Identification of Catalytic Amino Acids in the Human GTP Fucose Pyrophosphorylase Active Site

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ABSTRACT: GTP-L-fucose pyrophosphorylase(GFPP) catalyzes the reversible formation of the nucleotide-sugar GDP-β-L-fucose from guanosine triphosphate and β-L-fucose-1-phosphate. The enzyme functions primarily in the mammalian liver and kidney to salvage free fucose during the breakdown of glycoproteins and glycolipids. GFPP shares little primary sequence identity with other nucleotide-sugar metabolizing enzymes, and the three-dimensional structure of the protein is unknown. The enzyme does contain several sequences that could be nucleotide binding sites, but none of them are an exact match to consensus sequences. Using a combination of site-directed mutagenesis and UV photoaffinity cross-linking, we have identified five amino acid residues that are critical for catalysis. Some of these amino acids are found within the poorly conserved nucleotide binding consensus structures, while others represent new motifs. Two active site lysines can be cross-linked to photoaffinity probes. The site of cross-linking depends on the probe used. The identification of these critical residues highlights how distinct GFPP is from other nucleotide-sugar pyrophosphorylases.

Fucose is a deoxyhexose that is found in nearly all plant and animal species. The monosaccharide forms an integral part of complex carbohydrates, glycolipids, and glycoproteins (*1*). Fucose is made available during the synthesis of these structures via the sugar nucleotide GDP-β-L-fucose.

In most organisms, GDP-β-L-fucose is formed by the oxidation−reduction and epimerization of GDP-α-D-mannose (*2*). In mammals, a second pathway exists for the formation of GDP-β-L-fucose. Specifically, there is a salvage pathway that converts the fucose liberated from the breakdown of glycoproteins, glycolipids, and fucose-containing oligosaccharides into fucose-1-phosphate and subsequently to GDP-β-L-fucose. The first reaction is catalyzed by fucosyl kinase (*3*, *4*). The second reaction is catalyzed by a unique guanosine triphosphate fucose pyrophosphorylase (*5*). Overall, this salvage pathway accounts for approximately 10% of fucose metabolism in the average cell.

Guanosine triphosphate fucose pyrophosphorylase (GFPP, E.C. 2.7.7.30) catalyzes the formation of GDP-L-fucose from GTP and fucose-1-phosphate according to the following reaction:

$$\text{GTP} + \text{fucose-1-P} \leftrightarrow \text{GDP-L-fucose} + \text{PPi}$$

The reversible reaction is magnesium-dependent, although the enzyme is partially active when cobalt or manganese is substituted (*5*). The reaction is unusual in that, of the four canonical nucleoside triphosphates, only guanosine can be utilized efficiently to form a nucleotide-sugar (*5*). The GFPP active site though is more plastic than would have been imagined given the restricted base requirements (*6*). Surprisingly, the enzyme does not catalyze the formation of inorganic orthophosphate from the liberated pyrophosphate moiety as a way of energetically driving the reaction in the product formation direction. This feature may be a physiologically important mechanism for the regulation of the enzyme. Unlike other mammalian nucleotide-sugar metabolizing enzymes (e.g., see ref *7*), GFPP is a monomeric enzyme that shares little sequence conservation with other nucleotide-sugar pyrophosphorylases or other nucleotide-metabolizing enzymes.

Given the fact that the enzyme is highly divergent from enzymes with similar catalytic activity, it is not surprising that GFPP does not contain a recognized nucleotide binding site motif or other consensus structures involved in nucleotide-sugar recognition. No known nucleotide binding site consensus sequences (*8*−*13*) are found in the enzyme. Especially intriguing is the absence of a Nudix hydrolase motif (*14*) or other conserved structures found in sugar-nucleotide binding proteins (*15*). The enzyme however does contain four sequences that resemble Walker A sites (*16*). These sites were not detected by automated motif searching but were discovered upon closer inspection of the primary sequence. Divergent pseudostructures of the G-X_4-G-K-(T/S) consensus include: DIPECSGKTS (amino acids 391−400), GVQDNLKKSVKT (amino acids 472−483), its subset sequence NLKKSVKT (amino acids 476−483), and ELFSGNKT (amino acids 511−518). Obviously, these sequences are close to the consensus motif structure but are lacking critical features in both amino acid identity and spacing.

