Biosynthesis of Bacterial Glycogen

MUTAGENESIS OF A CATALYTIC SITE RESIDUE OF ADP-GLUCOSE PYROPHOSPHORYLASE FROM ESCHERICHIA COLI*

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Site-directed mutagenesis was used to explore the role of Lys-195 in ADP-glucose pyrophosphorylase from *Escherichia coli.* This residue, which is conserved in every bacterial and plant source sequenced to date, was originally identified as a potential catalytic site residue by covalent modification studies. Mutation of Lys-195 to glutamine produces an enzyme whose *Km* for glucose 1-phosphate is 600-fold greater than that measured for the wild-type enzyme. The effect on glucose 1-phosphate is very specific since kinetic constants measured for ATP, Mg++, and the allosteric activator, fructose 1,6-bisphosphate, are unchanged relative to those measured for the wild-type enzyme. Furthermore, the catalytic rate constant, *kcat*, for the glutamine mutant is similar to that of the wild-type enzyme. Taken together, the results suggest a role for Lys-195 in binding of glucose 1-phosphate and exclude its role as a participant in the rate-determining step(s) in the catalytic reaction mechanism.

To further study the effect of charge, shape, size, and hydrophobicity of the amino acid residue at position 195, a series of mutants were prepared including arginine, histidine, isoleucine, and glutamic acid. In every case, the kinetic constants measured for ATP, Mg++, and fructose 1,6-bisphosphate were similar to wild-type constants, reinforcing the notion that this residue is responsible for a highly localized effect at every case, the kinetic constants measured for ATP, Mg++, and the allosteric activator, fructose 1,6-bisphosphate, were similar to those measured for the wild-type enzyme. The effect on glucose 1-phosphate is 600-fold greater than that measured for the wild-type enzyme. The effect on glucose 1-phosphate is very specific since kinetic constants measured for ATP, Mg++, and the allosteric activator, fructose 1,6-bisphosphate, are unchanged relative to those measured for the wild-type enzyme. Furthermore, the catalytic rate constant, *kcat*, for the glutamine mutant is similar to that of the wild-type enzyme. Taken together, the results suggest a role for Lys-195 in binding of glucose 1-phosphate and exclude its role as a participant in the rate-determining step(s) in the catalytic reaction mechanism.

ADP-glucose pyrophosphorylase (ATP:α-glucose-1-phosphate adenyltransferase, EC 2.7.7.27) is a member of the nucleotidyl transferase class of enzymes, catalyzing the reversible phosphor transfer reaction between ATP and glucose 1-phosphate to yield ADP-glucose and pyrophosphate. The allosterically regulated bacterial enzyme is responsible for the first committed step in glycogen biosynthesis. As such, specifics of its active site chemistry are of interest from the point of view of understanding molecular details of allosteric regulation of catalysis.

What is currently known about this enzyme's catalytic site chemistry and mechanism can be briefly summarized. From steady-state kinetics and equilibrium binding studies substrates are known to bind in an ordered mechanism (1, 2). ATP binds first with positive cooperativity, followed by non-cooperative binding of glucose-1-P (2). Product release is ordered with pyrophosphate released first, followed by ADP-glucose (1). The enzyme apparently obeys a rapid equilibrium mechanism in which substrates bind in rapidly reversible binding steps, followed by slower chemical steps to achieve the transition state since the value measured for Glc-1-P *Km* from steady-state kinetics (3) is equal to the measured Glc-1-P dissociation constant, *Kd* in equilibrium binding experiments (2). Involvement of a covalent enzyme intermediate seems unlikely for this enzyme since Sheu et al. (4) demonstrated for the yeast enzyme, UDP-glucose pyrophosphorylase, that inversion of configuration of the α-phosphate of UTP occurs during formation of the product UDP-glucose. Also, no exchange of glucose 1-phosphate with ADP-glucose or pyrophosphate with ATP occurs in the absence of the second substrate. Little is known about catalytic site residues involved in substrate binding or catalysis for this enzyme. Covalent modification studies (5, 6) originally identified two potential catalytic site residues, Lys-195 and Tyr-114, the latter shown to be involved in ATP and ADP-glucose binding (7, 8). Reductive labeling of Lys-195 with pyridoxal phosphate resulted in loss of catalytic activity; ADP-glucose plus Mg2+ afforded protection against loss of activity (9). The importance of this residue to proper functioning of the enzyme is underscored by its conservation in every bacterial and plant source sequenced to date (10-12). A number of roles can be envisioned for a lysine residue at this particular catalytic site. Lysine may assist in transition state stabilization as, for example, has been suggested for adenylate kinase from *Escherichia coli* (13). Alternatively, lysine may be necessary in the initial binding of either substrate, or it may function in concert with its neighboring amino acid residues to properly fold the protein so as to provide the correct geometry of catalytic residues. Site-directed mutagenesis experiments were therefore undertaken to attempt to distinguish between these possibilities.

