Physicochemical and Kinetic Properties of Iodinated Yeast 3-Phosphoglycerate Kinase†

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ABSTRACT: The present studies have established that there is a critical tyrosyl residue in yeast 3-phosphoglycerate kinase. The iodination of this enzyme results in an inactivation following first-order kinetics. The extent of the modification is limited to only one tyrosyl residue. The monoiodotyrosine formation which leads to inactivation of the enzyme does not induce any significant conformational change as evidenced by hydrogen exchange and optical rotatory dispersion. The role of this tyrosine in the action of the yeast 3-phosphoglycerate kinase is studied. An effective protection against inactivation is observed with 3-phosphoglycerate, and the characteristic spectral effect of 3-phosphoglycerate binding cannot be detected in the modified enzyme. It is concluded that the essential tyrosyl residue may play a role in substrate binding.

3-phosphoglycerate kinase is considered to be a key enzyme in the glycolytic pathway since it is one to catalyze the first of the two ATP forming reactions. The enzyme from several origins has been studied with respect to catalytic properties. It has been shown that the single thiol group and the three methionyl residues in the yeast 3-phosphoglycerate kinase are not essential for activity (Krietsch and Büchner, 1970; Arvidsson and Larsson-Raznikiewicz, 1973; Markland et al., 1975). Furthermore, up to four histidyl residues can be carbethoxylated without loss of activity (Brevet et al., 1973). In contrast, it appears that a glutamyl residue is implicated in the transphosphorylation (Desvages et al., 1975), and as many as three lysine residues are functional in substrate binding (Markland et al., 1975).

In an earlier report (Roustan et al., 1973) we have shown that the interaction of 3-phosphoglycerate with yeast 3-phosphoglycerate kinase induces the spectral perturbation of an aromatic chromophore in the enzyme. In view of this fact, we studied the specific chemical modification of tyrosyl residues (Fattoum et al., 1975b) and we examined the effect of iodination on the properties of the enzyme.

Materials and Methods

3-Phosphoglycerate kinase was purchased from Boehringer Co. In some experiments this enzyme was chromatographed on carboxymethylcellulose (Brake and Weber, 1974). In both cases, the same stoichiometry for iodination was obtained. Its specific activity was 800–1000 units at 22 °C, pH 7.5. Glyceraldehyde-3-phosphate dehydrogenase from yeast (80 units/mg at 25 °C) was obtained from Boehringer Co., trypsin was from Seravac, pronase B grade from Calbiochem, aminopeptidase M from Rohm and Haas GmbH (Darmstadt), and pepsin from Nutritional Biochemical Corp. Nbs_2[1] was a Pierce Chemical reagent. Iodoacetic acid was recrystallized three times from ethyl ether–petroleum ether. ATP (sodium salt), ADP (lithium salt), and NADH (sodium salt) were from Calbiochem, and 3-phosphoglycerate was from Boehringer.

Monoiodotyrosine and diiodotyrosine were from Sigma. Tritiated water was obtained from New England Nuclear at a concentration of 100 mCi/g. The triiodide stock solution was labeled with carrier-free $Na^{125}I$ (80–100 mCi/ml); this was free of reducing agents and was supplied in dilute NaOH (Radiochemical Centre, Amersham). All other products were of the best available grade.

Protein Concentration and Enzyme Assay. 3-Phosphoglycerate concentration was determined from absorbance at 280 nm using $A_{1\ cm}^{1\%} = 5.0$ (Bücher, 1955). The concentration of modified protein was estimated either by uv absorbance or by the microbiuret method. A mol wt of 47 000 (Krietsch and Bücher, 1970) was used in the calculation. The enzymic activity was determined by the spectrophotometric method of Bücher (1947).

Physical Measurements. Difference spectra were done on a Cary Model 15 spectrophotometer as previously described (Roustan et al., 1968). Matched pairs of quartz cells of 0.437- or 0.150-cm light path were used, and the binding parameters were calculated from the difference spectra (Roustan et al., 1968, 1970).

Optical rotatory dispersion measurements were carried out on a FICA type spectropol I spectropolarimeter. All experiments were performed in 0.05 M Tris-acetate buffer, pH 7.5, at room temperature using a 1-cm light path cell. The refractive index of water at various wavelengths was taken from Fasman (1963). The rotatory parameters (a_0, b_0, λ_c) were calculated from dispersion curves in the visible range using the method of least-squares. The protein concentrations were 0.1–0.2 mg/ml and about 2 mg/ml in the uv and visible range, respectively.