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GFPP does contain two copies of a consensus structure that has been identified in the CaZY family 4 of retaining glycosyltransferases (*17*). The E-X_7-E motif was identified as being catalytically important, although chemical modification studies of other GDP-fucose metabolizing enzymes have implicated essential lysine residues (*18*). These sequences are identified as EKIGTLSCE (amino acids 289−297) and EMLIYKDVE (amino acids 566−574). Last, GFPP contains a single sequence that resembles the N-3 phosphate recognition site of kinesins (*19*). The D-X_2-G-X-E consensus structure is loosely identified as DVSVGE (amino acids 427−432).

Using a combination of site-directed mutagenesis and UV photoaffinity cross-linking, we have identified in this work several amino acids that are required, or partially required, to support the formation of GDP-β-L-fucose. Identification of key residues helps elucidate how GFPP supports the fucose salvage pathway, and it lays the foundation for further structure−function studies of this unusual enzyme.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis. All amino acid mutations were introduced by mutating the plasmid construct via site-directed mutagenesis. Mutagenesis was performed by following the instructions provided with the Transformer Site Directed Mutagenesis Kit (2nd version) from Clontech, Inc. Mutagenic oligonucleotides were synthesized by Gibco BRL as 30-mers with the altered nucleotide(s) centered in the sequence. Specific codon changes for each GAA to GCA (Glu to Ala), GAT to GCA (Asp to Ala), and AAA (or AAG) to GCA (Lys to Ala) mutant are in bold: E289A, 5′-TACTTGCTTTTTAT**GCA**AAAATAGGCACAC; E297A, 5′-GCACACTGAGCTGT**GCA**ATAGATGCCTATG; E432A, 5′-GATGTTTCAGTTGG**GCA**AAACTGCATTATT; E566A, 5′-TGCTGTCCATTGAA**GCA**ATGCTTATCTACA; E574A, 5′-TCTACAAAGATGTA**GCA**GATATGATAACTT; D77A, 5′-ATCACGTTTTTGTG**GCA**CCTGCTGGAGCCA; D184A, 5′-TGGTTACCTGTGCA**GCA**GATATTGAACTTT; D299A, 5′-TGAGCTGTGAAATA**GCA**GCCTATGGTGACT; D427A, 5′-CCAGATTGGGGCCT**GCA**GTTTCAGTTGGGG; K478A, 5′-TGCAAGACAACTTG**GCA**AAGAGTGTGAAAA; K479A, 5′-AAGACAACTTGAAA**GCA**AGTGTGAAAACGT; K482A, 5′- TGAAAAAGAGTGTG**GCA**ACATTGTCAGATA.

The selective oligonucleotide was designed to convert the *Nru* I restriction site of pET15b to a *Stu* I site. The sequence of the selective oligonucleotide was 5′-GTCTTGCTGGCG-T**AGGCCT**CGCGAGGCTGGA (*Stu* I site is in bold). All mutations were confirmed by DNA sequencing using the Applied Biosystems, Inc. model 373A automatic DNA sequencer. GFPP was purified as previously described (*6*).

Protein Stability Measurements. Stability measurements of the wild-type and mutant proteins were performed by measuring protein unfolding in the presence of urea via intrinsic tryptophan fluorescence in a Shimadzu RF5301 fluorometer. The excitation and emission wavelengths were 295 and 340 nm, respectively. Both excitation and emission monochromator slits were set at 1.5 nm. Protein (20 μM) was mixed with increasing amounts of urea (in the concentration range of 0−6.8 M), and the samples were incubated at room temperature for 10 h to ensure that unfolding equilibrium had been achieved. Relative fluorescence was converted into free-energy values according to the following relation (*20*):

$$\Delta G = -RT \ln[(y_f - y_i)/(y_i - y_u)]$$

where y_f and y_u are the relative fluorescence values for fully folded and fully unfolded GFPP, respectively, y_i is the relative fluorescence of the unfolding intermediates, T is the absolute temperature, and R is the gas constant. Linear regression and extrapolation of the relationship ΔG versus [urea] was employed to determine the free-energy value in the absence of denaturant (ΔG_{H2O}).

Enzymatic Assays. The procedure measures the hydrolysis of [γ-^{32}P]GTP to Norit nonadsorbable ^{32}PPi during the formation of GDP-β-L-fucose. The reaction conditions include in 100 μL: 50 μM [γ-^{32}P]GTP, 100 nCi; 20 mM Tris-HCl, pH 7.0; 10.0 mM $MgCl_2$; 200 μM GTP, 300 μM fucose-1-P; and various amounts of enzyme. Back reactions were prevented by the inclusion of 0.01 units of yeast inorganic pyrophosphatase. After 20 min at 37 °C, the reaction was terminated by addition of an acidic suspension of Norit A. The reaction was centrifuged at 8000*g*, and an aliquot of the supernatant was assayed via scintillation counting.

