Identification of Lysyl Residues Located at the Substrate-Binding Site in UDP–Glucose Pyrophosphorylase from Potato Tuber: Affinity Labeling with Uridine Di- and Triphosphopyridoxals†

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Received March 19, 1991; Revised Manuscript Received June 4, 1991

ABSTRACT: Uridine di- and triphosphopyridoxals were used to probe the substrate-binding site in potato tuber UDP–glucose pyrophosphorylase (EC 2.7.7.9). The enzyme was rapidly inactivated in time- and dose-dependent manners when incubated with either reagent followed by reduction with sodium borohydride. The inactivations were almost completely retarded by UDP–Glc and UTP but only slightly by α-D-glucose 1-phosphate. The complete inactivation corresponded to the incorporation of about 0.9–1.0 mol of either reagent per mole of enzyme monomer. Both reagents appear to bind specifically to the UDP–Glc-(UTP)-binding site. Structural studies of the labeled enzymes revealed that the two reagents modified the identical set of five lysyl residues (Lys-263, Lys-329, Lys-367, Lys-409, and Lys-410), in which Lys-367 was most prominently modified. The ratios of the amounts of labels incorporated into these residues were similar for the two reagents. Furthermore, linear relationships were observed between the residual activities and the amounts of incorporation into each lysyl residue. We conclude that the five lysyl residues are located at or near the UDP–Glc(UTP)-binding site of potato tuber UDP–Glc pyrophosphorylase and that the modification of these residues occurs in a mutually exclusive manner, leading to the inactivation of the enzyme.

Previously, we used UDP-PL,[1] a reactive analogue of UDP–Glc, to probe the active site in rabbit muscle glycogen synthase (Tagaya et al., 1985). This reagent inactivated the enzyme in accordance with the formation of a Schiff base. The enzyme was almost completely protected from inactivation by UDP–Glc. The label was bound to a lysyl residue in the sequence Glu-Val-Ala-Asn-Lys-Val-Gly-Gly-Ile-(Tyr). Mahrenholz et al. (1988) later identified this residue as Lys-38. *Escherichia coli* glycogen synthase uses ADP–Glc, in place of UDP–Glc, as the glucosyl donor. We have demonstrated, by using adenosine diphosphopyridoxal, the presence of Lys-15 close to the bound ADP–Glc (Furukawa et al., 1990). Despite the fact that the mammalian and bacterial enzymes show no significant sequence homology (Kumar et al., 1986), the sequence Lys-X-Gly-Gly (X represents an unspecified amino acid residue) containing the labeled lysyl residue is conserved in the two glycogen synthases as well as waxy maize starch synthase (Kloesgen et al., 1986). These results suggested the importance of this sequence for sugar nucleotide binding.

Walker et al. (1982) reported that the sequence Gly-X-X-X-X-Gly-Lys-Thr(or Ser) is conserved in many ATP- and GTP-binding proteins. We have demonstrated that the conserved lysyl residues in adenylate kinase (Tagaya et al., 1987; Yagami et al., 1988), *E. coli* H^+-ATPase (Noumi et al., 1987; Tagaya et al., 1988a), and Ha-*ras* oncogene product p21 (Ohmi et al., 1988) were specifically labeled by adenosine or guanosine polyphosphopyridoxals. Therefore, the motif of a lysyl residue in the glycine-rich region is general as the structural element of polyphosphate-binding loci.

The primary structure of potato tuber UDP–Glc pyrophosphorylase (EC 2.7.7.9) (Katsube et al., 1990), however, contains neither a Lys-X-Gly-Gly nor a Gly-X-X-X-X-Gly-Lys-Thr sequence, although the enzyme uses UDP–Glc and UTP as substrates. To probe the substrate-binding site of this enzyme, we have presently applied UDP-PL and UTP-PL. Both reagents similarly inactivated UDP–Glc pyrophosphorylase but, unexpectedly, labeled the identical set of *five* different lysyl residues. Kinetic studies revealed that those residues are specifically labeled by either reagent, in a mutually exclusive manner. The following paper (Katsube et al., 1991) provides evidence for the importance of these lysyl residues, especially of Lys-367, in the function of this enzyme.

