Identification of Lysyl and Glycyl Residues Located at the Active Site in Glycogen Synthase and Their Roles in the Catalytic Reaction

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CONTENTS

List of Abbreviations

AP_n-PL	adenosine polyphosphopyridoxal (*n* represents the number of phosphate moiety)
GP_n-PL	guanosine polyphosphopyridoxal (*n* represents the number of phosphate moiety)
UP_n-PL	uridine polyphosphopyridoxal (*n* represents the number of phosphate moiety)
bp	base pair(s)
EDTA	ethylenediaminetetraacetic acid
G-1-P	glucose-1-phosphate
G-6-P	glucose-6-phosphate
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid
HPLC	high performance liquid chromatography
IC_{50}	the concentration required for 50% inactivation of glycogen synthase
MES	2-(*N*-morpholino)ethanesulfonic acid
PCR	polymerase chain reaction
PTH	phenylthiohydantoin
K15Q	the *E. coli* glycogen synthase mutant, in which Lys15 is replaced by Gln (other mutant enzymes are abbreviated in the same manner).

SUMMARY

Glycogen synthases from *Escherichia coli* and mammalian muscle differ in many respects including the regulation, the sugar nucleotide specificity, and the primary structure. To compare the structure of the active sites in these enzymes, the affinity labeling study of the *E. coli* enzyme was carried out using adenosine diphosphopyridoxal as the reagent. The *E. coli* enzyme was inactivated in a time- and dose-dependent manner, when incubated with the reagent followed by sodium borohydride reduction. The inactivation was markedly retarded by ADP-glucose and ADP, suggesting that the reagent was bound to the substrate-binding site. The stoichiometry of the bound reagent to the enzyme was approximately 1 : 1. Sequence analysis of the labeled peptide isolated from a proteolytic digest of the modified protein revealed that Lys15 is labeled. Based on the geometry of the reagent, the ε-amino group of this residue might be located close to the pyrophosphate moiety of ADP-glucose bound to the *E. coli* enzyme, like that of Lys38 in the rabbit muscle enzyme which is labeled by uridine diphosphopyridoxal. The lysyl residues identified at their substrate-binding sites by affinity labeling are contained in the conserved tetrapeptide sequence, Lys-X-Gly-Gly, where X is an unspecified residue.

To elucidate the functional role of this conserved sequence, Lys15, Gly17, and Gly18 in the *E. coli* enzyme have been separately replaced by other amino acid residues via site-directed mutagenesis. Kinetic analyses of the Lys15 mutant enzymes showed that the ε-amino group of Lys15 is mainly involved in binding of the phosphate moiety adjacent to the glycosidic linkage in the substrate ADP-glucose, presumably through an ionic interaction. The mutant enzyme in which Ala was substituted for Gly17 had a catalytic rate constant three-orders of magnitude smaller than that of the wild-type enzyme with a slightly increased Michaelis constant for ADP-

glucose, whereas the Ala-for-Gly18 mutant showed a rate constant only 3.2-fold smaller. In addition, mutations of Gly17 and Gly18 resulted in a marked change in the reactivity of Lys15 toward affinity labeling reagents. These results suggest that the two glycyl residues in the conserved Lys-X-Gly-Gly sequence, in particular the one closer to the ADP-glucose-binding lysyl residue, participate in the catalysis by assisting the conformational change of the active site or stabilizing the transition state.

It has been found that the mutant enzyme in which Lys15 is replaced by Gln is inactivated by adenosine diphosphopyridoxal at concentrations higher than those required for the inactivation of the wild-type enzyme. ADP and ADP-glucose protected the mutant enzyme from inactivation, suggesting that the labeling reagent binds to the ADP-glucose-binding site. Sequence analysis revealed that the labeled residue is Lys277. Replacement of Lys277 by Gln resulted in a 140-fold decrease in k_{cat} without significant changes in the K_m values for ADP-glucose and glycogen. These results suggest that Lys277 at the active site participates in the catalytic reaction rather than binding of substrate.

CHAPTER I

General Introduction

Properties of Glycogen Synthases from Different Sources — Storage of carbohydrate, such as glycogen, is required in virtually all cell types for an energy source. Glycogen is a remarkably uniform molecule containing ≈ 4000 chains of α-1,4-linked glucose residues (1). The chains are on average 11-14 residues long and are branched by α-1,6-linkages. Synthesis of the α-1,4- and α-1,6-glucosyl linkage is carried out by glycogen synthase and branching enzyme, respectively. Glycogen synthase (EC 2.4.1.11) catalyzes the glucosyl transfer from nucleoside diphosphoglucose to the nonreducing end of glycogen. The mammalian enzyme, which has a subunit molecular weight of 85,000, utilizes UDP-glucose as the glucosyl donor (2). The regulatory mechanism of the mammalian enzyme has been extensively investigated. This enzyme is regulated by phosphorylation and dephosphorylation. This covalent modification leads to two forms that differ in their activation by glucose-6-phosphate (G-6-P). The dephosphorylated *I* form of the enzyme, at high UDP-glucose concentrations, is scarcely activated by G-6-P, whereas the phosphorylated *D* form has very low activity in the absence of this effector (3). The interconversion between the two forms of glycogen synthase is mediated by specific protein kinases and protein phosphatases (4) whose activities are regulated by an intracellular concentration of cyclic AMP (5). Seven different protein kinases are implicated in the phosphorylation at nine different sites *in vivo* (6).

Glycogen synthase from *Saccharomyces cerevisiae* also uses UDP-glucose as the glucosyl donor (7), and has a subunit molecular weight of 78,000-85,000. The yeast enzyme is also known to undergo reversible phosphorylation (8,9). The relationship between the reversible phosphorylation of glycogen synthase and the intracellular cyclic AMP level is unclear. It was reported that glycogen metabolism in yeast is regulated by the cyclic AMP-dependent pathways (10-12). However,

other reports showed that glycogen accumulation in yeast is regulated by mechanisms independent of the cyclic AMP levels (13,14).

The biosynthesis of bacterial glycogen occurs when growth is ceased by depletion of nutrients such as nitrogen, sulfur, and phosphate, and by an unfavorable pH for growth (15). It seems that glycogen is not necessary for the growth of bacteria, or rather plays a role in survival of the bacterial cells (16). Glycogen synthase from *E. coli* (EC 2.4.1.21), which prefers ADP-glucose to UDP-glucose and has a subunit molecular weight of 49,000 (17), is regulated by neither covalent nor noncovalent mechanism. ADP-glucose is a key compound to regulate bacterial glycogen synthesis. This compound is produced by the reaction of ATP with α-glucose-1-phosphate catalyzed by ADP-glucose pyrophosphorylase (18). This enzyme is activated by glycolytic intermediates and inhibited by AMP, ADP, and inorganic phosphate. The activity of ADP-glucose pyrophosphorylase directly correlates with the accumulation of bacterial glycogen (16). Accumulation of glycogen is also genetically controlled. In *E. coli*, the structural genes of glycogen synthase (*glg*A) and ADP-glucose pyrophosphorylase (*glg*C) are contiguous each other in the order of *glg*C-*glg*A (19). It is considered that the expression of these structural genes is simultaneously regulated by a repressor protein. Cyclic AMP receptor protein appears to modulate the expression of *glg*A and *glg*C through the repressor protein (20).

In higher plants (see ref. 21 for a review), synthesis of α-1,4-glucosidic linkage is carried out by starch synthase (EC 2. 4. 1. 21). This enzyme, which has a subunit molecular weight of 58,000, catalyzes the same reaction with bacterial glycogen synthase. The ADP-glucose-dependent pathway, consisting of the reactions catalyzed by ADP-glucose pyrophosphorylase, starch synthase, and branching enzyme, is predominant to starch synthesis in leaf as well as in non-photosynthetic

tissues such as endosperm and tubers. Regulatory systems for starch synthase have not been reported. The starch level in a cell correlates with the activity of ADP-glucose pyrophosphorylase. This enzyme is activated by 3-phosphoglycerate and inhibited by inorganic phosphate.

Thus, glycogen synthases from different sources using the same glucosyl donor are similar in their regulation mechanisms as well as their subunit molecular weights. ADP-glucose is used only for the synthesis of α-1,4-glucan, whereas UDP-glucose is used not only as the glucosyl donor in the synthesis of carbohydrates but also as the precursor of other sugar nucleotides such as UDP-galactose and UDP-glucuronate (22). In other words, UDP-glucose is not only a substrate for glycogen synthase. Therefore, it seems difficult that the glycogen synthesis is regulated only by UDP-glucose levels. It is reasonable that UDP-glucose-specific glycogen synthases have regulation mechanisms in their own molecules.

cDNAs encoding glycogen synthases from human muscle (23), rabbit muscle (24), and rat liver (25), and the genes encoding yeast (26) and *E. coli* (27) glycogen synthases, and maize starch synthase (28) were cloned and their corresponding amino acid sequences were deduced. The enzymes from mammalian and yeast, whose glucosyl donor is UDP-glucose, or the enzymes from *E. coli* and maize, whose glucosyl donor is ADP-glucose, share about 30-50 % sequence identities. However, there is no detectable overall sequence similarity between the enzymes that use different glucosyl donors. Thus, UDP-glucose- and ADP-glucose-specific glycogen synthases are different in structure as well as in regulation. If so, here arises a question that the structure-function relationships in the enzymes are also different between two groups, although essentially the same reaction is catalyzed. The regulatory mechanism of glycogen synthase has been extensively investigated as described above, but its catalytic mechanism has not been elucidated. For elucidation of the

catalytic mechanism, it is important to identify amino acid residues at the active site in glycogen synthase. As one of the most useful techniques for this purpose, it should be reasonable to use affinity labeling.

Nucleotidylyl Derivatives of Pyridoxal Phosphate — Pyridoxal phosphate, a coenzyme form of Vitamin B_6, has been used as a chemical modification reagent (29). This reagent modifies lysyl residues of proteins having the phosphate- or nucleotide-binding sites by the affinity of its negatively charged phosphate group. With a few exceptions, however, its specificity directing toward the active sites of enzymes was not satisfactory. In those cases, a few or more lysyl residues in the regions apart from the active sites of enzymes were labeled. To make pyridoxal phosphate more specific toward lysyl residues in the nucleotide-binding sites, Tagaya *et al.* (30) synthesized a uridylyl derivative of pyridoxal phosphate. This reagent, UP_2-PL, consists of UMP and pyridoxal phosphate conjugated through a pyrophosphate linkage. Contiguously, similar adenylyl and guanylyl derivatives were synthesized by them. This type of reagents have several advantages for affinity labeling of nucleotide-binding proteins. The reagents contain the whole structure of natural nucleotides, which promises the binding of the reagents to the nucleotide-binding sites of proteins. Fig. 1 schematically shows the reaction of AP_2-PL with the amino group of a protein. If a lysyl residue is located at the position close to the β- or γ- phosphate of the bound nucleotides, a Schiff base is formed between the 4-formyl group of pyridoxal moiety and the ε-amino group of the lysyl residue. The reagent is covalently fixed by sodium borohydide reduction. By the use of fluorescence derived from the pyridoxyl moiety of the reagent, the labeled lysine can be easily detected. This is advantageous to the identification of a labeled site.

Table I summarizes the proteins so far labeled by the reagents including uridylyl, adenylyl, and guanylyl derivatives with varying numbers of the phosphate group. The efficiencies of the reagents depend on the base- and phosphate-number-specificity of a nucleotide-binding protein. All the proteins summarized in Table I are specifically labeled by low concentrations of the reagents. The affinity of these reagents is, in general, considered to be 1000-fold higher than that of pyridoxal phosphate. In gluthathione synthetase, the locations of the lysyl residues labeled by the nucleotidylyl derivatives of pyridoxal phosphate were confirmed by X-ray crystallographic analyses (31).

Purpose of this Study — Tagaya *et al.* (30) modified rabbit muscle glycogen synthase using UP_2-PL as a reactive analog of the substrate UDP-glucose. A Lysyl residue located at the active site was specifically labeled by the reagent. Later, Mahrenholz *et al.* (32) identified this residue as Lys38. To elucidate the structure-function relationship of *E. coli* glycogen synthase, it is desirable to know whether or not the lysyl residue corresponding to Lys38 of the UDP-glucose-specific mammalian glycogen synthase is present in the ADP-glucose-specific *E. coli* enzyme. Fig. 2 shows the structures of adenine nucleotide derivatives of pyridoxal phosphate (AP_n-PL) in addition to that of ADP-glucose. AP_n-PL could be reactive analogs of ADP-glucose. Application of these reagents to the *E. coli* enzyme leads to a possibility to find the corresponding lysyl residue to Lys38 of the rabbit muscle enzyme.

In the studies described in this thesis, catalytically important amino acid residues of *E. coli* glycogen synthase were identified by affinity labeling and their roles in the catalytic function were investigated by site-directed mutagenesis. In Chapter II, AP_2-PL was applied to the *E. coli* enzyme. Lys15 was specifically modified by the reagent concomitant with inactivation of the enzyme. In comparison of the amino acid

sequence surrounding Lys15 with that of Lys38 in the rabbit muscle enzyme, a Lys-X-Gly-Gly sequence was found to be conserved (where X represents an unconserved amino acid residue). The roles of the amino acid residues in the conserved sequence in the enzymic function were investigated by site-directed mutagenesis, and the results are described in Chapter III. Lys15 is mainly involved in binding of ADP-glucose through an ionic interaction between its ε-amino group and the phosphate moiety adjacent to the glucose moiety of ADP-glucose. Two glycyl residues, particularly the residue near Lys15, play an important role in the catalytic reaction possibly by assisting conformational changes of the active site. It was also suggested in this chapter that basic amino acid residues other than Lys15 are involved in the catalytic reaction. In Chapter IV, attempts were made to identify other amino acid residues at the active site. The mutant enzyme, in which Lys15 is replaced by the amino acid residue unreactive to AP_2-PL, was treated with the reagent. Lys277 was specifically modified by AP_2-PL, and site-directed mutagenesis study of this residue showed a possibility that Lys277 is involved in the catalytic process. The structure-function relationship of glycogen synthase revealed by those studies is comprehensively discussed in Chapter VI. The usefulness of the new approach of affinity labeling combined with site-directed mutagenesis is also emphasized in this chapter.