UV Photoaffinity Cross-Linking. Substrates for the reaction, 8-azido-GDP-β-L-fucose and 8-azido-fleximer-GDP-β-L-fucose, were synthesized according to Pastuszak et al. (*5*) utilizing [^{32}P] fucose-1-P. Cross-linking reactions were carried out as described by Basu and Modak (*21*) with modifications described by Cheng et al. (*22*). Samples contained 50 mM HEPES (pH 7.0), 1.0 mg of purified GFPP protein, 200 μM GDP-β-L-fucose (or fleximer-GDP-β-L-fucose), and 50 μCi of [^{32}P] GDP-β-L-fucose (or [^{32}P] fleximer-GDP-β-L-fucose) in a total volume of 2.0 mL. This mixture was placed in the center of a glass evaporation dish, which was in turn placed on ice. The sample was irradiated with 254 nm UV radiation in a Bio-Rad UV cross-linking oven at full power. At various times, 50 μL aliquots were removed from the reaction and stored on ice. The protein was precipitated in the aliquots by the addition of 80% trichloroacetic acid (TCA). The pellets were washed in water, resuspended in a minimal volume of 10 mM Tris-HCl (pH 8.0), and subjected to liquid scintillation counting to determine the extent of cross-linking. A parallel reaction was conducted in the absence of radiolabel in order to measure residual enzyme activity. The aliquot from these reactions was diluted into linear enzyme assay range with 10 mM Tris-HCl (pH 8.0) and adjusted to 10 mM $MgCl_2$. Once the kinetics and optimal cross-linking time were determined, subsequent preparative reactions were treated as described, except that no aliquots were removed. At the conclusion of a preparative cross-linking reaction, the sample was dialyzed against 10 mM HEPES (pH 7.0).

Chemical Fragmentation and Amino Acid Analysis. Dialyzed cross-linked GFPP protein (0.25 mg) was digested with 600 μmol of cyanogen bromide (CNBr) in 400 μL of 0.3 N HCl at 25 °C for 10 h (*23*). The reaction was then diluted 2-fold with 10 mM HEPES (pH 7.0), and the material was applied to a BioGel P-10 column (20 cm × 0.77 cm^2), and fractions were collected. Peptide elution was monitored by absorbance at 280 nm. An aliquot of each fraction was measured for radioactivity via scintillation counting. Frac-

Table 1: Kinetic and Stability Measurements of GFPP Mutants[a]

mutant	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} s^{-1}$) $\times 10^3$	T_m	ΔG (°C)	$\Delta\Delta G$ (kcal mol^{-1})
wild-type	50.2 (1.8)	2.5 (0.1)	49.8	51.7	−3.8	0.0
wild-type*	39.9 (0.9)	3.5 (0.1)	87.7			
E289A	53.7 (1.1)	2.4 (0.1)	44.7	52.3	−3.9	0.1
E297A	80.5 (1.8)	0.2 (0.09)	2.55	50.2	−3.7	−0.1
E432A	127 (3.9)	0.05 (0.04)	0.44	48.5	−3.5	−0.3
E566A	49.6 (1.7)	2.6 (0.1)	52.4	51.5	−3.8	0.0
E574A	50.6 (1.4)	2.4 (0.1)	47.3	51.5	−3.8	0.0
D77A	289 (3.2)	0.001 (0.001)	0.003	52.9	−4.0	0.2
D184A	51.2 (1.3)	2.6 (0.1)	50.8	51.8	−3.8	0.0
D299A	50.1 (1.3)	2.6 (0.1)	51.8	51.6	−3.8	0.0
D427A	49.1 (1.6)	2.6 (0.2)	52.9	50.7	−3.8	0.0
K478A	52.2 (1.5)	2.3 (0.2)	44.1	50.2	−3.7	−0.1
K478A*	516 (5.1)	0.35 (0.05)	0.68			
K479A	51.1 (1.3)	2.2 (0.2)	43.1	51.2	−3.8	0.0
K482A	633 (4.8)	0.19 (0.05)	0.30	50.5	−3.7	−0.1
K482A*	39.2 (1.0)	3.6 (0.2)	91.8			
E297A/D77	nd[b]	nd[b]	nd[b]	50.1	−3.7	−0.1
E297A/E432A	422 (3.1)	0.001 (0.002)	0.002	48.2	−3.4	−0.4

