Rate Constants for D144N GAR TFase. Once purified, the N144 mutant GAR TFase was assayed and was found to be $\approx 10^4$ times less active than wild type at the pH optimum of 8.4. This represents a very large effect on the activity, and the small amount of activity that does remain can be attributed to background chromosomal GAR TFase.[3] A single isosteric polar residue change has thus inactivated the enzyme. It is unlikely that the protein is folded improperly owing to its identical behavior with wild type during purification.

In order to determine whether this mutation has affected the activity of the enzyme because Asp 144 was of importance to the catalytic mechanism or to the active-site integrity of the protein, the ability of the mutant to bind β-TGDDF, a specific and potent bisubstrate inhibitor of GAR TFase (Inglese et al., 1989), was determined. Titration of β-TGDDF with wild-type and D144N GAR TFase revealed that both proteins have similar affinities for the inhibitor, although the fluorescence enhancement of β-TGDDF when bound to the mutant GAR TFase is somewhat greater than for wild type (Table III). We conclude that the Asp to Asn mutation affects only the catalytic center of GAR TFase and not the residues or tertiary structure required for recognition and binding of substrates. The greater quantum yield achieved with the mutant enzyme can be rationalized by an environmental polarity change. Since nonpolar environments—the mutant has one less charge—tend to increase the population of excited state fluorophores, the mutant enzyme would tend to exhibit a greater quantum yield over the wild type (Lakowicz, 1983).

Labeling of D144N GAR TFase by N^{10}-(Bromoacetyl)-DDF. Once the primary reactive residue (Asp 144) was replaced by asparagine, it became of interest to determine if the affinity label would still react with the enzyme. Because the mutant enzyme is essentially inactive, any determination of labeling by N^{10}-(bromoacetyl)-DDF would have to be made by HPLC, where the labeling could be quantitated by measuring 254/280-nm absorbance ratios as previously described. The data from these experiments are shown in Figure 7. D144N GAR TFase still can be labeled by N^{10}-(bromoacetyl)-DDF to the extent of approximately 1 DDF chromo-

[3] A pH–rate analysis (*V* vs pH) of the mutant enzyme preparation gave a bell-shaped profile identical with that of wild type, indicating that this residual activity most likely due to contaminating wild-type GAR TFase (C. L. Borders, personal communication). The establishment of such contamination is of great importance when attempting to make quantitative measurements on mutant enzymes, as pointed out recently by Schimmel (1989).

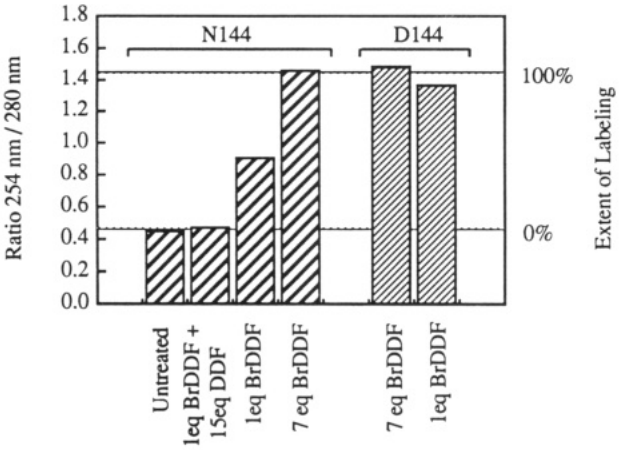


FIGURE 7: Bar graph showing the labeling of N144 GAR TFase by N^{10}-(bromoacetyl)-DDF and protection by DDF (dark hatched bars). The experiment was performed side by side with wild-type GAR TFase as a control (light hatched bars). The left *Y*-axis measures the 254/280-nm absorbance ratio (as determined on HPLC) of the protein after incubation as described under Methods with the indicated (*X*-axis) components. Both D144 and N144 GAR TFase have a 254/250-nm ratio of 0.48 and a ratio of 1.44 when labeled with 1 equiv of N^{10}-(bromoacetyl)-DDF (right *Y*-axis).