TABLE I

Proteins modified by nucleotidylyl derivatives of pyridoxal phosphate

Protein	Reagent	Reference
Rabbit muscle glycogen synthase	UP_2-PL	31
UDP-glucose pyrophosphorylase	$UP_{2,3}$-PL	33
Callose synthase	UP_2-PL	34
Lactate dehydrogenase	$AP_{2,3,4}$-PL	35
Alcohol dehydrogenase	AP_2-PL	36
Aldose reductase	AP_2-PL	37
F_1-ATPase	$AP_{3,4}$-PL	38-41
Amyloplast inner membrane ATPase	$AP_{3,4}$-PL	42
Ca^{2+}-ATPase	$AP_{3,4}$-PL	43, 44
Na^+, K^+-ATPase	$AP_{3,4}$-PL	45
Adenylate kinase	$AP_{3,4}$-PL	46-48
Pyruvate kinase	AP_2-PL	48
Hexokinase	AP_2-PL	48, 50
3-Phosphoglycerate kinase	AP_2-PL	48
Phosphorylase kinase	$AP_{3,4}$-PL	50
Pyridoxal kinase	AP_4-PL	51, 52
DNA-gyrase	AP_2-PL	53
Glutathione synthetase	AP_4-PL	30
Aminoacyl tRNA-synthetase	AP_3-PL	54
Ribulose phosphate kinase	AP_3-PL	55
ρ factor	AP_2-PL	56
*ras*p21 protein	$GP_{3,4}$-PL	57

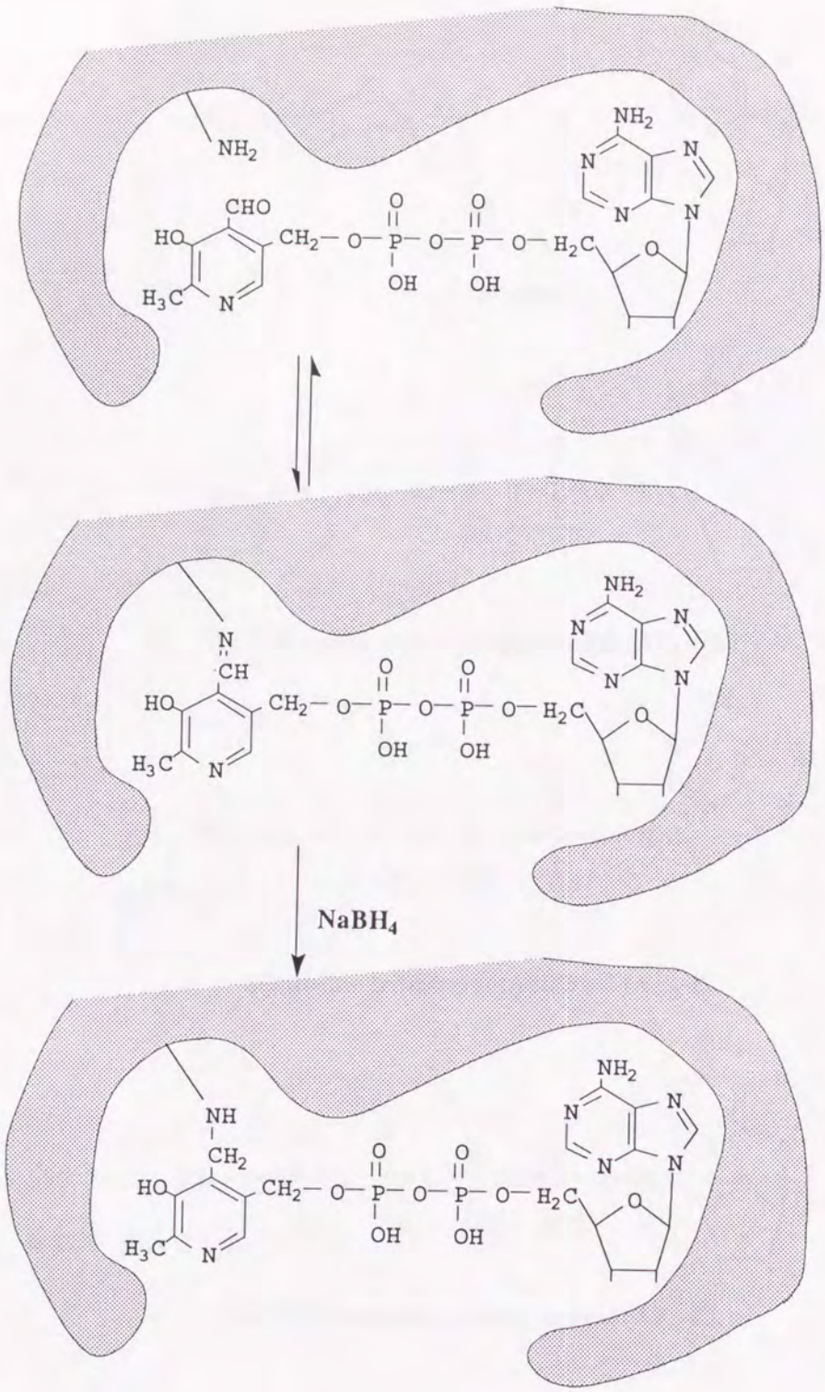
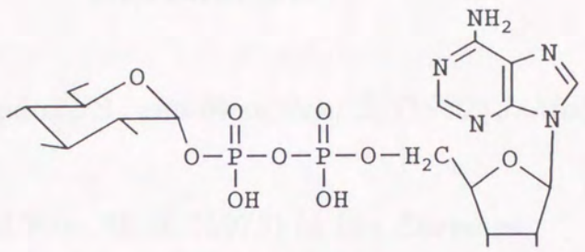
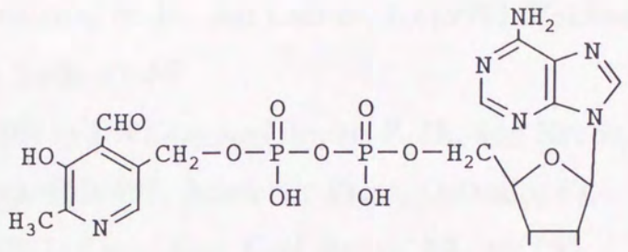


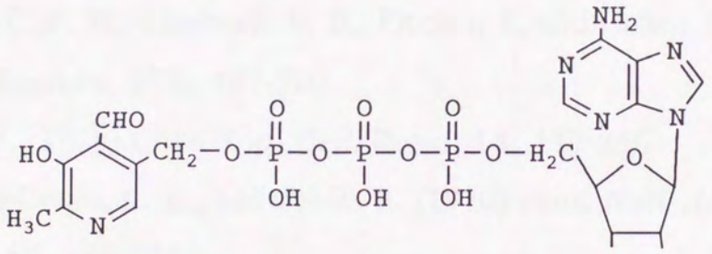
Fig. 1 **Reaction scheme of AP_2-PL with a protein.**



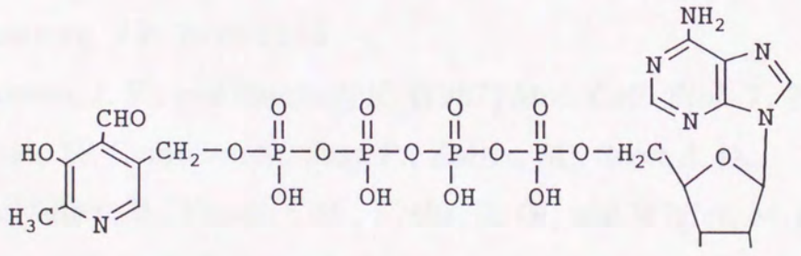
ADP-glucose



adenosine diphosphopyridoxal (AP_2-PL)



adenosine triphosphopyridoxal (AP_3-PL)



adenosine tetraphosphopyridoxal (AP_4-PL)

Fig. 2. **Comparative structures of ADP-glucose and adenylyl derivatives of pyridoxal phosphate.**

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CHAPTER II

Identification of Active Site Lys15 by Affinity Labeling with Adenosine Diphosphopyridoxal

INTRODUCTION

Bacterial glycogen synthase (EC 2.4.1.21) utilizes ADP-glucose as the glucosyl donor to synthesize an α-1,4-glucan chain in glycogen. The enzyme from *E. coli* has been purified to homogeneity, and its properties were studied (1). Its sugar nucleotide specificity is different from that of the mammalian enzyme which uses UDP-glucose instead of ADP-glucose. The subunit molecular weight of the bacterial enzyme is about one-half that found for the mammalian enzyme; 49,000 and 85,000 for the bacterial and mammalian enzymes, respectively. Unlike the mammalian enzyme, the bacterial enzyme is regulated by neither covalent nor noncovalent mechanism.

The nucleotide sequence of the *glg*A gene in *E. coli*, encoding glycogen synthase, was determined by Kumar *et al.* (2). The deduced amino acid sequence shows no significant homology with that of human muscle glycogen synthase which was also deduced from the nucleotide sequence (3). Therefore, the two glycogen synthases seem to be completely different molecular entities.

Tagaya *et al.* (4) showed that uridine diphosphopyridoxal, a reactive UDP-glucose analog, specifically modifies a lysyl residue located at the active site in rabbit muscle glycogen synthase. Later, Mahrenholz *et al.* (5) identified this residue as Lys38. Both the mammalian and *E. coli* enzymes catalyze essentially the same type of glucosyltransfer, although their properties are different. Therefore, it would be of interest to examine whether or not the active site structure of the rabbit muscle enzyme is conserved in the bacterial enzyme. To solve this problem, *E. coli* glycogen synthase has been modified with AP_2-PL (6,7), a potentially reactive analog of ADP-glucose.

EXPERIMENTAL PROCEDURES

Materials — Restriction endonucleases and a DNA ligation kit were obtained from Takara; α-amylases from *Bacillus subtilis* and human saliva were from Seikagaku Kogyo and Sigma, respectively; *Staphylococcus aureus* V8 protease and trypsin were from Worthington; and ADP-[^{14}C]glucose (8.77GBq/mmol) was from ICN Radiochemicals. AP_2-PL was synthesized as described previously (6). Oyster glycogen obtained from Nacalai Tesque was purified as described previously (8).

Construction of Plasmid pEGS — Plasmid pFY140 contains the entire *glg*A gene (9), but its direction is opposite for expression under the control of *lac* promoter. To achieve high levels of the *glg*A expression, pFY140 was digested with *Hind*III and ligated with pUC18. The direction of the *glg*A gene was checked by digestion of the resulting plasmid with *Pst*I. The plasmid in which the *glg*A gene is under control of *lac* promoter was designated pEGS.

Protein Concentration — Protein concentration was determined according to the method of Bradford (10), and calculated by using a molar absorption coefficient of 2.0×10^6 M^{-1} which had been determined on the basis of amino acid analysis of the purified protein.

Assay of Glycogen Synthase — Glycogen synthase was assayed by the filter assay method as described by Thomas *et al.* (11). The assay medium contained 50 mM *N*, *N*-bis(2-hydroxyethyl)-glycine (pH 8.5), 25 mM potassium acetate, 10 mM dithiothreitol, 0.5 mg/ml oyster glycogen, 0.5 mg/ml bovine serum albumin, and 0.7 mM ADP-[^{14}C]glucose. The medium was incubated at 30 °C for 10 min. Filter papers were placed in glass scintillation vials containing about 10 ml of toluene containing 0.4 % 2,5-diphenyloxazol and 0.01 % 1,4-bis[2-(5-phenyloxazolyl)]benzene, and counted with a Beckman 9000S scintillation counter. One unit of the

enzyme activity was defined as that amount which catalyzes the incorporation of 1 μmol of glucose into glycogen per minute under the above conditions.

Purification of Glycogen Synthase — pEGS was transformed into *E. coli* JM109 as described by Maniatis *et al.* (12). The cells were grown on 2 liters of L-broth containing 50 μg/ml ampicillin for 2 h, and isopropyl-β-D-thiogalactopyranoside was added at a final concentration of 0.5 mM. The cells were cultured for another 6 h, and collected by centrifugation. They were suspended in 50 mM glycylglycine buffer (pH 7.0) containing 0.2 mM dithiothreitol, and disintegrated with a French press (Otake Seisakusho, type 5501-M). The cell debris collected by centrifugation was discarded, and 2 mg of bacterial α-amylase and 500 units of salivary α-amylase were added to the supernatant to digest the glycogen. The solution was gently stirred overnight at the room temperature, and centrifuged at 78,000 x *g* for 90 min.

To the supernatant was added solid ammonium sulfate to 25 % saturation. After standing for 1 h, the precipitate formed was collected by centrifugation at 23,000 x *g*, dissolved in 50 ml of 50 mM Tris-HCl (pH 7.5) containing 0.2 mM dithiothreitol and 10 % glycerol (buffer A), and applied to Q-Sepharose (HR 16/10) which had been equilibrated with buffer A. The proteins were eluted with a linear gradient from buffer A to the same buffer containing 0.6 M sodium chloride over 50 min with a flow rate of 5 ml/min on the FPLC separation system (Pharmacia). Fractions containing glycogen synthase activity were pooled, and the enzyme was precipitated by adding ammonium sulfate to 70 % saturation. The precipitate was collected by centrifugation at 23,000 x *g* for 10 min, dissolved in a minimum volume of 50 mM 2-glycerophosphate buffer (pH 7.0) containing 0.2 mM dithiothreitol and 10 % glycerol, and dialyzed

against the same buffer overnight. Insoluble materials were removed by centrifugation, and the supernatant was used as the purified enzyme.

Gel electrophoresis — Sodium dodecylsulfate-polyacrylamide gel electrophoresis with 10 % acrylamide was carried out according to Laemmli (13).

Stoichiometry of Inactivation — Glycogen synthase (40 μM) was incubated with various concentrations of AP_2-PL at 20 °C for 30 min, reduced by sodium borohydride, and then applied to a centrifugal gel filtration column (14). The residual enzyme activity and the concentration of the enzyme in the passed-through fraction were measured. The amount of the bound label was determined by the fluorescence derived from the label (excitation at 330 nm and the emission at 395 nm) after denaturation of the enzyme with 0.5 M Tris-HCl (pH 8.5) containing 10 mM EDTA and 6M guanidine hydrochloride.

Isolation of AP_2-PL-Labeled Peptides — Glycogen synthase (3.0 μM) was incubated with 50 μM AP_2-PL in a total volume of 10 ml, at 20 °C for 30 min. Then, the protein was reduced with sodium borohydride, dialyzed against water, and finally lyophilized. The lyophilized protein was reduced with dithiothreitol, and *S*-pyridylethylated (15). The *S*-pyridylethylated enzyme was dialyzed against water, and lyophilized. The lyophilized protein was digested with trypsin in an enzyme-to-substrate ratio of 1 : 50 (w/w) at 37 °C for 24 h. The AP_2-PL-labeled peptides were purified on a Gilson HPLC system with the following solvents, (A) 0.1 % trifluoroacetic acid and (B) 0.088 % trifluoroacetic acid containing 60 % acetonitrile.

Sequence Study — Amino acid sequence was determined with an Applied Biosystems model 477A sequencer linked with an Applied Biosystems model 120A PTH analyzer.

Amino Acid Analysis — Amino acid was analyzed on a Hitachi 835 amino acid analyzer using *o*-phthaldialdehyde, after 6 N-HCl hydrolysis of samples in evacuated tubes. Proline was not quantitatively determined in this system.

RESULTS

Expression and Purification of Glycogen Synthase — In the presence of isopropyl-β-D-thiogalactopyranoside, *E. coli* JM 109 cells carrying pEGS overexpressed the protein with an apparent molecular weight of 48,000 under denatured conditions (Fig. 1, lane 3). This value corresponds to the subunit molecular weight of *E. coli* glycogen synthase (1). Nontransformed JM 109 itself did not produce this protein (Fig. 1, lane 2). In accordance with high expression of the 48-kDa protein, JM109 cells carrying pEGS showed glycogen synthase activity 240-fold higher than that of the nontransformed cells. Thus, it is concluded that this protein is the product of the *glg*A gene.

The overexpressed protein was purified as described under "Experimental Procedures". To achieve a good recovery of the enzyme in the ammonium sulfate fractionation, it is important that the harvested cells are suspended in the minimum volume of buffer in the preceding step. Table I summarizes the purification of the enzyme, and Fig. 1 depicts the results of sodium dodecylsulfate-polyacrylamide gel electrophoresis in each step of the purification. The apparent increase in the total enzyme activity at the Q-Sepharose step is probably caused from the depressed activity at the preceding step due to the contaminating amylase. The final preparation of the enzyme showed more than 90 % purity (Fig. 1, lane 6). The specific enzyme activity of the final

preparation is comparable to that of the enzyme purified from *E. coli* B cells (1). About 20 mg of glycogen synthase was obtained from 2 liters of the cell culture.

Inactivation of Glycogen Synthase by AP_2-PL — When the purified glycogen synthase was incubated with low concentrations of AP_2-PL followed by sodium borohydride reduction, the enzyme activity was lost in a time-dependent manner, and after 30 min, it reached a plateau dependent on the reagent concentration (Fig. 2). About 80 % of the original activity was lost during incubation with 50 μM AP_2-PL for 30 min. This pattern of inactivation is typical for inactivation by modification reagents containing pyridoxal phosphate (16,17), and is probably due to an equilibrium between the Schiff base and the free aldehyde.