[a] The values shown are the means of three replicate experiments. The standard deviation is in parentheses. $\Delta\Delta G = \Delta G(\text{wt}) - \Delta G(\text{mutant})$. [b] nd = No detectable enzyme activity. * = Fleximer GTP used as substrate.

tions containing radioactivity were pooled and lyophilized. An aliquot of the pool was visualized by SDS−PAGE and autoradiography. No other peptide fragments were radiolabeled. Amino acid sequence determination was performed by modified Edman degradation using an Applied Biosystems 470A gas-phase sequencer. The phenylthiohydantoin (PTH) derivatives were identified and quantitated by reverse-phase HPLC using an on-line Applied Biosystems 120A PTH analyzer. Radioactivity was determined at each Edman cycle via liquid scintillation counting.

RESULTS

Site-Directed Mutagenesis. Critical residues in the identified consensus motifs were systematically mutated to alanine residues in order to map amino acids that are catalytically important. The site-directed mutagenesis procedure resulted in a total of 12 alanine substitution mutants. All of the desired substitution mutants were confirmed by DNA sequencing, and the proteins were purified without incident using the standard purification procedure. Yields were similar to the wild-type enzyme, approximately 5 mg per liter of induced culture. Each homogeneous mutant enzyme was assayed for the ability to form GDP-β-L-fucose from GTP and fucose-1-P. Results from these enzymatic studies are presented in Table 1. Five glutamic acid residues were mutated to alanine. Of these, only two showed alterations in catalytic behavior. E297A is reduced in catalytic efficiency (k_{cat}/K_m) by 19.9-fold by a combination of a slight increase in K_m and a large decrease in k_{cat} relative to wild-type GFPP. A second mutant, E432A, has a larger effect on catalysis. This mutant reduces catalytic efficiency by 124.5-fold with a similar fold reduction in k_{cat} and K_m as E297A relative to wild-type GFPP. The other three glutamate to alanine mutants (E289A, E566A, and E574A) showed no statistically significant alterations in catalytic activity. All five of these mutants were fully folded and exhibited free-energy values within 0.3 kcal/mol of the wild-type enzyme. Hence, changes in catalytic efficiency are not due to overall loss of enzyme stability but are rather directly the result of the alanine substitution. E297 is found in the CaZY E-X_7-**E** consensus structure (mutation site in bold), and E432 is found in the nearly conserved kinesin phosphate recognition structure D-X_2-G-X-**E** (mutation site in bold).

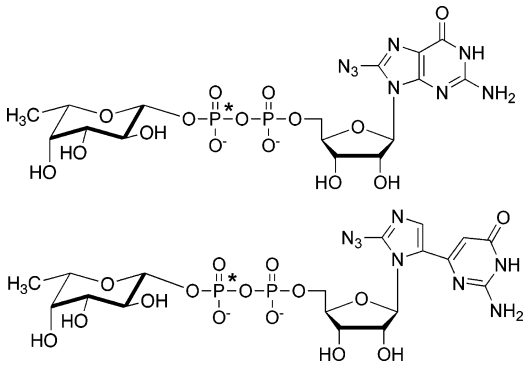

FIGURE 1: Structures of the two UV photoaffinity cross-linking probes used in this study. The top structure is 8-azido-GDP-β-L-fucose; the bottom structure is the fleximer guanine form of the same nucleotide sugar. The asterisk denotes the position of the radiolabel.

A total of four aspartate residues that were identified in the motifs or pseudomotifs were also mutated to alanine. Table 1 shows these results. Only the D77A mutant showed any significant changes in catalytic activity. This mutant had a greatly reduced k_{cat}/K_m (16 600-fold reduction). This mutant exhibited a 5.8-fold increase in the K_m and a 2500-fold decrease in k_{cat}. All of the aspartic acid substitution mutants are fully folded, and they have free-energy values identical to the wild-type enzyme with the exception of D77A, which is actually stabilized slightly ($\Delta\Delta G = 0.2$ kcal/mol) relative to wild-type GFPP. D77A is found as part of the D-X-X-G GTP binding motif. The E297A/D77A double mutant is completely devoid of measurable enzymatic activity, indicating that, of all the aspartate or glutamate residues mutated in the present study, these are the two that are most important for catalysis. The E297/E432A is nearly devoid of enzymatic activity with a 24 900-fold reduction in k_{cat}/K_m that is manifested by a 8.4-fold increase in K_m and a 2500-fold decrease in k_{cat}.