Experimental Procedures

Syntheses of UDP-PL and UTP-PL. UDP-PL was synthesized and purified as described previously (Tagaya et al., 1985). UTP-PL was synthesized from UDP and pyridoxal phosphate in essentially the same manner as UDP-PL. The crude UTP-PL synthesized was applied to a column (4.5 × 32 cm) of (aminoethyl)cellulose (Cl^- form). The material was eluted by 2000 mL of 10 mM HCl containing 10 mM LiCl at a flow rate of 8 mL/min. Fractions containing UTP-PL were pooled and neutralized with 1 N LiOH. The neutralized solution was concentrated to about 2 mL on a rotary evaporator below 25 °C. The concentrated solution was placed in a centrifugal tube, and 30 mL of cold acetone/methanol (4:1 by volume) was added at once. The precipitate collected by centrifugation was washed successively with acetone/methanol (4:1 by volume), acetone, and diethyl ether and dried in vacuo. After rechromatography on the same column, the trilithium salt of UTP-PL was dried in vacuo and stored at −20 °C. The yield was 38 mg.

Synthesis of ^{3}H-Labeled UDP-PL. To a freshly prepared UDP-PL solution (25 mg/3 mL) in 50 mM $KHCO_3$ (pH 8.0) was added 1 mL of [^{3}H]$NaBH_4$ (New England Nuclear, 10

† This investigation was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan and a grant from the Research Program on "Creation of New Materials through Intelligent Design" of ISIR, Osaka University.
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[1] Abbreviations: UDP-PL, uridine diphosphopyridoxal (P^1-uridine-5′ P^2-pyridoxal-5′ diphosphate); UTP-PL, uridine triphosphopyridoxal (P^1-uridine-5′ P^3-pyridoxal-5′ triphosphate); HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid; HPLC, high-performance liquid chromatography.

0006-2960/91/0430-8541$02.50/0

Ci/mmol, 25 mCi/mL in 10 mM NaOH), and the mixture was kept at 0 °C for 1 h and further at room temperature for 15 min. To this solution was added 0.3 mL of a freshly prepared nonradioactive 0.1 M $NaBH_4$ solution, and the mixture was left at room temperature for 1 h. To this solution was added 80 mg of the activated MnO_2. The mixture was stirred for 2 h and then centrifuged to remove the MnO_2. The supernatant was applied to a column of (aminoethyl)cellulose (Serva). After successive washing with water and 3 mM HCl containing 5 mM LiCl, [^{3}H]UDP-PL was eluted with a linear gradient of 70 mL of 3 mM HCl containing 5 mM LiCl and 70 mL of 3 mM HCl containing 50 mM LiCl at a flow rate of 0.4 mL/min. Fractions of 2 mL each were collected in test tubes containing 0.1 mL of 0.5 M HEPES (pH 8.0). Appropriate fractions were pooled and concentrated to about 4 mL. Although UDP-PL prepared contained about 3% UDP–pyridoxine, this contamination did not produce any problem. The radioactive product was diluted with nonradioactive UDP-PL and stored at −20 °C. The specific activity was 43 000 cpm/nmol.

Purification of UDP–Glc Pyrophosphorylase. UDP–Glc pyrophosphorylase from potato tuber was purified as described previously (Nakano et al., 1989). Before use, the enzyme was passed through a column of Sephadex G-25 that had been equilibrated with 25 mM HEPES (pH 8.0). The concentration of the enzyme was spectrophotometrically determined by using the absorbance at 280 nm at 1 mg/mL of 0.49 (Nakano et al., 1989). A molecular weight of 51 900 (Katsube et al., 1990) was used for calculation.

Enzyme Assay. The assay mixture (0.74 mL) contained 50 mM Tris-HCl (pH 8.0), 7 mM $MgCl_2$, 2 mM UDP–Glc, 5 mM PP_i, 20 μM glucose 1,6-bisphosphate, 0.3 mM $NADP^+$, 10 mM NaF, and 1 unit each of phosphoglucomutase and glucose-6-phosphate dehydrogenase. The assay was started by the addition of PP_i, and the increase in the absorbance at 340 nm was continuously monitored with a spectrophotometer at 30 °C. One unit of the enzyme activity was defined as the amount of enzyme that produces 1 μmol of glucose 1-phosphate/min under the above conditions.

