Identification and Modification of the Uridine-binding Site of the UDP-GalNAc (GlcNAc) Pyrophosphorylase*

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UDP-GalNAc pyrophosphorylase (UDP-GalNAcPP; AGX1) catalyzes the synthesis of UDP-GalNAc from UTP and GalNAc-1-P. The 475-amino acid protein (57 kDa protein) also synthesizes UDP-GlcNAc at about 25% the rate of UDP-GalNAc. The cDNA for this enzyme, termed AGX1, was cloned in Escherichia coli, and expressed as an active enzyme that cross-reacted with antiserum against the original pig liver UDP-HexNAcPP. In the present study, we incubated recombinant AGX1 with N₃-[³²P]-GlcNAc and N₃-[³²P]-GalNAc probes to label the nucleotide-binding site. Proteolytic digestions of the labeled enzyme and analysis of the resulting peptides indicated that both photoprobes cross-linked to one 24-amino acid peptide located between residues Val²¹⁶ and Glu⁴⁶⁷. Four amino acids in this peptide were found to be highly conserved among closely related enzymes, and each of these was individually modified to alanine. Mutation of Gly²⁰⁹ to Ala in the peptide almost completely eliminated UDP-GlcNAc and UDP-GalNAc synthesis, while mutation of Gly²²⁴ to Ala, almost completely eliminated UDP-GalNAc synthesis, but UDP-GlcNAc was only diminished by 50%. Both of these mutations also resulted in almost complete loss of the ability of the mutated proteins to cross-link N₃-[³²P]-GlcNAc or N₃-[³²P]-GalNAc. On the other hand, mutations of either Pro²⁰⁰ or Tyr²²⁷ to Ala did not greatly affect enzymatic activity, although there was some reduction in the ability of these proteins to cross-link the photofluorophore probes. We also mutated Gly¹¹¹ to Ala since this amino acid was reported to be necessary for catalysis (Mio, T., Yabe, T., Arisawa, M., and Yamada-Okabe, H. (1998) J. Biol. Chem. 273, 14392–14397). The Gly¹¹¹ to Ala mutant lost all enzymatic activity, but interestingly enough, this mutant protein still cross-linked the radioactive N₃-[³²P]-GlcNAc although not nearly as well as the wild type. On the other hand, mutation of Arg¹³⁶ to Ala had no affect on enzymatic activity although it also reduced the amount of cross-linking of N₃-[³²P]-GlcNAc. These studies help to define essential amino acids at or near the nucleotide-binding site and the catalytic site, as well as peptides involved in binding and catalysis.

UDP-GlcNAc pyrophosphorylase (UDP-GlcNAcPP)³ is a key enzyme in the biosynthesis of complex carbohydrates, since it is the enzyme that produces the activated form of GlcNAc, i.e. UDP-GlcNAc, for polymerization. This enzyme catalyzes the reversible reaction: UTP + GlcNAc-1-P ⇌ UDP-GlcNAc + PP. The enzyme was initially purified about 100-fold from Staphylococcus aureus, and that enzyme preparation utilized UDP-GalNAc (in the reverse direction) at about 2.8% the rate of UDP-GlcNAc (1). The enzyme was also partially purified from calf brain, and in that case, the enzyme also utilized UDP-glucose at about 36% the rate of UDP-GlcNAc (2). However, it was not clear from that report whether the calf brain enzyme had any activity with UDP-GalNAc, or whether the activity with UDP-glucose was due to the same enzyme, or to a contaminating pyrophosphorylase.

