Expression in *Escherichia coli* of UDP–Glucose Pyrophosphorylase cDNA from Potato Tuber and Functional Assessment of the Five Lysyl Residues Located at the Substrate-Binding Site†

Takuya Katsube, Yasuaki Kazuta, Katsuyuki Tanizawa, and Toshio Fukui*
The Institute of Scientific and Industrial Research, Osaka University, Ibaraki, Osaka 567, Japan
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ABSTRACT: The entire structural gene for potato tuber UDP–glucose pyrophosphorylase has been amplified from its cDNA by the polymerase chain reaction and inserted into the expression plasmid pTV118-N downstream from the *lac* promoter. *Escherichia coli* JM105 cells carrying thus constructed plasmid produced the enzyme to a level of about 5% of the total soluble protein upon induction with isopropyl β-D-thiogalactopyranoside. The recombinant enzyme purified to homogeneity in two column chromatographic steps was structurally and catalytically identical with the enzyme purified from potato tuber except for the absence of an N-terminal-blocking acetyl group. To examine functional roles of the five lysyl residues that had been identified by affinity labeling studies to be located at or near the active site of the enzyme [Kazuta, Y., Omura, Y., Tagaya, M., Nakano, K., & Fukui, T. (1991) *Biochemistry* (preceding paper in this issue)], they were replaced individually by glutamine via site-directed mutagenesis. The Lys-367 → Gln mutant enzyme was almost completely inactive, and the Lys-263 → Gln mutant enzyme had significantly decreased V_{max} values with perturbed K_m values for pyrophosphate and α-D-glucose 1-phosphate. Lys-329 → Gln also exhibited increased K_m values for these substrates but exhibited V_{max} values similar to those of the wild-type enzyme. The two mutant enzymes Lys-409 → Gln and Lys-410 → Gln showed catalytic properties almost identical with those of the wild-type enzyme. Thus, among the five lysyl residues, Lys-367 is essential for catalytic activity of the enzyme and Lys-263 and Lys-329 may participate in binding of pyrophosphate and/or α-D-glucose 1-phosphate.

UDP–glucose pyrophosphorylase (UTP:glucose-1-P[1] uridylyl-transferase) [EC 2.7.7.9] catalyzes pyrophosphorolysis of UDP–glucose forming UTP and glucose-1-P and also its reverse reaction, the synthesis of UDP–glucose from UTP and glucose-1-P, according to the ordered bi–bi mechanism. We have purified the enzyme to homogeneity from potato tuber and studied its structural and catalytic properties (Nakano et al., 1989). The enzyme consisting of an approximately 50-kDa single polypeptide utilizes UDP–glucose, but not ADP–glucose, as substrate and is not activated by 3-phosphoglycerate, indicating that the enzyme is distinct from ADP–glucose pyrophosphorylase catalyzing a similar nucleotidyl transfer. More recently, we have also isolated the cDNA encoding the potato UDP–glucose pyrophosphorylase from a cDNA library of immature potato tuber and determined its nucleotide sequence (Katsube et al., 1990). Comparison of the primary structure with those in a protein data bank has revealed significant homology only with the same enzyme from slime mold (Ragheb & Dottin, 1987).

To further study the reaction mechanism and active site structure of the enzyme, the availability of a large amount of the pure enzyme is prerequisite. The purification in a large quantity, however, seemed arduous due to the very time-consuming procedure of the enzyme purification from potato tuber (Nakano et al., 1989). Thus, we undertook construction of an expression plasmid for the cloned cDNA of the potato enzyme (Katsube et al., 1990). In this article, we report the PCR-based construction of an expression plasmid, efficient production of the enzyme in *Escherichia coli* cells, and purification and characterization of the recombinant enzyme.

Taking advantage of the expression system constructed, we have also started exploring the active site residues in the enzyme by site-directed mutagenesis. The five lysyl residues (Lys-263, Lys-329, Lys-367, Lys-409, and Lys-410) that had been identified by our affinity labeling studies on the potato enzyme with reactive substrate analogues uridine di- and triphosphopyridoxals (Kazuta et al., 1991) were selected as target residues and replaced individually by glutamine. Among the five lysyl residues, Lys-367 has been found to be indispensable for the function of UDP–glucose pyrophosphorylase.

Experimental Procedures

Materials. Phosphoglucomutase (rabbit muscle) and UDP–glucose dehydrogenase (bovine liver) were obtained from Sigma; glucose-6-P dehydrogenase (yeast) was from Oriental Yeast; and recombinant *Taq* DNA polymerase (AmpliTaq) and plasmids pTV118-N and pTV119-N were from Takara Shuzo. DEAE-cellulose was the product of Brown, and phenyl-Toyopearl was the product of Tosoh. All other reagents were of the highest grade available.