The inactivation was effectively prevented by substrates, ADP-glucose and ADP (Fig. 3). α-D-Glucose 1-phosphate and AMP offered only slight protective effects. These results suggest that AP_2-PL binds to the ADP-glucose (or ADP)-binding site.

Fig. 4 shows the stoichiometry of inactivation. When the degree of inactivation was plotted against the molar ratio of AP_2-PL to enzyme subunit, a straight line was obtained. Extrapolation of the line to 100 % loss of enzyme activity gave a value of approximately one, indicating that the specific binding of one mole of AP_2-PL to each enzyme subunit.

Isolation and Sequencing of the AP_2-PL-Labeled Peptide — To determine the site labeled by AP_2-PL, the AP_2-PL-labeled enzyme was reduced and *S*-pyridylethylated, and digested with trypsin, as described under "Experimental Procedures". The digest was chromatographed on the HPLC system using a VYDAC C4 column. The elution profile is shown in Fig. 5. One predominant fluorescent peak was observed, indicating the binding of the label to a specific site. Sequence analysis of

this labeled peptide revealed that its amino-terminal sequence is the same as that of the native protein (data not shown). This peptide was further digested by V8 protease and then applied to a VYDAC C18 column. The labeled peptide was eluted again as a single peak (Fig. 6). Sequence analysis of this peptide revealed that its structure is Met-Phe-Pro-Leu-Leu-X-Thr-Gly-Gly-Leu-Ala-Asp, where X represents an unidentified amino acid. This structure is consistent with the amino acid composition (Table II), and is identical to that from Met10 to Asp21 except for Lys15 in the complete amino acid sequence of *E. coli* glycogen synthase (2). Since an AP_2-PL-labeled lysyl residue cannot be positively identified (7), it is concluded that Lys15 is labeled by AP_2-PL.

DISCUSSION

The results of the investigation presented in this chapter provide evidence for the presence of Lys15 at the active site in *E. coli* glycogen synthase. Based on the geometry of the modifying reagent, the ε-amino group of Lys15 is probably located close to the pyrophosphate moiety of ADP-glucose in the *E. coli* enzyme, like that of Lys38 in the rabbit muscle enzyme which is labeled by UP_2-PL (4). Fig. 7 compares the amino-terminal sequences among glycogen synthases from *E. coli* (2), rabbit muscle (5), and human muscle (3). It was found that a sequence of Lys-X-Gly-Gly (X represents an unspecified residue) containing the labeled lysyl residue is conserved in the three enzymes, although the bacterial and mammalian muscle enzymes are not homologous (3). The conservation of Lys-X-Gly-Gly in the three enzymes may be a reflection on the importance of the sequence for sugar nucleotide binding.

A motif that several glycyl residues are present close to a reactive lysyl residue has been found in many nucleotide-binding proteins. In all the glutamate and leucine dehydrogenases, a Gly-Gly-X-Lys sequence is conserved (18-26). Piszkiewicz *et al.* (27) reported that pyridoxal phosphate modifies the conserved lysyl residue in glutamate dehydrogenase. On the other hand, Lys38 in rabbit muscle glycogen synthase was modified also by pyridoxal phosphate, if a higher concentration of the reagent was used (5). Therefore, the conserved lysyl residues in glycogen synthases and dehydrogenases seem to be equivalent. Walker *et al.* (28) found that a Gly-X-X-X-X-Gly-Lys sequence is conserved in several ATP- and GTP-binding proteins which include adenylate kinase (29), H^+-ATPase (28), and the *ras* oncogene product p21 (30). It has been demonstrated that the conserved lysyl residues in these proteins are specifically labeled by adenosine or guanosine polyphosphopyridoxals (7,31-34). Therefore, the motif of a lysyl residue in the glycine-rich region is general as the structural element of polyphosphate-binding loci.

It is not yet fully understand how the conserved sequence interacts with the polyphosphate moiety in the glycogen synthase. One possibility is that the conserved lysyl residue directly interacts with the polyphosphate moiety of substrate sugar nucleotide and the glycyl residues provide the flexibility in this locus, as observed in adenylate kinase (35). Another possibility is that the glycyl residues themselves interact directly with the polyphosphate moiety. In triose phosphate isomerase, the amide nitrogens of the two glycyl residues (Gly232 and Gly233) interact with the phosphate moiety of substrate triose phosphate (36). However, since this structure may be applicable only to monophosphate-binding sites, the former possibility is more likely. To understand the functional roles of the conserved lysyl and glycyl residues

in glycogen synthase, site-directed mutagenesis experiments were performed on these amino acid residues and the results are described in the next chapter.

TABLE I

Summary of purification of E. coli glycogen synthase [a]

Steps	Protein	Total Activity	Specific Activity	Purification
	mg	*unit*	*unit/mg*	*-fold*
Crude extract	700	6320	9.0	(1)
78,000 x *g* supernatant	552	4390	8.0	0.9
Ammonium sulfate precipitate	52	2200	42.3	4.7
Q-Sepharose	21	3700	177	20

[a] From a 2-liter culture of *E. coli* JM109 carrying pEGS.

TABLE II

Amino acid composition of AP_2-PL-labeled peptide

Amino acid	Found	Expected
Asp	1.14	1
Thr	0.92	1
Gly	2.78	2
Ala	1.02	1
Met	0.41	1
Leu	2.99	3
Phe	1.00	1
Lys	ND[a]	1
Pro	NQ[b]	1

[a] not detectable.

[b] not quantitatively determined.

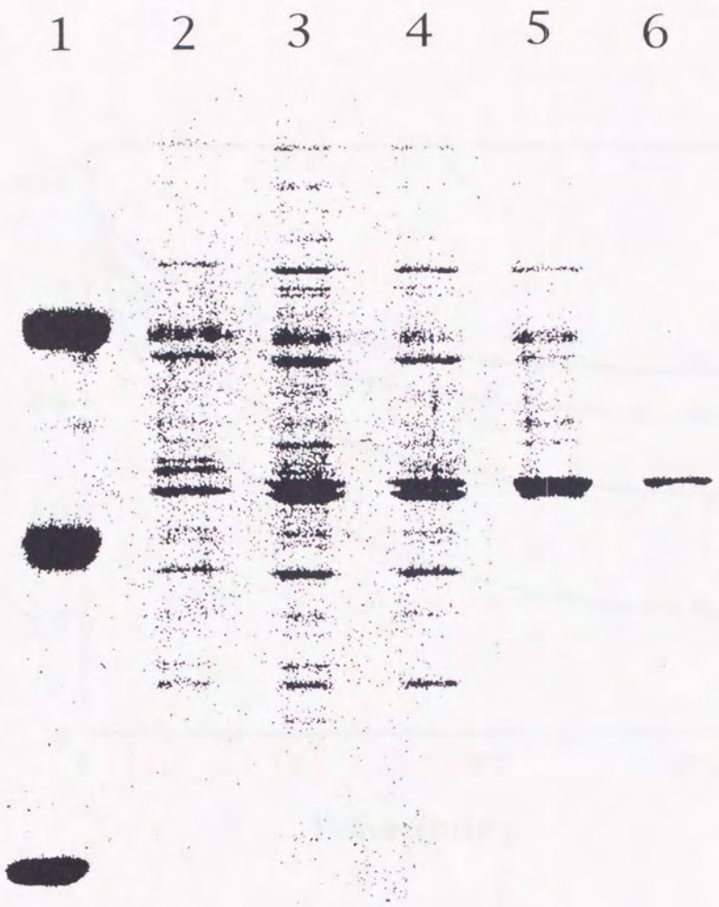

Fig. 1. **Sodium dodecylsulfate-polyacrylamide gel electrophoresis.** Lane 1, bovine serum albumin (M_r 67,000), egg albumin (43,000), and carbonic anhydrase (30,000); Lane 2, the crude extract from *E. coli* JM109 cells; Lane 3, the crude extract from *E. coli* JM109 cells carrying pEGS; Lane 4, the 78,000 x *g* supernatant; Lane 5, the ammonium sulfate precipitate; Lane 6, the eluant from Q-Sepharose.

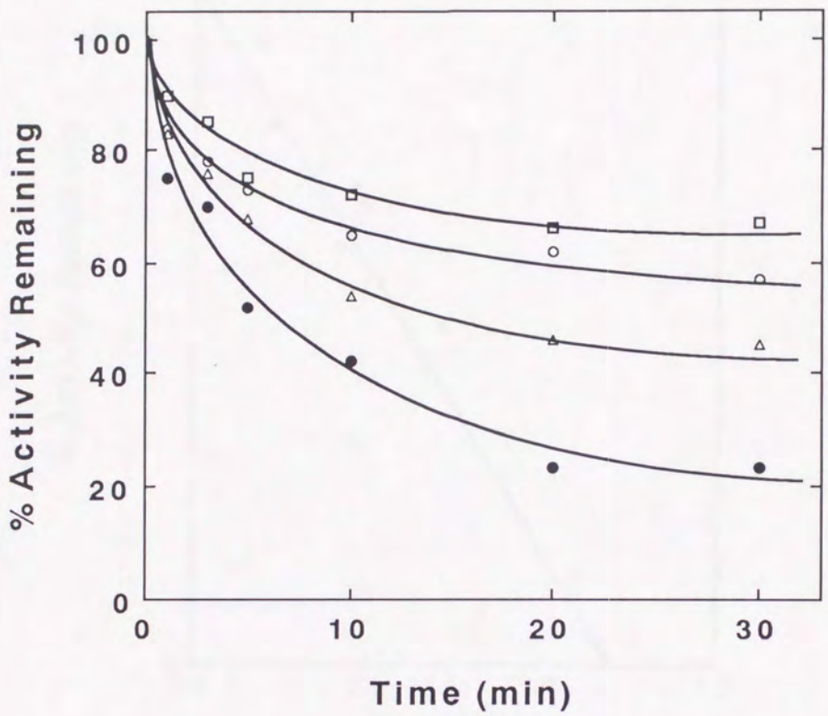

Fig. 2. **Inactivation of *E. coli* glycogen synthase by AP_2-PL.** The inactivation mixture (200 μl) contained 50 mM sodium glycerophosphate (pH 7.0), 0.2 mM dithiothreitol, 1 mM EDTA, 10 % glycerol, 2 μM enzyme, and AP_2-PL at 5 μM (□), 10 μM (○), 20 μM (△),or 50 μM (●). The mixture was incubated at 20 °C for various times, and 25 μl was withdrawn, mixed with 1.5 μl of 30 mM sodium borohydride. After dilution, the enzyme activity was measured.

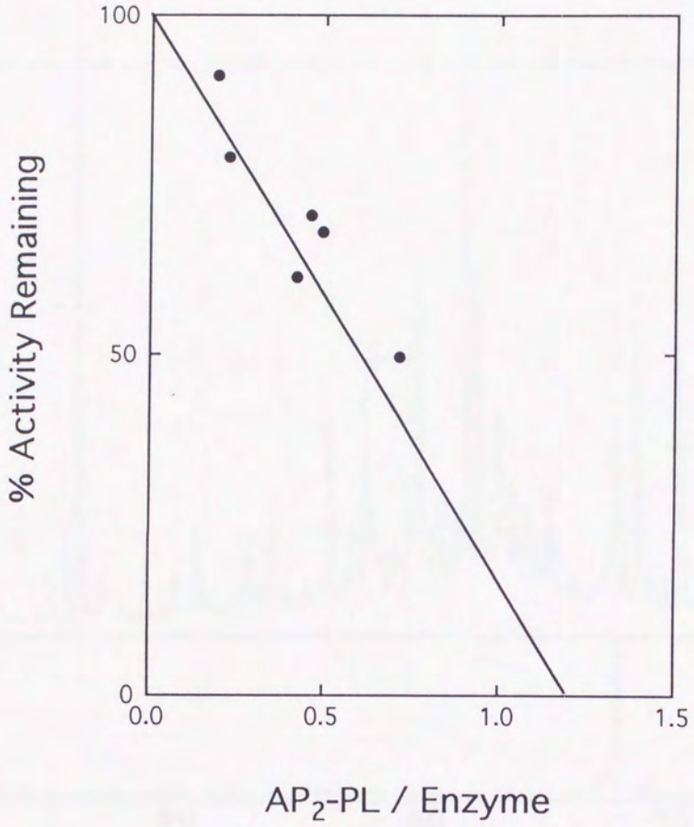

Fig. 4. **Stoichiometry of the inactivation.** Stoichiometry of the inactivation was measured as described under "Experimental Procedures".

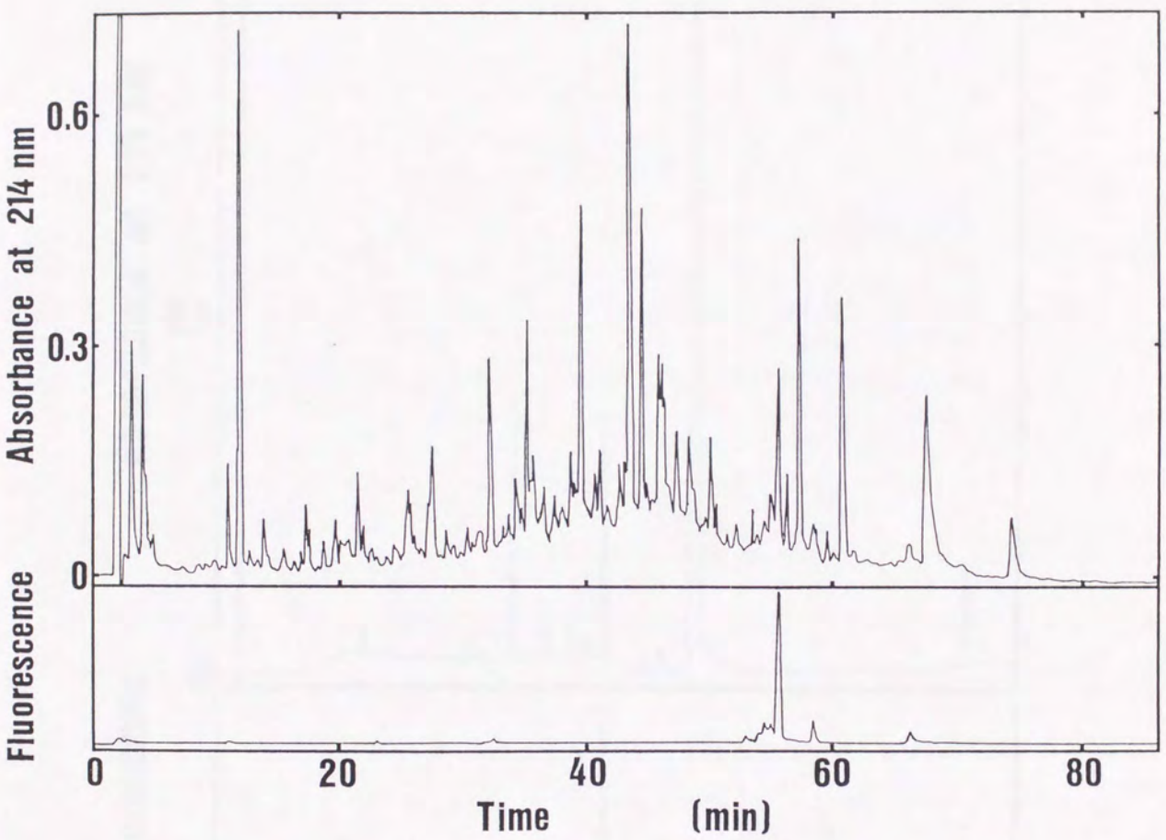

Fig. 5. **Elution profile of a tryptic digest of the AP_2-PL-labeled glycogen synthase.** The tryptic digest obtained as described under "Experimental Procedures" was applied to a VYDAC C4 column. The peptides were eluted with a linear gradient of Buffer (b) from 0 to 100 % over 50 min. Absorbance at 214 nm and the fluorescence (excitation at 330 nm and emission at 395 nm) of the effluent were continuously monitored.

