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Significance of Metal Ions in Galactose-1-Phosphate Uridylyltransferase: An Essential Structural Zinc and a Nonessential Structural Iron†

Sandaruwan Geeganage and Perry A. Frey*

Institute for Enzyme Research, The Graduate School, and Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin−Madison, 1710 University Avenue, Madison, Wisconsin, 53705

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ABSTRACT: Galactose-1-phosphate uridylyltransferase (GalT) catalyzes the reversible transformation of UDP-glucose and galactose-1-phosphate (Gal-1-P) into UDP−galactose and glucose-1-phosphate (Glc-1-P) by a double displacement mechanism, with the intermediate formation of a covalent uridylyl−enzyme (UMP−enzyme). GalT is a metalloenzyme containing 1.2 mol of zinc and 0.7 mol of iron/mol of subunits [Ruzicka, F. J., Wedekind, J. E., Kim, J., Rayment, I., and Frey, P. A. (1995) *Biochemistry 34*, 5610−5617]. The zinc site lies 8 Å from His 166 in active site, and the iron site lies 30 Å from the active site [Wedekind,J. E., Frey, P. A., & Rayment, I. (1995) *Biochemistry 34*, 11049−11061]. Zinc is coordinated in tetrahedral geometry by Cys 52, Cys 55, His 115, and His 164. His 164 is part of the highly conserved active-site triad His 164-Pro 165-His 166, in which His 166 is the nucleophilic catalyst. Iron is coordinated in square pyramidal geometry with His 296, His 298, and Glu 182 in bidentate coordination providing the base ligands and His 281 providing the axial ligand. In the present study, site-directed mutagenesis, kinetic, and metal analysis studies show that C52S−, C55S−, and H164N−GalT are 3000-, 600-, and 10000-fold less active than wild-type. None of the variants formed the UMP−enzyme in detectable amounts upon reaction with UDP−Glc in the absence of Gal-1-P. Their zinc content was very low, and the zinc + iron content was about 50% of that for wild-type GalT. Mutation of His 115 to Asn 115 resulted in decreased activity to 2.9% of wild-type, with retention of zinc and iron. In contrast to the zinc-binding site, Glu 182 in the iron site is not important for enzymatic activity. The variant E182A−GalT displayed about half the activity of wild-type GalT, and all of the active sites underwent uridylylation to the UMP−enzyme, similar to wild-type GalT, upon reaction with UDP−Glc. Metal analysis showed that while E182A−GalT contained 0.9 equiv of zinc/subunit, it contained no iron. The residual zinc can be removed by dialysis with 1,10-phenanthroline, with the loss in activity being proportional to the amount of residual zinc. It is concluded that the presence of zinc is essential for maintaining GalT function, whereas the presence of iron is not essential.

Galactose-1-phosphate uridylyltransferase (GalT,[1] EC 2.7.7.12) is an enzyme in the Leloir pathway of galactose metabolism (*1*). It catalyzes the nucleotide exchange between UDP−Glc and Gal-1-P to produce UDP−Gal and Glc-1-P by a ping pong kinetic mechanism (eqs 1 and 2).

$$\mathbf{E}\text{-His}^{166} + \text{UDP-Glc} \rightleftharpoons \mathbf{E}\text{-His}^{166}\text{-UMP} + \text{Glc-1-P} \quad (1)$$

$$\mathbf{E}\text{-His}^{166}\text{-UMP} + \text{Gal-1-P} \rightleftharpoons \mathbf{E}\text{-His}^{166} + \text{UDP-Gal} \quad (2)$$

His 166 in the active site of the enzyme from *Escherichia coli* attacks the α-phosphorus of UDP−Glc, displaces Glc-1-P, and forms the high-energy, covalent uridylyl−enzyme (UMP−enzyme) intermediate, which then reacts with Gal-1-P in eq 2 to produce UDP−Gal. Galactosemia, the human autosomal recessive genetic disease, results from the impairment of GalT activity, leading to toxic levels of galactose and Gal-1-P (*2*−*7*).

GalT from *E. coli* is a homodimer of molecular mass 80 kDa with 348 amino acid residues/subunit (*8*, *9*). It contains 1.21 mol of zinc/mol of subunits and 0.67 mol of iron/mol of subunits (*10*). Zinc is 8 Å from His 166 in the active site and is ligated by His 164 (*11*−*13*), which is part of the highly conserved sequence His 164−Pro 165−His166 (*14*, *15*). Cys 52, Cys 55, and His 115 complete the tetrahedral zinc coordination (Figure 1A) (*11*). Iron is ligated in a distorted square pyramidal geometry by three histidines (His 281, His 296, His 298) and Glu 182, which provides bidentate coordination. Iron is 30 Å away from the active site and is close to the subunit interface. While one site preferentially binds zinc and the other site prefers iron, both sites can

* To whom correspondence should be addressed at the University of Wisconsin−Madison. Phone: (608) 262-0055. Fax: (608) 265-2904. E-mail: frey@enzyme.wisc.edu.

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[1] Abbreviations: GalT, galactose-1-phosphate uridylyltransferase; UDP−Glc, uridine 5′-diphosphate glucose; Gal-1-P, galactose-1-phosphate; UDP−Gal, uridine 5′-diphosphate galactose; Glc, glucose; Glc-1-P, glucose-1-phosphate; NAD^+, nicotinamide adenine dinucleotide; $NADP^+$, nicotinamide adenine dinucleotide phosphate; PMSF, phenylmethanesulfonyl fluoride; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediamine tetraacetic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; ICP-MS, Inductively coupled plasma emission mass spectrometry.