Construction of Expression Plasmid. The entire region of the structural gene for the enzyme was first amplified by PCR (Mullis & Faloona, 1987) with the isolated cDNA (Katsube et al., 1990) as a template and the following two oligo-

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* The author to whom correspondence should be addressed.

[1] Abbreviations: glucose-1-P, α-D-glucose 1-phosphate; glucose-6-P, D-glucose 6-phosphate; PCR, polymerase chain reaction; IPTG, isopropyl β-D-thiogalactopyranoside; kbp, kilobase pair(s); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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nucleotides as primers, which were synthesized with an Applied Biosystems DNA synthesizer Model 381. The primers A1 and A2 corresponded to the sequences from nucleotide 130 to 149 and from nucleotide 1707 to 1685 (in the complementary sequence), respectively, in the cDNA structure (Katsube et al., 1990), and contained a new *Nco*I site (underlined), in which a mismatching base (asterisked) was included.

A1: 5′-CTTCTTCGCCATGGCTACTG-3′

A2: 5′-TTTGTATTGTATCCATGGTTCCA-3′

PCR was performed in a 100-μL solution containing 10 mM Tris-HCl (pH 8.9), 1.5 mM $MgCl_2$, 80 mM KCl, 0.5 mg/mL bovine serum albumin, 0.1% sodium cholate, 0.1% Triton X-100, 0.2 mM each deoxynucleotides (dATP, dGTP, dCTP, and dTTP), 1 μM each A1 and A2 primers, 0.5 ng of the cDNA in a λgt10 phage (clone *U4a*), and 5 units of *Taq* DNA polymerase with a program consisting of the cycle 1 (94 °C for 5 min, 50 °C for 2 min, and 72 °C for 4 min), cycles 2–26 (94 °C for 1 min, 50 °C for 2 min, and 72 °C for 4 min), and the last cycle (94 °C for 1 min, 50 °C for 2 min, and 72 °C for 20 min) in a thermal cyclic reactor (Hoei Science Co., Ltd., Tokyo, Model TC-100). The PCR product (about 1.6 kbp) was purified by filtration through a Centricon-100 cartridge (Amicon Corp.), digested with *Nco*I, and ligated into the expression vector pTV119-N. Although *E. coli* JM105 cells transformed with the resultant plasmid produced the enzyme efficiently on induction with IPTG (data not shown), nucleotide substitutions elicited during PCR were found at three positions when the entire region of the PCR product was sequenced by the dideoxy chain termination method (Sanger et al., 1977). Therefore, the 0.6-kbp *Nco*I–*Kpn*I fragment, in which all of the above three nucleotide substitutions were located, was then amplified by use of primer A1 and another primer A3 corresponding to the sequence from nucleotide 735 to 716 (in the complementary sequence), containing the unique *Kpn*I site (underlined).

A3: 5′-TGACCTGGAGGGTACCATCC-3′

PCR was carried out as described above, and the product was ligated into pTV118-N after double digestion with *Nco*I and *Kpn*I. The 1.0-kbp *Kpn*I-*Pst*I fragment excised from the first constructed plasmid was inserted into *Kpn*I and *Pst*I sites of the second constructed plasmid to yield the final plasmid designated as pTUG. The structure of pTUG was confirmed by restriction mapping and DNA sequencing.

Culture Conditions. *E. coli* JM105 cells carrying the expression plasmid pTUG were grown at 37 °C in 1.4 L of Luria broth (1.0% tryptone, 1.0% NaCl, 0.5% yeast extract) containing 50 μg/mL sodium ampicillin. After 2 h, IPTG was added to a final concentration of 0.5 mM, and the cultivation was continued for additional 8 h.

Purification of Recombinant Enzyme. Cells harvested by centrifugation (about 5 g, wet weight) were suspended in 50 mL of the buffer consisting of 5 mM Tris-HCl (pH 7.6), 0.2 mM EDTA, and 2 mM 2-mercaptoethanol (buffer A). After the cells were disrupted in a French pressure cell, the supernatant obtained by centrifugation was fractionated with ammonium sulfate. The proteins precipitated in 50–80% saturation of ammonium sulfate were dissolved in 10 mL of buffer A and dialyzed at 4 °C overnight against 4 L of the same buffer. The enzyme solution was applied to a DEAE-cellulose column (2 × 20 cm) previously equilibrated with buffer A and eluted with a linear gradient from 0 to 0.1 M NaCl in the same buffer (80 mL/80 mL each). Fractions with the enzyme activity were pooled and brought to 30% saturation of ammonium sulfate. The solution was applied on a phenyl-Toyopearl column (2 × 9 cm) equipped on a Pharmacia fast-protein liquid chromatography system and preequilibrated with 25 mM Tris-HCl buffer (pH 7.6) containing 1 mM EDTA and 30% saturation of ammonium sulfate. The enzyme was eluted with a 40-min linear gradient from 30 to 0% saturation of ammonium sulfate in the same buffer.