We purified the UDP-GlcNAcPP from pig liver cytosol to apparent homogeneity, and found that the purified enzyme contained two proteins with molecular masses of about 64 and 57 kDa. Interestingly enough, this purified enzyme also catalyzed the synthesis of UDP-GalNAc from UTP and GalNAc-1-P, and at saturating concentrations of GalNAc-1-P, the rate of synthesis of UDP-GalNAc was equal to that of UDP-GlcNAc (3). We sequenced three peptides from the 64-kDa protein and two from the 57-kDa protein and found that all of these sequences showed 100% identity to AGX1, a protein of unknown function from the sperm of infertile males (4). A second human protein of wider tissue distribution, but also of unknown function and called AGX2, has the identical sequence to AGX1 except that AGX2 has an additional 17-amino acid insert near the carboxyl terminus (5).

We cloned the cDNAs for AGX1 and AGX2 and expressed the active proteins in Escherichia coli. AGX1 migrated with the 57-kDa pig liver enzyme on SDS gels, and had about three times as much UDP-GalNAc synthetic activity as UDP-GlcNAc activity. On the other hand, AGX2 migrated with the 64-kDa pig liver protein and also had both UDP-GalNAc and UDP-GlcNAc activity, but at a ratio of about 1:8 (5). Thus, addition of the 17-amino acid insert changes the activity of the enzyme from synthesizing UDP-GalNAc to synthesizing UDP-GlcNAc (5).

In the current study, we have identified a 24-amino acid peptide involved in binding the uridine portion of the substrate, i.e. either UDP-GalNAc or UDP-GlcNAc. To do this, we synthesized N₃-[³²P]-GlcNAc and N₃-[³²P]-GalNAc (6, 7), and incubated both of these photoprobes with the recombinant AGX1 (i.e. UDP-GalNAcPP). Both photoprobes cross-linked specifically to amino acids in the active site, and the resulting radiolabeled peptide was identified and located in the protein sequence of AGX1. We then used this information to do site-phosphorylase; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PAGE, polyacrylamide gel electrophoresis; HSV-1 TK, human sarcoma virus type 1 thymidine kinase; AZTMP, 3′,5′-diazido-2′,3′-dideoxyuridine [³²P]5′-monophosphate.

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³ The abbreviations used are: UDP-GalNAcPP, UDP-GalNAc pyrophosphorylase; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PAGE, polyacrylamide gel electrophoresis; HSV-1 TK, human sarcoma virus type 1 thymidine kinase; AZTMP, 3′,5′-diazido-2′,3′-dideoxyuridine [³²P]5′-monophosphate.

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directed mutagenesis of four highly conserved amino acids, and determined the effect of amino acid changes on enzymatic activity and substrate binding. These studies indicated that two glycines in that peptide (at positions 222 and 224) are important in cross-linking the uridine portion of the substrate, as well as in enzyme catalysis. These glycines may play a direct role in binding and catalysis, or they may be important in protein conformation.

Another study had previously reported the identity between AGX1 and the UDP-GlcNAc pyrophosphorylase (8). That study indicated that Gly112, Arg116, and Lys123 were important for catalytic activity of this enzyme. We also confirmed that changing Gly111 (position 111 in our sequence) to Ala resulted in loss of catalytic activity, but importantly, this modification did not eliminate the ability of the mutant protein to cross-link the photoprobes. These studies begin to identify important amino acid residues and peptide sequences involved in substrate binding and catalysis.

**EXPERIMENTAL PROCEDURES**

**Materials**—The photoaffinity probes, N3-UDP-[32P]GlcNAc and N3-UDP-[32P]GalNAc, were prepared according to previously described methods (6, 7). UDP-[3H]GlcNAc and UDP-[3H]GalNAc were purchased from NEN Life Science Products Inc., or from American Radiolabel Chemical Co. Protein molecular weight markers and precast Tricine/SDS 10–20% polyacrylamide gels were purchased from Novex. Endoproteinases: Glc-U (V8), Asp-N, and Lys-C were obtained from Sigma. Mutants were generated using a Quickchange Mutagenesis Kit (Stratagene). Purified recombinant wild type and mutant AGX1s were prepared as described previously (5). Polyclonal antibody was prepared against the purified pig liver UDP-GlcNAc/GalNAc pyrophosphorylase as described previously (5).