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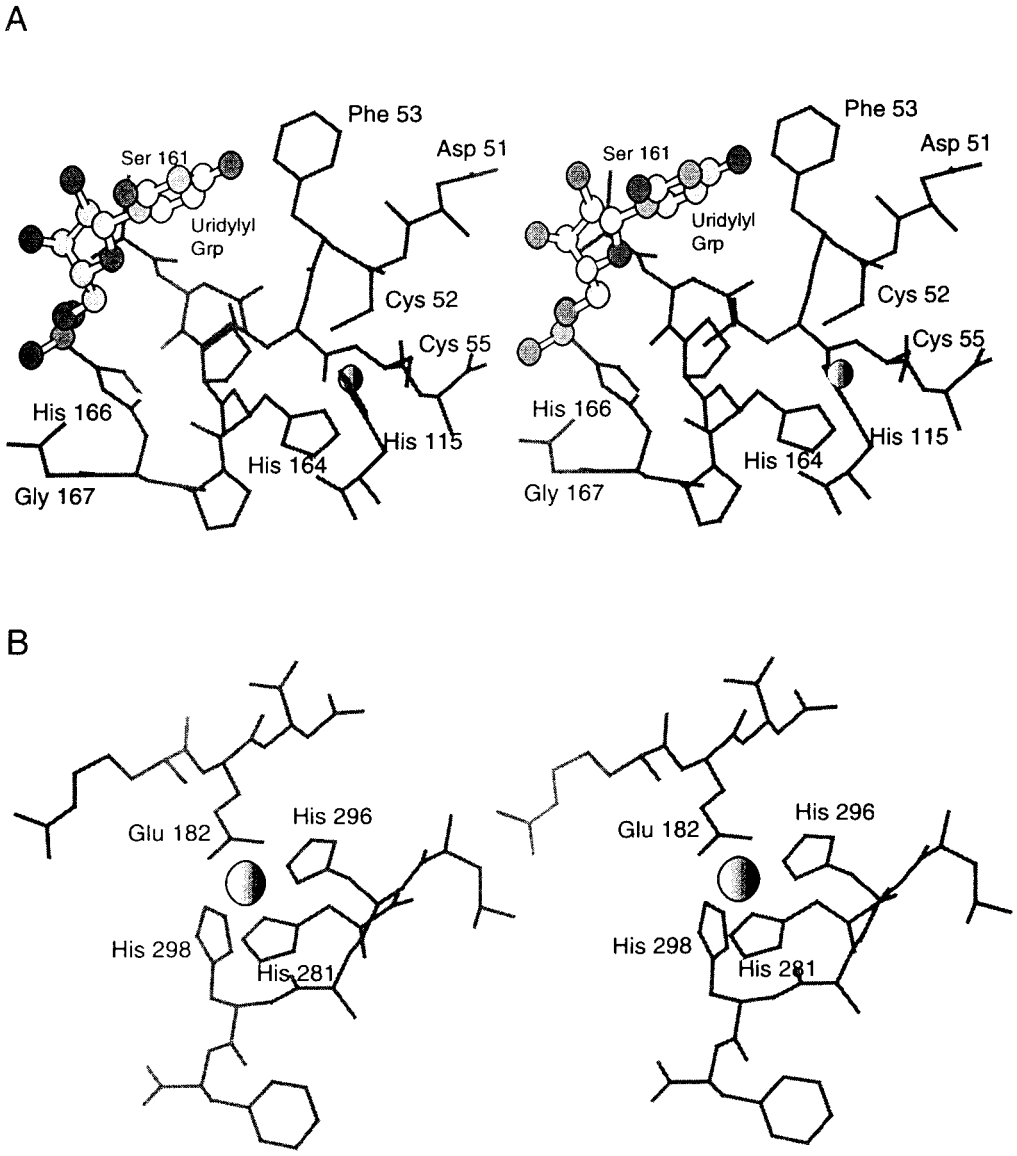

FIGURE 1: Stereoviews of the metal-binding sites of galactose-1-phosphate uridylyltransferase. (A) Zinc-binding site is shown from the structure of the uridylyl−enzyme intermediate (*12*). The UMP group, shown as a ball-and-stick model, forms a covalent linkage with His 166 N^e. His 164 N^d is ligated to zinc and active-site residues $C_{(160)}$ $SNPHP_{(165)}$ that are in a tight turn appear to be held in place by the ligation of His 164 to zinc. The thiolate groups of Cys 52 and Cys 55 are ligands to zinc in a rubredoxin knuckle motif and close a loop over the active site. His 115 completes the zinc coordination. (B) The iron-binding site of GalT shows His 296, His 298, and bidentate Glu 182 forming the base of a distorted square coordination pyramid. His 281 forms the axial ligand. Iron is shown as a sphere. This figure was created from X-ray coordinates from PDB file 1HXQ (*12*), using Molview (*25*).

accommodate either zinc or iron (*10*). Enzyme containing 2 equiv of iron seems to be less stable than the native enzyme or the enzyme with 2 equiv of zinc. The fact that each subunit contains 1.21 equiv of zinc suggest that at least some of the iron sites are generally occupied by zinc.

His → Asn variants of residues His 115 and His 164 have been made previously in experiments to identify the active-site nucleophile. The variant H115N−GalT displayed significant enzymatic activity, whereas H164N−GalT showed no activity (*14*). This raised the question whether the difference in activity was due to a difference in zinc content or whether His 164 played another critical role in the mechanism. Moreover, the iron ligand variants, H281N−, H296N−, and H298N−GalT were active, and this raised the question whether iron was essential for maintaining enzyme activity.

To answer these questions, it is necessary to be able to remove zinc or iron selectively from GalT. We constructed zinc ligand variants, C52S−, C55S−, H115N−, and H164N−GalT and the iron ligand variants E182Q− and E182A−GalT to enable selective removal of metal from the enzyme. Physicochemical analyses performed on these variants and described in the present work establish the significance of these metals for the structure and function of GalT.

MATERIALS AND METHODS

Materials. Glc-1-P, Gal-1-P, UDP−Glc, NAD^+, $NADP^+$, glucose-6-phosphate dehydrogenase, phosphoglucomutase, UDP−Glc dehydrogenase, β-mercaptoethanol, PMSF, Bicine, and Hepes were from Sigma. Affi-Gel Blue was from Bio-Rad. Amicon membranes and Centricon Concentrators were from Amicon. Q-Sepharose and Sephacryl-200 was from Pharmacia. All molecular biology supplies were from

Promega and Fisher. Slide-A-Lyzer dialysis cassettes were from Pierce.