Table I: Sequences of Synthetic Oligonucleotide Primers for Site-Directed Mutagenesis

Sequence[a]	Corresponding mutant
260 265	
-Ala-Asp-Val-Lys-Gly-Gly-	(wild-type)
GCT GAT GTC AAA GGT GGC	
5′-CT GAT GTC *CAA GGT GGC-3′	Lys-263→Gln
326 331	
-Pro-Asn-Pro-Lys-Glu-Val-	(wild-type)
CCC AAC CCA AAG GAA GTG	
5′-CC AAC CCA *CAG GAA GTG-3′	Lys-329→Gln
364 369	
-Leu-Pro-Val-Lys-Ala-Thr-	(wild-type)
CTT CCC GTG AAA GCA ACT	
5′-TT CCC GTG *CAA GCA ACT-3′	Lys-367→Gln
406 412	
-Pro-Glu-Phe-Lys-Lys-Val-Ala-	(wild-type)
CCT GAA TTC AAG AAG GTG GCC	
5′-CCT GAA TTC *CAG AAG GTG-3′	Lys-409→Gln
5′-AA TTC AAG *CAG GTG GCC-3′	Lys-410→Gln

[a] The mutated codons are underlined and mismatched bases are shown by asterisks.

Enzyme and Protein Assays. In assays during enzyme purification and in the determination of K_m values for UDP-glucose and PP_i, the initial rates of glucose-1-P formation from UDP-glucose were measured spectrophotometrically by monitoring the NADPH formation at 340 nm, accompanying the enzyme-coupled conversion to 6-phosphogluconate through glucose-6-P, as described previously [*Assay A* in Nakano et al. (1989)]. In the determination of K_m values for UTP and glucose-1-P in the synthetic reaction, the enzyme activity was determined by the uncoupled UDP-glucose dehydrogenase method [*Assay C* in Nakano et al. (1989)]. The protein concentration was measured by the method of Bradford (1976) using UDP-glucose pyrophosphorylase purified from potato tuber as the standard, the concentration of which was calculated on the basis of its absorption coefficient (0.49) at 280 nm and at 1 mg/mL determined previously (Nakano et al., 1989).

Site-Directed Mutagenesis. The *Kpn*I–*Bam*HI fragment (0.7 kbp) in the expression plasmid pTUG, which contains the target region for site-directed mutagenesis, was subcloned into M13 mp19. *E. coli* BW313 (*dut*⁻ *ung*⁻) cells were transfected with the M13 phage, and the single-stranded phage DNA containing uracil was purified from the culture supernatant. The oligonucleotide primers were designed to be complementary to this single-stranded template DNA and to contain appropriate mismatches as summarized in Table I. Synthesis of mutant DNA and selection were performed by the method of Kunkel et al. (1987), using a commercial kit (Mutan-K, Takara Shuzo). Nucleotide sequences of the mutant genes obtained were confirmed by the dideoxy chain termination method (Sanger et al., 1977). The 0.7-kbp *Kpn*I–*Bam*HI fragments containing the mutated sites were excised from the double-stranded M13 mp19 phage DNA, ligated into the

Table II: Purification of Recombinant UDP–Glucose Pyrophosphorylase from *E. coli* Clone Cells

	total act. (units)	total protein (mg)	sp act. (units/mg)	purification (*x*-fold)	recovery (%)
crude extract	33 600	605	55.5	(1)	(100)
ammonium sulfate	22 700	158	143	2.6	68
DEAE-cellulose	13 100	21.8	600	10.8	39
phenyl-Toyopearl	11 700	9.7	1210	21.8	35

*Kpn*I–*Bam*HI sites of plasmid pTUG, and transformed into *E. coli* JM105 cells. Cell culture and purification of the mutant enzymes were done under the same conditions as those for the wild-type enzyme.

CD Measurement. CD spectra were measured at 25 °C in 50 mM Tris-HCl buffer (pH 7.5) containing 2 mM EDTA and 2 mM 2-mercaptoethanol with a Jasco J-600 spectropolarimeter. The instrument was calibrated with ammonium (+)-10-camphorsulfonate, $\Delta\epsilon = +2.37\ M^{-1}\ cm^{-1}$ at 290.5 nm. In the calculation of the mean residue ellipticity, $[\theta]$, the mean residue weight was taken to be 111.0 for the enzyme protein. The CD spectra were obtained at a protein concentration of 0.3 mg/mL in a 2.0-cm light pass length cell and 0.1 mg/mL in a 0.1-cm light pass length cell in the wavelength region above and below 250 nm, respectively.