**Photoaffinity Labeling of AGX1**—Photoaffinity labeling was done using previously described methods (6). Briefly, incubations for labeling the proteins were as follows: 2–10 μg of purified wild type or mutant recombinant AGX1, in a final volume of 50 μl of 50 mM Tris buffer, pH 7.5, plus 5 mM MgCl2, were incubated for 30 s, on ice, with either N3-UDP-[32P]GlcNAc or N3-UDP-[32P]GalNAc. The reaction mixture was then exposed to short-wave UV light for 90 s, with agitation, to activate the azido group and covalently cross-link the probe to the protein. One ml of methanol, cooled to −80 °C, was added to stop the reaction, and the precipitated protein was isolated by centrifugation in a microcentrifuge at 10,000 × g for 10 min. The supernatant was discarded and the pellet was suspended in 20 μl of SDS gel-loading buffer, and subjected to SDS-PAGE on a 10% gel. Location of radioactive probes was determined by autoradiography (9, 10). To determine the efficiency of the labeling, some incubation mixtures contained the radioactive probe, plus various amounts of unlabeled UDP-GlcNAc or UDP-GalNAc. These reaction mixtures were processed as described above for the control incubations (5, 6).

**Enzymatic Digestions of Radiolabeled AGX1 and Isolation of Peptides**—The protein pellets from incubations of AGX1 and the photoprobes were isolated by centrifugation of the methanolic extracts, and suspended in 40 μl of digestion buffer (100 mM Tris buffer, pH 8.0, containing 0.1% SDS), rather than the SDS gel loading buffer. The sample mixture was divided into four equal aliquots, each of which was subjected to digestion at 37 °C for various times with 1 unit of one of the following proteases: endoproteinase Lys-C, V8 proteinase, endoproteinase Asp-N, or both endo-V8 and Asp-N. The protease digests were subjected to SDS-PAGE on precast SDS/Tricine gels in 10–20% polyacrylamide. Protein and peptide standards (MARK 12, Novex) were run on these gels to determine the sizes of the radiolabeled peptides obtained from each protease digestion. The location of the radiolabeled peptide was determined by subjecting the gels to autoradiography, while the standards were detected by Coomassie Blue staining. The predicted molecular masses of proteolytic peptides from AGX1 were determined using a Wisconsin Package Program.

**Site-directed Mutagenesis of Amino Acid Residues of AGX1**—Site-directed mutagenesis was performed on a pGEX-4T-2 plasmid with full-length AGX1 cDNA using a Quickchange Site-directed Mutagenesis Kit (Stratagene), according to the manufacturer’s instructions. Codons for Pro222, Gly223, Gly224, Tyr227, Gly111, and Arg115 were mutated to alanine by using the appropriate oligonucleotides.

**Western Blot Analysis**—Western blot analysis was done using standard technology. Wild type and mutant AGX1s were purified as described previously (5). Equal amounts of the various proteins were subjected to SDS-PAGE on 10% gels. The proteins from these gels were transferred electrophoretically to nitrocellulose membranes, and sequentially blotted.

**Measurements of Enzyme Activities and Determination of Km and Vmax**—Assays for UDP-GlcNAcPP and UDP-GalNAcPP activities were usually done in the forward direction (formation of UDP-HexNAc from HexNac-1-P and UTP). These assays were done with the various mutant proteins and the activities compared with that of purified wild type AGX1. The incubation mixtures contained the following components in a final volume of 100 μl: 50 mM Tris buffer, pH 7.5, 5 mM MgCl2, 2 mM [3H]UTP (55,000 cpm), 5 mM GlcNAc-1-P or GalNAc-1-P, and various amounts of the protein to be tested. After an incubation of 10 min at 37 °C, the incubations were terminated by heating at 100 °C for 30 s, and the mixture was applied to a column of DE-52. The UDP-HexNac were eluted with 70 mM (NH4)HCO3, could be readily separated from UTP which required much higher concentrations of bicarbonate for elution. Thus, the amount of radioactivity in the 70 μM elution was determined as a measure of activity.