1 The abbreviations used are Glc-1-P, glucose 1-phosphate; Fru 1,6-P2, fructose 1,6-bisphosphate; DTT, dithiothreitol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

2 N. Gentner and J. Preiss, unpublished results.

3 B. J. Smith-White, K. Ball, and J. Preiss, unpublished results.
**EXPERIMENTAL PROCEDURES**

**Reagents**—Glucose 1-phosphate, glucosamine 1-phosphate, xylose 1-phosphate, galactose 1-phosphate, deoxyribose 1-phosphate, mannose 1-phosphate, and glucuronic acid 1-phosphate were purchased from Sigma. [14C]Glucose 1-phosphate and [35S]ATP were from Amersham Corp. Enzymes for DNA manipulation and sequencing were from New England Biolabs or Boehringer Mannheim. Oligonucleotides were synthesized and purified by the Macromolecular Facility at Michigan State University. All other reagents were purchased at the highest quality available.

**Bacterial Strains and Media**—Bacterial strains used included E. coli MV1193 (Δlac-proAB) rpsL hsdR4 Δ(srl-rec)306: Tetl(α:Cm) lacZΔM15), E. coli C236 (Δ, ung, thi, relA/pCJ105 [Cm]), and E. coli K12 G6 MD3 (thr, his, thi, Str, Δ(mal A-aad)).

G6MD3 cells were grown in enriched medium which contained 1.1% K2HPO4, 0.85% KH2PO4, 0.6% yeast extract, 0.2% glucose, and 0.5% yeast extract, 0.5% NaCl. CJ236 cells were grown in LB medium containing 0.1 M potassium phosphate buffer, pH 7.5, containing 1 mM EDTA, 0.1 M potassium phosphate buffer, pH 7.5, containing 5 mM DTT, 0.1 M potassium phosphate buffer, pH 7.5, containing 200 mM glucose, and 0.1 M potassium phosphate buffer, pH 7.5, containing 0.5 M KCI, 5 mM DTT, 1 mM EDTA, and 10% glycerol. The column was washed with 60 ml of 0.1 M potassium phosphate buffer, pH 7.5, containing 5 mM DTT, 0.1 M potassium phosphate buffer, pH 7.5, containing 200 mM glucose, and 0.1 M potassium phosphate buffer, pH 7.5, containing 0.5 M KCI, 5 mM DTT, 1 mM EDTA, and 10% glycerol was used to elute the enzyme from the column. Fractions containing activity were pooled, and solid ammonium sulfate was added to 70% saturation. Active enzyme, which precipitated under these conditions, was resuspended in 0.05 M Tris-HCl buffer, pH 7.5, containing 1 mM EDTA, 0.5 mM DTT, and 10% glycerol, and dialyzed versus the same. Dialyzed enzyme was loaded onto an Amicon Green A column (1.5 x 12 cm) equilibrated with 25 mM sodium phosphate, pH 7.0, containing 0.5 M KCI, 5 mM DTT, and 10% glycerol. A step gradient containing three volumes of buffer B containing 0.3 M NaCl, followed successively with four volumes of buffer C containing 1.0, and 2.0 M KCI was used to elute enzyme. Active enzyme fractions were pooled, dialyzed versus 0.05 M Tris-HCl, pH 7.5, containing 1 mM EDTA, 0.5 M DTT, and 10% glycerol. Enzyme was then loaded onto a Mono Q HR 5/5 column using the Pharmacia Fast protein liquid chromatography system. The column was equilibrated with 0.05 M Tris-HCl, pH 7.5, containing 1 mM EDTA and 0.5 mM DTT. Elution of the enzyme was achieved using a gradient of KCl from 0 to 1 M in the equilibration buffer. At a flow rate of 0.5 ml/min, for the first two ml, the gradient used was 0–200 mM KCl. A gradient of 200–500 mM KCl was used for the next 20 ml. The column was washed with 2 ml 500 mM KCl followed by 1 ml 1 M KCl. Active enzyme fractions were pooled, and dialyzed versus 0.05 M Tris-HCl buffer, pH 7.5, containing 1 mM EDTA, 0.5 mM DTT, and 10% glycerol. The purification is summarized in Table I. Wild-type enzyme was purified from pOP12-transformed G6MD3 cells following essentially the same protocol. The purified and mutant and wild-type enzymes were judged to be homogeneous as only one protein band was detected on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, having a molecular mass of about 50,000.