Site-Directed Mutagenesis. Site-directed mutagenesis was performed by using the Bio-Rad Mutagene site-directed mutagenesis kit, which is based on the method of Kunkel (*16*). The *galT* gene has previously been cloned into the high expression vector pTZ18ROT (*17*). All oligonucleotide primers were purchased from Life Technologies. The mutagenic primers were 22 bases in length and contained the mutation (TGC → ACC in Cys 52 → Ser; TGC → TCT in Cys 55 → Ser; CAC → AAC in His 115 → Asn; CAT → AAT in His 164 → Asn; GAA → CAA in Glu 182 → Gln; GAA → GCA in Glu 182 → Ala) in the center. The codon change for Cys 55 → Ser introduced a *Pst*I site, and the Glu 182 → Gln or Gln 182 → Ala codon changes silenced a *Bbs*I site. Mutant colonies were identified by restriction digestion when present or by DNA sequencing. The mutant genes were sequenced in their entire length, by ABI Prism Ampli Taq sequencing in the University of Wisconsin—Madison Biotechnology Center to confirm the absence of adventitious mutations. Plasmids carrying mutated genes pTZ18ROC52S, pTZ18ROC55S, pTZ18ROH115N, pTZ18ROH164N, pTZ18ROE182Q, and pTZ18ROE182A were transformed into BL21(DE3)pLysS cells and overexpressed as described previously (*17*).

Protein Purification. Wild-type GalT was purified as described previously (*18*). The variants E182A− and H115N−GalT were purified by a minor modification of the same procedure (method 1). The ammonium sulfate precipitation steps were excluded and the supernatant fluid from the streptomycin sulfate precipitation was diluted to 4 mg mL^{-1} protein and applied directly to the Affi-Gel Blue affinity column. Fractions from the Q-Sepharose column were pooled, concentrated, frozen as pellets in liquid nitrogen, and stored in liquid nitrogen. SDS−PAGE analysis indicated that variant proteins purified under this protocol showed only one band in an overloaded gel upon Coomassie staining and appeared to be more than 95% pure.

Variants H164N−, C52S−, and C55S−GalT were purified by the following method (method 2). All buffers contained 10 mM β−mercaptoethanol and 1 mM PMSF. Cells (25 g) were lysed by sonication in 90 mL of 10 mM Hepes buffer at pH 7.5 containing 1 mM EDTA. Following centrifugation to remove cell debris, streptomycin sulfate was added to 3% final concentration, the mixture was stirred for 20 min at 4 °C and centrifuged. The supernatant fluid was treated with 35% ammonium sulfate and centrifuged. The pellet was discarded, and ammonium sulfate was added to 70% saturation and stirred for 20 min. After centrifugation, GalT activity remained in the pellet. The pellet was dissolved in a minimum volume of 10 mM Hepes buffer, pH 7.5, and desalted by passage through a Sephadex G-25 column (60 cm height, 5.5 cm diameter). The protein peak was collected and loaded onto a Q-Sepharose anion-exchange column (60 cm height, 4 cm diameter). Bound proteins were eluted with a linear salt gradient formed from 1 L each of 0.05 M NaCl and 0.25 M NaCl in 10 mM sodium−Hepes buffer at pH 7.5. GalT emerged as a single peak at about 0.2 mM NaCl. Fractions containing GalT were pooled and concentrated to less than 1 mL using an Amicon stirred cell and a Centricon concentrator. This was loaded onto a Sephacryl S-200 column (150 cm height, 2 cm diameter) and eluted with 10 mM Hepes buffer, pH 7.5. GalT emerged at 150 mL. Fractions from the column were pooled, concentrated, frozen as pellets by dripping into liquid nitrogen, and stored in liquid nitrogen. SDS−PAGE analysis indicated that GalT purified under this protocol showed a major GalT band in an overloaded gel upon Coomassie staining and appeared to be more than 90% pure.

Mass Spectrometry. Mass spectra of inactive variant proteins were obtained at the University of Wisconsin—Madison Biotechnology Center using a Perkin-Elmer SCIEX 365 Triple Quadrupole MS System. Protein samples were dialyzed overnight in 500 μL Slide-A-Lyzer dialysis cassettes against ammonium acetate, pH 6.9 (not adjusted), to remove small molecules such as β-mercaptoethanol and salts. Samples were diluted to 15 μM protein and treated with 2% acetic acid before injection. The error in the mass in this system was ±3 amu.

Metal Analysis. All metal analyses were performed at the University of Wisconsin—Madison Plant and Soil Analysis Laboratory by ICP-MS. Highly purified GalT was diluted in Nanopure water (Barnstead, 18.3 MΩ purity) and submitted for iron and zinc analysis. Each analysis was complemented by appropriate control experiments. Protein concentrations were measured spectrophotometrically using the extinction coefficient for GalT at 280 nm (72 400 M^{-1} cm^{-1}) (*10*).

Spectroscopy. UV-vis spectrophotometry was performed on a Shimadzu model 1601PC spectrophotometer. Circular dichroism spectra were obtained on an Aviv model 62ADS CD spectrometer at the Biophysics Instrumentation Facility of the Department of Biochemistry at the University of Wisconsin—Madison (supported by the University of Wisconsin—Madison and NSF Grant BIR-9512577). Native and variant proteins were diluted to 4 μM subunit concentration in 10 mM sodium phosphate buffer at pH 7.5, and spectra were obtained in a 1 cm path-length cuvette. Raw data were processed using the IGOR Pro 3.13 data analysis software from Wave Metrics Inc. (OR).

Enzymatic Assays. GalT activity was measured by a standard coupled assay (*19*). Assays were carried out in 1 mL cuvettes in pH 8.5 sodium-bicinate buffer in the presence of 10 mM β-mercaptoethanol. The formation of Glc-1-P was coupled to NADPH production by phosphoglucomutase and Glc-6-phosphate dehydrogenase. The formation of the uridylyl−enzyme was observed and quantified spectrophotometrically under the conditions of the standard assay with omission of Gal-1-P. Under these conditions, the amount of NADPH formed is equal to the amount of uridylyl−enzyme formed. The uridylyl−enzyme content of GalT was measured and quantified spectrophotometrically by adding Glc-1-P to the uridylyl−enzyme. The UDP−Glc formed was coupled to NADH production by UDP−Glc dehydrogenase. Under these conditions, 2 mol of NADH is produced/mol of UDP−Glc.