On the basis of the analysis of consensus motifs (and pseudoconsensus structures) in GFPP that may participate in substrate binding, four lysine residues were mutated to alanine. These results are also presented in Table 1. When GTP is used as the substrate, only K482A showed a loss of catalytic activity (166-fold reduction in k_{cat}/K_m, 12.6-fold increase in K_m, and a 13.2-fold reduction in k_{cat}). In the course of this study, all mutants were routinely screened using the fleximer GTP (flex-GTP) molecule as a substrate ((*6*); see the base portion of the molecules shown in Figure 1). All of the aspartate and glutamate mutants exhibited similar behavior regardless of which substrate (GTP or flex-GTP) was employed in the enzyme assay (data not shown). Surprisingly, when flex-GTP was used during the enzyme assay of the K482 mutant, the values of k_{cat}/K_m, K_m, and k_{cat} were essentially wild-type. The mutant K478A however exhibited a 73.2-fold reduction in k_{cat}/K_m (manifested by a 13-fold increase in K_m and a 10-fold decrease in k_{cat}). When GTP is used as substrate with the K478A mutant, the results are essentially the wild-type values. Hence, the identification of

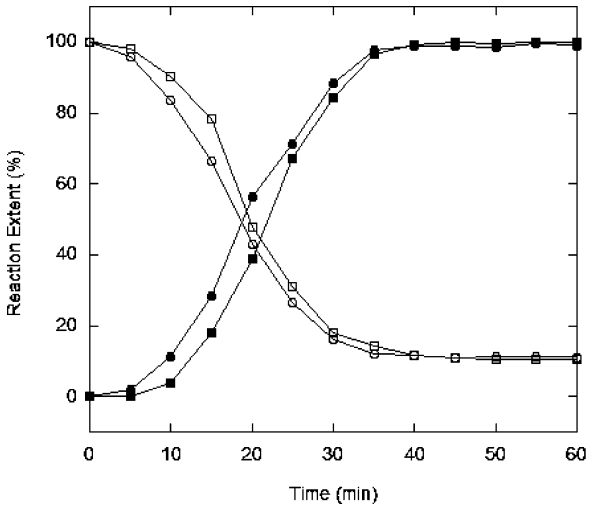

FIGURE 2: Radiolabeled GTP can be covalently incorporated into the GFPP active site, which results in a loss of enzyme activity. The photoaffinity cross-linking reaction was performed as described in Experimental Procedures. At the times indicated, aliquots were removed from the reaction and were assayed for the formation of the covalent photoadduct (closed symbols). An additional aliquot at the same timepoint was diluted into linear assay range and was assayed for residual enzymatic activity (open symbols). Circles, 8-azido-GDP-β-L-fucose; squares, 8-azido-fleximer-GDP-β-L-fucose.

the critical catalytic GFPP lysine residue(s) is dependent on the nature of the nucleoside triphosphate used in the assay. To try to elucidate this result further, GTP and flex-GTP were utilized in their 8-azido-NDP-fucose forms as photoaffinity probes.

UV Photoaffinity Cross-Linking. Radiolabeled N_3-GDP-β-L-fucose and N_3-fleximer-GDP-β-L-fucose can be covalently incorporated into the protein upon irradiation with 254 nm light. The structure of both photoaffinity probes is shown in Figure 1. In the reaction conditions used for the photoaffinity cross-linking, there is negligible basal enzymatic activity (data not shown). This makes it possible to undertake the cross-linking with [^{32}P]-labeled substrate instead of substrate labeled with ^{3}H or ^{14}C on the base moiety. Figure 2 shows that the incorporation proceeds as a function of time, and plateaus after approximately 40 min of irradiation. The cross-linking of either substrate with the protein results in a loss of enzymatic activity that is also a function of the irradiation time (Figure 2). The loss of enzymatic activity is presumably due to steric blocking of the GFPP active site by the formation of a covalent photoadduct. Inactivation of enzyme activity follows pseudo-first-order kinetics with a rate constant of 2.1×10^{-3} s^{-1} and a corresponding half-life of 18 min for reactions carried out using N_3-GDP-β-L-fucose. For reactions involving N_3-fleximer-GDP-β-L-fucose, the first-order inactivation rate constant was 1.5×10^{-3} s^{-1}, which corresponds to a half-life of 20 min. Both cross-linking reagents exhibited no appreciable differences in the kinetics of cross-linking or inactivation.