**Expression and Purification of Mutant and Wild-type Enzymes**—The glutamine mutase gene (K195Q) was subcloned into pUC19 at the Smal and PstI restriction sites. For expression of the K195Q enzyme, this plasmid was used to transform E. coli G6MD3, a mutant host with a genomic deletion of the glycogen biosynthetic genes. Cells were grown at 37 °C to a cell density of 5 x 10^9 cells/ml and inoculated with mutant phage at a multiplicity of infection of 10. Cultures were grown an additional 17 h at 37 °C when cells were harvested by centrifugation. Purification of these mutant enzymes was taken through the DEAE-Sepharose step as described.

**Enzyme Kinetics**—Enzymatic activity in the ADP-glucose synthesis direction at 37 °C was measured according to the method of Preiss et al. (3). For assay of wild-type enzyme reaction mixtures (final volume 200 μl) contained 0.1 mmol of [14C]glucose 1-phosphate (specific activity 500–1000 cpm/mmol), 0.3 μmol of ATP, 1.0 μmol of MgCl2, 0.3 μmol of fructose 1,6-bisphosphate, 20 μmol of HEPES buffer, pH 7.0, 100 μg of bovine serum albumin, and 1 μg (0.6 unit) of inorganic pyrophosphatase. For assay of mutant enzymes, the reaction components used were identical to wild-type, with the exception that 5.0 μmol of MgCl2 rather than 1.0 μmol was used. Fig. 2 illustrates the MgCl2 saturation curve for both the wild-type and K195Q mutant enzymes. It is clear that MgCl2 concentration-dependent inhibition occurs in the wild-type but not in the K195Q case. MgCl2 saturation curves for the other four mutants were virtually identical to that of the K195Q enzyme. In order to measure catalytic activity under optimal conditions, a MgCl2 concentration of 5 mM was used for wild-type enzyme measurements, and 25 mM MgCl2 was used for all reported mutant enzyme measurements. Control studies in which mutant enzymes were assayed at 5 mM MgCl2 yielded kinetic constants which were identical within the range of error to those obtained at 25 mM MgCl2. Vmax measurements were lower at 5 mM MgCl2.

**Mutant Lys-195 ADP-Glucose Pyrophosphorylases**

| Enzymes | Mutations | Kinetic Constants |
|---------|-----------|------------------|
| Wild-type | | Kcat=3.0±0.2 and Km=0.1±0.05 mM |
| Lys-195Q | Lys-195Q substitution | Kcat=2.5±0.1 and Km=0.1±0.05 mM |
| Arg-195 | Arg-195 substitution | Kcat=2.8±0.2 and Km=0.1±0.05 mM |
| His-195 | His-195 substitution | Kcat=3.2±0.3 and Km=0.1±0.05 mM |
| Ile-195 | Ile-195 substitution | Kcat=3.5±0.4 and Km=0.1±0.05 mM |
| Glu-195 | Glu-195 substitution | Kcat=3.8±0.5 and Km=0.1±0.05 mM |

**Fig. 1. Nucleotide sequence and encoded protein sequence of the ADP-glucose pyrophosphorylase gene in the region of Lys-195.** The synthetic oligonucleotides used to generate amino acid substitutions at position 195 (lower). The position 195 codons or anticodons are underlined, and the base substitutions are marked with asterisks.
with respect to sugar phosphate substrates other than glucose 1-phosphate. In a reaction volume of 200 μl was combined 0.26 pmol of ATP, either 0.1 pmol or 1.0 pmol of sugar phosphate substrate, 1.0 pmol (wild-type) or 5.0 pmol (K195Q) of MgCl₂, 0.3 pmol of fructose as double-reciprocal plots and the method of Wilkinson (20) was used following expressions for kinetic constants were used giving 50% of maximal activation, inhibition, or maximal velocity.