^{125}I solutions were counted in a Packard autogamma spectrometer. ^3H counting was carried out using Bray liquid scintillator in Intertechnique liquid scintillation spectrometer Model SL 30.

Iodination. 3-Phosphoglycerate kinase (42 μM) in 0.05 M Tris-acetate buffer, pH 7.5, at 12 °C was iodinated by the addition of 2.1 mM iodine (50-fold molar excess) which was freshly prepared in a stock solution of potassium triiodide in 0.10 M potassium iodide. The concentration of iodine was estimated spectrophotometrically before use (Cunningham and Nuenke, 1959). The reaction of iodine was stopped by the

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[1] Abbreviations used are: ATP, adenosine 5′-triphosphate; ADP, adenosine 5′diphosphate; NADH, reduced nicotinamide adenine dinucleotide phosphate; NbS_2, 5,5′-dithiobis(2-nitrobenzoate); EDTA, ethylenediaminetetraacetic acid; ORD, optical rotatory dispersion.

addition of 0.01 M thiosulfate. The excess reagent was removed by exhaustive dialysis.

Hydrogen Exchange Studies. Hydrogen exchange experiments were performed using the method of interrupted flow gel filtration (Schechter et al., 1969). The tritiation of enzyme samples was carried out as follows: 4 mg of protein in 1 ml of 0.05 M Tris-acetate, 0.1 mM EDTA buffer, pH 7.5, was incubated for 48 h at 4 °C with 0.5 mCi of [^{3}H]water. The tritiated enzyme samples were passed through a Sephadex G-25 (fine grade) column (2 × 100 cm) equilibrated with the same buffer at a flow rate of 60 ml/h. The column flow was interrupted at effluent volumes of 12, 45, and 75 ml for 10 min, 5 h, and 15 h, respectively. The protein fractions were determined from the absorbance at 280 nm, and the radioactivity of each fraction was measured. The number of ^{3}H atoms associated with the protein and the number of ^{3}H atoms back-exchanged were calculated according to Schechter et al. (1969).

Proteolytic Digestion of ^{125}I-Phosphoglycerate Kinase and Chemical Characterization of Modified Residues. The chemical nature of the iodine-sensitive side chain residues was determined after total proteolytic digestion of carboxymethylated ^{125}I-3-phosphoglycerate kinase. The iodinated enzyme was carboxymethylated following denaturation of the protein in 0.05 M Tris-acetate, 2 mM EDTA, 6 M guanidine chlorhydrate buffer, pH 8.5, then dialyzed and lyophilized (Fattoum et al., 1975a).

Two milligrams of treated enzyme was dissolved in 0.5 ml of 5% formic acid containing 0.1 mg of pepsin. After 12 h at 37 °C, the digest was lyophilized and subsequently dissolved in 0.5 ml of 0.2 M ammonium bicarbonate, pH 8.5, 0.1 mg of pronase was added, and the digestion was carried out at 37 °C overnight. Aminopeptidase M (0.500 mg) was added and the reaction was allowed to continue for 10 h. The iodinated products were identified by paper (Roche et al., 1951) or cellulose sheet chromatography. The chromatograms were developed in 1-butanol:acetic acid:water (78:5:17, v/v); the radioactive residues were located by autoradiography.

Peptide mapping was performed to localize the radioactive iodinated peptide. After carboxymethylation, the iodinated enzyme was digested with trypsin. Samples were dissolved in 0.2 M ammonium bicarbonate, pH 8.5, and submitted to trypsin at 37 °C with an enzyme:substrate molar ratio of 1:100 for 3 h, followed by an additional reaction overnight with an enzyme:substrate ratio of 1:50. Peptide maps were prepared on thin layer plates of silica gel according to the method of Sargent and Vadlamudi (1968).

Amino Acid Determinations. Tryptophan was titrated by the colorimetric method of Opienska-Blauth et al. (1963); the methionyl residues were determined according to Schirmer et al. (1970). The SH group was estimated by Nbs_2 (Ellman, 1959) in 1% sodium dodecyl sulfate, pH 8, or after carboxymethylation in 6 M guanidine chlorhydrate, pH 8.5, at 50 °C.

