Galactose-1-phosphate Uridylyltransferase: Identification of Histidine-164 and Histidine-166 as Critical Residues by Site-Directed Mutagenesis†

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ABSTRACT: Galactose-1-phosphate uridylyltransferase catalyzes the interconversion of UDP-glucose and galactose-1-P with UDP-galactose and glucose-1-P by a double-displacement mechanism involving the compulsory formation of a uridylyl enzyme intermediate. The uridylyl group is covalently bonded to the N^3 position of a histidine residue in the uridylyl enzyme. The *galT* gene of *Escherichia coli*, which codes for the uridylyltransferase and is contained in a plasmid for transformation of *E. coli*, has been sequenced, and the positions of the 15 histidine residues have been determined from the deduced amino acid sequence of this protein. Fifteen mutant genes, in each of which one of the 15 histidine codons has been changed to an asparagine codon, have been generated and used to transform the *E. coli* strain JM101. When extracts of the transformants were assayed for uridylyltransferase, 13 exhibited high levels of activity. Two of the extracts containing mutant uridylyltransferase exhibited less than control levels of activity. These mutant proteins, H164N and H166N, were overexpressed, isolated, and tested for their ability to form the compulsory uridylyl enzyme intermediate. Neither the H164N nor the H166N mutant proteins could form the intermediate. Thus, both His-164 and His-166 are critical for activity, and their proximity suggests that both are in the active site. One is the essential nucleophilic catalyst to which the uridylyl group is bonded in the intermediate, and the other serves an equally important, as yet unknown, function. The active-site sequence His(164)-Pro-His(166) is conserved in this enzyme from *E. coli*, humans, *Saccharomyces*, and *Streptomyces*.

The gene *galT* of *Escherichia coli* codes for the enzyme galactose-1-P uridylyltransferase (EC 2.7.7.12), which catalyzes the interconversion of UDP-glucose[1] and galactose 1-P with UDP-galactose and glucose-1-P (eq 1). This is one of

$$\text{UDP-glucose} + \text{galactose-1-P} \rightleftharpoons \text{UDP-galactose} + \text{glucose-1-P} \quad (1)$$

the reactions of the Leloir pathway of galactose metabolism, in which galactose is initially phosphorylated by ATP to galactose-1-P by the action of galactokinase. This is followed by the galactose-1-P uridylyltransferase reaction (eq 1). In subsequent steps phosphoglucomutase catalyzes the isomerization of glucose-1-P to glucose-6-P, and UDP-galactose 4-epimerase catalyzes the isomerization of UDP-galactose to UDP-glucose. In *E. coli*, the *gal* operon links the genes specifying galactokinase (*galK*), galactose-1-phosphate uridylyltransferase (*galT*), and UDP-galactose 4-epimerase (*galE*).

The catalytic pathway for the galactose-1-P uridylyltransferase reaction is known to follow eq 2a and 2b (Frey et

$$\text{E-His} + \text{UDP-glucose} \rightleftharpoons \text{E-His-UMP} + \text{glucose-1-P} \quad (2a)$$

$$\text{E-His-UMP} + \text{galactose-1-P} \rightleftharpoons \text{E-His} + \text{UDP-galactose} \quad (2b)$$

al., 1982). This is a double-displacement pathway involving ping-pong bi-bi kinetics with the formation and reaction of a covalent uridylyl enzyme (E-UMP) as the intermediate. The steady-state kinetics is consistent with the ping-pong bi-bi model, and the enzyme also catalyzes the exchange reactions required by eq 2a and 2b (Wong & Frey, 1974a,b).