Preparation and Analysis of UDP-PL- or UTP-PL-Labeled Peptides. UDP–Glc pyrophosphorylase (10 μM, 7.3 mL) was incubated with 20 μM UDP-PL or UTP-PL at 20 °C for 30 min. The reaction was stopped by adding a freshly prepared solution of $NaBH_4$. The solution was kept at 0 °C for 30 min. The labeled enzymes thus prepared were carboxymethylated, extensively dialyzed, and lyophilized. It was suspended in 0.4 mL of 0.1 M Tris-HCl (pH 7.5) containing 8 M urea and then diluted with 0.4 mL of 0.1 M Tris-HCl (pH 7.5). Lysyl endopeptidase (Wako Pure Chemicals) was added to the turbid solution in a 1:50 (mol/mol) ratio of protease to substrate. Digestion was performed at 36 °C for 12 h. Labeled peptides were separated on a Gilson HPLC system equipped with a Vydac C4 reverse-phase column at a flow rate of 1.0 mL/min. The solvent used was 0.1% trifluoroacetic acid (A) and 0.095% trifluoroacetic acid containing 90% acetonitrile (B). The absorbance at 215 nm and fluorescence (excitation at 325 nm and emission at 390 nm) of effluents were continuously monitored. The amino acid composition was determined, after hydrolysis with 6 N HCl in an evacuated tube, on a Hitachi 835 amino acid analyzer using *o*-phthalaldehyde. The amino acid sequence was determined with an Applied Biosystems Model 477A protein sequencer linked with an Applied Biosystems Model 120A PTH analyzer.

Incorporation of Labels into Lysyl Residues. UDP–Glc pyrophosphorylase (10 μM, 400 μL) was incubated with 20 μM UTP-PL at 20 °C for various time intervals, and the reaction was stopped by adding $NaBH_4$. After the mixtures were kept for 30 min at 0 °C, residual activities were measured. The solutions were concentrated and washed with 2 mL of 5 mM Tris-HCl (pH 7.5) on Amicon Centricon-30 cartridges three times. The concentrated solutions were then lyophilized. The labeled enzymes were suspended in 25 μL of 0.1 M Tris-HCl (pH 7.5) containing 8 M urea and diluted with 25 μL of 0.1 M Tris-HCl. To this solution was added 1 μg of lysyl endopeptidase. Digestions were performed at 36 °C. The digests were separated by HPLC as described above. The amounts of peptide eluted were determined by amino acid analysis. The amounts of UTP-PL incorporated were calculated from the areas of fluorescent peaks.

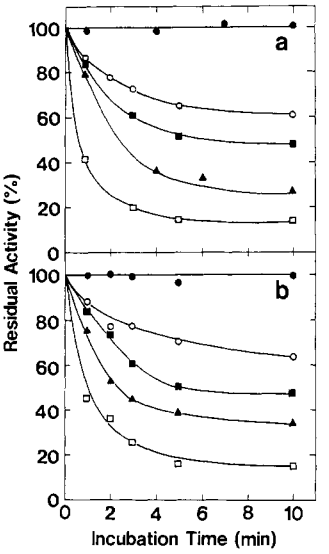

FIGURE 1: Time-courses of inactivation of UDP–Glc pyrophosphorylase by UDP-PL (a) and UTP-PL (b). The inactivation mixtures (200 μL) containing 50 mM HEPES, pH 8.0, 10.4 μg of enzyme, and either reagent at 0 μM (●), 1 μM (○), 3 μM (■), 5 μM (▲), or 10 μM (□) were incubated at 20 °C. At the indicated times, aliquots were withdrawn, reduced by 5 mM sodium borohydride, and diluted with 25 mM Tris-HCl, pH 8.0, and the residual activity was assayed.

RESULTS

Inactivation by UDP-PL and UTP-PL. Potato tuber UDP–Glc pyrophosphorylase was rapidly inactivated when incubated with low concentrations (1–10 μM) of either UDP-PL or UTP-PL followed by reduction with sodium borohydride. The time courses of inactivation by both reagents at various concentrations were quite similar (Figure 1). The absorbances at 430 nm in the difference spectra between the enzyme plus the reagent and the reagent alone increased in accordance with the inactivations by these reagents without sodium borohydride reduction (data not shown), suggesting the formation of a Schiff base (Shapiro et al., 1968). The addition of 5 mM $MgCl_2$ to the reaction mixtures showed no effect on inactivation.