The radiolabeled and cross-linked protein was subjected to cleavage with cyanogen bromide. Analysis of the primary amino acid sequence indicates that the protein should be cleaved 13 times by the reagent. The resulting fragments are predicted to range in size from 817 Da (7 amino acids) to 15.7 kDa (142 amino acids). The CNBr fragments were

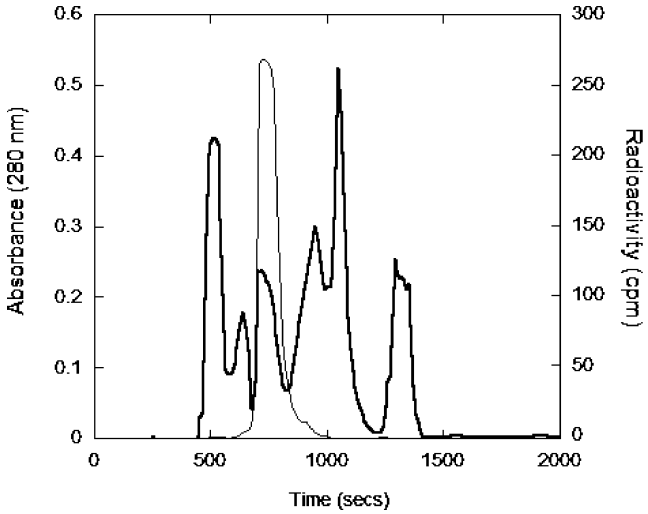

FIGURE 3: Elution profile of GFPP CNBr fragments through a Biogel P10 column. The absorbance profile is plotted, as a function of elution time, with a bold line; the radioactivity associated with the peptides is plotted in similar fashion using a thin line.

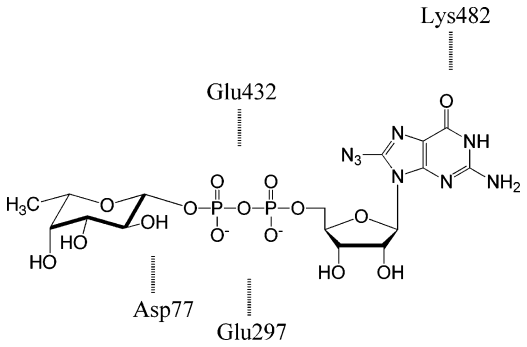

FIGURE 4: Hypothetical model depicting the possible interactions between GFPP amino acid residues identified in this study as being important to enzymatic activity and the GDP-β-L-fucose substrate. It is presumed that Glu and Asp residues interact with the phosphate moieties through either a metal ligand or function as a general base for the reaction (e.g., see ref *43*).

chromatographed through a Biogel P-10 column (Figure 3), and the peptide elution profile indicated that the radiolabel resided exclusively on a peptide that was approximately 8 kDa. This peptide corresponds to the predicted 8.4 kDa fragment that spans amino acids 470−544. Cleavage of that peptide with V8 protease in ammonium acetate indicated that the radiolabel was confined to the Nter region of the CNBr fragment (data not shown). Hence, it was possible to directly perform Nter amino acid sequencing on the isolated CNBr fragment without further chemical or proteolytic digestion. The results of the Nter amino acid analysis are shown in Table 2. When [^{32}P]-8-azido-GDP-β-L-fucose is used as the photoaffinity probe, no amino acid is detected in the ninth degradation cycle. Coincident with this cycle is 89% of the radioactivity. Thus, cycle 9 represents the position of the photoadduct. Cycle 9 corresponds to Lys478 and is therefore the site of cross-linking when GDP is the base component of the photoaffinity probe. A similar analysis was performed on GFPP that was cross-linked with [^{32}P]-8-azido-fleximer-GDP-β-L-fucose. Surprisingly, Lys478 is correctly identified in cycle 9, and there is no associated radioactivity in that cycle. Further rounds of Edman degradation (Table 2) reveal that the photoadduct is found at cycle 13 along with 84% of the radioactivity. This cycle corresponds to Lys482. Hence,

Table 2: Amino Acid Sequencing of the Isolated CNBr Peptide

cycle	amino acid	yield (N_3-GDP-fucose) pmol	yield (N_3-GDP-fucose) cpm	yield (N_3-fleximer-GDP-fucose) pmol	yield (N_3-fleximer-GDP-fucose) cpm
1	A470	222	0	313	0
2	F471	221	0	293	0
3	G472	218	0	302	0
4	V473	241	0	310	0
5	Q474	228	0	323	0
6	D475	237	0	327	0
7	N476	226	45	315	0
8	L477	224	230	334	0
9	X^{a} [a]	nd[b]	4346	312	0
10	K479	238	226	322	0
11	S480	229	38	329	61
12	V481	253	6	330	627
13	X^{b} [c]	236	0	nd[b]	5714
14	T483	231	0	321	412
15	L484	215	0	307	19

[a] X^{a} corresponds to K478. [b] nd = amino acid not detected. [c] X^{b} corresponds to K482.

when fleximerGDP is used in the photoaffinity probe, a different lysine is cross-linked. Both lysines are contained in the pseudo-Walker consensus structure NLKKSVKT (amino acids 476−483).