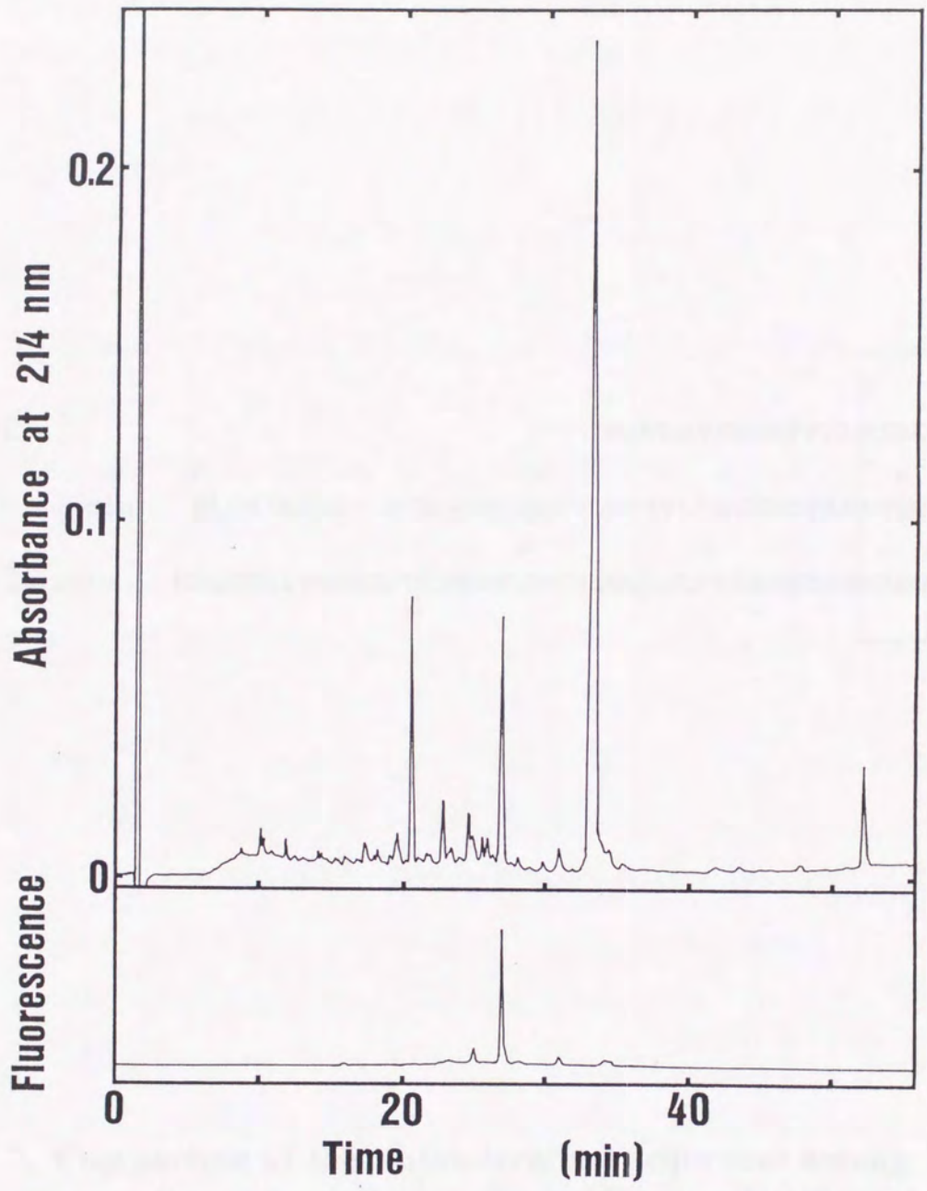

Fig. 6. **Elution profile of a V8 protease digest of the AP_2-PL-labeled peptide.** The V8 protease digest of the AP_2-PL-labeled peptide was applied to a VYDAC C18 column. The peptides were eluted with a linear gradient of Buffer (b) from 0 to 100 % over 50 min. Absorbance at 214 nm and the fluorescence (excitation at 330 nm and emission at 395 nm) of the effluent were continuously monitored.

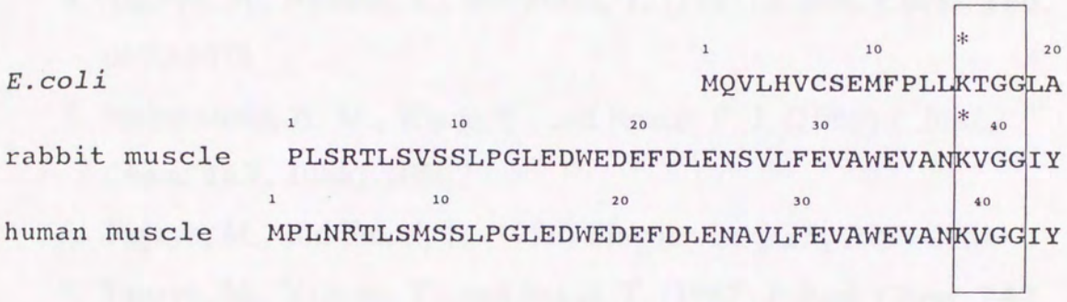

Fig. 7. **Comparison of the amino-terminal sequences among glycogen synthases from *E. coli* (2), rabbit muscle (5), and human muscle (3).** *Asterisks* represent lysyl residues labeled by either AP_2-PL or UP_2-PL.

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CHAPTER III

Role of the Conserved Lys-X-Gly-Gly
Sequence at the ADP-Glucose-binding Site

INTRODUCTION

Glycogen synthase catalyzes the glucosyl transfer from nucleoside diphosphoglucose to the nonreducing end of glycogen. This enzyme can be classified into two groups according to specificity for the glucosyl donor. The enzymes from *Escherichia coli* and higher plants (starch synthase), which utilize ADP-glucose as the glucosyl donor (1), have subunit molecular weights of 49,000-58,000 and are similar to each other in the amino acid sequence (about 30% identity) (2, 3). Mammalian and yeast glycogen synthases, which prefer UDP-glucose to ADP-glucose (4), have subunit molecular weights of 78,000-85,000 (5) and share about 50% sequence identity (6-9). However, there is no detectable sequence similarity between the ADP-glucose- and UDP-glucose-specific glycogen synthases.

As described in Chapter II, Lys38 in the rabbit muscle enzyme (10) and Lys15 in the *E. coli* enzyme, which were specifically labeled by UP_2-PL and AP_2-PL, respectively, are found in a tetrapeptide sequence, Lys-X-Gly-Gly (where X represents an unspecified residue) conserved in both enzymes. Since the nucleotidylyl derivatives of pyridoxal potentially react with lysyl residues at the polyphosphate-binding loci in nucleotide-binding proteins (11), the two labeled residues were suggested to be located at the substrate-binding site of glycogen synthases.

To elucidate the functional role of the conserved Lys-X-Gly-Gly sequence in glycogen synthase, the lysyl and two glycyl residues in the *E. coli* enzyme has been replaced by other amino acid residues via site-directed mutagenesis. The results reported in this chapter suggest that Lys15 is involved in binding of the phosphate moiety adjacent to the glycosidic linkage in the substrate ADP-glucose, through an ionic interaction. On the other hand, Gly17 presumably participates in the

catalysis by assisting conformational changes of the active site or stabilizing the transition state.

EXPERIMENTAL PROCEDURES

Materials — Vent DNA polymerase was obtained from New England Biolabs. Restriction endonucleases and a DNA ligation kit were obtained from Takara; plasmid pTrc 99A was from Pharmacia LKB Biotechnology; pBluescript II SK+ was from Toyobo; α-amylases from *Bacillus subtilis* and human saliva were from Seikagaku Kogyo. and Sigma, respectively; and ADP-[^{14}C]glucose (8.77 GBq/mmol) was from ICN Radiochemicals. An *E. coli* B mutant strain JP717 was kindly provided by Dr. Jack Preiss, Biochemistry Department, Michigan State University. AP_2-PL, AP_3-PL, and AP_4-PL were synthesized as described previously (12). Oyster glycogen purchased from Nacalai Tesque was purified as described previously (13).

Construction of Plasmid pEGS1 — Although pEGS was used in Chapter II for the overproduction of *E. coli* glycogen synthase, this plasmid appeared unfavorable for performing site-directed mutagenesis. Thus, pEGS1 was newly constructed as outlined in Fig. 1. A DNA fragment covering one-third of the *glg*A gene from its upstream region was amplified by PCR using pFY140, which contains the entire *glg*A gene and a part of the *glg*P gene (14), as a template and two synthetic primers designated as -38(+) and 585(-), generating a *Sma*I site at the 5'-end of the amplified fragment (Fig. 2). After digestion with *Sma*I and *Eco*RI (at position 568), the DNA fragment was subcloned into pBluescript II SK+. The resultant plasmid was then digested by *Nco*I and *Eco*RV, and ligated with the *Nco*I-*Eco*47III fragment from pFY140. The

*Eco*47III site is located at 181-bp downstream from the termination codon in *glg*A. The plasmid thus prepared (designated as pEGS-Blue) includes the entire glycogen synthase gene (*glg*A), but its direction is opposite with respect to the promoter. Therefore, the *Sma*I-*Sal*I fragment from pEGS-Blue was finally cloned into pTrc99A to give the expression plasmid designated as pEGS1.

Site-directed Mutagenesis — Site-directed mutagenesis was performed by the two-step PCR as described by Higuchi (15). First, two DNA fragments overlapping 15 or 16 bp each other and containing the same mutation were amplified separately using the synthetic primers shown in Fig. 2, paired with either -38(+) or 585(-). After excess primers remaining were removed by Centricon (Amicon), the two fragments were mixed and heated at 92 °C for 3 min for annealing. Using primers -38(+) and 585(-), the second PCR was carried out to produce a fragment contiguously covering the two regions amplified in the first reaction. The DNA fragment thus prepared was digested with *Sma*I and *Nco*I and subcloned into pEGS-Blue. The *Sma*I-*Sal*I fragment (encoding the entire enzyme) was cloned into pTrc99A for the expression of the mutant enzyme.

Enzyme Expression and Purification — An *E. coli* mutant strain JP717 lacking glycogen synthase activity (Preiss, J. personal communication) was used as a host cell for overproduction of the recombinant enzyme. The cells harboring the wild-type or mutant plasmids were cultured at 37 °C in Luria broth containing 50 μg/ml ampicillin. After 2 h of the precultivation, isopropyl β-D-thiogalactopyranoside was added at a final concentration of 2 mM, and the cells were cultured at 22 °C for another 12 h. Incubation at this temperature minimized the formation of inclusion bodies of the expressed glycogen synthase. The purification procedures of

the mutant enzymes were the same as those described for the wild-type enzyme (Chapter II).

Assay of Glycogen Synthase — Glycogen synthase activity was determined by the filter assay method as described by Thomas *et al.* (16). Steady-state kinetic parameters were determined by systematically varying the concentrations of both ADP-glucose and glycogen in the assay mixture, and the experimental data were analyzed on the basis of the sequential bi-bi mechanism by the least-squares method using a computer program.

pH Study — The buffer used in the pH study contained 50 mM MES, 50 mM HEPES, and 100 mM ethanolamine. The pH values of the assay mixture were adjusted with NaOH or HCl. For determination of pK values of ionizable groups in the Michaelis complex, $\log(V_{max})_{app}$ values measured with various concentrations of ADP-glucose at a saturating concentration (0.5 mg/ml) of glycogen in the pH range of 5.5-10.5 were plotted against pH. For determination of pK_i values for inhibition by ADP or AMP, pK_i values determined from the Dixon plot in the pH range of 6.6-10.5 were plotted against pH. Both plots were analyzed by the nonlinear regression computer program described by Cleland (17) according to the following equation.

$$\log y = \log \frac{c}{1 + [H]/K_1 + K_2/[H]} \qquad \text{eq. (1)}$$

where K_1 (K_a or K_{ia}) and K_2 (K_b or K_{ib}) are the dissociation constants for ionizable groups, y is $(V_{max})_{app}$ or $1/K_i$ at a particular pH, and c is the pH-independent constant of the parameter y.

Inactivation by Pyridoxal Phosphate and Its Nucleotidylyl Derivatives — The wild-type, G17A, or G18A enzyme (3 μM) was incubated with various concentrations of pyridoxal phosphate, AP_2-PL, AP_3-PL, or AP_4-PL at 20 °C for 30 min. After reductive fixation of the bound pyridoxal

compound by sodium borohydride, the residual enzyme activity was measured. For identification of the AP_2-PL- or AP_3-PL-labeled residue, the wild-type or G17A enzyme (10 μM each) was incubated with 200 μM AP_2-PL or AP_3-PL at 20 °C for 30 min, reduced with sodium borohydride, and *S*-pyridylethylated. The *S*-pyridylethylated protein was digested at 30 °C for 24 h with trypsin added in an enzyme-to-substrate ratio of 1 : 50 (w/w). Purification of the labeled peptides was performed on a Tosoh high performance liquid chromatography system using a Cosmosil 5C18 (Nacalai Tesque) column with the solvent system consisting of 0.1% trifluoroacetic acid (A) and 0.088% trifluoroacetic acid containing 60% acetonitrile (B). The peptides were eluted at a flow rate of 0.5 ml/min with a 40-min linear gradient of 0-100% B in solvent A. Fluorescence of the eluate (excitation at 330 nm and emission at 390 nm) was monitored.

Protein Concentration — Protein concentration was determined according to the method of Bradford (18) and calculated by using a molar absorption coefficient at 595 nm of 2.0 $x10^6$ M^{-1} as described in Chapter II.

RESULTS

Mutation of Lys15 — After constructing a new expression plasmid (pEGS1, Fig. 1) for the *E. coli* glycogen synthase, Lys15 was replaced by Glu, Gln or Arg by the two-step PCR with synthetic mutagenic primers (Fig. 2) to examine the effect of changing the charge or bulkiness of the side-chain. Table I summarizes the steady-state kinetic parameters of the wild-type and mutant glycogen synthases purified to homogeneity. The Michaelis constants for glycogen of the three Lys15 mutant enzymes were essentially identical with that of the wild-type enzyme. In contrast, their

K_m values for ADP-glucose were much larger than that of the wild-type enzyme. These results are consistent with the idea that Lys15 is located at the ADP-glucose-binding site as proposed in the preceding chapter. Substitution of Gln or Glu for Lys15 resulted in a 30 to 40-fold increase in the K_m values for ADP-glucose, while that of Arg had much smaller effect. Thus, the affinity for ADP-glucose appears to correlate with the charge of the amino acid residue at position 15. This suggests that the ε-amino group of Lys15 is involved in binding of ADP-glucose. The k_{cat} values of the three Lys15 mutants were 6-17% of the wild-type enzyme without apparent correlation with the character of amino acid residues, indicating that Lys15 is dispensable for the catalysis.

To further clarify the role of Lys15 in binding of ADP-glucose, pK values for ionizable groups in the Michaelis complex (Fig. 3a) and pK_i values for ADP and AMP in the competitive inhibition (Fig. 3b) were determined with the wild-type enzyme and K15Q enzyme having no charge on its side chain. ADP is a good competitive inhibitor with respect to ADP-glucose with K_i of about 30 μM at pH 8.5, whereas AMP is a poor one with K_i of about 0.5 mM at pH 8.5 (19). In the plot of $\log(V_{max})_{app}$ versus pH, both wild-type and K15Q enzymes showed similar convex profiles with pK_a = 6.0±0.03 and pK_b = 10.7±0.04, and pK_a = 6.5±0.06 and pK_b = 9.6±0.06, respectively (Fig. 3a), suggesting again that the ionization state of the ε-amino group of Lys15 is not directly related to the catalytic process. The slight shift of pK values in the K15Q enzyme profile may be due to a minor change of the active site structure caused by the mutation.