### Kinetic Characterization

**Kinetic Characterization**—Kinetic data were plotted as initial velocity versus substrate or effector concentration. Data were replotted as double-reciprocal plots and the method of Wilkinson (20) was used to determine Vₘₚ. Sigmoidal plots were replotted as Hill plots to obtain kinetic constants. Kinetic constants from hyperbolic plots were determined by the method of Wilkinson. For sigmoidal data the following expression for kinetic constants were used:

\[ k_{act} \times A_{act} + k_{inh} \times A_{inh} + k_{sub} \times A_{sub} = V \]

where:
- \( k_{act} \): catalytic rate constant
- \( A_{act} \): concentration of activator
- \( k_{inh} \): association constant of inhibitor
- \( A_{inh} \): concentration of inhibitor
- \( k_{sub} \): association constant of substrate
- \( A_{sub} \): concentration of substrate
- \( V \): velocity

Duplicate were run in each case; kinetic constants are expressed as the mean ± the difference from duplicate determinations.

#### Sugar Phosphate Specificity

The following assay was developed for measuring the activity of wild-type and K195Q mutant enzymes with respect to sugar phosphate substrates other than glucose 1-phosphate. In a reaction volume of 200 μl was combined 0.26 μmol of ATP, either 0.1 μmol or 1.0 μmol of sugar phosphate substrate, 1.0 μmol (wild-type) or 5.0 μmol (K195Q) of MgCl₂, 0.3 μmol of fructose 1,6-bisphosphate, 20 μmol of Hepes buffer, pH 7.0, and 1 μg of inorganic pyrophosphatase (0.6 units). Reaction mixtures were pre-equilibrated at 37 °C before addition of a 10-μl aliquot of enzyme to start the reaction. Reactions were terminated at desired time intervals up to 30 min by boiling for 1 min, followed by transfer to ice. Reaction mixtures were analyzed for total ATP content using a hexokinase, glucose-6-phosphate dehydrogenase-coupled assay. 100 μl of each mixture were analyzed for total ATP content using a hexokinase, NADP, 9 units of hexokinase, and 10 units of glucose-6-phosphate dehydrogenase. ATP consumption in a 30-min incubation had less than 0.01 units activity/mg enzyme; this was the lower limit of detectable activity. Extent of activation, inhibition, or maximal velocity.

### Thermal Stability

Enzyme samples were diluted to give the same final protein concentration, 0.68 mg/ml. Dilution buffer was 50 mM Tris, 1 mM EDTA, 0.5 mM DTT, 10% glycerol, pH 7.5. Potassium phosphate, pH 7.0, was added to give a final concentration of 30 mM phosphate. Individual samples (50 μl volume) of wild-type, K195Q, and K195E enzymes were heated simultaneously for 5 min in a water bath equilibrated at the specified temperature, then immediately placed on ice. They were assayed in the synthesis direction as described above.

### Results

**Kinetic Characterization of K195 Mutant Enzyme**—In the synthesis direction of assay, the glutamine mutant was characterized by a 600-fold greater glucose 1-phosphate Kₘ than the wild-type enzyme during the first phosphorylase only.

**Comparison of wild-type and K195Q mutant enzyme kinetic constants**

Reactions were performed at 37 °C as described under "Experimental Procedures." Data represent the average of two identical experiments ± average difference of the duplicates.

| Glucose 1-phosphate Kₘ (mM) | 0.025 ± 0.003 | 16.7 ± 3.8 |
|----------------------------|--------------|------------|
| ATP S₀.₅ (mM)              | 0.33 ± 0.016 | 0.19 ± 0.012 |
| MgCl₂ S₀.₅ (mM)            | 2.57 ± 0.20  | 3.44 ± 0.12 |
| Fructose 1,6-bis-phosphate | 25.4 ± 0.5   | 25.1 ± 2.3 |

| Vₘₚ (units/mg)             | 133 ± 7      | 58 ± 15    |
| kₘₚ (s⁻¹)                 | 111 ± 6      | 48 ± 13    |