Enzymatic activity could also be measured in the reverse direction. In this case, the incubation mixtures contained the following components in a final volume of 100 μl: 50 mM Tris buffer, pH 8.5, 5 mM MgCl2, 5 mM sodium pyrophosphate, 2 mM UDP-[3H]GlcNAc (or GalNAc) (35,000 cpm), and various amounts of the proteins to be tested. After an incubation of 10 min at 37 °C, the reactions were stopped by the addition of 0.5 ml of 5% trichloroacetic acid, and 0.5 ml of acid washed charcoal was added to absorb the nucleotides. The charcoal was removed by centrifugation, and the amount of radioactivity in the supernatant liquid was determined as a measure of the amount of HexNAc-1-P produced. Kinetic parameters, i.e. Km and Vmax/Km, were determined from Lineweaver-Burk plots obtained by varying the concentration of UDP-GlcNAc from 0.1 to 1 mM, and the concentration of UDP-GalNAc from 0.5 to 5 mM. In these experiments, the concentration of sodium pyrophosphate was kept constant at 5 mM.
RESULTS AND DISCUSSION

Identification of a Peptide Involved in Binding UDP-HexNAc—Recombinant AGX1 was incubated with either N$_2$-UDP-[32P]GlcNAc or N$_2$-UDP-[32P]GalNAc for 30 s and the mixtures were exposed to short wave UV light to activate the azido group. The protein was isolated and separated on SDS gels, and the amount of label was determined by exposure to film. The data in Fig. 1 indicate that the photo cross-linking of either of these probes (see Fig. 1, A and B) to the recombinant AGX1 increased with increasing concentrations of probe, and furthermore, this incorporation was saturable (not shown in this figure).

As shown in Fig. 2, the photo cross-linking of N$_2$-UDP-[32P]GlcNAc to AGX1 (lane 2) was inhibited in a concentration-dependent manner by adding increasing amounts of unlabeled UDP-GlcNAc (lanes 3–5) to the incubation mixtures. Photoincorporation of the N$_2$-UDP-[32P]GlcNAc probe was also inhibited by increasing amounts of unlabeled UDP-GalNAc (lanes 6–8). However, the amount of UDP-GalNAc required for inhibition of cross-linking was about 10 times greater than the amount of UDP-GlcNAc. These data are in agreement with the $K_m$ values for UDP-GlcNAc and UDP-GalNAc, and they strongly indicate that these photoaffinity probes are recognized as substrate analogs by the active UDP-GalNAcPP (AGX1). Similar results on cross-linking specificity were observed when the N$_2$-UDP-[32P]GalNAc probe was tested (data not shown). As expected since it is also a substrate, cross-linking of the azido probe was also inhibited by increasing amounts of UTP (lanes 9–11).

Since these photoprobes cross-linked in a specific manner to AGX1, it seemed likely that they would be useful as reagents to specifically label the substrate-binding site. Thus, large scale incubations were done with AGX1 and either N$_2$-UDP-[32P]GlcNAc or N$_2$-UDP-[32P]GalNAc, and after exposure to UV light, the two labeled proteins were isolated by centrifugation. Each protein was divided into four equal aliquots and each aliquot was digested with a specific proteases. Fig. 3A shows the results of an overnight digestion of UDP-GlcNAc-labeled enzyme...
with the various proteases (i.e. Lys-C, Asp-N, or V8). After a long incubation with each protease, most of the protein was digested to smaller peptides (as evidenced by Coomassie Blue staining), and only one or a few of these peptides were radio-labeled as shown in the figure. The resulting peptides were separated on SDS/Tricine gels and their migration was compared with various peptide standards.