Amino acid analysis was done on a TSM_1 Technicon and acid hydrolysis was carried out in sealed evacuated tubes with constant boiling 6 N HCl at 105 °C for 24 or 48 h.

Results

Effect of Iodine on Yeast 3-Phosphoglycerate Kinase

Specificity of Reaction. The enzyme in 0.05 M Tris-acetate buffer, pH 7.5, was incubated at 12 °C with 50 M excess of iodine. The data presented in Figure 1 show that no more than about two atoms of iodine may be incorporated per mol of enzyme. The chemical nature of the iodinated residue was determined after a total proteolytic digest of the carboxymethylated ^{125}I-3-phosphoglycerate kinase by chromatography (see Methods). All the organic iodine was present as monoiodotyrosine and diiodotyrosine. The monoiodohistidine was only detected at a minimal level. Apart from these residues, the results of the different analyses demonstrate that no oxidation of tryptophan and methionine had occurred during iodination (Table I). However, the single and nonessential thiol group may be reversibly oxidized. Its reduction can be obtained after treatment by 0.01 M mercaptoethanol at pH 9 for 2 h at 30 °C. Peptide mapping of the tryptic digest of carboxymethylated ^{125}I-3-phosphoglycerate kinase revealed one radioactive spot (Figure 2). After elution, this labeled product was subjected to a total proteolytic digest. Subsequent chromatography of the hydrolysate revealed that only [^{125}I]monoidotyrosine was present.

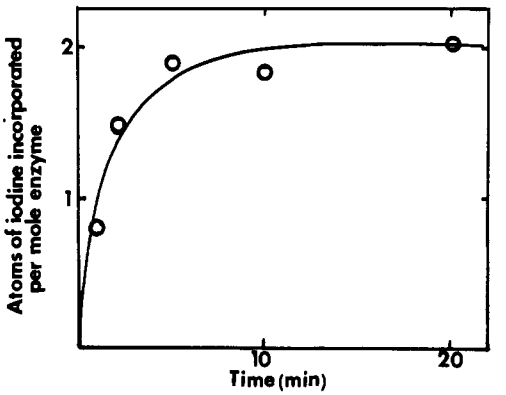

FIGURE 1: Time course of iodine incorporation. 3-Phosphoglycerate kinase (42 μM); 2.1 mM ^{125}I (2.4 × 10^6 cpm); 0.05 M Tris-acetate buffer, pH 7.5 (1 ml); temperature, 12 °C. The reaction of iodine was stopped by addition of 0.01 M thiosulfate.

TABLE I: Amino Acid Composition of Native and Iodinated 3-Phosphoglycerate Kinase.

Amino Acid	Native Enzyme[a]	Iodinated Enzyme[a] (86% Inhibited)
Aspartic acid	37.6	37.6
Threonine	16.9	16.5
Serine	20.8	21.5
Glutamic acid	38.2	38.6
Proline	17.4	18.6
Glycine	34.0	33.5
Alanine	40.0	40.4
Valine	33.4	32.3
Methionine	2.7	2.8
Isoleucine	20.6	20.9
Leucine	37.9	38.0
Tyrosine	6.9	6.8
Phenylalanine	18.0	18.0
Histidine	8.0	7.6
Lysine	40.0	39.8
Arginine	11.9	12.6
Cysteine[b]	0.9	0.8
Tryptophan[c]	2.3	2.4

[a] 48 h, 6 N HCl hydrolysis. Results calculated on the basis of 18 phenylalanine. [b] Determined by Nbs_2. [c] Determined according to Opienska-Blauth et al. (1963).