DISCUSSION

A search of the EMBL protein database revealed two GFPP sequences in addition to the human enzyme. Mouse (AJ276067) and rat (AJ276066) GFPP are 73 and 72% identical to the human enzyme, respectively, and 85% identical to each other (*24*). A Prosite (version 18.21) analysis of each protein revealed no exactly matching consensus sequences involved in nucleotide-sugar binding. This, coupled with a BLAST search, indicates that GFPP is a rather unique enzyme. However, two consensus structures did emerge from an analysis of the primary literature; a CaZY EX_7E catalytic motif is found in two places within the GFPP primary sequence. The sites are EKIGTLSCE (residues 289−297) and EMLIYKDVE (residues 566−574). A second group of consensus sequence was also found. The DXXG motif is one of the three canonical GTP binding motifs (*25*). This sequence is found in three positions in GFPP: DPAG (residues 77−80), DKPG (residues 184−187), and DAYG (residues 299−303). Surprisingly absent is the (N/T)KXD guanine base recognition motif (*26*, *27*), although this sequence is also absent on other guanine nucleotide metabolizing enzymes (e.g., see ref *27*).

Sequence conservation of binding motifs is especially high among nucleotide-sugar pyrophosphorylases. Analysis of the aligned primary sequences of the mammalian enzymes clearly delineates where active site residues are located within fully conserved consensus structures. These observations have been confirmed by a wide variety of experimental techniques. The three-dimensional structures of these enzymes that are complexed with substrate confirm the sequence and biochemical findings. Unlike GFPP, there are no unusual binding modalities or sequence variation in residues that participate in substrate binding or catalysis. Among the three known GFPP enzymes, the residues identified as being catalytically important are conserved. The divergence of GFPP from the broader family of nucleotide-sugar pyrophosphorylases may be related to differences in quaternary structure or regulation, as the monomeric GFPP functions in the fucose salvage pathway instead of the primary route for GTP-fucose production/utilization.

To determine whether any of the identified residues were catalytically important, each glutamate or aspartate residue in the consensus structures was systematically mutated to an alanine by site-directed mutagenesis. None of the mutations significantly alter the stability of the enzyme. Free-energy values of all the alanine substitution mutants are within a few tenths of a kcal mol^{-1} of the wild-type protein. Only one of the four EX_7E mutants, E297A, showed any variation in catalytic activity. The K_m is increased only 1.6-fold relative to wild-type when assayed for the ability to synthesize GDP-β-L-fucose; however, k_{cat} is reduced by a factor of 12.5. Clearly, glutamate 297 plays a role in the chemical step, perhaps acting as a general base for the reaction. Interesting, however, is the observation that catalytic activity is not abolished in this mutant. E297 is necessary for catalysis but is not the sole chemically important residue in the GFPP active site. Similar analysis revealed that a single aspartic acid mutant, D77A, affected catalysis. This mutant had a sizable effect on both the K_m (5.6-fold reduced) and the k_{cat} (2500-fold reduced). The double mutant E297A/D77A is completely devoid of measurable enzymatic activity, yet the mutant is still stable and folded. The usual role of the DXXG motif is to help coordinate phosphates, and the motif is typically found as part of a larger phosphate binding loop (*27*, *28*). Clearly, these two sites are important to substrate recognition and enzymatic activity, but the location and full nature of the GFPP active site remain a mystery. The enzyme does not contain the T(V/L)RD motif that was identified as a base recognition motif in human guanylate binding protein (*27*), the GXGTRXLPXTK motif (*29*), nor does it contain recognition motifs that have been identified in other nucleotide-sugar metabolizing proteins (*30*).

Active site glutamate residues can either function as metal ligands, a common theme in nucleotide binding, or as general bases to deprotonate the attacking water during the course of the reaction. Although it is assumed that GFPP binds a Mg^{2+}−GTP complex, no studies have been performed yet to look for enzyme-bound divalent cations. Nucleotide-sugar metabolizing enzymes that catalyze reversible reactions (like GFPP) need to coordinate phosphate species from the nucleoside triphosphate, the sugar−phosphate, as well as pyrophosphate. Hence, metal ligation between these species and glutamate or aspartate residues is most likely a key feature of the GFPP active site.