To see the reason why the inactivations ceased after a certain period of time, the enzyme was first incubated with 15 μM UDP-PL at 20 °C for 30 min and reduced with sodium borohydride (the residual activities were 10–20% of the original). After removal of the reagent by centrifugal gel filtration (Penefsky, 1977), the enzyme was reincubated with the same concentration of the reagent at 20 °C for 30 min. The resultant enzyme displayed less than 5% of the original activity. These results can be interpreted in terms of the presence of an equilibrium between the Schiff base and free aldehyde of

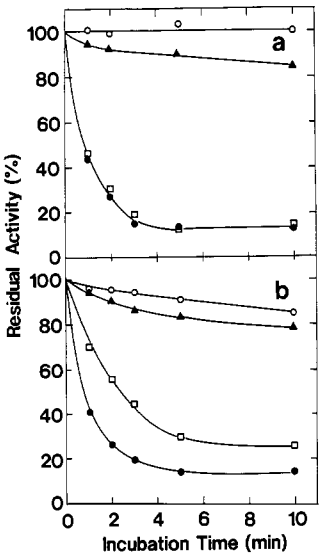

FIGURE 2: Effect of substrates on the inactivation of UDP–Glc pyrophosphorylase by UDP-PL (a) and UTP-PL (b). The inactivation mixtures (200 μL) containing 25 mM HEPES, pH 8.0, 10.4 μg of enzyme, and 20 μM either reagent (●) plus 2 mM UTP (○), 2 mM UDP–Glc (▲), or 2 mM glucose-1-P (□) were incubated at 20 °C. At the indicated times, aliquots were withdrawn, reduced by 5 mM sodium borohydride, and diluted with 25 mM Tris-HCl, pH 8.0, and the residual activity was assayed.

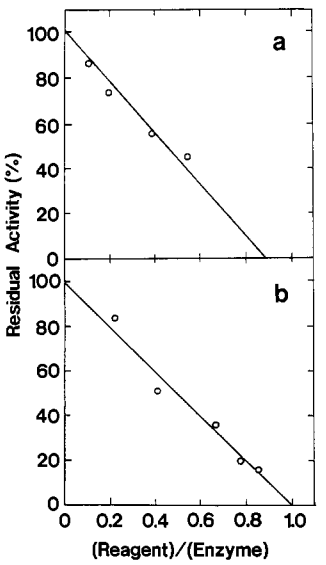

FIGURE 3: Relationship between the inactivation of UDP–Glc pyrophosphorylase by UDP-PL (a) and UTP-PL (b) and the amount of labels incorporated into the whole enzyme. Lines were drawn according to the method of least squares.

the pyridoxal moiety of the reagent in the enzyme.

The protective effect by substrates was investigated to elucidate where the reagents were bound. The enzyme was incubated with 20 μM UDP-PL or UTP-PL in the presence of 2 mM substrate. Similar patterns of protection were observed in the inactivations by both reagents (Figure 2); UTP and UDP–Glc almost completely protected the enzyme from inactivation, whereas glucose 1-phosphate offered little, if any, protective effect. These results suggest the binding of the reagents to the UDP–Glc(UTP)-binding site in the enzyme. The small effect of glucose 1-phosphate can be explained by the kinetic model in which glucose 1-phosphate is bound to the enzyme after UTP (Nakano et al., 1989).

Stoichiometry of Inactivation. The stoichiometry of the inactivation by UDP-PL was investigated by using the ^{3}H-labeled reagent. The enzyme was incubated with 20 μM [^{3}H]UDP-PL at 20 °C for 0.5, 1, 2, and 5 min, and the inactivation reaction was terminated by the addition of 5 mM sodium borohydride. The inactivation mixtures were applied to the gel filtration columns (Penefsky et al., 1977). The radioactivities, residual activities, and protein concentrations in the passed-through fractions were measured. The residual activity was plotted against the amount of UDP-PL bound to the enzyme (Figure 3a). Extrapolation of the line to zero activity indicated that the stoichiometry of inactivation is 0.9:1. When the enzyme was incubated with 20 μM [^{3}H]UDP-PL in the presence of 2 mM UTP, it was inactivated by 18% with the incorporation of 0.11 mol of the label/mol of enzyme monomer, confirming the specific labeling of the enzyme by the reagent.