### Mutant Lys-195 ADP-Glucose Pyrophosphorylases

**Table I**

| Volume (ml) | Total units | mg protein | Purification | Recovery % |
|-------------|-------------|------------|--------------|------------|
| Crude supernatant | 104 | 267 | 677 | 0.39 | 100 |
| Heat treatment | 107 | 259 | 251 | 1.02 | 97 |
| DEAE-Sepharose | 20 | 234 | 25 | 9.4 | 58 |
| Green A chromatography | 6.5 | 129 | ND | ND | 48 |
| FPLC | 3.5 | 109 | 7.2 | 15.3 | 39 |

| Volume (ml) | Total units | mg protein | Purification | Recovery % |
|-------------|-------------|------------|--------------|------------|
| Crude supernatant | 104 | 267 | 677 | 0.39 | 100 |
| Heat treatment | 107 | 259 | 251 | 1.02 | 97 |
| DEAE-Sepharose | 20 | 234 | 25 | 9.4 | 58 |
| Green A chromatography | 6.5 | 129 | ND | ND | 48 |
| FPLC | 3.5 | 109 | 7.2 | 15.3 | 39 |

### Mutant Lys-195 ADP-Glucose Pyrophosphorylases

**Table II**

| Glucose 1-phosphate Kₘ (mM) | 0.025 ± 0.003 | 16.7 ± 3.8 |
|----------------------------|--------------|------------|
| ATP S₀.₅ (mM)              | 0.33 ± 0.016 | 0.19 ± 0.012 |
| MgCl₂ S₀.₅ (mM)            | 2.57 ± 0.20  | 3.44 ± 0.12 |
| Fructose 1,6-bis-phosphate | 25.4 ± 0.5   | 25.1 ± 2.3 |

| Vₘₚ (units/mg)             | 133 ± 7      | 58 ± 15    |
| kₘₚ (s⁻¹)                 | 111 ± 6      | 48 ± 13    |

### RESULTS

**Kinetic Characterization of K195 Mutant Enzyme**—In the synthesis direction of assay, the glutamine mutant was characterized by a 600-fold greater glucose 1-phosphate Kₘ than the wild-type enzyme (Table II). In contrast, kinetic constants for substrate ATP, cofactor Mg²⁺, and allosteric regulator, Fru 1,6-P₂ were similar in mutant and wild-type cases; no more than a 2-fold difference was measured for each of these ligands. The catalytic rate constant, kₘₚ, is only 2-fold lower in the mutant. As indicated in the introduction, the Glc-1-P Kₘ for wild-type enzyme is equal to the equilibrium dissociation constant, Kₐ, for Glc-1-P (2). Observations that Glc-1-P Kₘ for the glutamine mutant is dramatically altered while kₘₚ is relatively similar to that of wild-type leads to the conclusion that Lys-195 is specifically involved in glucose 1-phosphate binding, with no role in the rate-determining step in the enzyme's catalytic mechanism. The small changes in ATP, Mg²⁺, and Fru 1,6-P₂ kinetic constants also indicate that replacement of Lys-195 with glutamine does not alter the overall folding integrity of the enzyme, leaving those ligand-binding sites intact.

There was an interesting difference between wild-type and K195Q in measurement of MgCl₂ dependence (Fig. 2). At high concentrations MgCl₂ acts as an inhibitor to the wild-type enzyme. This inhibition effect is completely absent in the K195Q mutant enzyme. Not pursued further here, the position 195 mutants will be valuable tools in future study of this inhibition effect. Because of this inhibition effect, kinetic constants reported in Tables II and IV were obtained under optimal conditions for wild-type (5 mM MgCl₂) and mutant enzymes (25 mM MgCl₂). Identical kinetic constants for mutant enzymes were obtained using either 5 or 25 mM MgCl₂, indicating a lack of effect of the chosen concentrations of MgCl₂ on these measurements. Vₘₚ measurements for the K195Q mutant, however, were slightly lower when measured...
FIG. 2. Steady-state kinetic measurements of MgCl₂ dependence for wild-type and K195Q enzymes in the synthesis direction of assay. Initial velocities (in nanomoles of ADP-glucose formed/10 min) for wild-type (open symbols) and K195Q (closed symbols) enzymes were determined at 37 °C as described under “Experimental Procedures.” The concentrations of ATP, Glc-1-P, and Fru 1,6-P₂ were 1.5, 0.5, and 1.5 mM, respectively. The amounts of wild-type and K195Q enzymes used were 0.022 and 1.4 μg, respectively.