Results

Construction of Expression Plasmid. We recently isolated a cDNA encoding UDP–glucose pyrophosphorylase from a cDNA library of immature potato tuber (Katsube et al., 1990). The nucleotide sequence around the initiation ATG codon was found to be consistent with the plant consensus sequence proposed by Heidecker and Messing (1986), which appeared unfavorable for the efficient gene expression in *E. coli*. Therefore, we initially tried to amplify by PCR the entire region of only the open reading frame in the cDNA, removing the 5′-upstream untranslated region and adding a new unique restriction site in the oligonucleotide primers to be used in PCR; synthetic oligonucleotides even containing mismatching bases for a template DNA can be used in PCR (Mullis & Faloona, 1987). The PCR product with an expected length of about 1.6 kbp (not shown) was ligated into the expression vector pTV119-N after digestion with *Nco*I (Figure 1). Of *E. coli* clones transformed with the hybrid plasmid, the one carrying the plasmid that was found to have been ligated in the correct orientation on restriction analysis showed appreciable UDP–glucose pyrophosphorylase activity in the cell-free extract [23 units/mg, cf. 3.7 units/mg in the extract of potato tuber (Nakano et al., 1989)]. However, when the entire region of the PCR-amplified fragment (1.6 kbp) was sequenced and compared with the cDNA sequence reported previously (Katsube et al., 1990), we found nucleotide substitutions at three positions, which were probably brought about by the imperfect fidelity of *Taq* DNA polymerase during PCR (Tindall & Kunkel, 1988); one was silent for amino acid residues [AAT (Asn-129) to AAC], but two were the mutations changing into other residues [CCA (Pro-66) to TCA (Ser) and ATT (Ile-153) to ACT (Thr)]. We purified this mutant enzyme to near homogeneity, but the enzyme was rapidly inactivated during the purification (data not shown). Thus, either or both Pro-66 and Ile-153 appeared to be important for stability of the enzyme and may be interesting as target residues for future site-directed mutagenesis studies.

Although the reason why these nucleotide substitutions were introduced in a relatively short region of the gene is unknown, we subsequently amplified by PCR only the 0.6-kbp *Nco*I–*Kpn*I fragment, in which all the above mentioned three nucleotide substitutions were included. The PCR product with an expected length was then doubly digested with *Nco*I and *Kpn*I and ligated into pTV118-N (Figure 1). DNA sequencing of the inserted fragment revealed the complete identity with the sequence of the corresponding region in the template cDNA (Katsube et al., 1990); no nucleotide substitutions were generated during PCR. Into *Kpn*I and *Pst*I sites of thus prepared plasmid, the 1.0-kbp *Kpn*I–*Pst*I fragment excised from the first constructed plasmid was finally inserted to form a plasmid designated pTUG (Figure 1). The construct of pTUG was again confirmed by restriction mapping.

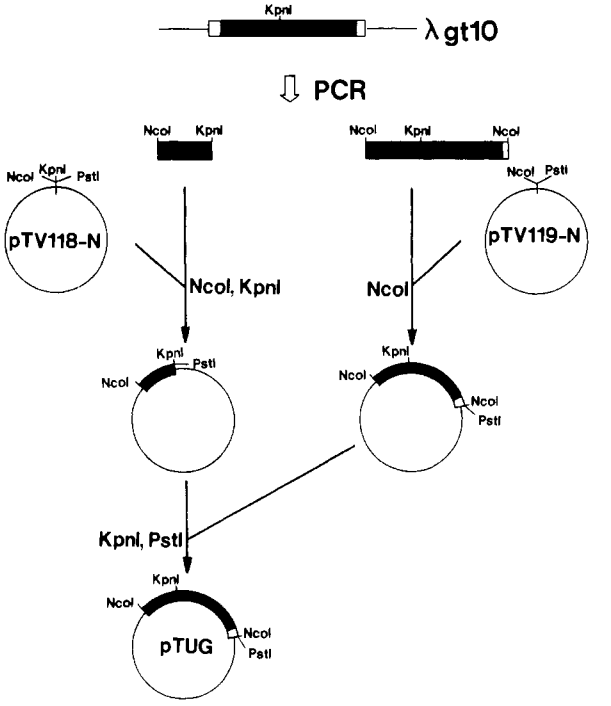

FIGURE 1: Construction scheme of the expression plasmid for potato UDP–glucose pyrophosphorylase. Only relevant restriction sites are shown. The closed and open areas represent the coding and noncoding regions, respectively, in the cDNA isolated (Katsube et al., 1990). See text for details.