Since the site of action of each of the different proteases is known, and the amino acid sequence of AGX1 is also known, we can predict the probable peptide sequence that is cross-linked, based on the size of the resulting radiolabeled peptides in each incubation and the overlapping sequences of the peptides produced by each protease (9, 10). The rationale for identifying the substrate-binding region by photolabeling is outlined in Fig. 3B and was used to identify overlapping radiolabeled peptides released from AGX1 by different proteases. The primary amino acid sequence of AGX1 was used to predict the molecular masses of peptides that would be released by each protease.

For example, the UDP-GlcNAc cross-linked AGX1 was digested overnight with Lys-C to give labeled peptides of 7.9, 6.3, and 5.0 kDa. Using the amino acid sequence of AGX1 to derive predicted peptide masses produced by Lys-C, the only peptide of 7.9 that could give rise to peptides of 6.3 and 5.0 kDa was in the region of the protein from amino acids 208–278. Likewise, the only peptide of 6.3 kDa that would yield the 5.0-kDa peptide was in the region from amino acid 216 to 271, by loss of a 1.49-kDa peptide by Lys-C action. The critical region could be further refined using V8 and Asp-N proteases. Thus Asp-N gave labeled peptides of about 11.2, 8.0, 6.5, and 5.5 kDa, with the 5.5-kDa peptide being the major species. The only peptide of 8.0 kDa that could give rise to a peptide of about 6.5 and 5.5 was in the region from amino acid 205 to 273. The 5.5-kDa peptide resulting from this digestion would be between amino acids 205 and 252. Finally, by the same analysis with V8 protease, it was deduced that the 5.7-kDa peptide must be between amino acids 190 and 240. Thus, the smallest peptide that could carry the labeled photoprobe and be compatible with the peptides produced by these three proteases would have to be between residues 216 and 240. The amino acid sequence of this peptide is presented in Fig. 4, and is from Val216 to Glu240. Essentially similar results, and the same peptide sequence, were obtained when labeling was done with N6-UDP-[32P]GalNAc (data not shown).

Fig. 4 also compares this peptide sequence with sequences from other related UDP-sugar pyrophosphorylases. This data indicates that there are a number of residues that are highly conserved, and thus it was important to determine whether modification of any of the conserved amino acids would affect the activity of the mutant protein to synthesize UDP-GlcNAc and UDP-GalNAc. We were also interested in knowing whether such changes would affect the ability of the protein to bind the UDP-GalNAc substrates.

Mutant proteins were produced by site-directed mutagenesis in which selected amino acids were modified to alanines. Alainine was chosen since it does not prevent or disrupt formation of Ω-helix or β-pleated sheet secondary structures (11). Thus, we chose to modify Pro220, Gly222, Gly224, and Tyr227. The results of these alterations on the ability of these proteins to catalyze the synthesis of either UDP-GlcNAc or UDP-GalNAc are shown in Fig. 5 (A and C). In these incubations, each mutant protein was incubated with either GlcNAc-1-P (Fig. 5A) or GalNAc-1-P (Fig. 5C) and [3H]UTP, and the formation of UDP-GlcNAc or UDP-GalNAc was determined. It can be seen that mutation of Gly222 resulted in an almost complete loss in the ability of that mutant protein to synthesize the corresponding UDP-HexNAc, whereas change of Gly224 resulted in considerable, but not complete loss in activity. On the other hand, mutation of Pro220 to alanine resulted in only a slight decrease in activity, while change of Tyr227 to alanine did not greatly affect the ability of that mutant protein to synthesize the UDP-HexNAcs. It should be noted that all of these comparisons of enzymatic activity were on the basis of specific activity measurements with the purified mutant AGX1s.

Similar results were obtained when these various mutant UDP-GlcNAcPPs were assayed in the reverse direction. These results are presented in Fig. 5 (B and D). In this case, the Gly224 mutant appeared to retain more activity with UDP-GalNAcPPs than with UDP-GalNAc. However, the results were generally similar with either the forward or the reverse assay.