Steady-state kinetic parameters for E182A−GalT at 27 °C were measured using the coupling enzyme systems (phosphoglucomutase, Glc-6-P dehydrogenase, forward direction; UDP−Glc dehydrogenase, reverse direction) described above. In the forward direction, the rate of formation of Glc-1-P was measured at varying UDP−Glc and Gal-1-P concentrations. In the reverse direction, the rate of formation of UDP−Glc was measured at varying UDP−Gal and Glc-1-P

Table 1: Expression of Metal Ligand Variants of GalT

variant	level of expression	cell extract activity (unit mg protein^{-1})	approximate protein yield (mg/50 g of cells)	specific activity-purified protein (unit mg protein^{-1})
wild-type	high	16.3	250	180
C52S	low	6×10^{-4}	20	0.06
C55S	low	8×10^{-4}	35	0.3
H164N	low	1.6×10^{-4}	30	0.018
H115N	high	4×10^{-1}	100	2.9
E182Q	high	5.73	nd[a]	nd
E182A	high	21.7	250	88.5
no plasmid	na[a]	8×10^{-5}	na	na

[a] ND, not determined. NA, not applicable.

concentrations. Necessary control experiments were performed to ensure that the GalT was the rate-limiting enzyme in the assay. Measured rates were normalized for the amount of enzyme added and computer fitted to the following equation for the ping pong kinetic mechanism (*20*):

$$v = V[A][B]/\{K_a[B] + K_B[A] + [A][B]\}$$

where *A* is either UDP−Glc or UDP−Gal and *B* is either Gal-1-P or Glc-1-P, respectively.

Metal Activation of Variant Enzymes. Attempts to activate the variant enzymes by addition of missing iron or zinc were carried out either by simple incubation with equimolar $FeSO_4$ or $ZnSO_4$ in sodium-bicinate 100 mM buffer containing 1 mM β−mercaptoethanol or by dialysis against these buffers.

Metal Chelation Studies. Metal chelation studies were done as described previously with the exception of using Slide-A-Lyzer dialysis cassettes 500 μL volume (*10*). Variant enzymes (40 μM subunits) were dialyzed against 2000 volumes of buffer (0.05 M Hepes at pH 7.5, 0.05 M NaCl, and 10 mM β−mercaptoethanol). Chelators, EDTA, and 1,10-phenanthroline, when present, were at 2 and 5 mM, respectively. All buffer stocks were scrubbed of metal by passing through a Chelex 100 column. Nanopure water (18.3 MΩ) was used to make all buffers, and all experiments were carried out either in metal scrubbed glassware or in metal free plastic containers. All buffers were analyzed by ICP-MS to confirm that there were no contaminating metals.

RESULTS

Preparation and Preliminary Assays of Variant GalTs. The variants C52S−, C55S−, H115N−, H164N−, E182Q−, and E182A− GalT were constructed as described in the Experimental Section. The resulting mutated genes were overexpressed in *E. coli.* Specific activities of the cell extracts are listed in Table 1. These results show that the mutated GalTs can be grouped in two classes. Three of the zinc ligand variants C52S−, C55S−, and H164N−GalT were poorly expressed and displayed very low activities. Typically, on an SDS−PAGE gel, wild-type cell extract displays a thick band corresponding to overexpressed GalT at about 10% of the total cellular protein. In contrast, the above three variants showed only faintly distinguishable bands on an SDS−PAGE gel. The other zinc ligand variant, H115N−GalT, was well expressed and displayed higher activity in cell extract. The variants of the iron ligand Glu 182 displayed even higher activities, comparable to wild-type enzyme activity, and also very high levels of expression.

The zinc ligand variant H115N−GalT and iron ligand variant E182A−GalT were purified by the same procedure as native GalT, and the specific activities in Table 1 of purified enzymes were obtained. Both variants showed substantial enzymatic activity. H115N−GalT displayed 1.5% of wild-type activity, while E182A−GalT was about half as active as wild-type GalT, despite missing a bidentate iron ligand. The E182Q-GalT was not purified.

We first attempted to purify the H164N−GalT using the same procedure as for the wild-type enzyme, which includes binding to and elution from an Affi-Gel Blue affinity column. When the cell extract was loaded onto the column, all H164N−GalT passed through the affinity column along with the nonbinding proteins. Similar observations were made with C52S− and C55S−GalT. The binding of GalT to Affi-Gel Blue Cibacron Blue F3GA resin is likely to be dependent on the enzyme structure. Therefore it is possible that, because of these mutations in the zinc ligands, there are structural perturbations that prevent these variants from binding to the affinity column. Alternatively, they bind weakly and are displaced by tighter binding proteins in the cell extract.

To obtain pure enzyme for physical studies, we developed a new purification method for C52S−, C55S−, and H164N−GalT, as described in the Materials and Methods. GalT obtained from the Q-Sepharose column contained one major higher molecular weight contaminating band. We were able to separate that and obtain >90% pure GalT by Sephacryl-200 chromatography. Standard activity measurements showed that purified C52S−, C55S−, and H164N−GalT were 3000-, 600-, and 10000-fold less active than wild-type GalT, respectively.

Mass Spectra of Inactive GalT Variants. In a typical purification of native GalT, the progress is monitored by enzymatic assays and identification of protein containing column fractions by measurements of A_{280}. When an inactive variant such as H164N−GalT is purified, the presence of the protein is monitored by SDS−PAGE analysis of column fractions and by measurements of A_{280}. The low level of expression of the zinc ligand variants posed the danger of either copurifying a contaminant or purifying the wrong protein. To confirm that the purified proteins were actually mutated GalT, we obtained electrospray ionization triple quadrupole mass spectra of the C52S−, C55S−, H164N−, and wild-type GalTs. Mass spectra showed only one protein component in our preparations and the molecular masses were 39 502, 39 502, 39 491, and 39 515 amu, respectively. These values agreed within experimental error for the variant and native GalT molecular masses. The calculated molecular mass for native GalT is 39 644 amu based on the amino acid sequence. The consistent difference of 129 amu between calculated and experimental values maybe a result of a missing N-terminal Met residue or a number of rearrangements during ionization.