Gene Expression in E. coli and Purification of the Recombinant Enzyme. A crude extract from *E. coli* JM105 cells transformed with pTUG and grown in the presence of IPTG exhibited a high level of UDP–glucose pyrophosphorylase activity (55.5 units/mg). On the basis of the specific activity of the purified enzyme (1210 units/mg, see below), the amount of enzyme in the cell extract corresponded to about 5% of that of the total soluble protein. The enzyme was found to be inducibly formed by addition of IPTG; in the absence of IPTG, the specific activity in the cell extract was only 11 units/mg. Thus, the *lac* promoter in the vector pTV118-N functioned efficiently for expression of the potato enzyme gene placed 3′-downstream. SDS–PAGE and following immunoblot analyses showed a protein band that was reactive with an antiserum raised against the purified UDP–glucose pyrophosphorylase from potato tuber and exhibited an electrophoretic mobility identical with that of the potato enzyme (Figure 2).

The efficient gene expression in *E. coli* enabled us to purify readily the recombinant enzyme by ammonium sulfate fractionation and merely two column chromatographic steps as

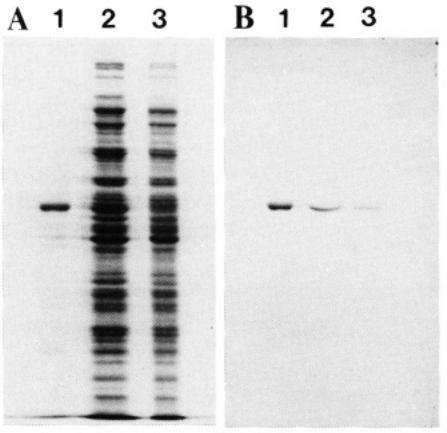

FIGURE 2: SDS-PAGE and immunoblot analyses of cell extracts from *E. coli* transformed with pTUG. Each sample was electrophoresed in duplicate on the same 10% gel slab according to Laemmli (1970). Half of the gel (A) was stained for proteins with Coomassie Brilliant Blue, and the other half (B) was electrotransferred to a nitrocellulose filter and subjected to immunoblot analysis with use of a rabbit antiserum raised against the purified enzyme from potato tuber (Nakano et al., 1989) and an anti-rabbit-IgG goat antibody conjugated with peroxidase (Bio-Rad). Lane 1: the purified enzyme from potato tuber (about 1 μg). Lane 2: a crude extract of the clone cells grown in the presence of IPTG (total protein, about 20 μg). Lane 3: a crude extract of the clone cells grown in the absence of IPTG (total protein, about 20 μg).

described under Experimental Procedures (Table II). The homogeneity of the purified enzyme was >95%, as judged from SDS-PAGE analysis, and its specific activity was even higher than that (899 units/mg) of the enzyme purified from potato tuber (Nakano et al., 1989). The purification from a 1.4-L culture of the cloned cells took only 2–3 days and provided about 10 mg of the pure enzyme, although further attempts to increase the final yield (35%) are needed.

Characterization of the Recombinant Enzyme. In contrast to the enzyme purified from potato tuber, the N-terminal residue (Ala) of which is blocked with an acetyl group (Katsube et al., 1990), the direct Edman degradation of the purified recombinant enzyme afforded unequivocally an N-terminal partial sequence of Ala-Thr-Ala-Thr-Thr-, indicating that its N-terminus is unblocked. The sequence is identical with that of the potato enzyme without the blocking group and the one predicted from cDNA sequence following the translation initiator Met (Katsube et al., 1990). These results show that the enzyme produced in *E. coli* clone cells had undergone cotranslational removal of the initiator Met as had the potato enzyme but not posttranslational acetylation at the newly formed N-terminal Ala.

The recombinant enzyme had a molecular mass of about 53 000 daltons as estimated from the mobility in SDS-PAGE described above, which agrees well with the size of the potato enzyme (Nakano et al., 1989). In addition, both the recombinant and potato enzymes exhibited identical CD spectra, from which the α-helix content in the enzyme was calculated to be above 12% by the method of Chen et al. (1972).

The K_m values of the recombinant enzyme for substrates were determined from the results of initial velocity studies in both degradation and synthesis of UDP-glucose. From the double-reciprocal plots of the enzyme activity against the substrate concentrations (data not shown), the K_m values for each substrate were calculated to be 0.15 mM for UDP-glucose, 0.14 mM for PP_i, 0.08 mM for UTP, and 0.13 mM for glucose-1-P, the V_{max} value being about 1300 units/mg in the degradation of UDP-glucose. All these values are essentially identical with those determined for the enzyme purified from potato tuber (Nakano et al., 1989). Therefore, the recombinant enzyme is identical with the enzyme purified from potato tuber not only in structural but also in catalytic properties except for the absence of an N-terminal blocking acetyl group.