In order to determine whether these changes in activity were due to differences in the ability of the mutant enzymes to bind

![Fig. 4. Predicted sequence of peptide of AGX1 labeled by affinity probes and comparison with other known sequences.](image)

**Fig. 5.** Enzymatic activity of various mutant AGX1 preparations made by site-directed mutagenesis of conserved amino acids. Recombinant proteins were prepared and pyrophosphorylase activity was tested in the forward direction with GlcNAc-1-P (A) or GalNAc-1-P (C) + UTP, and in the reverse direction with UDP-GlcNAc (B) or UDP-GalNAc (D) + inorganic pyrophosphate, as described under "Experimental Procedures." Mutations are proline to alanine (P220A), glycine to alanine (G222A or G224A), and tyrosine to alanine (Y227A).

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**Identification and Modification of UDP-GalNAcPP**

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![Image](image)
the UDP-HexNAc substrates, the $K_m$ values of the mutant proteins for UDP-GlcNAc were determined, and are presented in Table I. Mutants P220A and Y227A had $K_m$ values that were essentially the same as those of the wild type AGX1 (approximately 0.8 mM), whereas G224A showed a $K_m$ of about 2.2 mM and G222A had such a low binding affinity that it was not possible to obtain an accurate $K_m$ value. Because the determination of $K_m$ values reflect both substrate binding and catalysis, the apparent decreases in UDP-GlcNAc affinity could actually be due to changes in catalytic activity, binding affinity, or both.

In order to be certain that the mutated amino acid changes were really affecting binding rather than catalysis, we sought to separate these two steps. One way to determine relative binding affinities without involving the catalytic step is to use the photoaffinity substrates (i.e. N$_3$-UDP-$[^{32}P]$GlcNAc and N$_3$-UDP-$[^{32}P]$GalNAc), and to evaluate their ability to label the mutant proteins. However, prior to doing the cross-linking studies, it was important to be certain that each recombinant mutant protein was produced at the same rate in the various E. coli strains. Fig. 6 demonstrates, by SDS-gel electrophoresis and Western blotting, that each of the AGX1 recombinant proteins reacted equally well with the polyclonal antibody prepared against the purified pig liver UDP-HexNAcPPs suggesting that the single amino acid changes did not grossly affect protein conformation. In addition, each of the E. coli strains, with the exception of the G224A strain, grew at about the same rate, and produced approximately the same amount of recombinant AGX1 (data not shown). These findings indicate that changes in Pro$^{220}$, Gly$^{222}$, or Tyr$^{227}$ do not affect synthesis, targeting, or turnover of the corresponding AGX1s. In the case of mutation G224A, that E. coli strain grew significantly slower and had only about 25% of the amount of recombinant protein found in the other strains. We do not know at this time why this particular amino acid substitution affects production of the protein, but it is clear from other data presented here that substitution of glycine 224 with alanine also greatly affects the catalytic site had been altered. Mio et al. (8) had previously reported that modification of Gly$^{112}$ to alanine resulted in loss of catalytic activity. We confirmed that modification of Gly$^{111}$ to alanine in the AGX1 sequence did cause almost complete loss of catalytic activity. We confirmed that modification of Gly$^{111}$ to alanine in the AGX1 sequence did cause almost complete loss of catalytic activity.

Fig. 7 demonstrates that the results of labeling with the photoprobe corresponded well with the activity measurements shown in Fig. 5, as well as with the $K_m$ measurements presented in Table I. That is, very little binding of the UDP-GlcNAc probe (Fig. 7A), or of the UDP-GalNAc probe (Fig. 7B), was detected with the G222A or the G224A mutant proteins (lanes 3 and 4). On the other hand, the other two mutant proteins, P220A (lane 2) and Y227A (lane 5), did bind the probe, although labeling was significantly lower then with the wild type AGX1 (lane 1).