Kinetics of Tyrosine Iodination. Figure 3 shows the progress of the iodination of tyrosine vs. time. From these data it is clear that only one tyrosyl residue is modified. At the beginning of

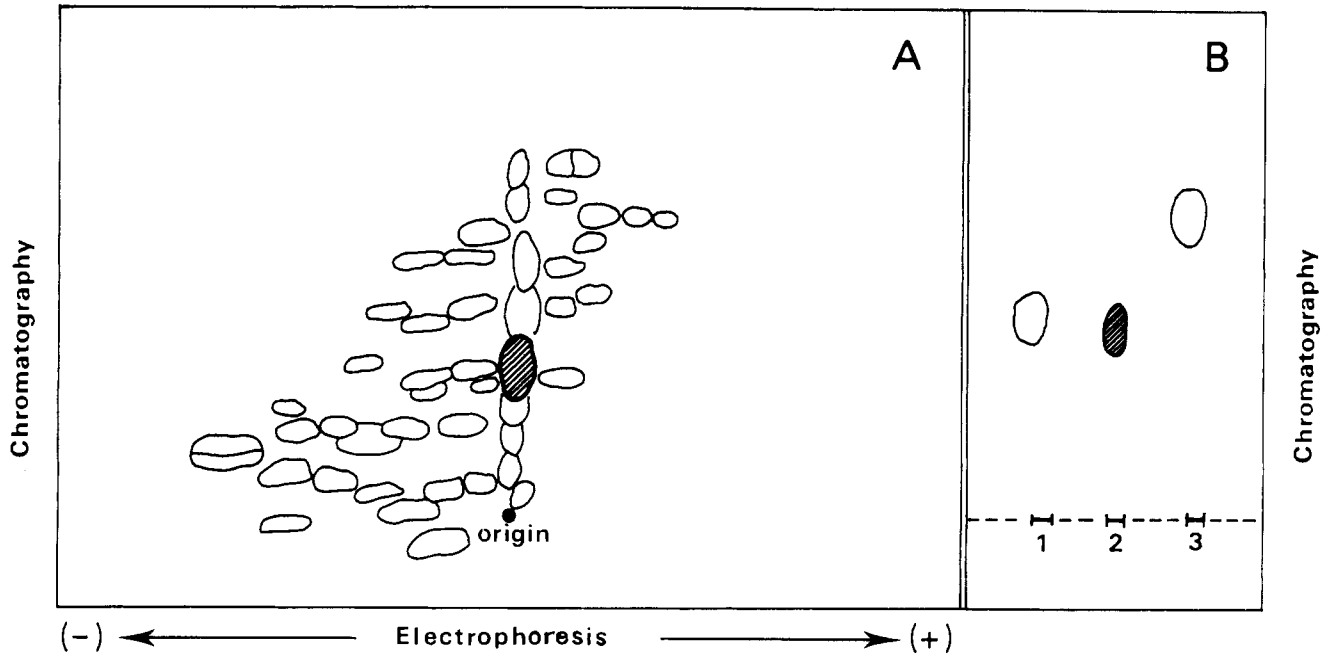

FIGURE 2: Characterization of the iodinated residue. (A) Peptide mapping of tryptic digest of carboxymethylated ^{125}I-3-phosphoglycerate kinase. Electrophoresis in 4% pyridine:0.4% acetic acid:water (3:1:1, v/v). O, Ninhydrin-positive spots; ●, ^{125}I tryptic peptide. (B) Chromatography of ^{125}I tryptic peptide after total proteolytic digest. Solvent: 1-butanol:acetic acid:water (78:5:17 v/v); 1, monoiodotyrosine; 2, radioactive sample; 3, diiodotyrosine.