To determine the stoichiometry of the inactivation by UTP-PL, the enzyme was modified with the reagent in a similar manner. The fluorescences derived from the pyridoxyllysine moiety (excitation at 320 nm; emission at 390 nm), residual activities, and protein concentrations were measured. The residual activity was plotted against the amount of the bound reagent (Figure 3b). Extrapolation of the line to zero activity indicated that the stoichiometry of inactivation by UTP-PL is 1.0:1.

Table I: Structures of UDP-PL-Labeled Peptides[a]

peak	sequence	positions	labeled site
I	T-L-A-D-V-X-G-G-T-L-I-S-Y-E-G-K	258–273	K-263
II	M-E-I-I-P-N-P-X-E-V-D-G-V-K	322–335	K-329
III-1	X-V-A-N-F-L-G-R	410–417	K-410
IV-1	S-N-P-S-N-P-S-I-E-L-G-P-E-F-X-K	395–410	K-409
V-1	L-P-V-X-A-T-S-D-L	364–372	K-367
VI-1	L-P-V-X-A-T-S-D-L	364–372	K-367

[a] One-letter abbreviations are used. X represents an unidentified residue that was concluded to be the labeled lysyl residue. Positions are in the complete sequence of potato tuber UDP–Glc pyrophosphorylase (Katsube et al., 1990).

Identifications of Labeled Sites. To identify the lysyl residue(s) labeled by UDP-PL or UTP-PL, the enzyme was modified with either reagent followed by carboxymethylation and then cleaved by lysyl endopeptidase. The digest was separated by HPLC as described under Experimental Procedures. Fluorescent peptides were eluted as three major peaks accompanied by three minor peaks for each labeled enzyme. The elution profiles were reproducible, and the multiple fluorescent peaks did not result from the incomplete digestion of the labeled enzymes. The peaks were termed peaks I–VI for the UDP-PL-inactivated enzyme and peaks I′–VI′ for the UTP-PL-inactivated enzyme, in the order of elution. The two elution profiles were similar; peaks I–VI appeared to correspond to peaks I′–VI′, respectively. The peak I–VI peptides were analyzed as follows.

Peak I. Automated sequence analysis determined the structure of the peak I peptide as shown in Table I. Since the phenylthiohydantoin derivative of UDP-PL-labeled lysine was not detected in the sequence analysis (Tagaya et al., 1985), we concluded that the residue of cycle 6 was the labeled lysine.

Peak II. The peak II material was rechromatographed by HPLC using the ammonium acetate system. Automated sequence analysis of the purified peptide determined its structure as shown in Table I. The residue of cycle 8 was the labeled lysine.

Peak III. The peak III material was digested by trypsin (Millipore Corp.) and then purified by HPLC using the trifluoroacetic acid system. Only one fluorescent peak (peak III-1) was detected by HPLC. The structure of peak III-1

Table II: Relative Amounts of Labels Incorporated into the Five Lysyl Residues[a]

reagent	addition	residual activity (%)	relative amounts of labels (%)				
			K-263	K-329	K-367	K-409	K-410
UDP-PL	none	15	23	9	59	6	3
UDP-PL	1 mM UTP	60	23	13	52	9	3
UTP-PL	none	15	20	9	61	7	3
UTP-PL	1 mM UTP	53	24	11	51	10	4

[a] See text for the experimental procedure.

was determined as shown in Table I. The residue of cycle 1 was the labeled lysine.

Peak IV. The peak IV material was rechromatographed on a C18 column and then cleaved by trypsin. The digest was separated on a C18 column again. One major fluorescent peak (peak IV-1) was detected. The structure of the peak IV-1 peptide is shown in Table I. The label was on the residue of cycle 15.

Peaks V and VI. Direct sequence analyses of the peak V and VI peptides gave the same amino-terminal sequence. We interpreted that they were probably produced by heterogeneous cleavage by lysyl endopeptidase. Therefore, both peptides were further digested by chymotrypsin (Millipore Corp.) and subjected to separation by HPLC using the trifluoroacetic acid system. Two fluorescent peaks were detected in each peptide digest; peaks V-1 and V-2 and peaks VI-1 and VI-2. The peak V-2 peptide was again purified by HPLC using the ammonium acetate system. The structures of the peak V-1 and VI-1 peptides were the same as shown in Table I, whereas the peak V-2 and VI-2 peptides had another leucyl residue at the carboxyl terminus of the peak V-1 and VI-1 peptides, respectively.