Extensive biochemical evidence supports the assignment of an essential histidine residue as the nucleophilic catalyst at the active site. Diethyl pyrocarbonate completely inactivates the free enzyme at pH 6 but is only a sluggish inactivator of the uridylyl enzyme. The inactivated enzyme is reactivated by hydroxylamine, and either UDP-glucose or UDP-galactose completely blocks inactivation by diethyl pyrocarbonate (Wong et al., 1977). The covalent uridylyl enzyme can be isolated in either an active or a denatured form, and the active form reacts with glucose-1-P to form UDP-glucose (Wong & Frey, 1974b; Wong et al., 1977). The uridylyl moiety is known by chemical degradation of the [^{32}P]UMP-enzyme (to [N^3-^{32}P]phosphohistidine) to be bonded to the imidazole ring of a histidine residue (Yang & Frey, 1979). The stereochemistry is consistent with the kinetic mechanism. The reaction proceeds with overall retention of configuration at the α phosphorus (Sheu et al., 1979). Each step, shown in eq 2a and 2b, proceeds with inversion of configuration at phosphorus, giving overall retention at phosphorus (Arabshahi et al., 1986).

The purpose of this study is to characterize the active site by locating the essential histidine residue. In the past, the uridylyltransferase has proven to be difficult to work with. It is sensitive to proteolysis and, until recently, only small amounts of the protein could be isolated at one time. In addition, when the protein is uridylylated and subsequently digested with trypsin, the uridylyl moiety is found in a large insoluble peptide fragment. For these reasons site-directed mutagenesis is here applied to the characterization of the active

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[1] Abbreviations: NADPH, reduced nicotinamide adenine dinucleotide phosphate; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl thiogalactoside; XG, 5-bromo-4-chloro-3-indoyl β-D-galactopyranoside; ATP, adenosine triphosphate; dNTP, 2′-deoxynucleoside triphosphate; RF, replicative form; DEAE, diethylaminoethyl; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; glucose-1-P, glucose 1-phosphate; galactose-1-P, galactose 1-phosphate; UDP-glucose, uridine 5′-diphosphoglucose; UDP-galactose, uridine 5′-diphosphogalactose; Tris, tris(hydroxymethyl)aminomethane; [α-^{35}S]dATPαS, 2′-deoxyadenosine 5′-*O*-([1-^{35}S]-1-thiotriphosphate).

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site. The nucleotide sequence of the *galT* gene of *E. coli* is known (Lemaire & Muller-Hill, 1986; Cornwell et al., 1987), and from the deduced amino acid sequence the positions of the 15 histidine residues are also known.

To identify the essential histidine, we generated 15 mutant genes, in each of which a single histidine codon is changed to an asparagine codon. We chose asparagine to replace histidine because it is in certain ways a structurally conservative replacement. We found 13 of the expressed mutant proteins to be enzymatically active. The absence of activity associated with two mutant proteins led us to identify His-164 and His-166 as residues that are essential for the activity of this enzyme. One of these must be the active-site nucleophilic catalyst, and the other must have a different, but also critical, function.

Materials and Methods

Chemicals and Reagents. All restriction enzymes, DNA polymerase I (Klenow fragment), T4 DNA polymerase, T4 DNA ligase, isopropyl thiogalactoside (IPTG), and 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (XG) were from Boehringer-Mannheim; deoxynucleotides and dideoxynucleotides were from Pharmacia. [α-^{35}S]dATPαS, 600 Ci/mmol, was from Amersham.

Bacterial Strains, Plasmids, and Bacteriophage. E. coli JM101 (lac pro, supE, thi [F'traD36, pro AB, lac I^{q}z M15]) and M13mp9 were from BRL. *E. coli* BL21 (DE3) (F$^-$ *hsdS,gal*) was a gift from Dr. W. Studier (Brookhaven National Laboratories, Upton, NY). *E. coli* RZ1032 (HfrKL16, Po145 [*lys*A(61–62)], *dut*1, *ung*1 *thi*1, *rel*A1, zbd-279::Tn10, *sup*E44) was a gift from Dr. T. Kunkle (National Institute of Environmental Health Sciences, Research Triangle Park, NC). Plasmid pI24 was a gift from Dr. S. Adhya (NIH, Bethesda, MD). Plasmids pGem 3fl and pGem 4z and helper bacteriophage R408 were from Promega Biotech.