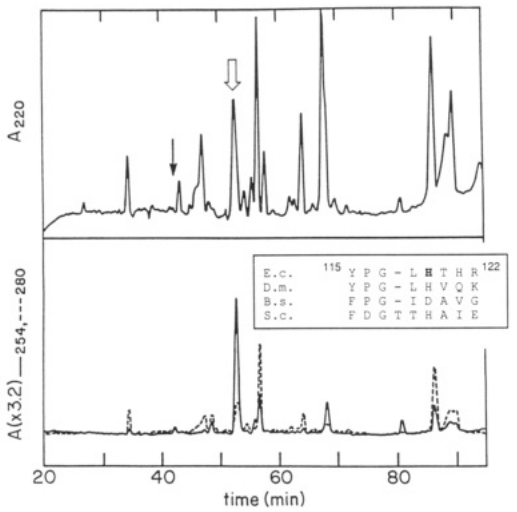


FIGURE 8: Reverse-phase HPLC profile of a trypsin digest of N144 GAR TFase labeled with N^{10}-(bromoacetyl)-DDF. The solid arrow indicates expected elution point of N^{10}-(hydroxyacetyl)-DDF, and the open arrow indicates the label-containing peptide. Condition C as described under Methods was used for elution of peptides. The sequences shown represent overlapping conserved regions in other transformylase species.

phore per enzyme monomer. Protection from labeling by DDF indicates that the affinity label is reacting at the active site.

Trypsin Digestion of Labeled D144N GAR TFase. Trypsin would serve two purposes in a first attempt at isolating a modified peptide from D144N GAR TFase. If the label is hydrolyzed, as in the original experiment with wild-type enzyme, to N^{10}-(hydroxyacetyl)-DDF, the linkage is probably another ester; if the label is not hydrolyzed, a peptide could be directly isolated and sequenced.

Figure 8 shows the chromatogram of the trypsin digest and clearly indicates that the label is not hydrolyzed from the modified peptide. After isolation the single labeled peptide was rechromatographed to give a homogeneous peptide sample containing the DDF moiety. This peptide was subjected to automated Edman degradation analysis and corresponded to the shown sequence in which the fifth cycle was blank. The peptide is generated from cleavage at K114 and R122, and the fifth cycle corresponds to His 119. This histidine residue was found to be invariant in three of the four species of GAR TFase (Figure 8). His 119 is 25 residues removed from Asp 144 in the primary sequence and may represent a neighboring residue to Asp 144 in the tertiary structure.

Homology between GAR TFase and Other Folate-Utilizing Enzymes. To examine whether the sequence we have identified as being at the active site of GAR TFase (≈residues 119–144) may include part of a folate-binding consensus sequence, we conducted a search for shared amino acid sequences between *E. coli* GAR TFase and other proteins in GenBank (version 6). This search revealed a short stretch of residues (110–145) in the transformylase that shared partial sequence homology with DHFR from several sources. The partially homologous

```
                    (His-119)*                  (Asp-144)*
E. coli GAR TFase   SLLPKYPGLHTHRQALENGDEEHGTSVHFVTDELDG
                     ||| |||      |       || |    |   |
Human DHFR          KLLPEYPGVLSDVQ------EEKGIKYKFEVYEKNN
                     ||| |||      |       || |    |   |
Bovine DHFR         KLLPEYPGVPLDVQ------EEKGIKYKFEVYEKNN
                     ||| |||      |       || |    |   |
Mouse DHFR          KLLPEYPGVLSEVQ------EEDGIKYKFEVYEKKD
                      ||  |||     |       || |    |   .
Chicken DHFR        KLLTEYPGVPADIQ------EEDGIQYKFEVYQKSV
                      ||    |     |       || |    |   .
Porcine DHFR        KLLSECSGVPSDVQ------EEKGIKYKFEVYEKNN
```

region from the DHFR corresponds to the βG and βH β-sheets (residues 157–186) at the C-terminal end of the protein that are not involved in the active-site region or folate-binding pocket (Volz et al., 1982). The corresponding area from *E. coli* GAR TFase encompasses both Asp 144 and His 119 as compared above. The evolutionary significance of this homology, if any, is presently unclear.