Table I summarizes the structures of UDP-PL-labeled peptides as well as the labeled lysyl residues identified. The peak I′–VI′ peptides were similarly analyzed (data not shown). The combined results indicated that the two sets of five lysyl residues labeled by UDP-PL and UTP-PL were identical: Lys-263, Lys-329, Lys-367, Lys-409, and Lys-410.

Incorporation of Label into Each Lysyl Residue. The amounts of UDP-PL or UTP-PL incorporated into the five lysyl residues were calculated from the intensities of the fluorescence in peaks I–VI (Table II). Lys-367 was most prominently labeled, followed by Lys-263 and Lys-329. The labelings of Lys-409 and Lys-410 were small. At the residual activity of 15%, the patterns of labeling were almost the same for both reagents. Similar patterns were also observed in the presence of 1 mM UTP, showing no protection by substrate of particular lysyl residues.

The amounts of UTP-PL incorporated into each lysyl residue were determined at different periods of incubation time. The plot of the residual activity against the amounts of the label per enzyme monomer gave a set of straight lines for each lysyl residue (Figure 4). The relative amounts of labeling of the five lysyl residues were independent of the degree of inactivation of the enzyme, excluding the possibility of nonspecific labeling. Extrapolations of the lines to zero activity indicated that the amounts of label incorporated into Lys-367, Lys-263, Lys-329, Lys-409, and Lys-410 per enzyme monomer were 0.62, 0.24, 0.07, 0.04, and 0.03 mol/mol of enzyme monomer, respectively. A sum of these values is 1.00, showing that the modifications of the lysyl residues stoichiometrically proceed in total. These results suggest that UTP-PL labels the five different lysyl residues in a mutually exclusive manner, leading to the complete inactivation of the enzyme.

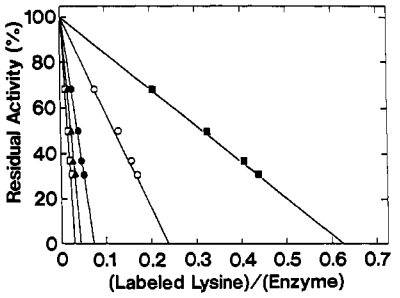

FIGURE 4: Relationship between the inactivation of UDP–Glc pyrophosphorylase by UTP-PL and the amount of labels incorporated into individual lysyl residues. The relative amounts of labels incorporated were calculated as the percentage of the total: K-263 (○), K-329 (●), K-367 (■), K-409 (▲), and K-410 (□).

Discussion

UDP-PL and UTP-PL modified potato tuber UDP–Glc pyrophosphorylase in a similar manner, contrary to our expectation. Preferential bindings of both reagents to the UDP–Glc(UTP)-binding site in the enzyme are shown by the following criteria. (i) The two reagents at low concentrations efficiently inactivated the enzyme in parallel with the formation of a Schiff base. (ii) The inactivation by each reagent was markedly protected by UDP–Glc and UTP. (iii) The binding of 0.9–1.0 mol of each reagent to 1 mol of enzyme monomer resulted in the complete loss of enzyme activity. Nonspecific labeling was not observed when the enzyme was modified in the presence of UTP. (iv) The inactivation showed saturation behavior in respect to reagent concentrations, showing that the reagent binds reversibly to the enzyme.

Surprisingly, the sequence analyses of the labeled peptides identified as many as five different lysyl residues (Lys-263, Lys-329, Lys-367, Lys-409, and Lys-410) as the labeled sites, even though the complete inactivation corresponded to the incorporation of 0.9–1.0 mol of either reagent per mole of the enzyme. Not only did both reagents modify the identical set of five different lysyl residues but also the ratios in the amounts of labels incorporated into these residues were almost the same for the two reagents. These results suggest that the five lysyl residues are located at or near the UDP–Glc(UTP)-binding site of the enzyme and that the modification of these residues occurs in a mutually exclusive manner.

