which had a different reaction sequence from the well known rabbit muscle enzyme, was studied.

MATERIALS AND METHODS

Equilibrium centrifugation

The molecular weight of yeast phosphoglucomutase was determined by means of the meniscus depletion method of YPHANTIS[10]. The equilibrium centrifugation experiments were carried out with a Beckman model E analytical ultracentrifuge equipped with a Rayleigh interference optical system.

Crystalline yeast phosphoglucomutase

The enzyme was crystallized as described in the previous paper[9].

Assay of enzyme activity

Unless otherwise specified, the reaction mixture contained 0.2 μmole of glucose 1-phosphate, 5 nmoles of glucose 1,6-diphosphate, 10 μmoles of Tris–HCl buffer (pH 7.5) and 10 μg of bovine serum albumin in a final volume of 1.0 ml. After the incubation at 25° for 10 min, the reaction was initiated by the addition of 5 μl of the enzyme solution. The reaction was terminated by the addition of 2.5 ml of 1 M H_2SO_4. Acid-labile phosphate was determined by means of the BARTLETT method[11].

Reagents

Crystalline bovine serum albumin, norleucine and cysteic acid were purchased from Sigma, and glucose 1-phosphate and glucose 1,6-diphosphate from Boehringer Mannheim. For the experiments involving substrate titration of the enzyme modified with 1-anilino-8-naphthalene sulfonate (ANS), glucose 1-phosphate was purified chromatographically by means of RAY's method[5]. ANS was a commercial product. *p*-Chloromercuribenzoate (PCMB) was used after purification[12] and *N*-bromosuccimide was recrystallized from water.

Protein determination

The turbidimetric method[13,14] was used to relate protein concentration to absorbance of the enzyme at 280 nm. The crystalline bovine serum albumin was used for the standard.

Amino acid analysis

The enzyme was hydrolyzed at 110° for 22 h or 70 h in 6 M HCl. Amino acid analyses were performed with a Yanagimoto amino acid autoanalyzer LC-5S. For determination of the half-cystine content of the enzyme, a performic acid oxidation was carried out as described by HIRS[15].

The tryptophan content of the enzyme was determined from the absorbance at 280 nm and 294.4 nm of the enzyme in 0.1 M NaOH[16].

N-bromosuccimide oxidation

An absorbance difference at 280 nm per mole of tryptophan oxidized by *N*-bromosuccimide, $4 \cdot 10^3$ (see ref. 17), was used both in acetate and in urea.

Fluorimetric titration with ANS[18]

Fluorescence intensity (at 470 nm) of ANS bound by the enzyme was measured with a Hitachi fluorescence spectrophotometer, model MPF-2A. The excitation wavelength was 400 nm. Although fluorescence of ANS bound by the enzyme was maximally excited at 370 nm, it was excited at 400 nm in order to decrease the effect of quenching by absorption. The following equation was used for determination of the number (N) of ligand bound by the enzyme and the dissociation constant K.

$$\frac{[S]}{\alpha} = \frac{1}{1-\alpha} K + N[E]$$

Here $[S]$ and $[E]$ represent the initial concentration of the ligand and that of the enzyme, respectively. α is defined as $x/N[E]$, where x is the concentration of the sites of the enzyme bound by the ligand. K and N can be obtained from the plot of $[S]/\alpha$ *versus* $1/1-\alpha$.

RESULTS

Molecular weight

The plot of the logarithm of fringe displacement *versus* (radius)^2 was linear. The molecular weight was calculated from the slope of the plot. The partial specific volume of the enzyme, 0.75, was used for the calculation. The average of triplicate measurements is presented in Table I. These data give a molecular weight of 69 500. This value was close to that of the enzymes from other origins[6-8,19].

TABLE I

MOLECULAR WEIGHT DETERMINATION

The concentrations of the enzyme were 0.017, 0.035 and 0.05% for each speed. Ionic strengh was adjusted with NaCl to 0.1. Temperature was 10°.

Speed (rev./min)	*Molecular weight*
26 000	70 100
28 000	68 800
Average	69 500

Molecular extinction coefficient of the enzyme

The molecular extinction coefficient at 280 nm, $8.2 \cdot 10^7$ cm^2/mole, was determined by means of the turbidimetric method.