Uridylylation and Deuridylylation of Metal Ligand Variant GalTs. We added UDP−Glc to purified H115N−GalT and E182A−GalT at pH 8.5 at 27 °C, and both underwent uridylylation rapidly. The amount of Glc-1-P released showed that all of the active sites in the E182A−GalT were uridylylated while 94% of the active sites were uridylylated in H115N−GalT. Extended reactions of H115N- and E182A−GalT with UDP−Glc displayed biphasic kinetics corresponding to rapid uridylylation of active sites to form the uridylyl−

Table 2: Metal Content of GalT Metal Ligand Variants

variant	zinc (mol/mol)[a]	iron (mol/mol)	zinc + iron (mol/mol)	% active sites uridylylated	specific activity (unit mg protein^{-1})
C52S	0.44 ± 0.06	0.26 ± 0.11	0.70 ± 0.11	0	0.06
C55S	0.36 ± 0.01	0.37 ± 0.06	0.72 ± 0.06	0	0.3
H164N	0.48 ± 0.01	0.39 ± 0.17	0.87 ± 0.17	0	0.018
H115N	1.17 ± 0.01	0.53 ± 0.14	1.70 ± 0.14	94	2.9
E182A	0.86 ± 0.03	0[b]	0.86 ± 0.03	100	85.8
wild-type[c]	1.21 ± 0.09	0.67 ± 0.14	1.88 ± 0.14	100	180

[a] Moles per mole of enzyme subunits. [b] Below the detection limit of ICP-MS under same conditions as other analyses. [c] From Ruzicka et al. (*10*).

enzyme, followed by slow hydrolysis of the uridylyl−enzyme to UMP and free enzyme. This phenomenon had been observed in wild-type and other variants of GalT (*21*, *22*). Uridylyl-E182A−GalT can be isolated and treated with Glc-1-P to produce UDP−Glc. All uridylylated active-sites undergo the second step of the mechanism rapidly. In essence, H115N- and E182A−GalTs behave similarly to the wild-type enzyme as far as uridylylation and deuridylylation are concerned.

The three mutated GalTs showing low activity did not undergo observable uridylylation upon extended treatment with UDP−Glc. These results indicated that most of the active sites in C52S−, C55S−, and H164N−GalT were nonfunctional. The slow observable assay rate may be due either to a very small percentage of active sites, below the detection limit of our assays, undergoing uridylylation and hydrolysis, or to a trace of wild-type contamination in the enzyme preparation. BL21(DE3)pLysS may not be a *galT* deletion strain. However, our previous work with inactive variants have not revealed any wild-type contamination (*21*). Even if the very low activities observed were wild-type contamination, the conclusion that C52S−, C55S−, and H164N−GalTs lack functional active sites remains supported by the results of active-site titration experiments showing that they cannot be uridylylated.

Metal Content of Uridylyltransferase Metal Ligand Variants. We subjected all GalTs with mutated metal ligands to metal analysis using ICP-MS in order to determine whether there was a relationship between the varying levels of activity and metal content. The results of metal analysis for variant and native GalTs are shown in Table 2. The three inactive zinc ligand variants, C52S−, C55S−, and H164N−GalT contain 0.8 less zinc/subunit than wild-type GalT. Within experimental error, these proteins contained half of the metal content relative to the wild-type enzyme. Inasmuch as in each variant a zinc ligand is missing and levels of zinc are dramatically reduced, it appears that the loss of metal ions is largely from the zinc site. None of the three variants reacting with UDP−Glc produce a burst of Glc-1-P under single turnover conditions, suggesting that uridylylation of active sites does not take place. The zinc sites in these three proteins are presumably unoccupied, causing the active sites to be nonfunctional. Experiments described below further support this conclusion.

The three zinc ligand variants retain on average 0.4 mol/mol subunits of zinc. This residual zinc is likely to represent partial occupancy of the iron site by zinc. The fact that native GalT contains more than 1 equiv of zinc/subunit (1.21 mol/mol subunits) and less than 1 equiv of iron/subunit supports this interpretation (0.67 mol/mol subunits). Also, Ruzicka et al. have shown that it is possible to generate active enzyme containing 2 equiv of iron or zinc (*10*). Therefore, the presence of zinc in the iron site is not detrimental to the enzymatic activity.

H115N−GalT remains different from the other zinc ligand variants. Its zinc and iron sites appear to be occupied, resulting in a subunit with 94% of its active sites being functional. The overall activity is 34-fold less than wild-type, but that could result from minor structural perturbations due to the mutation. His 115 is not conserved between human and *E. coli* GalTs. The human enzyme contains a serine at this position, and a Ser → Leu mutation is a commonly observed galactosemic variant resulting in 10-fold less activity relative to wild-type (*23*). Wells and Fridovich-Keil propose that the histidine at the adjacent 112 position (His 132 in Human GalT) might serve as a zinc ligand in the human enzyme (*23*).

The absence of the bidentate iron ligand Glu 182 in the variant E182A−GalT results in a protein that contains no detectable iron. We carried out several metal ion analyses at higher protein concentrations and were unable to detect any iron in E182A−GalT. Its zinc content is less than that of wild-type GalT, and the sum of zinc + iron is about half that of the wild-type enzyme, indicating that this mutation has resulted in the absence of any metal ion in the iron site. However, E182A−GalT retains 50% of wild-type activity despite a loss of a bidentate ligand and all iron from the iron site. All of its active sites are also functional, as indicated by the uridylylation of the enzyme by UDP−Glc in the absence of Gal-1-P. Interestingly, the amount of zinc in E182A−GalT, all of which is presumably in the zinc site, is 0.86 ± 0.03 mol/mol subunits. The remaining zinc in C52S−, C55S−, or H164N−GalT, all of which are at the iron site is 0.43 ± 0.06 mol/mol subunits. Addition of these two gives the wild-type zinc level of 1.21 ± 0.09 mol/mol subunits, which provides indirect evidence that the iron site may be partially occupied by zinc.