In contrast, the pK_i profile for ADP of the K15Q enzyme was quite different from that of the wild-type enzyme (Fig. 3b). The wild-type enzyme showed a convex pK_i profile, giving pK_{ia} and pK_{ib} values of 7.0±0.06 and 10.1±0.03, respectively. By analyzing the pK_i profile of the

wild-type enzyme at different temperatures (at 20 °C and 30 °C), the enthalpy changes for ionization (ΔH_{ion}) were calculated to be 0.5 kcal/mol for the group in the acidic side and 12.2 kcal/mol for the group in the alkaline side. The ΔH_{ion} value of the latter group is in good agreement with the value of an amino group in proteins (10-13 kcal/mol) (17), while the small ΔH_{ion} value of the former group may be ascribable to the second deprotonation from the β-phosphate group (pK≒6.3) of ADP. On the other hand, the pK_i values for ADP of the K15Q enzyme did not change in a pH range of 6.7-10.4. The pK_i profile for AMP of the wild-type enzyme was also independent on pH (Fig. 3b). These results clearly show that the ε-amino group of Lys15 with pK of about 10 is involved, in its protonated form, in the ionic interaction with the β-phosphate group of ADP.

Mutation of Gly17 and Gly18 — Three mutant enzymes were then prepared, in which Gly17 and Gly18 are individually or simultaneously replaced by Ala (Fig. 2), to see the effect of minimum change in the side-chain bulkiness on the catalytic properties. All the Gly mutants were purified to homogeneity and analyzed for their kinetic parameters (Table I). The most noteworthy change in kinetic parameters was that the k_{cat} value of the G17A enzyme was three-orders of magnitude smaller than that of the wild-type enzyme, though that of the G18A enzyme was only 3.2-fold smaller. On the other hand, changes in the K_m values for ADP-glucose and glycogen were trivial. The parameters of the double mutant enzyme (G17A/G18A) were similar to those of the G17A enzyme. CD studies excluded a possibility that the conformations of the G17A and G18A enzymes are grossly altered (data not shown). These results suggest that Gly17 plays an important role in the catalytic process, but not in the binding of ADP-glucose, while the role of Gly18 in the catalysis is much less significant.

Affinity Labeling of Gly17 and Gly18 Mutants — The affinity labeling reagent AP_2-PL specifically modified Lys15 in *E. coli* glycogen synthase (Chapter II). The reactivity of a lysyl residue in proteins toward the formyl group of pyridoxal phosphate or its derivatives appears to be dependent on p*K* of the amino group, which in turn is affected by both its position and microenvironment in the protein (11). Only the deprotonated form of an amino group is capable of forming the Schiff base. The position of the lysyl residue relative to that of the formyl group in the bound reagent may also influence the rate of the Schiff base formation. To investigate changes in the microenvironment around the ADP-glucose-binding Lys15 caused by the mutations of Gly17 and Gly18, the inactivation profiles by pyridoxal phosphate and its adenine nucleotidylyl derivatives of the mutant enzymes were compared with those of the wild-type enzyme. As shown in Fig. 4, the G17A and G18A enzymes, like the wild-type enzyme, were inactivated effectively by AP_2-PL. The concentrations of AP_2-PL required for 50% inactivation (IC_{50}) were 10, 90, and 25 μM for the wild-type, G17A, and G18A enzymes, respectively. The differences in the IC_{50} values of the three enzymes for AP_2-PL corresponded fairly well with the differences in their K_m values for ADP-glucose (Table I). Interestingly, the wild-type enzyme was inactivated by pyridoxal phosphate with an IC_{50} value of 80 μM, whereas the G17A and G18A enzymes were not inactivated by this reagent up to 100 μM. On the contrary, the wild-type enzyme was not inactivated by AP_3-PL, whereas the G17A and G18A enzymes were inactivated by this reagent with IC_{50} values of 150 and 60 μM, respectively. Neither the wild-type nor the mutant enzymes were inactivated by AP_4-PL. These results indicate that both mutations of Gly17 and Gly18 significantly change, in a similar way, the reactivity of Lys15 toward the pyridoxal derivatives, if it is labeled also in the mutant enzymes.

Thus, labeled lysyl residue(s) in the AP_2-PL- or AP_3-PL-modified mutant enzyme (G17A) were then determined. The G17A enzyme inactivated with AP_2-PL or AP_3-PL was cleaved with trypsin, and the labeled peptide was purified by HPLC as described under "Experimental Procedures". A single significantly fluorescent peak, whose retention time was identical with that of the AP_2-PL-labeled peptide derived from the wild-type enzyme, was observed in the elution profiles of both modified mutant enzymes (Fig. 5). Sequence analysis revealed that both AP_2-PL- and AP_3-PL-labeled peptides start from Met1 and Lys15 is actually labeled (Table II).

DISCUSSION

The study presented in this chapter has been undertaken to elucidate the functional role of a sequence motif, Lys-X-Gly-Gly (X represents an unspecified amino acid residue), conserved in the mammalian and bacterial glycogen synthases (10), although the residue corresponding to Lys15 of the *E. coli* enzyme is Arg in the yeast enzyme (9). Replacement of Lys15 of the *E. coli* enzyme by Gln or Glu resulted in a marked increase in K_m values for ADP-glucose, while that by Arg lead to only a 6-fold increase. Considering the appreciably high activities (k_{cat} values) of the Lys15 mutant enzymes, Lys15 is not essential for the catalysis. Nevertheless, the ε-amino group of Lys15 is important for efficient binding of ADP-glucose, most likely through an ionic interaction with the phosphate moiety adjacent to the glycosidic linkage in ADP-glucose. In agreement with this, the inhibition constant for ADP of the K15Q enzyme and that for AMP of the wild-type enzyme were independent on pH and much larger than the pH-dependent constants for ADP of the wild-type

enzyme (Fig. 3b). Loss of the ionic interaction caused by the replacement of Lys15 by Gln or Glu, therefore, probably lowers the affinities for ADP and ADP-glucose. On the other hand, $(V_{max})_{app}$ values of the K15Q enzyme, like the wild-type, are pH-dependent, suggesting that ionizable groups other than Lys15 are involved in the catalytic process.

The importance of the two conserved glycyl residues (Gly17 and Gly18) for the catalytic function is to quite different extents. The G17A enzyme had essentially no activity, whereas the G18A enzyme showed a k_{cat} value nearly 40% of that of the wild-type enzyme. In spite of this marked difference, the changes in the reactivity of Lys15 with affinity labeling reagents caused by the mutations of Gly17 and Gly18 are similar with each other. Replacement of Gly17 or Gly18 by Ala makes Lys15 capable of reacting with AP_3-PL, but not with pyridoxal phosphate (Fig. 4). This may reflect the positional and/or microenvironmental change of Lys15 (ε-amino group) at the ADP-glucose-binding site by the mutations. However, this change must be very subtle, since AP_4-PL reacts with Lys15 of neither the wild-type enzyme nor Gly17 and Gly18 mutant enzymes. The fact that replacements of Gly17 and Gly18 by Ala result in only small increases in the K_m value for ADP-glucose also suggests that the perturbation at the ADP-glucose-binding site by the mutations of Gly17 and Gly18 is limited. On the other hand, two possible explanations can be given to the dramatic decrease in the k_{cat} value of the G17A enzyme. In triose phosphate isomerase, the amide nitrogens of the two glycyl residues (Gly232 and Gly233) interact with the phosphate moiety of substrate triose phosphate (20). The amide nitrogen or carbonyl of Gly17 may also directly participate in the catalytic process, for example, by stabilizing the transition state of the glucosyl transfer reaction through a hydrogen-bonding interaction. Alternatively, the main-chain flexibility

provided by Gly17 may be critical for a conformational change during the catalysis.

TABLE I

Kinetic parameters of wild-type and mutant glycogen synthases

Enzyme	K_m for ADP-Glc	K_m for glycogen	k_{cat}
	mM	*mg/ml*	s^{-1}
wild-type	0.12 ± 0.01	26 ± 3	694 ± 28
K15Q	3.9 ± 0.3	44 ± 2	120 ± 10
K15E	5.6 ± 1.0	52 ± 1	44 ± 3
K15R	0.82 ± 0.06	28 ± 1	82 ± 6
G17A	0.64 ± 0.05	36 ± 5	0.58 ± 0.03
G18A	0.37 ± 0.07	17 ± 2	260 ± 47
G17,18A	0.34 ± 0.06	32 ± 1	0.14 ± 0.02

TABLE II

The structures of AP_2-PL- and AP_3-PL-labeled peptide derived from the G17A mutant enzyme

Cycle	AP_2-PL-labeled peptide		AP_3-PL-labeled peptide	
	Residue	Yield	Residue	Yield
		pmol		*pmol*
1	Met	248	Met	118
2	Gln	138	Gln	150
3	Val	102	Val	106
4	Leu	136	Leu	105
5	His	55	His	22
6	Val	84	Val	72
7	ND[a]	—	ND	—
8	Ser	88	Ser	34
9	Glu	28	Glu	47
10	Met	113	Met	28
11	Phe	75	Phe	65
12	Pro	65	Pro	54
13	Leu	50	Leu	43
14	Leu	70	Leu	98
15	ND[b]	—	ND	—
16	Thr	18	Thr	16
17	Ala	43	Ala	67
18	Gly	56	Gly	88
19	Leu	53	Leu	67
20	Ala	19	Ala	62

[a] This residue corresponds to Cys-7. *S*-Pyridylethylated cysteine cannot be identified on the system used for phenylthiohydantoin-amino acid analysis. [b] This residue corresponds to Lys-15. The lysyl residue labeled by nucleotidylyl derivatives of pyridoxal cannot be identified (10, 12).

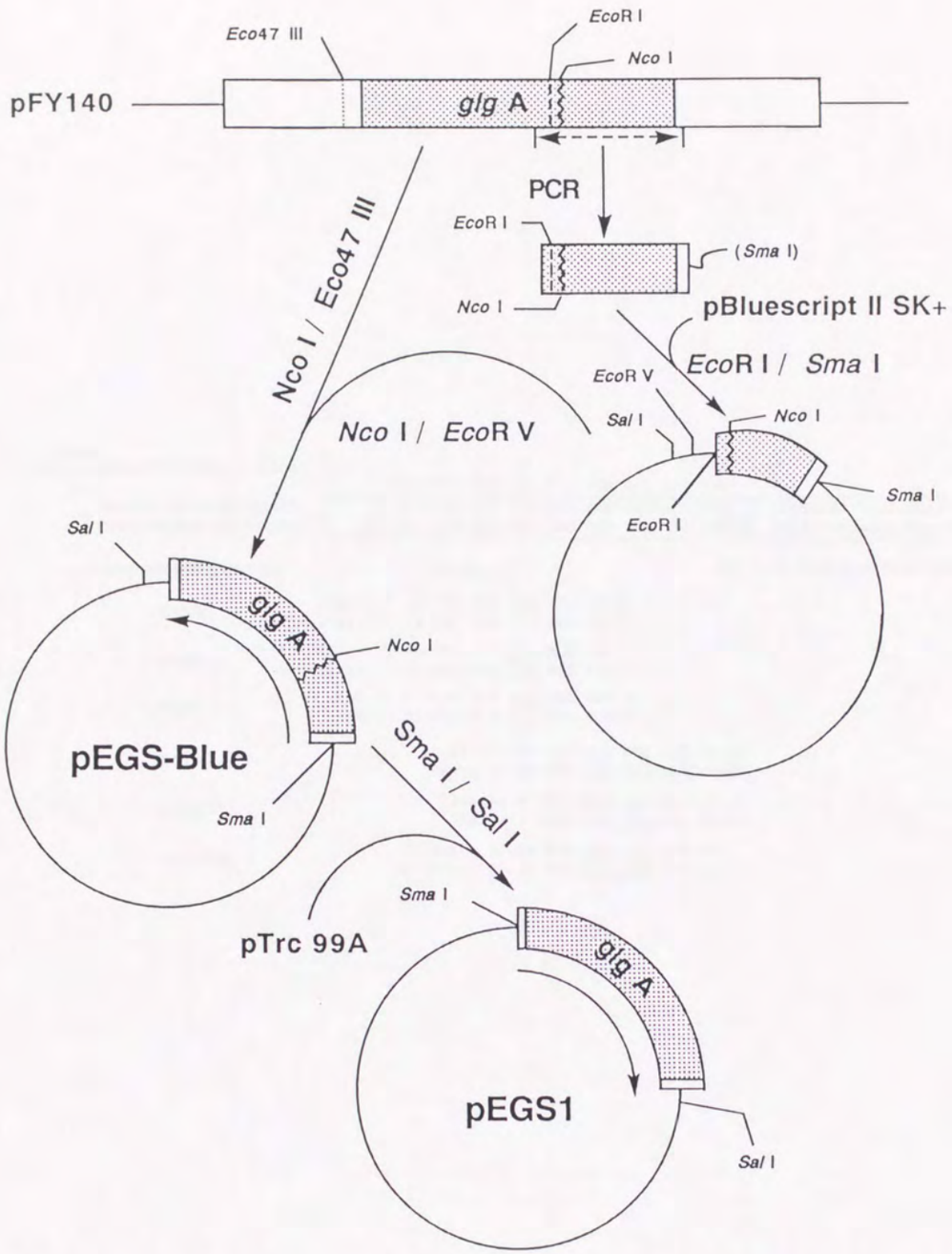

FIG. 1. **Construction scheme for the expression plasmid of *E. coli* glycogen synthase (*glg*A).** Only relevant restriction sites are indicated, and lengths of DNA are shown arbitrary. Restriction fragments are shown by bars and marked differently for an easier understanding of the cloning procedure; lines are the regions from vector DNA.

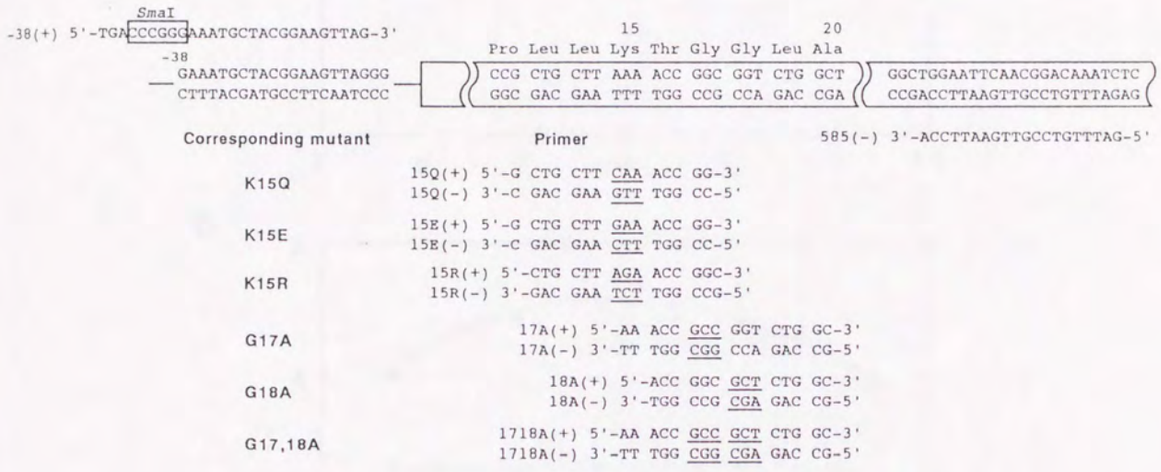

FIG. 2. **Sequences of primers for PCR and site-directed mutagenesis of glycogen synthase.** The mutated codons are underlined. For replacement of Lys15 by Gln, primers -38(+) and 15Q(-), and primers 585(-) and 15Q(+) were used in the first PCR, and primers -38(+) and 585 (-) were used in the second PCR. Construction of other mutant genes was carried out in a similar way.