Expression and Purification of Mutant Enzymes. Using the synthetic oligonucleotide primers shown in Table I, we have prepared mutant plasmids that express the enzymes containing single amino acid substitutions for Lys-263, Lys-329, Lys-367, Lys-409, and Lys-410 with Gln. Gln was chosen as a substituting residue because it has a side chain comparable in size and structure to Lys but has no positive charge. These mutations were confirmed by sequencing of the single-stranded M13 mp19 DNAs. The *Kpn*I–*Bam*HI fragments containing the desired mutations were returned to the same sites of the expression plasmid pTUG, which were then transformed into *E. coli* JM105 cells. The *E. coli* cells were grown in the presence of 0.5 mM IPTG. UDP-glucose pyrophosphorylase activities in the cell extracts from the Lys-329 → Gln, Lys-409 → Gln, and Lys-410 → Gln mutants were similar to that in the extract of cells expressing the wild-type enzyme (50 units/mg), whereas that from the Lys-263 → Gln mutant was only about 1/50 of that from the wild-type enzyme, and the activity in the Lys-367 → Gln mutant cell extract was as low as the background activity in the cell extract of *E. coli* carrying no plasmid. Therefore, the latter two lysyl residues seemed to be important for the enzyme activity. To investigate the kinetic properties of the five mutant enzymes, they were purified to homogeneity by the same method as the wild-type enzyme. The purified mutant enzymes were free from the enzyme due to the host *E. coli* cells, since the *E. coli* enzyme was eluted at fractions completely different from the recombinant potato enzyme during the purification (in the DEAE-cellulose chromatography, data not shown).

CD Spectra of the Wild-Type and Mutant Enzymes. To examine whether single mutations at five different lysyl residues had deleteriously affected the overall conformation of the enzyme protein, CD spectra of the five mutant enzymes purified were measured in comparison with that of the wild-type enzyme. Their spectra, after correction at the same protein concentration, overlapped completely with that of the wild-type enzyme in the entire wavelength region from 200 to 320 nm (data not shown). This suggests that conformational changes of the protein caused by the amino acid replacements, if any, are very subtle.

Kinetic Analysis of Mutant Enzymes. K_m values of the mutant enzymes were determined for both substrates in the degradation (UDP-glucose and PP_i) and those in the synthesis (glucose-1-P and UTP), assuming that the recombinant wild-type and mutant enzymes catalyze the reaction by the ordered bi–bi mechanism as does the enzyme from potato tuber (Nakano et al., 1989). If the uridylyl transfer is the rate-limiting step in the reaction and all the other equilibria are adjusted rapidly, then K_m values for the two substrates are obtained from the initial reaction velocities measured at various concentrations of one substrate to be determined in the presence of a fixed, saturating concentration of another substrate (Cleland, 1963). However, since the addition of excess PP_i depletes Mg^{2+} ions, which are essential for the enzyme activity (Nakano et al., 1989), from the assay solution by forming an insoluble complex, concentrations of PP_i were restricted not to exceed that of Mg^{2+} ions used in the assay (5 mM). Hence, initial velocities in the degradation of UDP-glucose were measured at 5 mM PP_i and at 2 mM UDP-glucose. Therefore, the PP_i-binding site in the Lys-263

Table III: Kinetic Parameters of Wild-Type and Mutant Enzymes

enzyme	V_{max}[a] degradation (units/mg)	V_{max}[a] synthesis (units/mg)	K_m[a] UDP–Glc (mM)	K_m[a] PP_i (mM)	K_m[a] G-1-P (mM)	K_m[a] UTP (mM)
wild type	1300	780	0.15	0.14	0.13	0.076
Lys-263 → Gln	40	10	0.066	14	1.4	0.074
Lys-329 → Gln	920	440	0.19	3.1	0.99	0.17
Lys-367 → Gln	(0.15)	(0.38)	(0.12)	(2.0)	(8.0)	(0.33)
Lys-409 → Gln	1000	720	0.17	0.17	0.19	0.11
Lys-410 → Gln	1200	950	0.22	0.16	0.23	0.10

[a] Values in parentheses were determined with an amount of the mutant enzyme more than 1000 times that of the wild-type enzyme in the assay.