DNA Manipulations. Restriction endonucleases were used as directed by the manufacturers. The DNA was ligated overnight in a 0.02-mL reaction mixture containing 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM DTT, 0.4 mM ATP, and 1 unit of T4 DNA ligase at 25 °C.

Construction of galT Plasmids. Figure 1 illustrates the construction of plasmids and site-directed mutagenesis. The plasmid pI24 is a pBR322 derivative that contains the galactose operon. The *galT* gene was excised by digestion with *Hin*dIII and *Pvu*I, and the *Pvu*I end was made blunt by treatment with exonuclease III prior to the *Hin*dIII treatment. The fragment was purified from DEAE-81 paper by the method of Danner (1982), cloned into *Hin*dIII- and *Sma*I-digested M13mp9(RF). The recombinant was named mTLC 5800.

An overexpression vector was constructed from two commercially available plasmids. A 2821 base pair *Pvu*II fragment containing the gene for ampicillin resistance and the f1 origin of replication was isolated from pGEM 3fl and ligated with the 380 base pair *Pvu*II fragment from pGEM 4z, which contained the T7 promoter, the *lac* promoter, and the gene coding for the α peptide of β-galactosidase. The latter gene also contained a multicloning region. A transformant carrying the f1 origin of replication in the + orientation was selected and named pTLC-v.

Wild-type and mutant transferase genes were isolated from their respective M13 bacteriophages by digestion of the M13 RF form with *Hin*dIII and *Eco*RI, purification of the fragment, and ligation into the *Hin*dIII- and *Eco*RI-digested pTLC-v vector. The plasmid containing the wild-type *galT* gene was named pTLC 5800, and the plasmids containing the

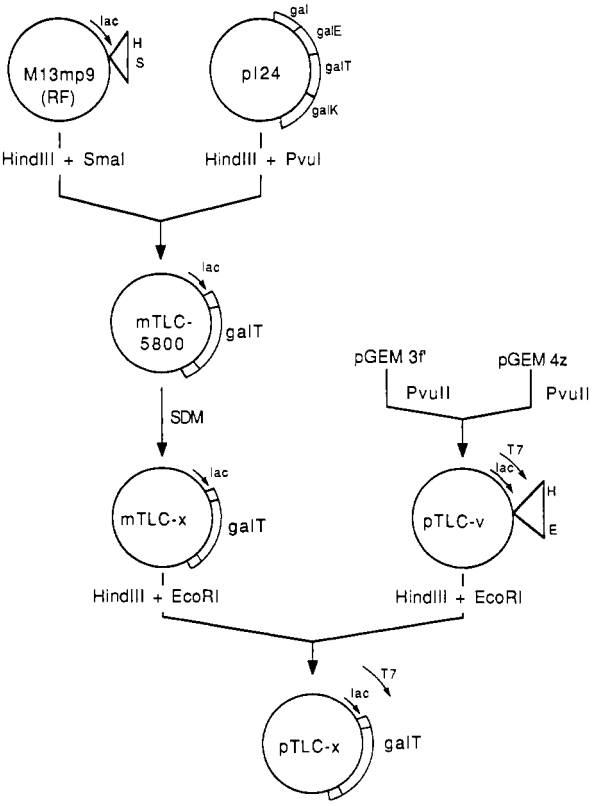

FIGURE 1: Cloning, site-directed mutagenesis, and overexpression of the gene *galT* of *E. coli*. Details are provided in the text under Results. The abbreviation mt refers to mutation, and SDM refers to site-directed mutagenesis.

mutant genes were designated pTLC H164N, etc.

The identities of the mutant plasmids were confirmed by nucleotide sequencing using the dideoxy method of Sanger. The plasmids, in the *E. coli* strain B121(DE3), were isolated and transformed into JM101. A 1% inoculum of these cells, containing the appropriate plasmid, was infected to 0.1% (10^{11} plaque-forming units/mL) with an inoculum of the helper bacteriophage R408 and allowed to grow for 4 h. The single-stranded forms of the plasmids were isolated as described below for the template for site-directed mutagenesis. Subsequently, the DNA was sequenced using the dideoxy method and [α-^{35}S]dATPαS.