Many nucleotide metabolizing enzymes and nucleotide-sugar metabolizing enzymes in particular contain active site lysine residues (*31*, *32*). In addition, lysine residues have been observed in nucleotide associated cross-links (e.g., see refs *22*, *33*) if they are normally interacting with the base moiety via a hydrogen bond. The UV photoaffinity cross-linking technique has proven successful in locating active sites of other nucleotide-sugar metabolizing enzymes (e.g., see ref *32*). In fact, Pastuszak et al. (*5*) indicated that GFPP can be efficiently cross-linked with 8-N_3-^{32}P-GDP-fucose but did not indicate the site of cross-linking on the enzyme. Here, the interplay between lysines and the substrate base moiety is shown to be important with GFPP. The active site lysines represent an expansion of the Walker A-type consensus sequence beyond what has been demonstrated for other nucleotide binding proteins. Clearly, the observation that

GFPP can utilize the NLKKSVKT sequence indicates that the nucleotide binding mode is subtly different than that used by proteins utilizing the G-X_4-G-K-(T/S) motif. Current site-directed mutagenesis work involves a more extensive study of the NLKKSVKT sequence in order to elucidate other binding determinants.

GFPP clearly can utilize either Lys478 or Lys482 during substrate recognition. The mutant K478A results in loss of activity when GTP is used as a substrate, but the mutant retains full activity when synthesizing nucleotide-sugars with a fleximer GTP substrate. Conversely, full activity is retained in the K482 mutant when utilizing GTP but is inactive in the presence of fleximerGTP. Since the fleximerGTP molecule is not a canonical nucleoside triphosphate, the catalytically important active site lysine is 478.

The fleximers were originally designed as potential antiviral compounds (*34*), with sights set on improving binding interactions with enzyme active sites for known inhibitors. It quickly became apparent however, that the molecule could be useful as an enzyme active site probe (*35*). The inherent flexibility of the structurally unique nucleoside analogue permits the pyrimidine ring to rotate about the imidazole ring via the extra carbon−carbon bond. As expected, the increase in rotational degrees of freedom allows the fleximer base to sample alternative binding modalities. This allows the base moiety to interact with secondary amino acids residues not previously involved in the mechanism of action within the binding site. Case in point, with GFPP, the fleximer adopts a conformation that allows it to be cross-linked to a lysine that is not within cross-linking distance to a canonical guanine base. Interestingly, the fleximer base apparently does not adopt multiple conformations in the GFPP active site as evidenced by cross-linking to a single lysine, but clearly the two ring components have rotated with respect to one another.

The fleximer nucleotide sugar is also a better substrate than GTP, having a catalytic efficiency more than twice that for GTP. Although this is not physiologically relevant, it is clear from these studies that the fleximer nucleosides will be quite useful in other protein structure−function investigations. These flexible nucleoside analogues may, in fact, aid in new antiviral drug design by revealing alternative binding modes that can help direct alternative inhibitor design.

More notably, the fleximerGTP was able to retain biological activity despite mutations to active site residues critical to catalysis. Given the recent reports (*36*−*39*) of tenofovir's and etravirine's ability to evade viral resistance mutations due to their inherent flexibility, this finding is clearly significant. The number of these novel active site probe reagents is also increasing, so a wide range of fleximer mimics for canonical nucleosides are now available (*40*, *41*).

This work does not eliminate the possibility that other lysine residues are involved in substrate recognition or catalysis. Human GFPP contains 39 lysines, so a site-directed mutagenesis approach to completely map active site lysines is impractical. A similar problem presents itself with the number of possible active site arginines. GFPP contains 22 arginines, residues that have been implicated in being functionally important residues in other nucleotide-sugar pyrophosphorylases (*42*). Ultimately, structural information will be required to fully understand the nature of the GFPP active site. Crystallization studies are in progress, but the enzyme is refractory to producing diffraction quality crystals. Still, this work sheds the first light on increasing our understanding of the GFPP active site architecture by identifying several catalytically important residues. GFPP is an unusual enzyme that shares very limited sequence conservation with other nucleotide-sugar pyrophosphorylases, and this study expands our understanding of GFPP substrate recognition and catalysis in a manner that begins to dissect how the enzyme functions in mammalian fucose metabolism. Further studies are planned, and the results of those will be reported in due course.

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