Circular Dichroism Spectra of GalT Variants. To assess whether there are major secondary structural differences between the metal ligand variants and wild-type enzymes, CD spectra were obtained. The spectra of C52S− and C55S−GalT essentially overlap one another, while the spectrum of H164N−GalT shows a minor deviation. However, all three spectral shapes are significantly different from the spectrum of wild-type GalT (Figure 2). These results show that the loss of zinc causes a perturbation in the secondary structure of GalT, confirming that zinc is a "structural" metal.

Interestingly, the CD spectrum of E182A−GalT overlaps that of the wild-type enzyme, consistent with the fact that E182A−GalT retains substantial enzymatic activity. The loss

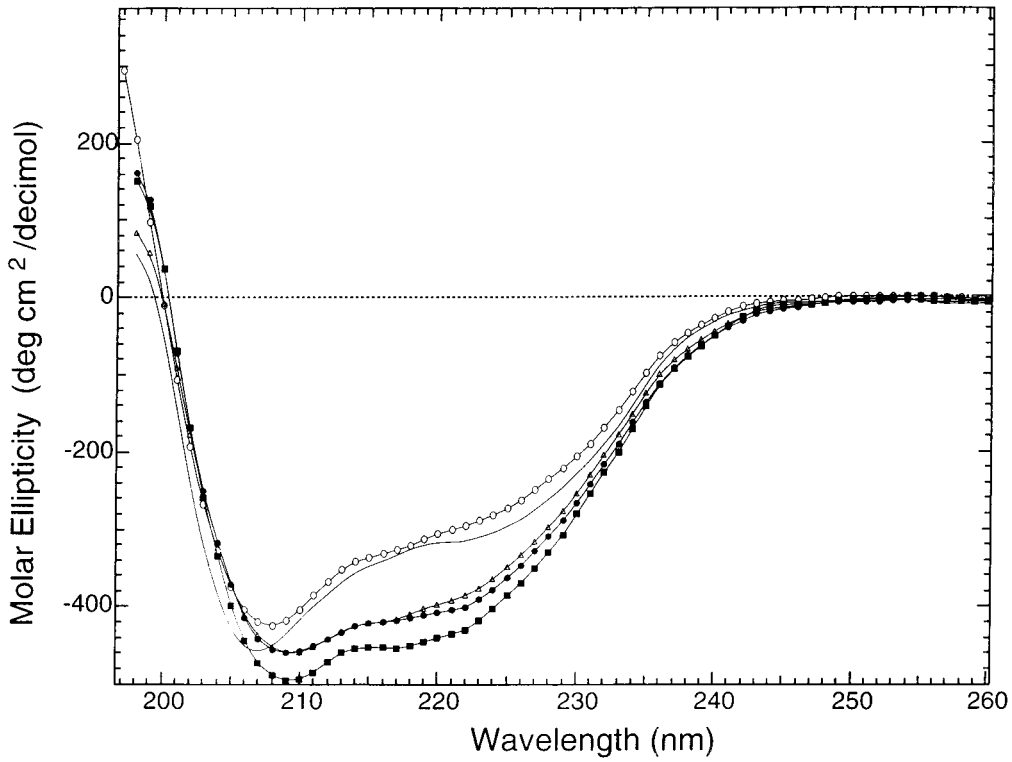

FIGURE 2: Circular dichroism spectra of wild-type and variant GalT. Wild-type (open circles) and E182A−GalT (line- no marker) spectra overlap one another, indicating no major secondary structure changes between the wild-type enzyme and the iron depleted variant. Zinc depleted variants C52S− (closed circles), C55S− (open triangles), and H164N−GalT (closed squares) show changes in their shapes indicating a perturbation in their secondary structures. The greatest deviation is in H164N−GalT, while the spectra for C52S− and C55S−GalT are overlapped with each other.

of the ligand and the loss of iron does not appear to affect the secondary structure of the protein.

While CD spectra offer a qualitative assessment of structural perturbations by the loss of zinc, one needs to obtain crystal structures of these variants to reach definite conclusions on the exact structural changes brought about by the loss of metal ligands and metals. The extreme low level of protein expression in the zinc ligand variants presents a problem in obtaining large enough quantities of protein for crystallographic analyses. However, high levels of expression of E182A−GalT makes structural studies feasible.

EDTA Dialysis of Metal Ligand Variant GalTs. Wild-type GalT contains tightly bound zinc and iron, and dialysis with millimolar EDTA does not remove the metals (*10*). To assess whether the remaining metals in the C52S−, C55S−, H164N−, and E182A−GalTs are as tightly bound, we dialyzed 200 μM of each variant in Hepes buffer containing 2 mM EDTA for 24 h. If a metal ion is present in a site missing a ligand, EDTA should be able to remove that metal. None of the variants showed a decrease in their metal contents or activities (data not shown). The metal content values remained the same as those reported in Table 2 for enzyme as purified. These results suggested that the residual metals in the variants are tightly bound, presumably in the iron site in C52S−, C55S−, and H164N−GalT and in the zinc site in E182A−GalT.

Metal Activation of Inactive Metal Ligand Variants. We attempted to increase the enzyme activity of C52S−, C55S−, or H164N−GalTs by treatment with equimolar $ZnSO_4$, either by incubation with or dialysis against zinc-containing buffers. Periodic assays for enzyme activity failed to indicate any increase. Similar results were obtained in experiments done with E182A−GalT and buffers containing $FeSO_4$. These results indicate that the structural perturbation and the zinc loss caused by C52S−, C55S−, and H164N−GalTs are irreversible, and the enzyme, once folded without zinc, does not unfold and capture it.