| Sugar-phosphate concentration in assay | Wild-type | K195Q |
|--------------------------------------|-----------|-------|
| mM  | unit/mg | unit/mg |
| Glucose 1-phosphate  | 0.5       | 50⁰  | 1.3 ± 0.1 |
|      | 5.0      | 64 ± 2 | 17.7 ± 0.6 |
| Glucosamine 1-phosphate | 0.5   | 0.12⁰  | <0.01 |
|      | 5.0      | 18.2 ± 0.6 | 0.12 ± 0.03 |
| Xylose 1-phosphate  | 0.5       | 0.19⁰  | <0.01 |
|      | 5.0      | 6.1 ± 0.2 | <0.01 |
| Galactose 1-phosphate | 0.5   | <0.01  | <0.01 |
|       | 5.0      | 2.4 ± 0.3 | <0.01 |
| Deoxyribose 1-phosphate | 0.5   | <0.01  | <0.01 |
|       | 5.0      | <0.01  | <0.01 |
| Mannose 1-phosphate | 0.5   | <0.01  | <0.01 |
|       | 5.0      | <0.01  | <0.01 |
| Glucuronic acid 1-phosphate | 0.5   | <0.01  | <0.01 |
|       | 5.0      | <0.01  | <0.01 |

* Single determination.

Table III

Sugar-phosphates as substrates for wild-type and K195Q mutant enzymes

Reactions were performed at 37 °C as described under “Experimental Procedures.” Data represent the average of two identical experiments ± average difference of the duplicates. The lower limit of detection for the assay was 0.01 unit/mg.

Sugar-phosphates as substrates for wild-type and K195Q mutant enzymes

| Sugar-phosphate | Concentration (mM) | Wild-type | K195Q |
|-----------------|-------------------|-----------|-------|
| Glucose 1-phosphate | 0.5       | 50⁰  | 1.3 ± 0.1 |
| Glucosamine 1-phosphate | 0.5   | 0.12⁰  | <0.01 |
| Xylose 1-phosphate  | 0.5       | 0.19⁰  | <0.01 |
| Galactose 1-phosphate | 0.5   | <0.01  | <0.01 |
| Deoxyribose 1-phosphate | 0.5   | <0.01  | <0.01 |
| Mannose 1-phosphate | 0.5   | <0.01  | <0.01 |
| Glucuronic acid 1-phosphate | 0.5   | <0.01  | <0.01 |

The inhibitor AMP shows a similar trend in Kᵢ measurements to that of the glucose 1-phosphate Kᵢ values, although the magnitudes of the differences in AMP Kᵢ values are smaller than were seen for Glc-1-P Kᵢ values (Table IV). The observations that AMP but not ATP binding is affected by mutations at position 195 will be addressed under “Discussion.”

**Thermal Stabilities of Wild-type, K195Q, and K195E Proteins**—The thermal stability experiment (Fig. 3) was done under nonreversible conditions which do not allow thermodynamic parameters to be calculated; however, the data was gathered simultaneously for all three enzymes thereby allowing direct comparison of the three. Clearly, even for the worst case substitution in terms of effect on Glc-1-P Kᵢ (glutamic acid for lysine), the protein retains the same heat inactivation profile as the wild-type protein. Position 195 is obviously not critical to the stability of the native folded state. Evidence of this was also observed in the relatively uniform kinetic constants obtained for ATP, Mg²⁺, and Fru 1,6-P₂ for the five mutants, indicating a lack of effect of amino acid replacement at position 195 on these ligand-binding sites.
binding site but that the sugar-phosphate portions of these that ATP binds. This agrees well with earlier kinetic and complex does not interact with Lys-195 at all. In contrast, the phosphate oxygen in an orientation away from the e-amino group. The adenine portion of AMP and ADP-glucose share the same group. The possibility of its having a role in transition state negatively charged phosphate oxygen and positively charged e-amino group. The adenine portion of AMP and ADP-glucose show full site binding suggesting a covalent modification work which suggested that the adenine moiety of ATP, ADP-glucose, and AMP has virtually no effect on ATP binding. ATP may bind such a site in the activated and unactivated conformations. There is a high degree of specificity for the size, shape, and charge of one amino acid, lysine, to adequately maintain the native conformation of the enzyme. Kinetic results indicate that the nature of the substitution at position 195 affected AMP- or ADP-glucose binding, yet had virtually no effect on ATP binding. ATP may bind such that the triphosphate portion of the molecule anchors the phosphate oxygen in an orientation away from the e-amino group of lysine 195. In such an orientation, the ATP-Mg\textsuperscript{2+} complex does not interact with Lys-195 at all. In contrast, the phosphate portion of AMP or ADP-glucose may be rotated up into the region of Lys-195 so as to allow interaction between a negatively charged phosphate oxygen and positively charged e-amino group of lysine 195. The adenine portion of AMP and of ADP-glucose could occupy the same site on the protein that ATP binds. This agrees well with earlier kinetic and covalent modification work which suggested that the adenine moieties of ATP, ADP-glucose, and AMP share the same binding site but that the sugar-phosphate portions of these ligands may occupy unique sites (2, 21-23). It is also in agreement with steady-state kinetic data which shows AMP to act noncompetitively with respect to glucose 1-phosphate, indicating that both compounds can bind to the enzyme simultaneously. Moreover, earlier binding studies (2) with the wild-type enzyme has shown that ATP binding exhibits half-site reactivity with only two molecules of substrate binding to one molecule of the homotetrameric enzyme. However, ADP-glucose and AMP show full site binding suggesting a binding conformation/site for AMP and ADP-glucose different from that for ATP. The large increases in glucose 1-phosphate \( K_r \) when position 195 is replaced by residues other than lysine explains the absolute conservation of lysine in all pyrophosphorylases sequenced to date. The shape, size, and charge properties of lysine are clearly necessary for proper functioning of the enzyme under physiological concentrations of glucose 1-phosphate. This is an interesting example of a highly specific function for an amino acid residue at the catalytic site of an enzyme. There is a high degree of specificity for the size, shape, and charge of one amino acid, lysine, to adequately bind substrate. However, there is also a large degree of tolerance for amino acid substitutions in terms of the ability of the protein to maintain its native folding properties including maintenance of a closely neighboring binding site (ATP-binding site).