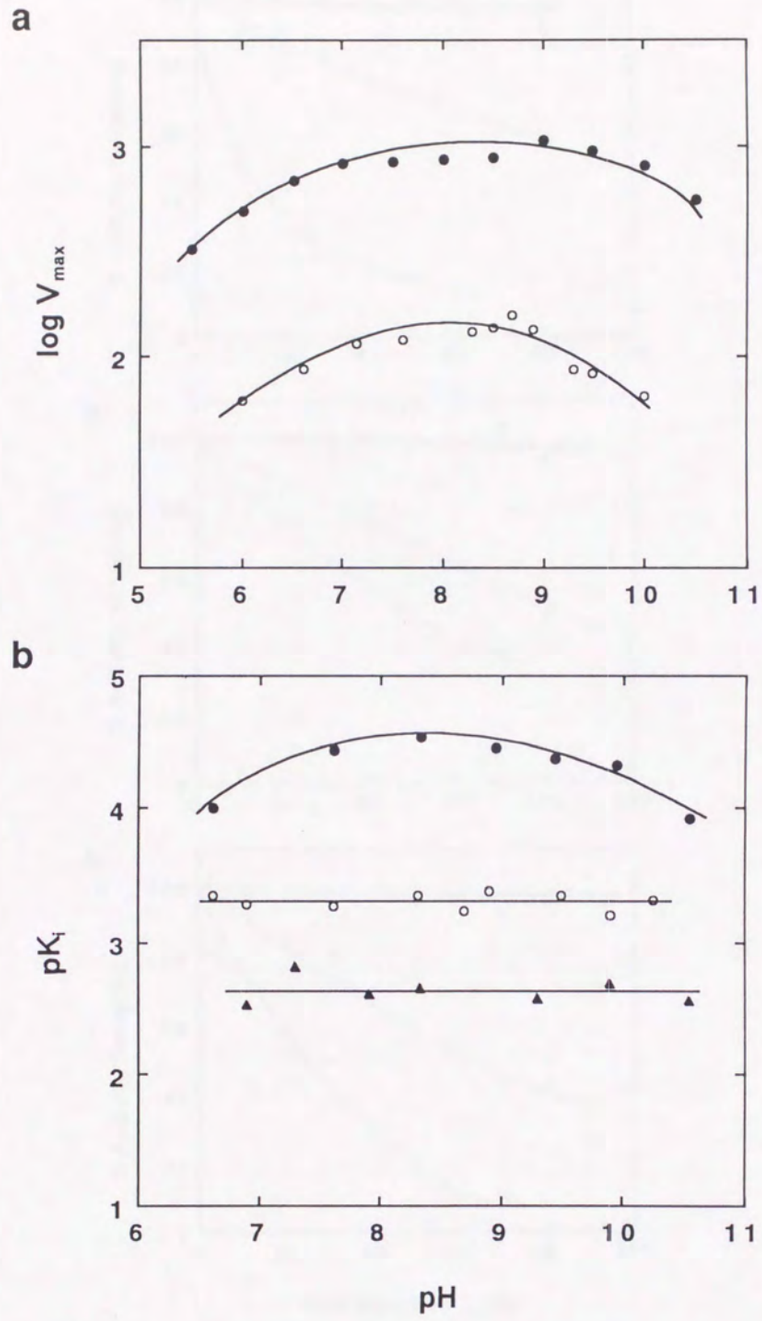

FIG. 3. **pH profiles for kinetic parameters of the wild-type and K15Q enzymes.** **a**, $\log(V_{max})_{app}$ values of the wild-type (●) and K15Q (○) enzymes were plotted against pH. **b**, pK_i values for ADP of the wild-type (●) and K15Q (○) enzymes, and those for AMP (▲) of the wild-type enzyme were plotted against pH.

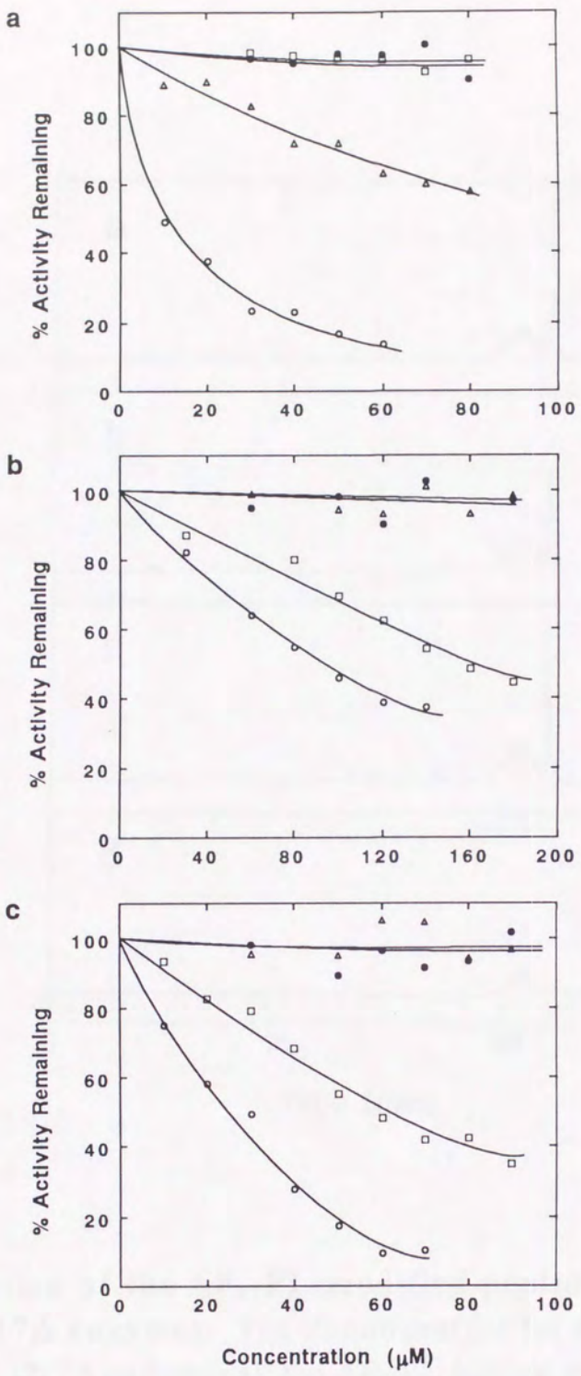

FIG. 4. **Inactivation of the wild-type and Gly17 and Gly18 mutant enzymes by pyridoxal derivatives.** The wild-type enzyme (**a**), G17A enzyme (**b**), or G18A enzyme (**c**) was incubated with pyridoxal phosphate (△), AP_2-PL (○), AP_3-PL (□), or AP_4-PL (●). See "Experimental Procedures" for details.

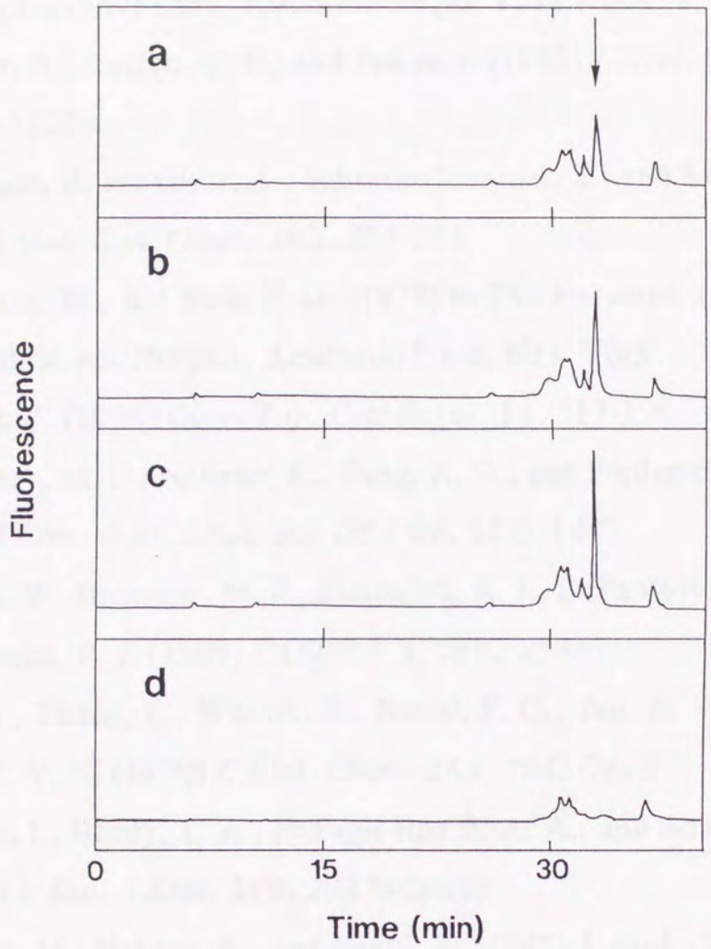

FIG. 5. **Isolation of the AP_n-PL-modified peptides in the wild-type and G17A enzymes.** The elution profiles for the AP_3-PL-labeled peptide in the G17A enzyme (**a**), the AP_2-PL-labeled peptide in the G17A enzyme (**b**), the AP_2-PL-labeled peptide in the wild-type enzyme (**c**), and the unlabeled G17A enzyme (**d**). The fluorescence derived from the pyridoxyllysine moiety (excitation at 330 nm and emission at 390 nm) was monitored. The position of the labeled peptide eluted is indicated by an arrow.

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CHAPTER IV

Identification of Active Site Lys277 by Affinity Labeling of Lys15 Mutant Enzyme

INTRODUCTION

E. coli glycogen synthase catalyzes glucosyltransfer from ADP-glucose to the nonreducing end of glycogen. In the catalytic properties it shows some similarities to mammalian glycogen synthases (1). However, the mammalian enzymes, which prefer UDP-glucose to ADP-glucose as the glucosyl donor, have subunit molecular weights about twice twice as much as that of the *E. coli* enzyme and the two enzymes share no detectable overall sequence similarity (2, 3). Lys38 in the mammalian enzyme is specifically labeled by UP_2-PL (4), and Lys15 in the *E. coli* enzyme is also specifically labeled by AP_2-PL, as described in Chapter II. Interestingly, Lys15 and Lys38 were found in the conserved tetrapeptide sequence of Lys (labeled)-X-Gly-Gly (where X represents an unspecified residue). In Chapter III, site-directed mutagenesis of the *E. coli* enzyme was performed to clarify the role of the conserved tetrapeptide sequence. Kinetic analyses of the mutant enzymes in which Lys15 is replaced by other amino acid residues revealed that the lysyl residue is mainly involved in binding of the phosphate moiety adjacent to the glycosidic linkage of ADP-glucose, presumably through an ionic interaction. The glycyl residue which is closer to the lysyl residue in the conserved sequence participates in the catalytic reaction by assisting conformational changes of the active site or stabilizing a transition state.

In this chapter, affinity labeling of the K15Q enzyme with AP_2-PL has been undertaken. It was expected that lysyl residue(s) other than Lys15 located at the ADP-glucose-binding site may be labeled by AP_2-PL, as a result of the replacement of Lys15 by an amino acid residue unreactive to the reagent. Lys277 has been found to be specifically labeled by AP_2-PL in the K15Q enzyme. Site-directed mutagenesis study has shown that this

residue is important for the catalytic reaction, but not for the binding of ADP-glucose.

EXPERIMENTAL PROCEDURES

Materials — AP_2-PL, AP_3-PL, and AP_4-PL were synthesized according to the method of Tagaya and Fukui (5). All other materials were obtained from the sources as described in Chapters II and III.

Purification of Enzymes — The purification procedure of mutant glycogen synthase was the same as that of the wild-type enzyme (Chapter II). *E. coli* JP717, which originates from *E. coli* B strain and lacks glycogen synthase activity, was used as the host cell for expression of the mutant enzymes (Chapter III).

Protein Concentration — Protein concentration was determined according to the method of Bradford (6) and calculated by using a molar absorption coefficient at 595 nm of 2.0×10^6 M^{-1}.

Enzyme Assay — Glycogen synthase activity was measured by the filter assay method as described by Thomas *et al.* (7). Steady state kinetic parameters were determined by plotting the reciprocals of initial reaction rates measured by the systematic variations of the concentrations of ADP-glucose and glycogen in the assay mixture.

Inactivation of the K15Q Enzyme by AP_2-PL — K15Q mutant glycogen synthase (0.6 μM) was incubated with varying concentrations of AP_2-PL at 20 °C. After reduction by sodium borohydride at 0 °C for 10 min, the residual activity of the modified enzyme was measured. For the isolation of the AP_2-PL-labeled peptide, the inactivation mixture (100 μL) containing 4 nmol of the K15Q enzyme and 1 mM AP_2-PL was incubated with or without 20 mM ADP-glucose at 20 °C for 30 min and followed

by sodium borohydride reduction. The labeled enzyme was *S*-pyridylethylated, dialyzed, and lyophilized. The lyophilized protein was dissolved in 200 μL of 0.1 M Tris-HCl containing 2 M urea, and trypsin was added with an enzyme-to-substrate ratio of 1 : 50 (w/w). Digestion was performed at 30 °C for 24 h. The labeled peptide was isolated on a Tosoh HPLC system with the following solvents: A, 0.1% trifluoroacetic acid, and B, 0.088% trifluoroacetic acid containing 60% acetonitrile.

Amino Acid Sequence Analysis — Amino acid sequence was determined with an Applied Biosystems model 477A protein sequencer linked with an Applied Biosystems model 120A PTH analyzer.

Site-directed Mutagenesis — Site-directed mutagenesis of Lys277was performed by a two-step PCR according to the method of Higuchi *et al.* (8) as described in Chapter III. This method requires two complement primers for constructing one mutation site. The primers used were summarized in Table I. Plasmid pTrc99A (9) was used for the expression of the mutant enzymes.

RESULTS

Inactivation of the K15Q Enzyme by AP_2-PL — Lys15 in *E. coli* glycogen synthase is important for binding of the substrate ADP-glucose, but not essential for enzyme activity (Chapter III). To search other lysyl residue(s) at the active site, the mutant enzyme in which Lys15, a residue reactive to the labeling reagent, is replaced by an unreactive glutaminyl residue via site-directed mutagenesis was modified with AP_2-PL. When incubated with AP_2-PL at concentrations in a range from 200 to 800 μM followed by sodium borohydride reduction, the K15Q enzyme was inactivated in a dose- and time-dependent manner (Fig. 1a). The inactivation reaction ceased within 20-30 min, as observed in the modifications of other enzymes with the nucleotidylyl derivatives of pyridoxal (10). This cessation probably reflects the formation of an equilibrium between the Schiff base and free aldehyde of the pyridoxal moiety of the reagent. Only the enzyme species forming the Schiff base is irreversibly inactivated by borohydride reduction. The concentration of AP_2-PL required for 50% inactivation was about 500 μM, which is approximately 30-times that required for 50% inactivation of the wild-type enzyme (Chapter II). However, this difference is in good agreement with the difference in the K_m values for ADP-glucose between the wild-type and K15Q enzymes (Chapter III). The requirement of higher concentrations of AP_2-PL for the inactivation of the K15Q enzyme is probably due to its lower affinity for adenine nucleotides.

Next, the effect of substrate on inactivation by AP_2-PL was examined. A high concentration (20 mM) of substrate was added to the inactivation mixture containing 800 μM AP_2-PL, because the K15Q enzyme showed an affinity for ADP-glucose markedly lower than the wild-type enzyme as described above (Chapter III). As shown in Fig. 1b, the inactivation of

the K15Q enzyme was almost completely protected by ADP and ADP-glucose, suggesting specific binding of AP_2-PL at the ADP-glucose- or ADP-binding site of the K15Q enzyme.