Oligodeoxynucleotides. Oligodeoxynucleotides for mutagenesis were synthesized by using a Biosearch 8600 DNA synthesizer or purchased from the University of Wisconsin Biotechnology Center. Each oligomer was 18 nucleotides long with the mismatch base located in the center. Following electrophoresis on a 20% polyacrylamide urea gel, they were purified by a standard protocol (Atkinson & Smith, 1984).

Site-Directed Mutagenesis. Site-directed mutagenesis was carried out by the method of Kunkle (1985) with the following modifications. The template was created by transfecting RZ1032 with mTLC 5800 and isolating the single-stranded DNA as follows: Two milliliters of 2xYT broth (16 g of tryptone, 10 g of yeast extract, 5 g of sodium chloride per liter of water) was inoculated to 1% with a fresh overnight culture of RZ1032 and with the appropriate bacteriophage and shaken for 4 h at 37 °C. The culture was centrifuged for 15 min in a Beckman microfuge; the supernatant fluid was transferred to a fresh microfuge tube and again centrifuged for 15 min. One milliliter of the resulting supernatant fluid was added to a clean microfuge tube containing 0.25 mL of a solution consisting of 20% PEG 6000 and 7.5 M ammonium acetate. After incubation for 20 min at 25 °C, followed by centrifu-

gation for 20 min, the supernatant was discarded and the pellet recentrifuged for 1 min. Residual fluid was removed and discarded by using a glass capillary. Tris–EDTA buffer (0.1 mL of 10 mM Tris-HCl at pH 8.0 containing 1 mM Na_2EDTA) was added to the pellet, and the mixture was incubated on ice for 20 min. After centrifugation for 20 min, the pellet was discarded, and 0.05 mL of buffer-saturated phenol was added to the supernatant. The mixture was incubated at 65 °C for 20 min and centrifuged for 5 min, and the aqueous layer was removed and extracted with chloroform. The incubation on ice and the subsequent incubation at 65 °C removed any adventitious contamination of small oligomeric primers that can give false priming at a later stage. The DNA was recovered by ethanol precipitation using ammonium acetate as the salt and resuspended in 0.02 mL of Tris–EDTA buffer.

The phosphorylated mutagenic oligomer (120 pmol, 0.0012 mg), template (0.2 pmol, 0.001 mg), and buffer (40 mM Tris-HCl at pH 7.5 containing 20 mM $MgCl_2$ and 1.0 mM DTT) in a total volume of 0.012 mL were incubated at 65 °C for 20 min and allowed to cool to room temperature. Extension buffer (0.035 mL of 20 mM Tris, pH 7.5, containing 10 mM $MgCl_2$, 1.0 mM DTT, 1.0 mM all four dNTP's, and 0.4 mM ATP) was added to the annealed mixture. The extension reaction was initiated by addition of 2 units of T4 DNA ligase and 1 unit of T4 DNA polymerase. After 4 h at 37 °C, aliquots were used to transform JM101 that was made competent via the $CaCl_2$ protocol (Davis et al., 1980). Clear plaques were selected and screened for mutant formation by single-lane DNA sequencing using the dideoxy method and [α-^{35}S]dATPαS.

Purification of the Wild-Type and Mutant Enzymes. The plasmids pTLC 5800, pTLC H164N, and pTLC H166N were transformed into BL21 (DE3) to overproduce the enzymes using the T7 promoter. To obtain cells for protein purification, 2 L of 2xYT broth containing 0.1 mg/mL ampicillin was inoculated to 1% with an overnight culture of cells containing the appropriate plasmid. The culture was shaken at 37 °C until the A_{600} reached 1.0 (approximately 3–4 h). IPTG was then added to a final concentration of 0.3 mM to induce gene expression, and the cells were shaken for an additional 3 h. The cells were harvested by centrifugation and frozen in liquid nitrogen.