Efforts are in progress to crystallize the pyrophosphorylase enzyme in both its activated and unactivated conformations. Structural analysis of these enzyme forms should provide more information on the feasibility of the conclusions drawn here, as well as highlight other catalytic site residues critical to binding and catalysis.

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REFERENCES

1. Paule, M. R., and Preiss, J. (1971) J. Biol. Chem. 246, 4602-4609
2. Haugen, T. H., and Preiss, J. (1979) J. Biol. Chem. 254, 127-136
3. Preiss, J., Shen, L., Greenberg, E., and Gentner, N. (1966) J. Biol. Chem. 5, 1833-1845
4. Sheu, K.-F., Richard, J. F., and Frey, P. A. (1979) Biochemistry 18, 5548-5556
5. Parsons, T. F., and Preiss, J. (1978) J. Biol. Chem. 253, 7638-7645
6. Lee, Y. M., and Preiss, J. (1986) J. Biol. Chem. 261, 1058-1064
7. Lee, Y. M., Mukherjee, S., and Preiss, J. (1986) Arch. Biochem. Biophys. 244, 585-595
8. Kumar, A., Tanaka, T., Lee, Y. M., and Preiss, J. (1988) J. Biol. Chem. 263, 14634-14639
9. Parsons, T. F., and Preiss, J. (1978) J. Biol. Chem. 253, 6197-6202
10. Baeccker, P. A., Furlong, C. E., and Preiss, J. (1983) J. Biol. Chem. 258, 5084-5088
11. Leung, P. S. C., and Preiss, J. (1987) J. Bacteriol. 169, 4355-4360
12. Anderson, J. M., Hnilo, J., Larson, R., Okita, T. W., Morell, M., and Preiss, J. (1989) J. Biol. Chem. 264, 12238-12242
13. Reinstein, J., Schlichting, I., and Wittinghofer, A. (1990) Biochemistry 29, 7451-7459
14. Okita, T. W., Rodriguez, R. L., and Preiss, J. (1981) J. Biol. Chem. 256, 6944-6952
15. Romeo, T., and Preiss, J. (1989) J. Bacteriol. 171, 2773-2782
16. Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 488-492
17. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) Methods Enzymol. 154, 367-382
18. Ozaki, H., and Preiss, J. (1972) Methods Enzymol. 28, 409-413
19. Kumar, A. Ghosh, P. Lee, Y. M., Hill, M. A., and Preiss, J. (1989) J. Biol. Chem. 264, 10464-10471
20. Wilkinson, G. N. (1961) Biochem. J. 80, 324-332
21. Gentner, N., and Preiss, J. (1968) J. Biol. Chem. 243, 5882-5891
22. Larson, C. E., Lee, Y. M., and Preiss, J. (1986) J. Biol. Chem. 261, 15402-15409
23. Larson, C. E., and Preiss, J. (1986) Biochemistry 25, 4371-4376