→ Gln mutant enzyme, which has a very high K_m value for PP_i (see Table III), is unsaturated when kinetic constants for UDP–glucose are determined. This limitation inevitably gave the V_{max} and K_m values of the Lys-263 → Gln mutant enzyme with significant experimental errors (±30%) in the degradation; values in other determinations, however, had small errors (±2%). In the synthesis of UDP–glucose, fixed, saturating concentrations of a substrate were 5 mM for glucose-1-P and 2 mM for UTP, except for the determinations for the Lys-263 → Gln and Lys-367 → Gln mutant enzymes, both of which have a high K_m value for glucose-1-P; the fixed concentrations of glucose-1-P were 15 and 10 mM, respectively.

Among the five mutant enzymes whose kinetic parameters are summarized in Table III, the Lys-367 → Gln mutant enzyme had almost no activity. Although it is uncertain if the slight activity, which was detectable only when a very large amount of the mutant enzyme (more than 1000 times the amount of the wild-type enzyme) was used in the assay, is experimentally valid, V_{max} values of about 1/10 000 of those of the wild-type enzyme were obtained; the K_m values, however, showed an increase of only 20-fold for PP_i and 8-fold for glucose-1-P in comparison with those of the wild-type enzyme. The Lys-263 → Gln mutant enzyme had a 30-fold decrease in the V_{max} value in the degradation and a 80-fold decrease in the synthesis of UDP–glucose. Its K_m for PP_i showed a 100-fold increase and that for glucose-1-P showed a 10-fold increase. The mutation of Lys-329 into Gln resulted in 20-fold and 8-fold increases in the K_m values for PP_i and glucose-1-P, respectively, without greatly affecting V_{max} values. Finally, both Lys-409 → Gln and Lys-410 → Gln mutant enzymes showed no significant changes in V_{max} values in both reactions and in K_m values for all substrates. The equilibrium constant (K_{eq}) calculated according to the Haldane equation

$$K_{eq} = \left(\frac{V_{max,degradation}}{V_{max,synthesis}}\right)^2 \frac{K_{m,glucose\text{-}1\text{-}P} K_{m,UTP}}{K_{m,UDP\text{-}glucose} K_{m,PP_i}}$$

was 1.3 with the wild-type enzyme and ranged from 1.0 to 1.8 with the five mutant enzymes, confirming relatively small experimental errors in the steady-state kinetic constants determined for each mutant enzyme.

Discussion

We have achieved an efficient gene expression in *E. coli* of potato tuber UDP–glucose pyrophosphorylase. The recombinant enzyme overproduced in the clone cells could be purified readily to homogeneity. Since the purification requires only two column chromatographic steps, it is also easy to obtain the purified enzyme in a large quantity, facilitating our studies on the enzyme. Single crystals of the recombinant enzyme were obtained by dialysis in the buffered ammonium sulfate solution, and we have started to collect their X-ray diffraction data. Furthermore, site-directed mutagenesis studies to explore functional residues in the enzyme also have been made feasible.

A series of a new type of affinity labeling reagents have been developed in this laboratory to be used for specific modification of lysyl residues in various nucleotide-binding proteins. For example, uridine diphosphopyridoxal, a reactive analogue of UDP–glucose, has been used successfully to identify the essential lysyl residue at the active site of glycogen synthase *a* from rabbit muscle (Tagaya et al., 1985). Recently, Kazuta et al. (1991) of this laboratory employed uridine di- and triphosphopyridoxals for affinity labeling of UDP–glucose pyrophosphorylase from potato tuber. Both reagents modified the enzyme in a strictly stoichiometric fashion (1 mol of the label/mol of enzyme) at very low concentrations, but surprisingly enough, the label was distributed in not less than five lysyl residues (Lys-263, Lys-329, Lys-367, Lys-409, and Lys-410) with either reagent, leading to a suggestion for the presence of a cluster of five lysyl residues at or near the substrate-binding site of the enzyme (Kazuta et al., 1991). In order to examine the functional roles of the five lysyl residues, we have replaced these residues individually by Gln through site-directed mutagenesis.

Of the five mutant enzymes prepared, the Lys-367 → Gln mutant enzyme was shown to be almost completely inactive in both degradation and synthesis of UDP–glucose; its catalytic efficiency in terms of V_{max}/K_m values (provided the kinetic constants determined are meaningful) is in the order of 10^{-5}–10^{-6} of that of the wild-type enzyme. Among the five lysyl residues, Lys-367 is the residue that always suffered most prominently in the modification by the affinity labeling reagents (Kazuta et al., 1991) and is solely conserved in the corresponding region in the primary structure of the slime mold enzyme (Ragheb & Dottin, 1987; Katsube et al., 1990). These findings strongly suggest that Lys-367 is essential for the catalytic activity of UDP–glucose pyrophosphorylase.