The enzymes were isolated by using a modification of a previously published procedure (Arabshahi et al., 1986) in which the Affi-gel blue column chromatography was eliminated. By use of this procedure, up to 60 mg of 80–90% pure protein could be obtained from a 2-L growth. The purity of each enzyme was determined by densitometry tracings of the SDS–polyacrylamide gels. In addition, the purity of the wild-type enzyme was confirmed by comparison with the known specific activity of the pure enzyme.

Enzymatic Assays. Galactose-1-P uridylyltransferase activity was assayed by using a standard coupled assay, in which the formation of glucose-1-P is coupled to NADPH formation by using phosphoglucomutase and glucose-6-P dehydrogenase (Wong & Frey, 1974a). The formation of the uridylyl enzyme was observed and quantitated spectrophotometrically by using the standard assay solution and excluding galactose-1-P. Under these conditions the amount of NADPH produced is equal to the amount of the uridylyl enzyme formed (Wong et al., 1977).

Results

Preparation and Preliminary Assays of Mutant Uridylyltransferases. Each of 15 mutant genes of *galT* was produced by changing a single codon for histidine (CAC or CAT) to a codon for asparagine (AAC or AAT) by site-directed mutagenesis of the template that is described under Materials and Methods. Asparagine was chosen to replace histidine in the mutant proteins because it is considered to be a structurally conservative change. The 15 mutant genes, each of which had one histidine codon replaced by an asparagine codon, were separately transformed into the *E. coli* strain JM101, and several tranformants of each were selected. The single-stranded DNA was prepared, and the desired mutants were characterized by nucleotide sequencing. Once identified, the transformants containing the desired mutations were plaque purified and transfected into JM101.

Table I: Activities of Mutant Uridylyltransferases

mutant	activity[a]	%[b]	mutant	activity[a]	%[b]
wild type[c]	15.5	100	H166N	0.08	0.5
background[c]	0.1	0.7	H218N	10.5	70
H9N	23	150	H240N	10.3	68
H11N	9.7	64	H281N	0.45	3
H27N	2.5	17	H292N	8.8	59
H48N	7.9	53	H296N	7.3	49
H93N	11	73	H298N	1.2	8
H115N	1.1	7	H342N	5.9	39
H164N	0.06	0.4			

[a] Activity is expressed as standard activity units per milligram of protein in the cell extracts. [b] Activity as a percentage of that of the wild-type extract. [c] The wild-type extract was obtained from cells transformed with M13 carrying the wild-type gene. The background activity was obtained in extracts of cells transformed with M13 carrying no *galT* gene. All other extracts were obtained from cells transformed with M13 carrying the indicated mutant genes.

Cell extracts of these transformants were prepared and assayed for galactose-1-P uridylyltransferase to compare the activities of the 15 mutant enzymes. To obtain cells for extraction of mutant enzymes, the mutant or wild-type transformants were inoculated into media, the expression of the mutant or wild-type enzyme was induced with IPTG, and the cells were harvested 2 h after induction.

Table I presents the activities of galactose-1-P uridylyltransferase in the extracts containing the wild-type and 15 mutant enzymes. All activity measurements reflect the sum of activity of the wild-type enzyme specified by the chromosomal gene in *E. coli* JM101 plus the activitiy of the enzyme specified by the gene carried on the M13 vector used to transform JM101. The control extract is one prepared from cells of JM101 infected with a M13 bacteriophage that did not contain a uridylyltransferase gene. The activity in this extract is considered to be a background activity and is 0.7% of the activity expressed by the transformant carrying the wild-type gene on M13mp9, which is assigned the 100% activity for the fully active, wild-type enzyme in the extract. Therefore, the enzyme is produced from the vector at 140 times the level at which it is produced from the chromosomal gene.