Amino acid composition

The amino acid composition of the yeast enzyme did not exhibit large differences from the compositions of the enzymes from rabbit muscle[20] and *E. coli*[6], except that the amounts of serine and tyrosine were relatively large, and those of methionine and arginine relatively small (Table II).

The tryptophan and tyrosine contents of the enzyme, as determined spectrophotometrically, were 7.2 and 25.9, respectively. The tyrosine contents determined by this method agreed approximately with the data from amino acid analysis.

TABLE II

AMINO ACID COMPOSITION OF THE ENZYME

Amino acid	*Residues per mole of the enzyme*	*Amino acid*	*Residues per mole of the enzyme*
Aspartic acid	68	Leucine	36
Threonine	33	Tyrosine	23
Serine	35	Phenylalanine	26
Glutamic acid	52	NH_3	77
Proline	26	Lysine	42
Glycine	50	Histidine	9
Alanine	40	Arginine	15
Valine	33	Half-cystine*	6
Methionine	3	Tryptophan**	7
Isoleucine	39		

* Estimated as cysteic acid.
** Estimated spectrophotometrically.

PCMB titration

Fig. 1 shows that 3.0 nmoles of the native enzyme are saturated with 12.3 nmoles of PCMB. This indicated that four sulfhydryl groups existed on the surface of the native enzyme.

The activity of the enzyme was not fully suppressed by PCMB. This suggested that the sulfhydryl groups did not form the active center of the enzyme.

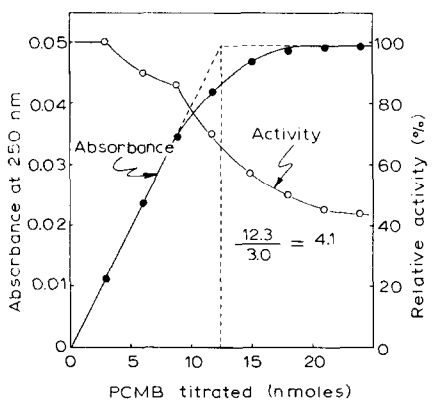

Fig. 1. PCMB titration of the enzyme. The titration was carried out in 10 mM Tris buffer (pH 7.5) with 3.0 nmoles of the enzyme. After the incubation of the enzyme and PCMB for 20 min, the absorbance at 250 nm was recorded. Activity was determined by assaying 5 μl of the enzyme solution which had been removed from the cuvette. ●, absorbance at 250 nm; ○, relative activity.

The titration of the enzyme was also performed in 5 M urea. About five sulfhydryl groups of the urea-denatured enzyme were modified with PCMB.

It was suggested from the results of amino acid analysis and PCMB titration that four sulfhydryl groups existed on the surface of the enzyme, and that one or two were located inside the enzyme molecule.

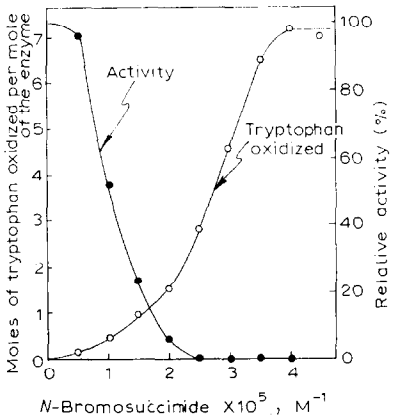

Fig. 2. *N*-Bromosuccimide oxidation of the enzyme. The oxidation was performed in 50 mM acetate buffer (pH 4.0). The concentration of the enzyme was 1.12 μM. Activity was determined by assaying 5 μl of the enzyme solution which had been removed from the cuvette. ○, moles of tryptophan oxidized per mole of the enzyme; ●, relative activity.

N-bromosuccimide oxidation of the enzyme

Fig. 2 shows that seven tryptophan residues of the native enzyme are oxidized by *N*-bromosuccimide. Seven tryptophan residues of the urea-denatured (5 M) enzyme were also oxidized by *N*-bromosuccimide. These results coincided well with the values obtained from the absorbance of the enzyme in 0.1 M NaOH and suggested that all the tryptophan residues existed on the surface of the enzyme.