Table 3: Steady-State Kinetic Parameters for Wild-Type GalT and E182A−GalT at 27 °C

	WT[a]	E182A
	Forward Direction	
k_{cat} (s^{-1})	780 ± 19	653 ± 52
$K_m^{UDP-Glc}$ (mM)	0.200 ± 0.016	0.390 ± 0.035
$K_m^{Gal-1-P}$ (mM)	0.303 ± 0.033	0.770 ± 0.068
	Reverse Direction	
k_{cat} (s^{-1})	283 ± 19	177 ± 10
$K_m^{UDP-Gal}$ (mM)	0.121 ± 0.037	0.048 ± 0.005
$K_m^{Glc-1-P}$ (mM)	0.157 ± 0.007	0.194 ± 0.017

[a] From ref *19*.

Steady-State Kinetic Parameters for E182A-GalT. Initial rates at varying substrate concentrations in the forward and reverse directions were measured at 27 °C for the E182A−GalT. These rates give parallel lines in double reciprocal plots, indicating that the variant follows a ping pong kinetic mechanism similar to the native GalT. Kinetic parameters for wild-type GalT and E182A−GalT are very similar (Table 3). K_m values are in the same order of magnitude as wild-type enzyme and k_{cat} values for E182A−GalT in the forward and reverse directions are essentially the same as wild-type values. Therefore, the loss of Glu 182 as an iron ligand and the complete loss of iron from the enzyme do not affect the mechanism, substrate binding, turnover or active-site func-

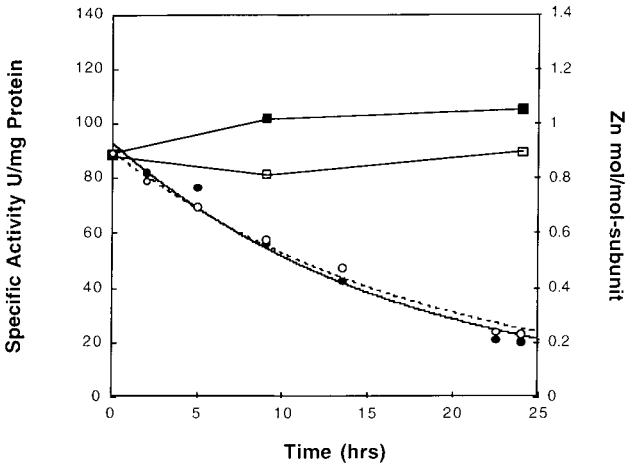

FIGURE 3: Removal of zinc from E182A−GalT by dialysis against 1,10-phenanthroline. The specific enzymatic activity (closed circles) decreases with dialysis against 1,10-phenanthroline during 24 h in a profile that overlaps with the residual zinc content of the variant (open circles). Control experiments in the absence of the chelator show that the enzyme activity (open squares) and metal content (closed squares) remain essentially the same over the same time period. The iron content at each time point was also measured was below the detection limit of ICP-MS. These results indicate that the residual activity of E182A−GalT is dependent on its zinc content, and the presence of iron is not necessary for enzymatic activity.

tion. Neither the presence of iron nor occupancy of the iron site is essential for GalT activity.

Removal of Zinc from E182A-GalT by Dialysis against Phenanthroline. The tightly bound zinc and iron in wild-type GalT can be removed by dialysis against 5 mM 1,10-phenanthroline. The half-time for zinc + iron removal under these conditions is approximately 4 days (*10*). The residual activity of the native enzyme under these conditions is proportional to the remaining zinc + iron content of the enzyme. Ruzicka et al. proposed that the presence of zinc or iron or zinc + iron is essential for maintaining enzyme activity (*10*).

We performed an identical experiment with E182A−GalT, which contained no iron but retained 50% activity. The results are shown in Figure 3. Under exactly the same conditions, zinc is removed much faster from E182A−GalT than from the wild-type enzyme. The half-time for this process is about 10 h. As zinc is dialyzed away into 1,10-phenanthroline, the activity decreases and the residual activity is correlated with the residual zinc content. In the control experiment where no chelator was added, enzyme activity and the zinc content remain essentially the same. These results conclusively show that zinc is the essential structural metal, the removal of zinc results in a loss of enzyme activity, and the residual activity is proportional to the remaining zinc levels. Iron and occupancy of the iron site do not seem to be critical for GalT structure or function.

Affi-Gel Blue Chromatography of Purified C52S−, C55S−, and H164N−GalT Variants. We have investigated further the binding properties of C52S−, C55S−, and H164N−GalTs to the Aff-Gel Blue affinity column. A small, known amount of variant protein purified by method 2 (Materials and Methonds) was loaded onto a small Affi-Gel Blue column and eluted with 5 mM sodium-bicinate buffer at pH 8.5. Once the nonbinding protein had been eluted, the bound protein was eluted by washing with 1 M NaCl, 5 mM sodium-bicinate at pH 8.5. None of the H164N−GalT was bound to the affinity column, whereas C52S- and C55S−GalTs contained equal amounts of binding and nonbinding proteins. Their activities differed, with the binding proteins displaying activities an order of magnitude higher than the nonbinding proteins. However, their metal contents remained essentially the same. These results suggest that changing His 164 to Asn causes a significant perturbation and the largest deviation from the chromatographic properties of the wild-type enzyme. The changes Cys 52 to Ser and Cys 55 to Ser are significant enough to remove zinc from its binding site, but not as dramatic as that of His 164 to Asn. This is also manifested in the higher levels of activities seen in the C52S− and C55S−GalTs. H115N−GalT binds to the affinity column with apparently the same affinity as the native enzyme, and it also displays a much higher activity.

These results raise the question why it was not possible to use the affinity column step to purify the C52S− and C55S−GalTs. The most likely possibility is that, due to their low expression and perhaps weaker interaction with the affinity resin, they may not be able to compete with other binding proteins present in the cell extract. In these latter experiments, homogeneous proteins were loaded on the column, and the competition for binding was much less than would be the case in a crude extract.

DISCUSSION

Ruzicka et al. have previously shown that the metal ions in GalT do not participate in catalysis; however, their presence is critical for maintaining enzymatic activity (*10*). Zinc and iron are extremely tightly bound to GalT, and several days are required to remove them by dialysis against 1,10-phenanthroline (*10*). Both metal ions are removed at the same rate in such experiments, and this results in a loss of enzymatic activity. The residual enzyme activity can be correlated to remaining zinc + iron levels. These results presented three possible scenarios for metal dependence of GalT: the activity could require zinc, iron, or both zinc and iron. However, it was not possible to distinguish among the three possibilities. Attempts to remove zinc selectively from the native enzyme with a chelator were not successful (F. J. Ruzicka, personal communication). 8-Hydroxyquinoline removed 94% of iron from the enzyme and that resulted in a 45% loss of enzyme activity (*10*). Nonetheless, these results did not eliminate the possibility that both sites could be partially occupied by zinc, resulting in 45% residual activity.