Identification of Labeled Residue — To confirm specific binding of AP_2-PL at the ADP-glucose-binding site and determine the labeled site(s), the K15Q enzyme modified with the labeling reagent in the presence or absence of ADP-glucose was *S*-pyridylethylated and digested by trypsin as described under "Experimental Procedures". The digests were applied to a reverse phase column on an HPLC system, and the absorbance at 214 nm and fluorescence (excitation at 330 nm and emission at 395 nm) derived from pyridoxyllysine were monitored. When the enzyme was modified by AP_2-PL in the absence of ADP-glucose, one predominant fluorescent peak was observed (Fig. 2a). The amount of this fluorescent peak markedly decreased when the enzyme was labeled in the presence of ADP-glucose (Fig. 2b). These results suggest that a lysyl residue at the ADP-glucose-binding site is specifically labeled by AP_2-PL, although the enzyme was incubated with a high concentration of the labeling reagent. Sequence analysis revealed that the structure of the labeled peptide is Ala-Glu-Asn-X-Arg, where X represents a residue not positively identified. This structure is identical to that from Ala274 to Arg278 except for Lys277 in the complete amino acid sequence of *E. coli* glycogen synthase (2). Since the AP_2-PL labeled lysyl residue cannot be positively identified (10), it was concluded that Lys277 is specifically labeled by AP_2-PL.

Reactivities of Lys277 to AP_2-, AP_3-, and AP_4-PL — Reactivities of lysyl residues located at the adenine nucleotide-binding sites toward the nucleotidylyl derivatives of pyridoxal vary in enzymes (10). In adenylate kinase, Lys21 is labeled by any of AP_2-PL, AP_3-PL, and AP_4-PL (11, 12). The reactivities of Lys15 in *E. coli* glycogen synthase to these labeling reagents are strikingly different from those of Lys21 in adenylate

kinase. Lys15 in glycogen synthase is labeled by AP_2-PL, but not by AP_3-PL or AP_4-PL (Chapter III). Only when Gly17 or Gly18 is replaced by Ala, Lys15 in the mutant enzymes is labeled by AP_3-PL as well as AP_2-PL (Chapter III). It was interested in whether Lys277 is labeled by AP_3-PL and AP_4-PL or not. Fig. 3 shows that the K15Q enzyme was inactivated by neither AP_3-PL nor AP_4-PL up to 1 mM, twice as much as the concentration required for 50% inactivation by AP_2-PL.

Sequence comparison — Lys15 labeled by AP_2-PL in the modification of the wild-type *E. coli* glycogen synthase is located at the tetrapeptide sequence of Lys-X-Gly-Gly, which is conserved, except for X, in both mammalian and higher plant enzymes (3, 13), although the lysyl residue is replaced by an arginyl residue in the yeast enzyme (14). When the sequence around Lys277 in *E. coli* glycogen synthase was compared with the amino acid sequences of the mammalian (3) and yeast enzymes (14), both of which show no overall sequence similarity to the *E. coli* enzyme, no similar region was found. However, as shown in Fig. 4, a sequence around Lys277in the *E. coli* enzyme is well conserved in maize starch synthase, which shows about 30% amino acid identity to the *E. coli* enzyme (13). This conservation suggests that Lys277 in *E. coli* glycogen synthase plays an important role in enzyme catalysis.

Replacement of Lys277 — To reveal the role of Lys277, the glycogen synthase mutant enzyme in which Lys277 is replaced by Gln was prepared via site-directed mutagenesis. Gln was chosen to examine the effect of removal of a positive charge at position 277 without significant change in side-chain bulkiness. Table II summarizes the kinetic properties of the wild-type and K277Q enzymes. The K_m values for ADP-glucose and glycogen changed little. In contrast, the k_{cat} value of the K277Q enzyme was 140-times as small as that of the wild-type enzyme. These results

suggest that Lys277 is involved in the catalytic reaction rather than binding of ADP-glucose.

DISCUSSION

As described in Chapter II, Lys15 in *E. coli* glycogen synthase is specifically labeled by AP_2-PL. Site-directed mutagenesis studies showed that this lysyl residue is mainly involved in binding of the substrate ADP-glucose, but not essential for enzyme activity (Chapter III). Obviously, amino acid residue(s) other than Lys15 are involved in the catalytic reaction of this enzyme. In this chapter, it has been found that Lys277 in the K15Q enzyme is specifically labeled by AP_2-PL. The results of site-directed mutagenesis have demonstrated that this residue, which is conserved in maize starch synthase (2, 13), actually plays an important role in the catalytic reaction.

One possible explanation why Lys277 is not labeled by AP_2-PL in the modification of the wild-type enzyme, but labeled in the modification of the K15Q enzyme is that Lys15 is located close to the formyl group of the labeling reagent bound to the wild-type enzyme, whereas Lys277 is located relatively far. However, this explanation encounters difficulty in interpreting the finding that Lys277 in the K15Q enzyme, like Lys15 in the wild-type enzyme, is labeled by AP_2-PL, but not by AP_3-PL or AP_4-PL. The labeling of Lys277 only by AP_2-PL in the K15Q enzyme rather suggests that Lys277, as well as Lys15, is located adjacent to the pyrophosphate moiety of the labeling reagent, although a possibility that mutation of Lys15 results in a movement of Lys277 close to the formyl group of the labeling reagent cannot be excluded. Probably the reactivity of Lys15 toward the formyl group of AP_2-PL is much higher than that of

Lys277. Once AP_2-PL binds to the more reactive Lys15, the ADP-binding site is occupied, therefore Lys277 is not labeled by the second molecule of the labeling reagent.

The mutant enzyme, in which Lys277 is replaced by Gln, was prepared to see the effect of removal of the positive charge at the position of Lys277 with a minimum change of the side-chain bulkiness on the enzyme reaction. The kinetic parameters of the mutant enzyme shows the positive charge of this residue is dispensable for the binding of the substrates but important for the catalytic reaction. The role of the ε-amino group of Lys277 in the catalytic process will be discussed in the following chapter.

TABLE I

Sequence of synthetic primers for site-directed mutagenesis

Primer	Sequence
	275 280
	-Ala-Glu-Asn-Lys-Arg-Gln-Leu-
	GCG GAA AAT AAG CGC CAG TTA
	CGC CTT TTA TTC GCG GTC AAT
	*
277Q+	5'-GCG GAA AAT CAG CGC CAG T-3'
	*
277Q-	3'-CGC CTT TTA GTC GCG GTC A-5'
641+	5'-CATCCAATTGCCATGGTCAT-3'
1109-	3'-CCACGACGTCCTTCCAAAG-5'

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

TABLE II

Kinetic parameters of wild-type and mutant glycogen synthases

Enzyme	K_m for ADP-glucose	K_m for glycogen	k_{cat}
	mM	*μg/ml*	s^{-1}
wild-type	0.12±0.01	26±3	694 ±28
K277Q	0.17±0.03	11±1	4.9±1.6

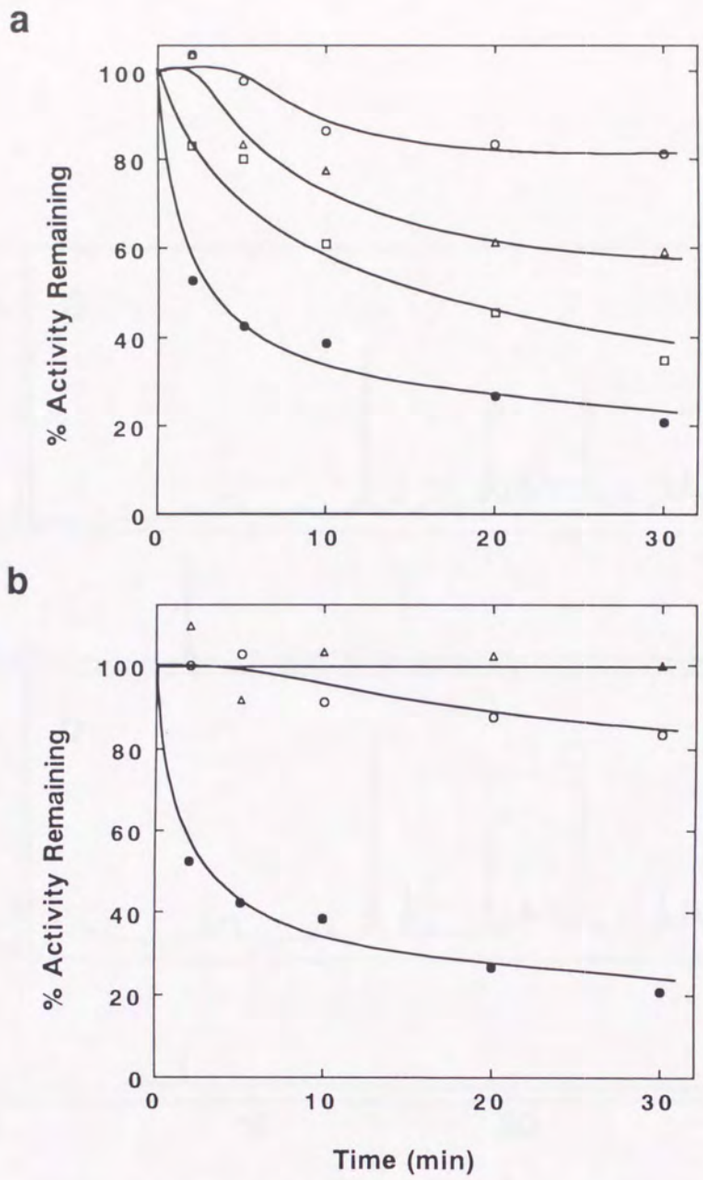

Fig. 1. **Inactivation of K15Q enzyme by AP_2-PL. a,** effect of the concentration of the reagent. The inactivation mixture (150 μl) contained 50 mM sodium glycerophosphate (pH 7.0), 2 mM dithiothreitol, 10% glycerol, 0.6 μM K15Q enzyme, and AP_2-PL at 200 μM (○), 400 μM (△), 600 μM (□), or 800 μM (●). The mixture was incubated at 20 °C for various times, and 25 μl was withdrawn, and mixed with 1.5 μl of 30 mM sodium borohydide. After dilution, the enzyme activity was measured. **b,** effect of substrates on the inactivation. K15Q enzyme was incubated with 800 μM AP_2-PL in the presence of 20 mM ADP-glucose (○), 20 mM ADP (△), or none (●). Other conditions were essentially the same as above.

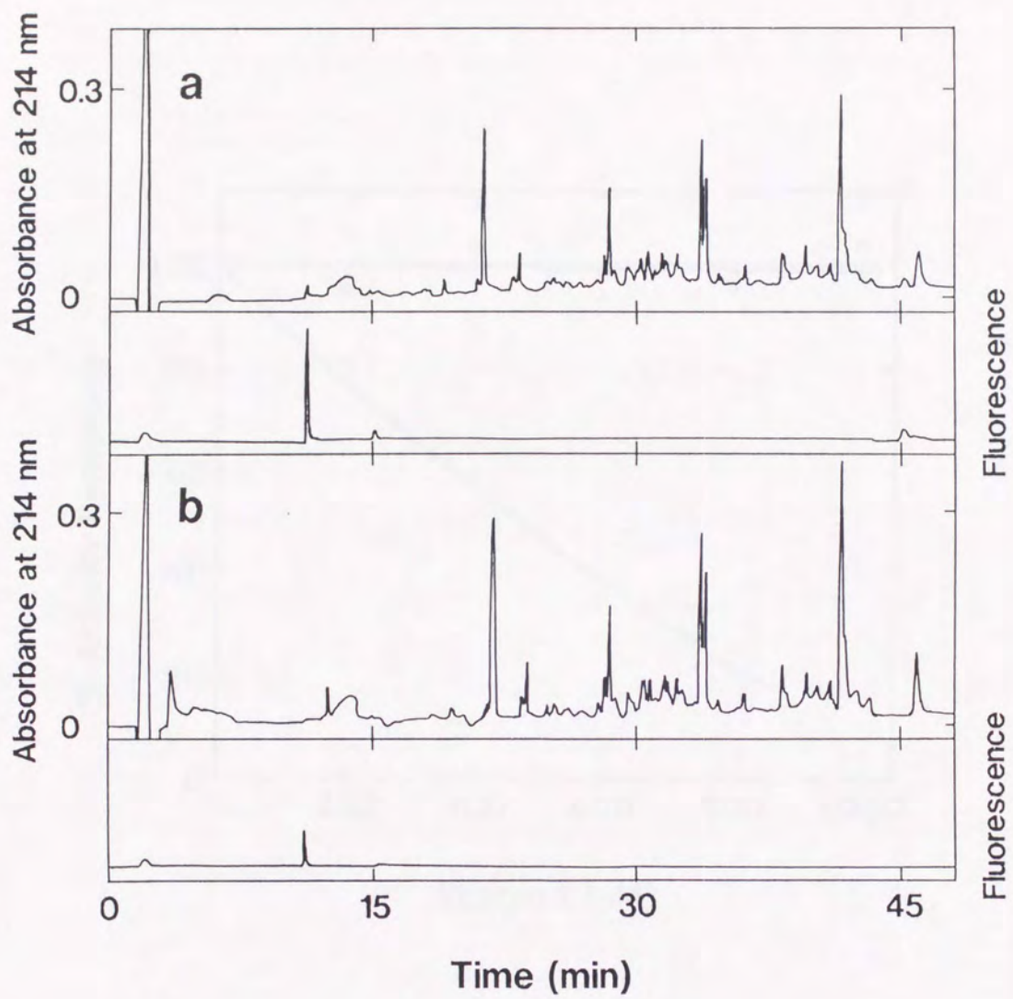

Fig. 2. **Elution profiles of trypsin digests of the AP_2-PL-labeled K15Q enzyme.** The trypsin digest of K15Q enzyme which was labeled by AP_2-PL in the absence (a) or the presence (b) of ADP-glucose was applied to a Cosmosil 5C18-AR column (Nacalai Tesque). The peptides were eluted with a linear gradient of buffer (B) from 0 to 100% over 40 min. Absorbance at 214 nm and the fluorescence (excitation at 330 nm and emission at 395 nm) of the effluent were continuously monitored.

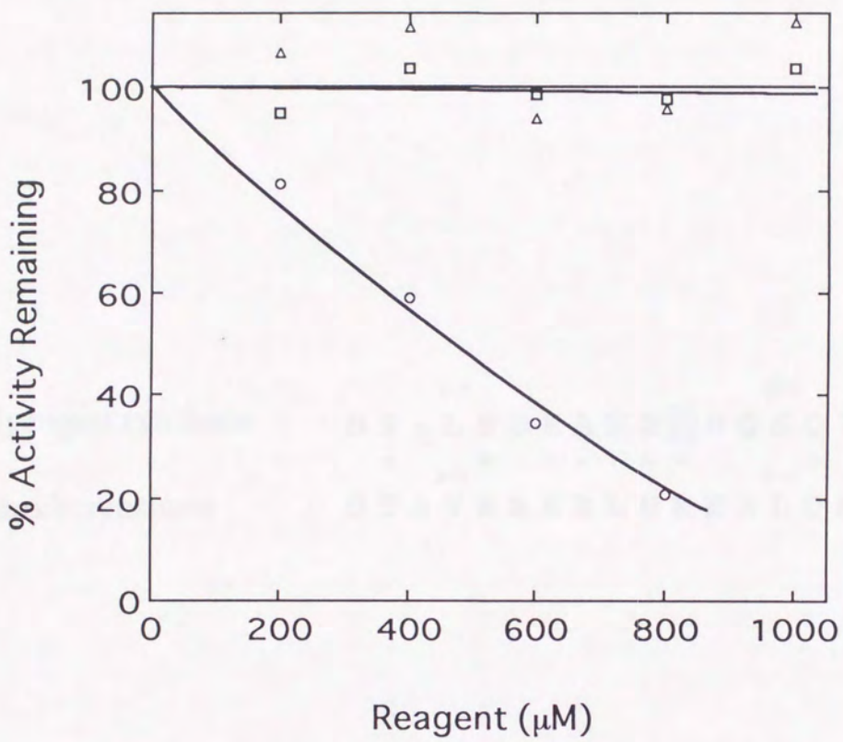

Fig. 3. **Inactivation of K15Q enzyme by AP_n-PL.** The K15Q enzyme was incubated with various concentrations of AP_2-PL (○), AP_3-PL (△), or AP_4-PL (□) at 20 °C for 30 min, and reduced by sodium borohydide at 0 °C for 10 min. The residual activity of the labeled enzyme was measured after dilution.