Fig. 2 also shows that the enzyme is fully inactivated by *N*-bromosuccimide and that the activity is decreased to about 15% of that of the native enzyme by oxidation of one tryptophan residue per mole of the enzyme. The inactivation by *N*-bromosuccimide was approximately proportional to the decrease of the absorbance; 5 and 48% of the activity was lost on oxidation of 0.09 and 0.45 mole of tryptophan per mole of the enzyme, respectively. This suggested that the inactivation by *N*-bromosuccimide was due to oxidation of the tryptophan moiety of the enzyme and that one tryptophan residue of the enzyme played an important role in the activity of the enzyme.

The effect of glucose 1-phosphate and glucose 1,6-diphosphate on the inactivation by *N*-bromosuccimide was examined in pH 4.0 and in pH 7.5 (optimal pH for the enzyme activity). The substrate and the coenzyme were not able to protect the enzyme from inactivation by *N*-bromosuccimide.

Titration with hydrophobic probe

The hydrophobic region of the enzyme was studied with a hydrophobic probe, ANS. Fig. 3a and 3b show that the number of ANS units bound by the enzyme was not influenced by the addition of the substrate and the coenzyme, and that the dissociation constant was decreased by the substrate and the coenzyme. The dissociation constants, K, were 1.0 μM and 9.5 μM in the presence and in the absence of the substrate and the coenzyme, respectively. On the other hand, N was 8.5 under both conditions.

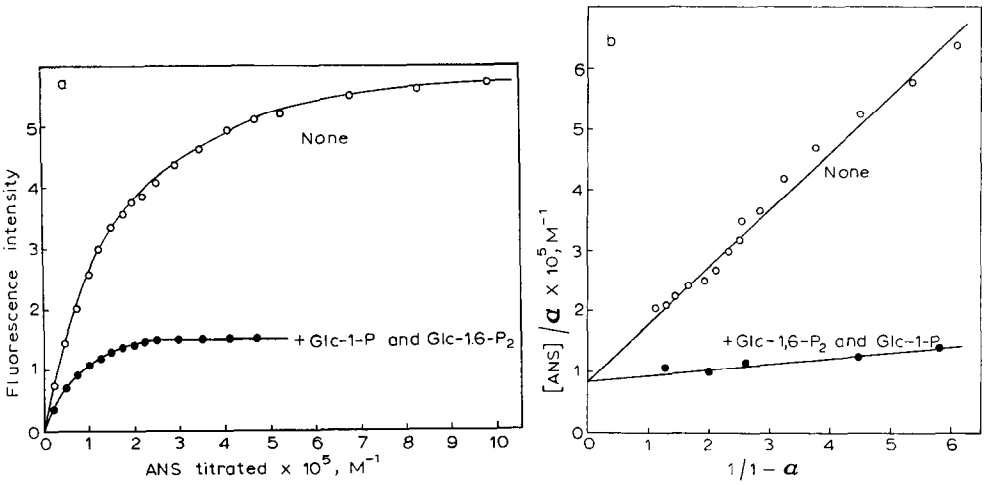

Fig. 3. Fluorimetric titration of the enzyme by ANS. The titrations were carried out in 10 mM Tris buffer (pH 7.5) by adding the solution of ANS to the enzyme solution in the presence of 1.0 mM glucose 1-phosphate and 6.0 μM glucose 1,6-diphosphate (●) or in the absence of the substrate and the coenzyme (○). The fluorescence intensities of ANS in the absence of the enzyme were subtracted from those of ANS in the presence of the enzyme. The values of α were obtained from the ratio of the individual increase to the maximum increase of fluorescence intensity.

Fig. 3a also shows that the fluorescence of ANS bound by the enzyme was strongly quenched by the addition of the substrate and the coenzyme. The quenching by the substrate and the coenzyme was specific for the enzyme, since the fluorescence intensity of ANS bound by bovine serum albumin was not influenced by glucose 1-phosphate and glucose 1,6-diphosphate. These observations suggested that the state of a hydrophobic region of the enzyme was changed by the addition of the substrate and the coenzyme.