Results from the present study unambiguously show that of the two structural metals in GalT, only zinc is necessary to maintain enzyme activity. As shown in Figure 1A, the GalT active-site architecture is such that active-site residues $C_{(160)}$ $SNPHP_{(165)}$ are in a turn that wraps around the diphosphate portion of the substrate where the chemistry occurs (*11*−*13*). His 166 is the nucleophile at the start of a β−sheet following this turn. The backbone carbonyl group of His 164 is only 2.5 Å from the $N^{\delta 1}$ of His 166, and it forms a hydrogen bond with the $N^{\delta 1}$ proton (*12*). We have previously identified this as an important mechanistic feature in GalT that facilitates the stabilization of the uridylyl−enzyme and efficient deuridylylation (*22*). By ligating to Zn(II), His 164 apparently stabilizes the structure of this

region and maintains the proper alignment of active-site residues for efficient catalysis.

Cys 52 and Cys 55 are on a surface loop of the subunit and coordinate to zinc in a rubredoxin knuckle motif (*11*). Cysteine thiolates are also involved in a network of hydrogen bonding that provide structural stability to the active site. The loss of either of the two cysteine ligands should increase the conformational flexibility of the loop and, as shown by the present experiments, weaken the ligation of zinc.

The active site of GalT is very close to the surface of the subunit, and a part of the active site is made by a loop from the second subunit (*13*). This site performs a number of difficult tasks. It carries out an apparently energetically unfavorable uridylylation reaction to form the high-energy uridylyl−enzyme intermediate. It excludes water from the active site to protect the uridylyl−enzyme from hydrolysis until the second substrate binds. The presence of zinc may facilitate the proper alignment of catalytic residues that participate in nucleophilic catalysis, and it may allow the turns from Asp 49 to Gly 63 to be closed over the active site. Loss of zinc by any of the three zinc ligand variants may perturb the active site, expose it to water, and make it nonfunctional, as seen by the inability of these three variants to form the uridylyl−enzyme.

His 115 is somewhat buried in the structure and most likely does not make an essential contribution to maintaining zinc in its position. Asparagine at this position is able to maintain zinc in its bound state and results in H115N−GalT displaying activity in all of its active sites. However, as seen by its lower activity relative to wild-type GalT, even H115N−GalT must incorporate a minor structural perturbation in the active site. These results show the importance of the presence of properly coordinated zinc, for GalT function.

Another issue that may be addressed is the low expression of C52S−, C55S−, and H164N−GalTs. We have observed that native GalT is intrinsically susceptible to proteolytic degradation. During cell lysis, to obtain significant protein yields, it is important to add EDTA and PMSF to inhibit protease activity. Variants depleted in zinc may display even more exposed protease recognition sites. This would increase susceptibility to proteolysis. All other variants, including H115N−GalT, that contain a full complement of zinc are very efficiently overexpressed.

The most surprising finding of this study is the high level of activity displayed by E182A−GalT, despite the absence of a metal ion bound at the iron site. This site is close to the subunit interface, and it has been hypothesized that iron may play a role in GalT dimer formation (*11*). From the crystal structure of the UDP−Glc bound H166G-GalT, we know that the active site of this enzyme consists of residues from the second subunit (*13*). Therefore, to be active, the enzyme has to be a dimer. The fact that E182A−GalT displays high activity suggests that the presence of iron or the occupancy of the iron site is not necessary for either dimer formation or activity.

It has been reported recently that the human enzyme also contains similar amounts of zinc per subunit as the *E. coli* enzyme (*23*). Two of the zinc ligands, His 164 and Cys 55 are conserved between human, rat, and *E. coli* enzymes. His 115 is replaced by Ser, and Cys 52 is replaced by Asn between these species. It is possible that two different residues complete the coordination of zinc in the human enzyme. Regardless of which residues coordinate zinc, it is likely that, even in the human enzyme, zinc plays a similar role in maintaining active-site structure and facilitating efficient catalysis.

Recently, the variant H184Q−GalT has been observed in a case of human galactosemia (nucleotide changes in the human GalT gene web site; http://www.emory.edu/PEDIATRICS/medgen/research/db.htm). His 184 in human GalT corresponds to His 164 in the *E. coli* enzyme. A Gln residue at His 164 position should cause the enzyme to lose zinc and all activity, as has been observed in the case of H164N−GalT in the present work.

There have been no reports regarding whether the human enzyme contains iron. However, all the residues that coordinate iron, His 281, His 296, His 298, and Glu 182, are conserved between the human enzyme and the *E. coli* enzyme. A Gln variant at His 296 (H319Q in humans) has been reported as a completely inactive galactosemic variant (*24*). Interestingly a H296N−GalT variant previously constructed in this laboratory displayed 50% of native GalT activity in cell extracts (*14*). Therefore, it is possible that the human enzyme has evolved without the iron site, which, based on these results, is not critical for enzyme function. The reported loss of activity of H319Q-GalT may be due to another structural deficiency.

In conclusion, the present work has shown that, of the two structural metals in GalT, only zinc is needed for maintaining the enzyme activity and secondary structure. Three zinc ligands, Cys 52, Cys 55, and His 164 are critical for maintaining zinc levels in the enzyme while His 115 is not critical. The difference in activity previously observed between the H164N− and H115N−GalTs is due to their varying zinc content (*14*). An iron depleted E182A−GalT variant shows a high level of enzymatic activity. The zinc in this variant can be removed, and the residual activity is proportional to the residual amount of zinc. The high levels of activity of H281N−, H296N−, and H298N−GalTs previously constructed (*14*), most likely arise from the fact that the presence of iron is not essential for maintaining GalT activity.

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