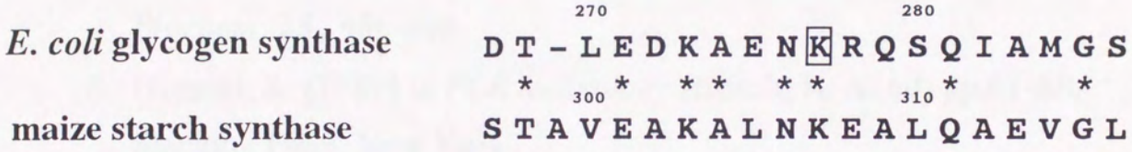

Fig. 4. **Comparison of the amino acid sequences between *E. coli* glycogen synthase and maize starch synthase.** Gaps (*hyphens*) were introduced for maximum matching. *Numbers* above sequences are residue numbers in each enzyme. *Asterisks* indicate identical residues. The lysyl residue labeled by AP_2-PL is *boxed*.

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CHAPTER V

Comprehensive Discussion

In this chapter, a new approach of affinity labeling progressed in the present study is discussed first. Then, the feature of *E. coli* glycogen synthase as a nucleotide-binding protein is also discussed. Finally, possible reaction mechanism of glycogen synthase is described in the comparison with that of glycogen phosphorylase.

Combined Application of Affinity Labeling and Site-directed Mutagenesis — Affinity labeling has been widely used to examine the structure of the active sites of enzymes. Although the binding of an labeling reagent is promised by its affinity moiety, amino acid residues reactive to the reagent are labeled regardless of the importance for enzyme reaction. In UDP-glucose pyrophosphorylase, for example, five lysyl residues (Lys263, Lys329, Lys,367, Lys409, and Lys410) were labeled by UP_2-PL or UP_3-PL (1). Mutagenesis studies (2) revealed that Lys367 is essential for the catalytic reaction, and Lys263 and Lys329 participate in binding of the substrates. However, Lys409 and Lys410 appear to be involved in neither the catalytic process nor binding of the substrates. In glutathione synthetase, Lys17, Lys18, Lys144, and Lys148 are labeled by AP_3-PL (3). Crystallographic analysis showed that Lys18 is located at the γ-glutamylcysteine-binding site, whereas Lys17, Lys_{144}, and Lys148 are all located in the mouth of the nucleotide-binding crevice. AP_3-PL seems to be trapped by lysyl residues located at the mouth when it enters into or leaves from the nucleotide-binding crevice.

In the present study, affinity labeling was performed on the mutant enzyme in which the amino acid residue most reactive to the reagent is replaced by an unreactive amino acid residue. As a result, a catalytically important amino acid residue which had not been modified in the affinity labeling of the wild-type enzyme was identified at the active site of the enzyme. This new application of affinity labeling combined with site-

directed mutagenesis would be applicable to other proteins and provide a fertile information on the structure-function relationship of enzymes.

Role of Lysyl and Glycyl Residues in Nucleotide-binding Site — Walker *et al.* (4) reported that a Gly-X-X-X-X-Gly-Lys sequence ("glycine-rich region") is conserved in several ATP- and GTP-binding proteins which include adenylate kinase (5), H+-ATPase (4), and the *ras* oncogene product p21 (6). Since the glycine-rich sequence forms a loop structure in these proteins and plays a crucial role in binding of phosphate moieties of adenine and guanine nucleotides, it is also called a "phosphate-binding loop (P-loop)" (7). The conserved lysyl residues in these proteins were specifically labeled by adenosine or guanosine polyphosphopyridoxals (8-12). The motif of a lysyl residue in the glycine-rich region seems to be general as a structural element of polyphosphate-binding loci. In adenylate kinase, a segment of Gly-Gly-Pro-Gly-Ser-Gly-Lys-Gly constructs a loop structure with the turn at Pro17 (13). (The residue numbers of the amino acids follow the amino acid sequence of the porcine muscle enzyme.) AP_2-, AP_3-, and AP_4-PL modified the same lysyl residue, Lys21, in the glycine-rich loop (8,9). Enzyme inactivation caused by the modification of Lys21 was retarded most effectively by the addition of ATP. These observations were interpreted as the evidence for the location of the ε-amino group of Lys21 close to the γ-phosphate of bound MgATP. Reinstein *et al.* (14) reported that the mutant enzyme in which Lys21 was replaced by Gln had essentially no enzyme activity. The positive charge of the ε-amino group of Lys21 might play an essential role in the catalytic process.

The glycine-rich loop containing Lys21 in adenylate kinase has high mobility compared with the other part of the enzyme molecule. The loop remarkably moved during the transition between two crystal forms, being regarded as an induced-fit of the enzyme (15). It was suggested that the

conformational flexibility of this loop is important for the enzyme activity of this enzyme. The mutant adenylate kinase in which Pro17 is replaced by Gly or Val *via* site-directed mutagenesis had enzyme activity comparable with the wild-type enzyme, but markedly reduced affinities for the two substrates (16). Replacement of Gly15 or Gly20 by Ala results in similar changes in the catalytic property (17). These results suggest that, in adenylate kinase, the loop structure retained by Pro17 is important for the binding of substrates and, in addition, the two glycyl residues are required for providing the flexibility of the loop structure.

The roles of Lys15 and Gly17 in the conserved Lys-X-Gly-Gly sequence of *E. coli* glycogen synthase revealed in this study are opposed to those of lysyl and glycyl residues in the consensus "P-loop" sequence. In adenylate kinase, as described above, the two conserved glycyl residues (Gly17 and Gly20) are important for binding of the substrates, whereas Lys21 plays an essential role in the catalytic process. In contrast, Gly17 in *E. coli* glycogen synthase is essential for catalytic activity, rather than binding of the substrate, whereas Lys15 is mainly involved in binding of the substrate. The reactivities of the conserved lysyl residues in the two enzymes toward affinity labeling reagents are also different. Lys-21 in adenylate kinase is labeled by any one of AP_2-PL (8), AP_3-PL, and AP_4-PL (9). However, Lys15 in glycogen synthase is labeled only by AP_2-PL. When Gly17 or Gly18 is replaced by Ala, Lys15 in the mutant enzymes becomes reactive with AP_3-PL. It seems likely that the ε-amino group of Lys21 in adenylate kinase is more mobile than that of Lys15 in the *E. coli* glycogen synthase. This difference in the mobility of lysyl residues may be related to the differences in their positions in the respective consensus sequence and in their roles in the catalytic function.

Comparison of Catalytic Mechanism of Glycogen Synthase with that of Glycogen Phosphorylase — Glycogen phosphorylase catalyzes the

transfer of glucosyl moiety from G-1-P to the nonreducing end of glycogen using pyridoxal phosphate as the coenzyme. However, the enzyme functions *in vivo* in the direction of glycogen degradation because the concentration of P_i far exceeds that of G-1-P (18). Its kinetic mechanism is rapid equilibrium random bi-bi (19,20). Two hypotheses concerning the role of the coenzyme have been proposed; pyridoxal phosphate functions as an acid-base (21,22) or an electrophile (23-25). In the acid-base mechanism, the coenzyme 5'-phosphate becomes protonated and functions as an acid providing proton to the substrate phosphate dianion and the reaction is completed by nucleophilic attack of the 4-OH of the terminal glucose of the acceptor polysaccharide on the carbonium ion. In the electrophilic mechanism, the coenzyme 5'-phosphate is tightly coordinated by positive charges and constrained into a trigonal bipyramidal configuration, where the empty apical position is carrying a positive charge. This position is attacked by a nucleophilic O of the substrate phosphate and the resulting pseudopyrophosphate bond is expected to withdraw an electron from the substrate phosphate and thus labilize the glycosidic bond.

The affinity labeling with bis(pyridoxal phosphate) of rabbit muscle glycogen phosphorylase showed the presence of Lys574 at the active site of this enzyme (26). The lysyl residue is conserved also in potato tuber starch phosphorylase and modified by the same reagent (27). It was proposed based on the results of x-ray crystallographic studies that the ε-amino group of Lys574 interacts with the phosphate group of either the coenzyme or substrate G-1-P (28,29). Moreover, Hu and Johnson recently observed that Lys568 and Arg569 as well as Lys574 participate in the interaction with both phosphate groups ( ). In both acid-base and electrophile mechanisms, these interactions should be the motive factor to labilize the α-C1-O1 glycosidic bond of G-1-P. The α-configuration in

C1 is retained through enzyme catalysis (31), suggesting a double displacement mechanism involving the formation of either a β-glycosyl-enzyme covalent or carbonium ion intermediate (32). The amino acid residue to stabilize these intermediate must present near the glycosidic bond of G-1-P.

Information on the structure-function relationship in *E. coli* glycogen synthase obtained in the present studies are summarized as follows. Lys15, which is specifically labeled by AP_2-PL (Chapter II), is located at the active site of *E. coli* glycogen synthase. Site-directed mutagenesis studies (Chapter III) revealed that this lysyl residue is mainly involved in binding of ADP-glucose through an ionic interaction of its ε-amino group with the phosphate moiety close to the glycosidic linkage of ADP-glucose. When Lys15 is replaced by an unreactive residue, Lys277 is specifically labeled by AP_2-PL (Chapter IV). Site-directed mutagenesis study on this residue revealed that its positive charge is indispensable for the catalytic reaction. The analyses of the two glycine (Gly17 and Gly18) mutants showed that the two glycyl residues, in particular Gly17, play an important role for the catalytic function.

Fig. 1 shows a proposed model for the active site of *E. coli* glycogen synthase based on the results described above. In the catalytic reaction of glycogen synthase, glucosyl transfer to glycogen is considered to proceed via the nucleophilic attack of the 4-hydroxyl group of the terminal glucose of glycogen on C1 carbon of nucleoside diphosphoglucose. Takagi *et al.* (25) discovered that the glucosyl moiety of pyridoxal diphosphoglucose when the compound was reconstituted with apophosphorylase was transferred to the nonreducing end of glycogen. This finding showed that the catalytic reaction can occur even if the two phosphate groups of the coenzyme pyridoxal phosphate and the substrate G-1-P are linked through a pyrophosphate linkage. The structure of

pyridoxal diphosphoglucose is similar to that of ADP-glucose or UDP-glucose. It is therefore possible that the catalytic mechanisms of glycogen synthase and glycogen phosphorylase resemble each other. In this sense, it is assumed that, at the active site of glycogen synthase, there are basic amino acid residues which play the following roles; (i) reducing the electron density of the C1 carbon of ADP- or UDP-glucose through interaction with the pyrophosphate moiety, (ii) promoting or stabilizing the carbonium intermediate at C1 position of ADP- or UDP-glucose, and (iii) increasing the nucleophilicity of the 4-hydroxyl group of the nonreducing terminal glucose of glycogen. The present results suggest that, in *E. coli.* glycogen synthase, Lys15 plays the role of (i). Lys277 may play the role of (ii) or (iii), or increase the basicity of these catalytic residues. On the other hand, two possibilities on the role of Gly17 can be considered based on the nature of glycine. Gly17 may assist conformational changes of the active site in the catalytic reaction, or may stabilize a transition state through hydrogen bonding with its α-amide proton. In the G17A mutant, the enzyme activity decreased more than three-orders of magnitude without significant change of the affinity for substrates, suggesting that the off-rate of the product may be considerably lowered. Gly17 would be important for the movement of the active site at a transition state and/or in the product-release.

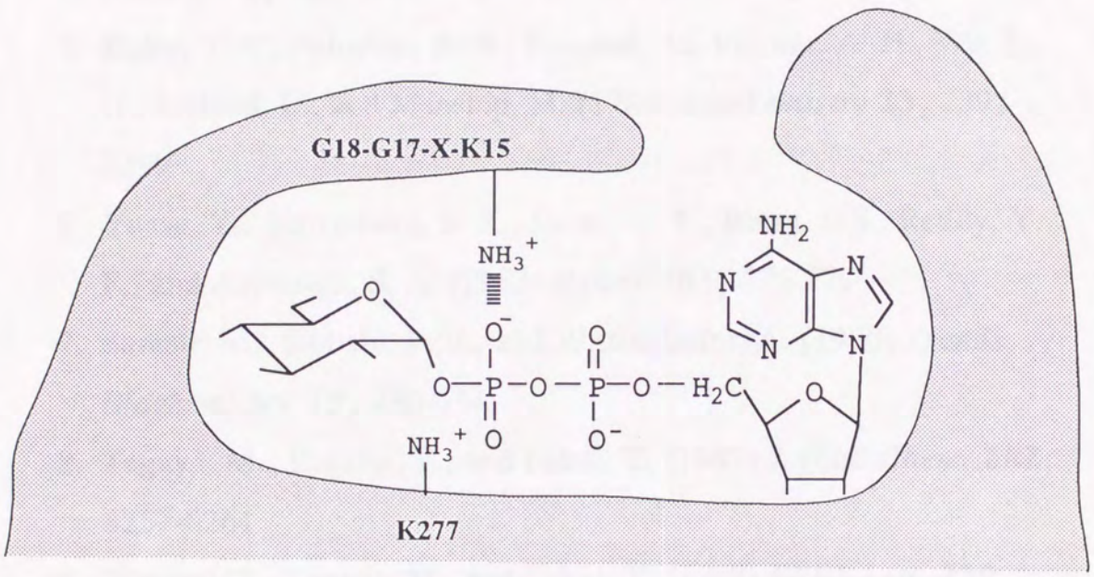

Fig. 1 **A hypothetical model for the active site of *E. coli* glycogen synthase.**

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List of Publications

(1) Papers Related to the Thesis:

Identification of Lysine 15 at the Active Site in *Escherichia coli* Glycogen Synthase: CONSERVATION OF A Lys-X-Gly-Gly SEQUENCE IN THE BACTERIAL AND MAMMALIAN ENZYMES

Koji Furukawa, Mitsuo Tagaya, Masayori Inouye, Jack Preiss, and Toshio Fukui

(1990) *J. Biol. Chem.* **265,** 2086-2090

Role of the Conserved Lys-X-Gly-Gly Sequence at the ADP-Glucose-binding Site in *Escherichia coli* Glycogen Synthase

Koji Furukawa, Mitsuo Tagaya, Katsuyuki Tanizawa, and Toshio Fukui

(1993) *J. Biol. Chem.* in press

Identification of Lys277 at the Active Site of *Escherichia coli* Glycogen Synthase: APPLICATION OF AFFINITY LABELING COMBINED WITH SITE-DIRECTED MUTAGENESIS

Koji Furukawa, Mitsuo Tagaya, Katsuyuki Tanizawa, and Toshio Fukui

(1993) *J. Biol. Chem.* submitted for publication

(2) Other Published Papers:

Fibrinogen Nagoya, a Replacement of Glutamine-329 by Arginine in the γ-Chain That Impairs the Polymerization of Fibrin Monomer

Toshiyuki Miyata, Koji Furukawa, Sadaaki Iwanaga, Junki Takamatsu, and Hidehiko Saito

(1989) *J. Biochem. (Tokyo)* **105,** 10-14