Thirteen of the extracts exhibited activities ranging from 4.5 times the control to 153 times the control activity. Of the 15 mutants only 2, H164N and H166N, showed less activity than the control. Inasmuch as the reaction mechanism for the uridylyltransferase absolutely requires the formation of the uridylylated enzyme intermediate, and a mutant lacking the active-site histidine could not form the intermediate, elimination of the essential histidine should result in a mutant exhibiting no activity. For this reason H164N and H166N were examined further.

Isolation and Further Characterization of H164N and H166N. To isolate the two mutant proteins that exhibited less than control levels of enzymatic activity, it was necessary to overproduce them at high enough levels to allow them to be

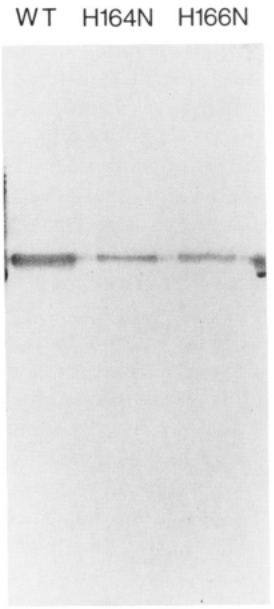

FIGURE 2: SDS–polyacrylamide gel electrophoresis of uridylyltransferase and mutant proteins H164N and H166N.

detected solely by protein concentration measurements during their purification. An overexpression vector constructed from two commercially available plasmids was used for this purpose (see Materials and Methods). This expression vector allowed the production of single-stranded plasmid DNA when cells were infected with a helper bacteriophage, providing an easily obtainable template for nucleotide sequencing. Second, the orientation of a multicloning site allowed ease in subcloning. The gene for the α fragment of β-galactosidase, which contained the multicloning region, allowed the use of the blue/white screen for the detection of inserted DNA. Finally, the two promoters allowed induction of the mutant genes by either T7 RNA polymerase or IPTG. The wild-type and mutant genes were isolated from their M13 vectors by digestion with the restriction enzymes *Hin*dIII and *Eco*RI. The mutant *galT* genes were then inserted into the *Hin*dIII- and *Eco*RI-digested expression vector, which allowed their production to be under the control of the T7 promoter.

These plasmids were transformed into the bacterial strain BL21(DE3), which contains on its chromosome the gene for T7 RNA polymerase under the control of the promoter *lacUV5*. The transformants were grown and protein production was induced by addition of IPTG, which induced the formation of the T7 RNA polymerase which, in turn, induced expression of *galT*. Under the conditions used, up to 60 mg of protein (80–90% purity estimated by gel electrohoresis) could be obtained from a 2-L growth. The variations in purity were attributed to the fact that during chromatography the location of the mutant enzymes was determined by A_{280} measurements, and broader cuts were made than would have been justified by quantitative assays of activity.

Figure 2 shows a SDS–polyacrylamide gel electrophoretogram of the purified wild-type and mutant uridylyltransferases. The left lane contains the wild-type enzyme (83% pure), the center lane contains the mutant protein H164N (90% pure), and the right lane contains the mutant protein H166N (80% pure). No difference in electrophoretic mobility could be observed among these three proteins in repeated tests.

The purified wild-type enzyme and mutant proteins H164N and H166N were tested for their reactivity with UDP-glucose in forming the uridylyl enzyme by using the spectrophotometric procedure of Wong et al. (1977). In this method the standard

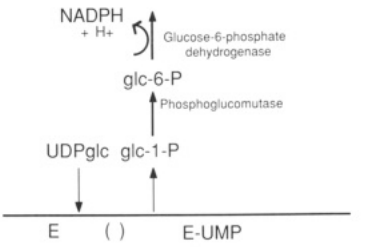

FIGURE 3: Reaction scheme for measurement of uridylyl enzyme formation.