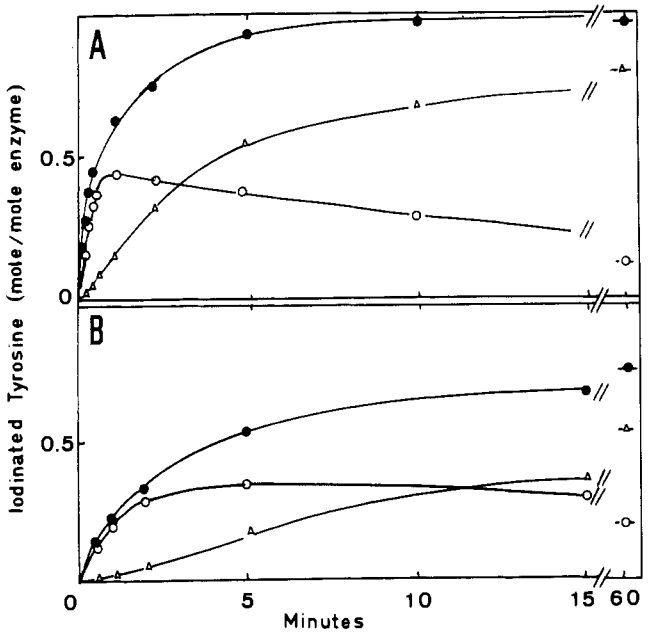

FIGURE 3: Kinetics of tyrosine iodination. The amount of monoiodotyrosine and diiodotyrosine formed was determined after total proteolytic digest of ^{125}I protein samples and chromatography (see Methods). (A) Iodination in absence of substrates. (B) Iodination in presence of 11 mM 3-phosphoglycerate and 11 mM ADP. Monoiodotyrosine plus diiodotyrosine (●-●); monoiodotyrosine (O-O); diiodotyrosine (Δ-Δ).

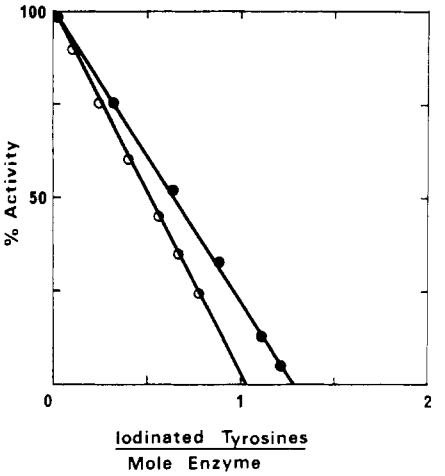

FIGURE 4: Residual activity of 3-phosphoglycerate kinase vs. mol of modified tyrosine (mono- plus diiodotyrosine). Iodination in absence of substrates (●-●) and after preincubation with 3-phosphoglycerate (11 mM) and ADP (11 mM) (O-O).

the reaction, the kinetics of tyrosine iodination may be analyzed by an irreversible process with pseudo-first-order equations (Seon et al., 1970).

$$\text{tyrosine} + \text{iodine} \xrightarrow{k_1} \text{monoiodotyrosine}$$

$$\text{monoiodotyrosine} + \text{iodine} \xrightarrow{k_2} \text{diiodotyrosine}$$

The constant rate k_1 was found to be 1.7 M^{-1}. The theoretical curve for the relationship between the maximum amount of monoiodotyrosine formed and the value of the ratio k_1/k_2 is obtained from

$$\frac{\text{monoiodotyrosine}_{max}}{\text{tyrosine}_{orig}} = \frac{(k_1/k_2)^{k_2}}{k_2 - k_1}$$

(Seon et al., 1970).

This allows the determination of the values of $k_1/k_2 = 1.6$ and $k_2 = 1.1$ M^{-1}.

Inactivation of 3-Phosphoglycerate Kinase by Iodine

Kinetics. Iodine treatment of 3-phosphoglycerate kinase at pH 7.5 produced a loss of enzymic activity which increased with time. The kinetics of this inactivation is of pseudo-first-order with k_{app} of 1.7 M^{-1} at 12 °C. The rate of the inhibition

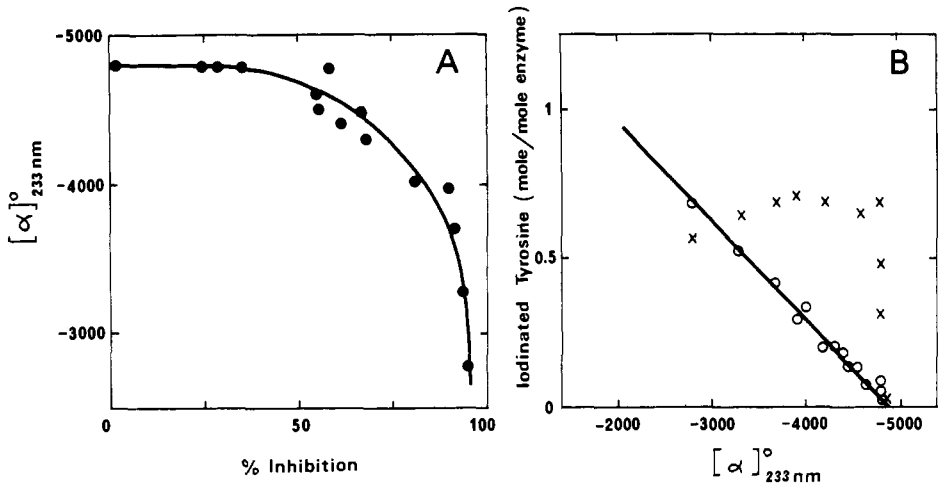

FIGURE 5: Effect of iodination on rotation at 233 nm of 3-phosphoglycerate kinase. (A) Variation of $[\alpha]_{233\,nm}$ vs. enzyme inactivation. (B) Amount of iodinated tyrosine as monoiodotyrosine (X) or diiodotyrosine (O) vs. $[\alpha]_{233nm}$.