CONCLUSION

Possible Roles of Labeled Residues in GAR TFase. The proximity of D144 to the formyl group, as deduced from

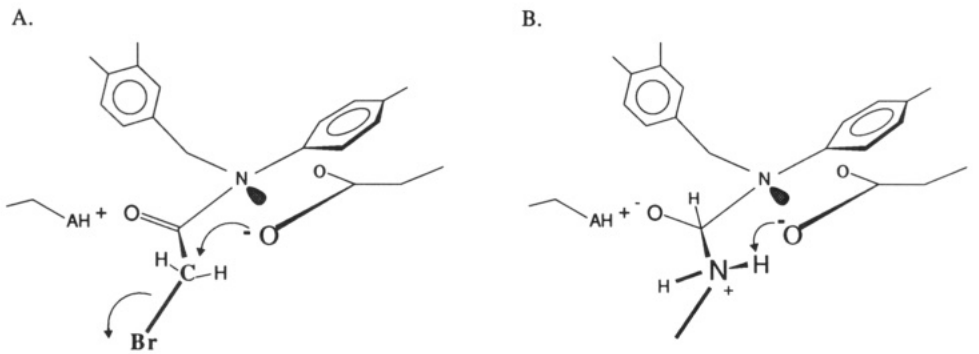


FIGURE 9: Plausible one-base active-site model for GAR TFase incorporating Asp 144 as the general base: (A) N^{10}-(bromoacetyl)-DDF bound in proximity to Asp 144; (B) the putative intermediate between GAR and N^{10}-formyl-DDF in proximity to Asp 144. AH^+ represents an electrophilic group present to stabilize the transition state.

analogy to that of the N^{10}-(bromoacetyl)-DDF–D144 interaction, suggests a possible role of D144 as an active-site base. This base may be involved in the deprotonation of the primary aminium group of GAR or possible deprotonation of the proposed hemioxyorthoamide tetrahedral transition state in the reaction. The overlap of the bromomethyl group with the amino group of the putative tetrahedral transition state (Smith et al., 1982) for the formyl transfer is compared in Figure 9. Assuming a one-base mechanism, Asp 144 may also be used to deliver the proton from the amine of the tetrahedral complex to N^{10} of the cofactor. The general-base-catalyzed aminolysis of model C^1-tetrahydrofolates at the formate oxidation level has been shown by Bullard et al. (1974). This is in accord with the more general finding that the aminolysis of amides is both acid- and base-catalyzed (Jencks, 1969). The use of the aspartyl or glutamyl group as a general base in enzymes has been documented in several cases: the E165 residue in triosephosphate isomerase (Hartman, 1971; Alber et al., 1981; Straus et al., 1985), the D184 residue in cAMP protein kinases (Buechler & Taylor, 1988; Yoon & Cook, 1987; Bramson et al., 1984), and the E401 residue in pig kidney medium chain acylCoA dehydrogenase (Powell & Thorpe, 1988).

The role of His 119 either as a spectator or functioning amino acid in catalysis at the active site of GAR TFase will have to await further analysis. For now, all that can be inferred is that His 119 appears to reside in close proximity to Asp 144 in the folded protein.

The identification of residues at the active site of GAR TFase represents the first such topological study made on this enzyme. Current work is aimed at obtaining an X-ray crystallographic structure for this enzyme (Stura et al., 1989). When available, this combined approach will provide the means for identifying which residues are important contributors to catalysis.

ACKNOWLEDGMENTS

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Registry No. Asp, 56-84-8; His, 71-00-1; GAR TFase, 9032-02-4.

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Zinc Interactions with Regulatory Dimers from *Escherichia coli* Aspartate Transcarbamoylase†