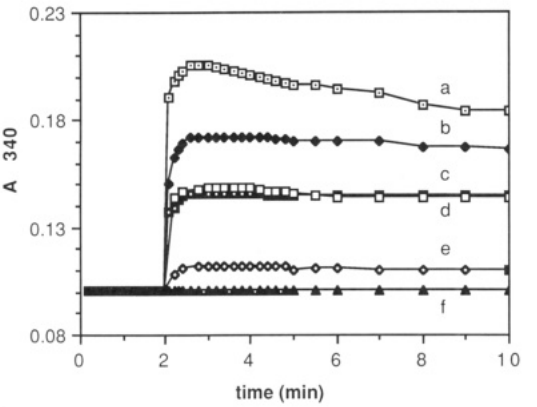

FIGURE 4: Analysis of uridylyl enzyme formation. The figure shows the A_{340} changes upon addition of 0.5 μmol of UDP-glucose to five assay reaction mixtures containing uridylyltransferase at levels of 28.2 nmol (line a), 19.4 nmol (line b), 9.7 nmol (line c), 9.3 nmol (line d), and 2.3 nmol (line e). Line f is the change in A_{340} elicited by mutant protein H164N at levels of 1.58, 3.15, 6.3, and 19 nmol. Line f is also the change in A_{340} elicited by the mutant protein H166N at levels of 2.32, 4.65, 7.75, and 28 nmol. The details of the procedure are described under Materials and Methods.

assay mixture was modified slightly to allow uridylyl enzyme formation to be detected by enzymatically trapping the glucose-1-P formed in a single turnover (Figure 3). Galactose-1-P was excluded from the reaction mixture, so that only one turnover could occur. And the amount of enzyme or mutant protein was large relative to assay levels (milligram rather than microgram levels). The phosphoglucomutase and glucose-6-phosphate dehydrogenase in the reaction mixture allowed the glucose-1-P formed to be measured as NADPH. By this method the concentration of active sites could be accurately determined independent of the enzymatic activity of the mutant enzyme preparations. This method avoided the problem of attempting to relate the activities of the wild-type and mutant proteins, which was complicated by the presence of wild-type enzyme from the host in the purified mutant proteins. The small degree of wild-type contamination, estimated from Table I at less than 1%, would not be detected in an active-site titration.

Figure 4 illustrates the data obtained in the active-site titration of the wild-type and mutant transferases. The figure compares the change in A_{340} with time after the addition of UDP-glucose to varying amounts of the wild-type transferase and the mutant proteins H164N and H166N. Lines a–e show the increase in A_{340} when UDP-glucose is added to the wild-type uridylyltransferase at several enzyme levels in five separate reaction mixtures. Line f represents the same experiment as depicted in lines a–e except for the replacement of wild-type enzyme by either of the mutant proteins H164N or H166N. The addition of UDP-glucose did not induce a change in A_{340} in the presence of either mutant protein preparation over a period of 2 h at any of the four amounts of protein used for each mutant. The wild-type enzyme clearly demonstrates the

production of NADPH, as indicated by the increase in A_{340}, whereas neither of the two mutant enzymes can form the uridylylated enzyme. We estimate that in these experiments we would have detected uridylyl enzyme formation by the mutant proteins if they had reacted at a rate of 10^{-6} times that of the wild-type enzyme.

We conclude that both His-164 and His-166 are critical residues in galactose-1-P uridylyltransferase. One of them must be the active-site nucleophile, and the other must have some other critical function.

Discussion

Of the 13 active mutant uridylyltransferases generated by His → Asn mutation, 11 are approximately within an order of magnitude as active as the wild-type enzyme. Two of the 13 deserve further comment. The mutant enzyme H9N exhibits 50% more activity in the crude extract than wild type, and the mutant enzyme H281N exhibits 3% of the wild-type activity. The others are simply structural variants that are somewhat less active than the wild-type enzyme.