TABLE II: Effect of the Inactivation on the Conformation of 3-Phosphoglycerate Kinase.

Inhibition (%)	Back-Exchanging Hydrogen Atoms at Indicated Intervals				ORD Parameters			
	Protein	15 h	5 h	⅙ h	a_0 (deg)	$-b_0$ (deg)	λ_c (nm)	$-[\alpha]_{233nm}$ (deg)
0	14	0	11	35	223	148	252	4800
35	18	0	16	34	224	141	252	4800
80	16	0	13	30	223	133	250	4000
95	16	0	12	32	210	41	223	2800

TABLE III: Effect of Various Ligands on the Inactivation of 3-Phosphoglycerate Kinase.

Ligands	Concentrations (mM)	Protection (%)
ATP-Mg	10	46
ADP	20	40
3-phosphoglycerate	20	57
3-phosphoglycerate + ADP	20	57
Pyrophosphate	10	23
Tripolyphosphate	10	36

was similar to that observed for the monoiodotyrosine formation.

This process was affected by temperature. The rate of inactivation was determined at 8, 12, 16, and 20 °C. The plot of log k_{app} vs. $1/T$ is linear, and the activation energy is equal to about 20 kcal/mol. A value of 24 kcal/mol for *N*-acetyltyrosine monoiodination has been reported by Mayberry et al. (1965).

Stoichiometry. Figure 4 illustrates the correlation between the number of modified tyrosine and the level of inactivation. The total loss of activity is related to 1 ± 0.2 iodinated tyrosine (the mean of several experiments). Therefore, a single tyrosyl residue is responsible for the complete inhibition of 3-phosphoglycerate kinase.

Effect of Tyrosine Modification on the Conformation of 3-Phosphoglycerate Kinase

To analyze whether a conformational change takes place concomitantly with the chemical modification of the critical residue, some physical properties of the iodinated enzyme were determined. These included the ORD behavior and the tritium exchange technique.

Figure 5 shows the values of rotation at 233 nm corresponding to various samples which are inactivated to different extents. This value is not affected up to 60% inhibition, whereas a large decrease of $[\alpha]_{233nm}$ is observed when the enzyme is completely inactivated. Therefore, this variation is not directly related to the inhibition. Furthermore, variation of $[\alpha]_{233nm}$ is correlated with diiodotyrosine but not with monoiodotyrosine formation (Figure 5). Moreover, b_0 and λ_c parameters are also significantly altered only when the enzyme is inactivated by as much as 90% (Table II).

The results from hydrogen-exchange experiments on native and modified enzymes are listed in Table II. The number of tritium atoms back-exchanged at different times within the iodinated enzyme was close to that found in the native enzyme. The same result was obtained for the number of tritium atoms still associated with the protein.

Effect of Substrates

Protection. The effect of several substrates or analogues on iodination has been tested. It is clear from the results in Table III that the best protection against inhibition is observed with 3-phosphoglycerate, but no further protection is obtained when ADP is added to 3-phosphoglycerate.

An important effect is also observed with pyrophosphate and tripolyphosphate.

From the kinetics shown in Figure 3B, the constant rates from iodination in the presence of ADP plus 3-phosphoglycerate are $k_1 = 0.33\ M^{-1}$, $k_2 = 0.35\ M^{-1}$ and the ratio $k_1/k_2 = 0.9$ compared to 1.6 without substrates. So, the protection