We also examined the binding of N$_3$-UDP-$[^{32}P]$GlcNAc to two other mutant proteins in which amino acids at the proposed catalytic site had been altered. Mio et al. (8) had previously reported that modification of Gly$^{112}$ to alanine resulted in loss of catalytic activity. We confirmed that modification of Gly$^{111}$ to alanine in the AGX1 sequence did cause almost complete loss of catalytic activity, and the data in Table I indicate that the $K_m$ for UDP-GlcNAc could not be measured with this mutant en-

### Table I

| Enzyme    | $K_m$ (mM) |
|-----------|------------|
| AGX-1     | 0.7        |
| P220A     | 0.8        |
| G222A     | 2.2        |
| G224A     | 0.8        |
| Y227A     | 0.8        |

![Image](image1.png)

**Fig. 6.** Western blot analysis to determine cross-reactivity and amount of recombinant mutant proteins produced in various E. coli cultures. One $\mu$g of purified AGX1, or the mutant proteins, were subjected to SDS-PAGE. The proteins were transferred to nitrocellulose membranes and blotted with anti-AGX1 antibodies. Lane 1 is AGX1, while lanes 2–5 are P220A, G222A, G224A, and Y227A.

![Image](image2.png)

**Fig. 7.** Photoaffinity labeling of AGX1 and mutant proteins with N$_3$-UDP-[$^{32}$P]GlcNAc. Each recombinant AGX1 (5 $\mu$g of protein) was incubated with radioactive probe, exposed to UV light, and subjected to SDS-PAGE. Gels were exposed to film to visualize radioactive proteins. Lane 1 is wild type AGX1 + probe. Other lanes represent the mutant proteins; i.e. lane 2, P220A; lane 3, G222A; lane 4, G224A; lane 5, Y227A.

![Image](image3.png)

**Fig. 8.** Photoaffinity labeling of AGX1 and mutant proteins with N$_3$-UDP-[$^{32}$P]GlcNAc. Each recombinant AGX1 (5 $\mu$g of protein) was incubated with the radioactive probe, exposed to UV light, and subjected to SDS-PAGE. Gels were exposed to film to visualize radioactive proteins. Lane O, AGX1 without exposure to UV; lane 1, AGX1 with exposure to UV. In all other lanes, the incubations were exposed to UV light, i.e., lane 2, P220A; lane 3, G222A; lane 4, G224A; lane 5, Y227A; lane 6, G111A; lane 7, R115A.
zyme. However, as shown in Fig. 8 (lane 6), this modification did not prevent the cross-linking of the N₅-UDP-[³²P]GlcNAc probe. These results indicate that Gly¹¹¹ is somehow involved in, or necessary for, catalysis, but is not directly involved in the uridine-binding site. On the other hand, modification of Arg¹¹⁵ to alanine did not affect the catalytic activity of the AGX1 mutant proteins, nor the $K_m$ for UDP-GlcNAc (see Table 1), although it did affect the cross-linking of the probes to a significant degree (Fig. 8, lane 7). In this particular experiment, Y227A did not label with the probe. That is probably an operational error since in most other experiments, this mutant enzyme did become labeled (for example, see Fig. 7, lane 5).

The studies described in this report were designed to use the photoaffinity substrate analogs as tools to identify the peptide that becomes cross-linked, and is therefore presumed to be involved in binding that region of the substrate. The proteolitic mapping methodology to do such studies was developed by the Drake laboratory to isolate the binding site for TMP in the HSV-1 thymidine kinase (HSV-1 TK). In that study, the investigators used the thymidylate analog [³²P]5-azido-dUMP, and were able to localize the photoprobe to a 20-amino acid peptide from Ile¹¹² to Tyr¹³².²

Evidence that the photolabeling methodology does give reliable and valuable information about the binding site was shown in those studies by the fact that several amino acids in that peptide were subsequently shown to interact with the 5-position of deoxypyrimidines in x-ray crystal structures of HSV-1 TK (12, 13). Specifically, Gln²²⁵ was shown to hydrogen bond with the N-3 and O-4 atoms of the thymine base. Unpublished studies have shown that mutation of this Gln²²⁵ residue results in loss of thymidine kinase activity and minimal photoincorporation of 5-azido-dUMP photoprobe.²