His-9 is not a highly conserved residue in the four species in which the sequences are known. It is present in the uridylyltransferases from *E. coli*, humans (Reichardt & Berg, 1988), and *Saccharomyces cerevisiae* (Tajima et al., 1985). However, it is not conserved in the uridylyltransferase from *Streptomyces lividans* (Adams et al., 1988), which contains a glycine residue at position 9, or that from *Saccharomyces carlsbergensis*, which contains a tyrosine residue at positon 9 (Citron & Donelson, 1984). The replacement of histidine with asparagine at position 9 in the uridylyltransferase of *E. coli* gives a modest activity increase in the extract. This may be due either to intrinsically higher activity for the mutant enzyme or to enhanced expression of the mutant protein.

One mutant enzyme, H281N, exhibited only 3% of the wild-type activity. It is difficult to conceive of how His-281 could be the active-site nucleophile. Activity at the level of 3% of that of wild type is many orders of magnitude higher than what must be expected for a change of the active-site histidine to asparagine. The mechanism requires a ping-pong pathway with the compulsory formation of a uridylyl enzyme. If His-281 were the active-site nucleophile, we would have to postulate that Asn-281 in H281N acts as the nucleophile in place of histidine. The nucleophilic reactivities of amides are many orders of magnitude lower than that of imidazole, whereas the reaction of the mutant enzyme is slower by only a factor of 33 than the reaction of the wild-type enzyme. Therefore, it is highly unlikely that Asn-281 would react in this way. Furthermore, His-281 is not conserved in the human enzyme, which has alanine at this position.

The activity of the mutant enzyme H281N is actually high when considered on the basis of the rate enhancement factors by which enzymes increase reaction rates. The rate enhancement factor for galactose-1-P uridylyltransferase from *E. coli* is *greater* than 10^9. Since the activity of the mutant is 3% that of the wild-type enzyme, it must catalyze the reaction at a rate more than 3×10^7 times faster than that of the nonenzymatic reaction. The turnover number for the wild-type uridylyltransferase is 1600 s^{-1}, and that for the mutant enzyme H281N is 48 s^{-1}. Therefore, the mutant enzyme is a fairly active enzyme in its own right, which could hardly be the case if its catalytic mechanism were undermined by loss of its essential nucleophilic catalytic group.

Since neither of the mutant proteins H165N nor H166N can form the uridylyl enzyme intermediate, both are critical for enzymatic activity, and one of them is the active-site nucleophile. The analysis for uridylyl enzyme formation in Figure 4 is a powerful test of function. The extracts and purified mutant enzymes exhibit low levels of activity owing to the presence of host uridylyltransferase, which copurifies with the mutant proteins. If the observed activity were due to very low activity in the mutant proteins, they should undergo complete uridylylation at a slow rate. As shown by Figure 4, they do not, which means that the mutant proteins lack all capacity to function as uridylyltransferases and cannot undergo the first step of the reaction (eq 2a). Uridylylation of the low levels of wild-type activity in the extracts would not have been detected in Figure 4.

His-164 and His-166 are the only histidine residues that are conserved in the uridylyltransferases of all four species for which sequence information is available (*E. coli*, human, *Streptomyces*, and *Saccharomyces*). In addition, these histidines are located within a well-conserved sequence extending from position 151 to position 169. This region contains nine identities and eight positions in which the interspecies variations are highly conservative. This must be the active-site region of the structure. The conserved sequence His(164)-Pro-His(166), preceded by Pro, Gly, or Lys in the four species, may be a β turn at the active site in the structure of the enzymes (Chou & Fasman, 1978). We know of no other enzyme that contains two essential histidines proximal to each other in the sequence and both in the active site.

In summary, 15 mutant enzymes of galactose-1-phosphate uridylyltransferase have been generated, in each of which one histidine is replaced by an asparagine. Thirteen of these are active and 2, H164N and H166N, are inactive. Neither of the inactive mutant enzymes can be uridylylated by UDP-glucose. Either His-164 or His-166 is the active-site nucleophile, and the other plays another essential role in the active site.

Registry No. His, 71-00-1; Asn, 70-47-3; galactose-1-phosphate uridylyltransferase, 9016-11-9.

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

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

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

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

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

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