Nucleotidyl derivatives of pyridoxal phosphate have been used as the affinity label for the nucleotide-binding site in various proteins. We identified a single lysyl residue as the major labeled site in the modification of rabbit muscle and *E. coli* glycogen synthases (Tagaya et al., 1985; Furukawa et al., 1990), rabbit muscle adenylate kinase (Tagaya et al., 1987; Yagami et al., 1988), rabbit muscle phosphorylase kinase γ-subunit (Tagaya et al., 1988b), and Ha-*ras* oncogene product p21 (Ohmi et al., 1988). On the other hand, labeling of more than one lysyl residue is not rare. In the F_1 of *E. coli* H^+-ATPase, Lys-201 in the α-subunit and Lys-155 in the β-subunit were preferentially labeled in the absence of Mg^{2+} (Noumi et al., 1987). In Ca^{2+}-transporting ATPase from sarcoplasmic reticulum, adenosine triphosphopyridoxal specifically labeled Lys-684 in the presence of Ca^{2+} but labeled Lys-684 plus Lys-492 in the absence of the metal ion (Yamamoto et al., 1988, 1989). Furthermore, Lys-103 and Lys-110 of the B-subunit were preferentially labeled in the modification of *E. coli* DNA gyrase (Tamura & Gellert, 1990). In *E. coli* methionyl- and valyl-tRNA synthetases, three different lysyl residues were labeled even though affinity labeling yielded a covalent 1:1 enzyme–reagent complex (Hountondji et al., 1990). Referring to the crystallographic structure, they dis-

cussed the roles of each lysyl residue in the binding of ATP to the enzyme.

Potato tuber UDP–Glc pyrophosphorylase is rich in lysyl residues (Katsube et al., 1990): 42 Lys, compared with 9 Arg and 5 His, in a total of 478 amino acid residues. In the complete amino acid sequence (Katsube et al., 1990), all of the labeled lysines are located in the carboxyl half. Only Lys-367 that was most prominently labeled is conserved in the equivalent position of the slime mold enzyme (Ragheb & Dottin, 1987), which is homologous to the potato enzyme. The sequences surrounding this lysyl residue are also highly conserved in the two enzymes. By contrast, the other four lysyl residues are not found in the corresponding positions of the slime mold enzyme. Even though there are 14 lysyl residues conserved in the two enzymes, none of them, except for Lys-367, were labeled by either reagent. These results suggest the particular importance of Lys-367 in the function of this enzyme.

In the following paper, Katsube et al. (1991) report the catalytic properties of the five mutant enzymes of potato tuber UDP–glucose pyrophosphorylase in which the above five lysyl residues were replaced individually by glutamine via site-directed mutagenesis. The Lys-367 → Gln enzyme was almost completely inactive, whereas the Lys-263 → Gln enzyme had significantly decreased V_{max} values with perturbed K_m values for pyrophosphate and glucose 1-phosphate. The Lys-329 → Gln enzyme also exhibited increased K_m values for these substrates but exhibited V_{max} values similar to those of the wild-type enzyme. The Lys-409 → Gln and Lys-410 → Gln enzymes showed catalytic properties similar to those of the wild-type enzyme. They concluded that Lys-367 is essential for catalytic activity of the enzyme and that Lys-263 and Lys-329 may participate in binding of pyrophosphate and/or glucose 1-phosphate.

Most of the sequences surrounding the five labeled lysines contain prolyl and/or glycyl residues nearby (Table I). Since these two residues favor a turn structure (Schulz & Schirmer, 1979), the labeled lysines might be located close to turns in the folding of the polypeptide chain of the enzyme. The secondary structure of this enzyme computed according to Chou and Fassman (1978) showed that Lys-329 and Lys-367 are located at the turn-favor region and that the rest are between an α-helix and a β-sheet. On the other hand, Rossman et al. (1974) found the common structure for the nucleotide-binding sites of lactate dehydrogenase and related enzymes. This fold has a $\beta\alpha\beta$ structure, and nucleotides bind to the edge of this structure. Lysyl residues that interact with a nucleotide should be located close to the turns in the Rossman fold. The deduced locations of the UDP-PL- and UTP-PL-labeled lysines in UDP–Glc pyrophosphorylase thus satisfy this requirement.

Acknowledgments

We thank Dr. Katsuyuki Tanizawa for his fruitful discussions and critical reading of the manuscript and Mr. Koji Furukawa for the synthesis of [^{3}H]UDP-PL.

Registry No. UDP-PL, 97654-04-1; UTP-PL, 135145-98-1; [^{3}H]-UDP-PL, 135145-99-2; UDP-Glc pyrophosphorylase, 9026-22-6; lysine, 56-87-1.

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