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ABSTRACT: Zn^{2+} is tetrahedrally bonded to the 4 nonadjacent thiols of each regulatory chain (M_r 17 000) near r–c contacts between catalytic (c) and regulatory chains (r) in aspartate transcarbamoylase (ATCase; c_6r_6). This paper reports on Zn^{2+} interactions with r dimer in the absence of stabilizing r–c contacts. After r_2 and c_3 subunits were separated, –SH groups of r_2 were titrated with *p*-(hydroxymercuri)benzenesulfonate (PMPS) at pH 7.0. The concomitant release of Zn^{2+} (2 equiv/r dimer) was quantitated with 4-(2-pyridylazo)resorcinol (PAR) and was a linear function of PMPS added until 8 mercaptide bonds per r_2 were formed. Breakage of 1 of 4 Zn^{2+}–sulfur bonds in a Zn^{2+} binding cluster therefore makes the other three bonds more labile. From stopped-flow measurements, the PMPS-promoted Zn^{2+} release from r_2 or mercaptide bond formation with 10- to 20-fold excess PMPS/r_2–SH at pH 7.0 was first order with an Arrhenius activation energy E_a = 10 kcal/mol and a half-time $t_{1/2}$ = 9 ± 2 ms at 20 °C without inhibitory anions present. The rate of mercurial-promoted Zn^{2+} release from r_2 is at least 77 times faster than that from intact c_6r_6 [Hunt, J. B., Neece, S. H., Schachman, H. K., and Ginsburg, A. (1984) *J. Biol. Chem. 259*, 14793]; this indicates that Zn^{2+} binding clusters are more accessible to attack by PMPS than are those in ATCase. The addition of a 25-fold excess of the multidentate fluorescent chelator quin-2 to r_2 gave a rate of Zn^{2+} dissociation that was 1/210th of that observed with excess mercurial. Furthermore, the $Zn(PAR)_1$ complex was identified as the active species in the transfer of Zn^{2+} from $Zn(PAR)_2$ to aporegulatory subunits, with $k = (8 \pm 3) \times 10^5$ M^{-1} s^{-1} at pH 7.0 and 15 °C for this second-order association reaction. Although kinetic results are dependent on the mechanisms involved, an affinity constant $K'_A = (1.3 \pm 0.6) \times 10^{12}$ M^{-1} for Zn^{2+} binding to r dimer at pH 7.0 and 20 °C in the absence and presence of 100 mM KCl could be determined spectrally by rapid equilibration with the high-affinity, sensitive metalloindicators indo-1 and quin-2. This K'_A value is based on the assumptions that Zn^{2+} binding sites in r_2 are equivalent (noninteracting) and that apo-r_2 does not dissociate; if apo-r_2 dissociates, $K'_A \approx 10^{14}$ M^{-1}. Within experimental error, the K'_A value was independent of [indo-1]/[r_2] ratios from 36 to 3 with 0.3–8 μM r_2. Thus, $\Delta G' \simeq -16$ kcal/mol at 20 °C (pH 7.0) for sequential formation of 4 Zn^{2+}–thiol bonds (inclusive of energy changes for polypeptide chain transitions). CTP (1 mM ± 1 mM Mg^{2+}) and Mg·ATP (1 mM) increased $K'_A \leq \sim 1.2$- and ~1.7-fold, respectively, for r_2 binding Zn^{2+}.

A goal of studying Zn^{2+} interactions with aspartate transcarbamoylase (ATCase)[1] from *Escherichia coli* (EC 2.1.3.2) and with isolated regulatory subunits of ATCase is to gain information on the structural roles of Zn^{2+} in the intact enzyme and in the assembly of ATCase. Assembly processes and protein–protein interactions depend on the correct folding of polypeptide chains, and Zn^{2+} binding to the four nonadjacent Cys residues of each regulatory chain will restrict folding pathways and constrain the final conformations assumed. Recently, the Zn^{2+} domain of each regulatory chain of ATCase was shown to be homologous with the "Zn finger" structure of transcriptional factor IIIA from immature oocytes of *Xenopus laevis* (Berg, 1988), which is the first discovered example of an important class of DNA-binding proteins containing tandem repeats of "Zn finger" structural motifs for

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[1] Abbreviations: ATCase or c_6r_6, aspartate transcarbamoylase; c_3, catalytic trimer subunit; r_2, regulatory dimer subunit containing 2 equiv of specifically bound Zn^{2+} unless designated as apo-r_2; PAR, 4-(2-pyridylazo)resorcinol; indo-1, 1-[2-amino-5-(6-carboxyindol-2-yl)phenoxy]-2-(2′-amino-5′-methylphenoxy)ethane-*N,N,N′,N′*-tetraacetic acid; quin-2, 2-[[2-[bis(carboxymethyl)amino]-5-methylphenoxy]methyl]-6-methyl-8-[bis(carboxymethyl)amino]quinoline; PMPS, *p*-(hydroxymercuri)benzenesulfonate; neohydrin, 1-[3-(chloromercuri)-2-methoxypropyl]urea; 2-ME, 2-mercaptoethanol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.