The mapping technique has also been successful in identifying the AZT-monophosphate-binding site on HIV-1 integrase (10). In a study using the analog AZT-monophosphate, the AZT-monophosphate-binding site of HIV-1 integrase was elucidated and confirmed by molecular modeling and site-directed mutagenesis. In all of these studies, including the one reported here, the assumption is that the azido probe, when activated, will covalently cross-link to a functional group of one amino acid in the substrate-binding site. Thus, identification of the peptide containing the labeled probe will determine at least part of the substrate-binding region. That assumption appears to also be valid in the experiments described in our report, since site-directed mutagenesis of several of the highly conserved amino acids in the cross-linked peptide has dramatic effects on binding of substrate and catalytic activity.

The peptide identified in our study and proposed to be the uridine-binding site had considerable homology to sequences in various other uridine diphosphate sugar pyrophosphorylases, such as the UDP-GlcFPs from yeast and bovine and human liver, and also the UDP-GlcNAcPP from yeast. These sequences showed considerable similarity in the type of amino acid in a specific position, as well as in a number of identical amino acids. Conversion of either of two glycines, i.e. Gly²²² and Gly²²⁴, to alanine resulted in almost complete loss of catalytic activity as well as loss of most of the ability of these mutant proteins to cross-link with the probes. It is possible that Gly²²² or Gly²²⁴ could be the actual site of cross-linking. However, it is much more likely that these glycines provide critical conformational roles for orienting neighboring or adjacent amino acids into the uridine binding pocket. As shown in Fig. 4, the resulting structural pocket formed by these glycines is highly conserved. Thus, substitution of alanine residues must confer new steric and rotational constraints that alter the conformation of this pocket and prevent binding of the UDP-HexNAc substrate. Interestingly, mutation of another amino acid that could serve as a conformational determinant, i.e. Pro²³⁰, had minimal effects on substrate binding and enzymatic activity.

The fact that both the UDP-GalNAc probe and the UDP-GlcNAc probe gave the same labeled peptides from each protease digestion is strong evidence that this cross-linking does involve the substrate-binding site, and that it is specific for the uridine portion of the molecule. It will be important to develop other photoaffinity probes where the cross-linker is attached to the sugar rather than the nucleotide base so that amino acids involved in sugar recognition can be identified. We would expect to see a difference in the nature of the peptide that becomes labeled when the cross-linking is due to the sugar portion of the substrate.

Sequence comparisons by Mio et al. (8) indicated that there was another region among UDP-sugar pyrophosphorylases that was highly conserved, and for many of the UDP-GlcNAcPPs this region was generally from amino acid 105 to amino acid 132. Substitution of alanine for Gly¹¹¹, Arg¹¹⁶, or Lys¹²³ greatly diminished enzymatic activity of the yeast UDP-GlcNAcPP. These investigators suggested that Gly¹¹² could be a binding site for glucose-1-P because this mutation caused an increase in the $K_m$ value for glucose-1-P, but did not greatly affect the affinity for UDP. These results are interesting in relation to our studies, as reported here. We modified Gly¹¹¹ in our sequence and found that the mutant enzyme lost almost all enzymatic activity, but was still able to cross-link the photoaffinity probe, N₅-UDP-[³²P]GlcNAc, to some extent. Since this probe is cross-linked via its nucleoside base, our results are complimentary to those of Mio and co-workers (8) and suggest that at least part of the uridine recognition region is at or near Gly²²² and Gly²²⁴, whereas the sugar-binding region is probably near Gly¹¹¹ or Gly¹²². Once we have a UDP-HexNAc with the photoactivatable group on the sugar, we will be able to directly test this hypothesis.

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