On the other hand, Lys-263 is the residue that was also prominently modified by the affinity labeling reagents next to Lys-367 (Kazuta et al., 1991). Although significant activities of the Lys-263 → Gln mutant enzyme clearly show that Lys-263 is dispensable for activity, the K_m values for PP_i and glucose-1-P were drastically perturbed by its substitution into Gln, suggesting that Lys-263 may participate in binding of PP_i and/or glucose-1-P in the binary complex. With regard to the Lys-329 → Gln mutant enzyme, even though its V_{max} values were affected to a lesser extent, the K_m values for PP_i and glucose-1-P were 20 and 8 times higher than those, respectively, of the wild-type enzyme. Thus, Lys-329 might also be involved somehow in binding of PP_i and/or glucose-1-P but not in catalysis. The reaction of UDP–glucose pyrophosphorylase proceeds by the ordered bi–bi mechanism, in which MgUTP and glucose-1-P are bound and $MgPP_i$ and UDP–glucose are released in this order (Nakano et al., 1989). Therefore, significant conformational changes probably occur in the enzyme upon binding of the first substrate to be able to accommodate the second substrate. It might thus be possible to speculate that Lys-263 and Lys-329 are involved in

binding after such conformational changes. However, the direct involvement in binding is not yet warranted, since, in general, dissociation constants for substrates are not equal to K_m values and their determination requires thorough kinetic analyses (Cleland, 1963). It is interesting to note that both Lys-263 and Lys-329 are not conserved in the corresponding regions of the slime mold enzyme (Ragheb & Dottin, 1987; Katsube et al., 1990), which has an about 10 times lower specific activity than the potato enzyme (Pannbacker, 1967). The low activity may be partly explained by the absence of Lys-263 and Lys-329 in the slime mold enzyme.

The Lys-409 → Gln and Lys-410 → Gln mutant enzymes had steady-state kinetic constants almost identical with those of the wild-type enzyme, indicating that both Lys-409 and Lys-410 do not participate in catalysis. However, this does not necessarily show that these two lysyl residues are located apart from the active site.

In conclusion, among the five lysyl residues identified by the affinity labeling studies to be located at or near the active site, Lys-367 was found to be essential for the catalytic activity of UDP-glucose pyrophosphorylase. Although it is unknown how Lys-367 functions in the catalysis, the amino group of its side chain may interact with a phosphate group of the substrate during the transition state as has been shown by an NMR study of adenylate kinase (Fry et al., 1986), in which the amino group of Lys-21 may interact with the α-phosphate group of the bound substrate MgATP.

Registry No. Lys, 56-87-1; PP_i, 14000-31-8; UDP-glucose phosphorylase, 9026-22-6; glucose-1-P, 59-56-3.

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Metabolism of Low-Density Lipoprotein Free Cholesterol by Human Plasma Lecithin–Cholesterol Acyltransferase†

Phoebe E. Fielding,*,‡,§ Takashi Miida,‡ and Christopher J. Fielding‡,∥

Cardiovascular Research Institute and Departments of Medicine and Physiology, University of California Medical Center, San Francisco, California 94143

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ABSTRACT: The metabolism of cholesterol derived from [^{3}H]cholesterol-labeled low-density lipoprotein (LDL) was determined in human blood plasma. LDL-derived free cholesterol first appeared in large α-migrating HDL (HDL_2) and was then transferred to small α-HDL (HDL_3) for esterification. The major part of such esters was retained within HDL of increasing size in the course of lecithin–cholesterol acyltransferase (LCAT) activity; the balance was recovered in LDL. Transfer of preformed cholesteryl esters within HDL contributed little to the labeled cholesteryl ester accumulating in HDL_2. When cholesterol for esterification was derived instead from cell membranes, a significantly smaller proportion of this cholesteryl ester was subsequently recovered in LDL. These data suggest compartmentation of cholesteryl esters within plasma that have been formed from cell membrane or LDL free cholesterol, and the role for HDL_2 as a relatively unreactive sink for LCAT-derived cholesteryl esters.

High-density lipoprotein (HDL) is now recognized to be highly heterogeneous in size as well as lipid and apoprotein composition. An important part of HDL metabolism involves transfers of lipids, particularly free cholesterol, between different HDL subfractions (Eisenberg, 1984; Patsch & Gotto, 1987).

There are two major sources for the free cholesterol appearing in HDL: the surface of secreted lipoproteins, particularly very low and low-density lipoproteins (VLDL and LDL), and cell membranes. An earlier report showed that different HDL subfractions were active in the transport of free cholesterol from these sources (Miida et al., 1990). Cellular cholesterol was initially transferred to the pre-β-migrating HDL whose protein moiety contained only apolipoprotein A-I. LDL free cholesterol was transferred mainly to the major

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‡ Cardiovascular Research Institute.

§ Department of Medicine.

∥ Department of Physiology.

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