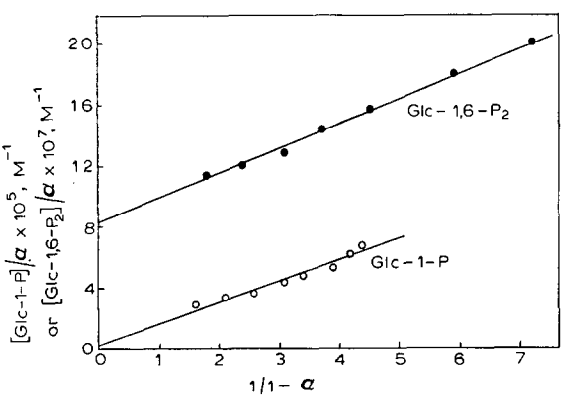

Fig. 4. Titration of glucose 1-phosphate and glucose 1,6-diphosphate to the enzyme modified with ANS. Quenching of ANS attached to the enzyme was measured by the addition of glucose 1-phosphate or glucose 1,6-diphosphate. The enzyme was saturated with 0.1 mM ANS. The titrations of glucose 1-phosphate (○) and glucose 1,6-diphosphate (●) were carried out in Tris buffer (pH 7.5) with 0.82 μM of the enzyme. The values of α were obtained from the ratio of the individual decrease to the maximum decrease of fluorescence intensity.

Binding studies of the substrate and the coenzyme

The quenching of ANS attached to the enzyme was also brought about by the individual addition of the substrate or the coenzyme. The enzyme saturated with ANS was titrated with glucose 1-phosphate or glucose 1,6-diphosphate. Fig. 4 shows that the dissociation constants of glucose 1-phosphate and glucose 1,6-diphosphate to the enzyme are 14 μM and 0.16 μM, respectively, and that the number of moles of glucose 1,6-diphosphate bound by the enzyme is 1.0. The number of moles of glucose 1-phosphate bound by the enzyme was not able to be determined, since the dissociation constant of glucose 1-phosphate to the enzyme was large compared with the concentration of the enzyme used. The K values obtained by titration experiments agreed tolerably well with the K_m values obtained by the kinetic studies (K_m for glucose 1-phosphate, 4.0 μM; K_m for glucose 1,6-diphosphate, 0.14 μM)[9].

The enzyme activity was not influenced by saturation of the enzyme with 0.1 mM of ANS in the presence of 0.2 mM glucose 1-phosphate and 0.2 μM glucose 1,6-diphosphate. This suggested that the affinities of the substrate and the coenzyme for the enzyme were not largely influenced by ANS.

DISCUSSION

It was suggested that the tryptophan residue of the enzyme played an important role in the activity of the enzyme. However, the residue did not probably exist on the substrate- and coenzyme-binding sites of the enzyme. It may be supposed that the residue is required for a catalytic step in the enzyme reaction or for the maintenance of the highly ordered structure of the enzyme.

The quenching of ANS attached to the enzyme was individually induced by the substrate and the coenzyme. The dissociation constants of the substrate and the coenzyme to the enzyme agreed tolerably well with the K_m values obtained in kinetical experiments. This provided further evidence for a "sequential" mechanism of the yeast enzyme reaction. If the reaction of yeast enzyme proceeds *via* a "ping-pong" mechanism, the enzyme must exist in the dephospho-form, because the reaction did not proceed without the addition of the coenzyme[9]. The substrate should not combine with the dephospho-enzyme in a "ping-pong" mechanism, unless the substrate concentration is increased in order to bring about the substrate inhibition. In the yeast enzyme, the substrate inhibition constant was 1.0 mM[9]. It was impossible that the inhibition constant was decreased to 14 μM by the binding of ANS to the enzyme, since the activity was not inhibited by ANS in the presence of 0.2 mM glucose 1-phosphate and 0.2 μM glucose 1,6-diphosphate. Therefore, the dissociation constant of the substrate to the enzyme measured by fluorimetric titration will not mean the substrate inhibition constant, but the K_m value.

The "sequential" mechanism is divided into the terms "random sequential" and "ordered sequential". Although the kinetic experiments[9] had probably suggested the former, the latter had not fully been excluded. The results of fluorimetric titration also suggested a "random sequential" mechanism for a reaction of the yeast enzyme, since the substrate and the coenzyme were individually able to attach to the enzyme.

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