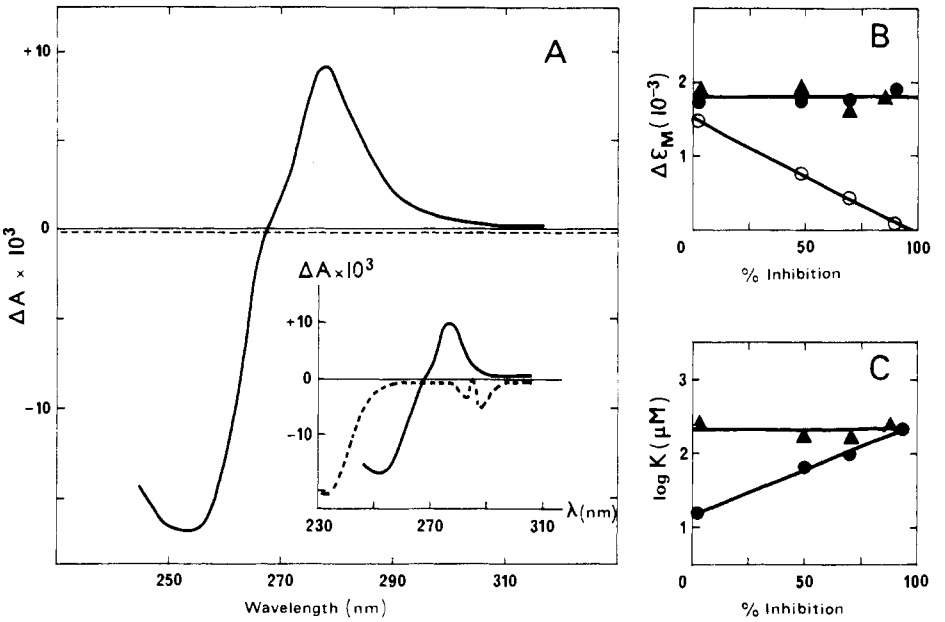

FIGURE 6: Interaction of substrates with modified 3-phosphoglycerate kinase. Difference spectra were performed in 0.05 M Tris-acetate buffer, pH 7.5, at 18 °C. (A) To 38 μM iodinated 3-phosphoglycerate kinase (95% inactivated) was added 1.1 mM 3-phosphoglycerate (- - -) or 0.22 mM ATP-Mg (—). Inset, to 40 μM native enzyme was added 0.087 mM 3-phosphoglycerate (- - -) or 0.07 mM ATP-Mg (—). (B) The $\Delta\epsilon_M$ for 3-phosphoglycerate (O-O), ATP-Mg (●-●) and ADP (▲-▲) interaction is plotted vs. inactivation. (C) Plots of dissociation constants for ATP-Mg (●-●) and ADP (▲-▲) interaction vs. inactivation.

induced by substrates can be explained by a decrease of the two iodination rates. However, the presence of substrates does not affect the extent of iodination (Figure 4).

Binding of Substrates. The inactivation of the enzyme by iodination of one essential tyrosine does not alter the shape of the difference spectra produced by the interaction of ATP and ADP (Figure 6). $\Delta\epsilon_M$ of the minimum peak at 252 nm remains unchanged at any level of inactivation (Figure 6). The dissociation constant for ADP binding is not altered by this chemical modification, whereas the K_D of ATP-Mg is increased by an order of magnitude (Figure 6). ATP-Mg is bound to a lesser extent to the iodinated protein. As previously described (Roustan et al., 1973), the interaction of 3-phosphoglycerate with the native enzyme induces a spectral perturbation of tyrosyl groups in the protein (Figure 6). Tyrosine-modified enzyme fails to give the spectrum characteristic of 3-phosphoglycerate binding (Figure 6). The $\Delta\epsilon_{230nm}$ at a high 3-phosphoglycerate concentration(Figure 6)decreases concomitantly with inhibition.

Discussion

The iodination reaction of 3-phosphoglycerate kinase results in the chemical modification of a single tyrosyl residue per mole of enzyme; this stoichiometry is established by measuring the maximum extent of radioactive monoiodotyrosine and diiodotyrosine formed. The revelation of only one radioactive spot in the peptide map can be taken as another proof for the specificity and the stoichiometry of the reaction. In addition, no oxidation of methionine and tryptophan occurs, so the effect of iodine appears to be very limited to tyrosyl group.

From the results obtained by iodine labeling, the modification of a single tyrosyl group leads to total loss of the catalytic activity. The rate constant of this inactivation is found to be identical with that of monoiodotyrosine formation. Furthermore, the activation energy of this reaction is compatible with that of *N*-acetyltyrosine monoiodination (Mayberry et al., 1965).

These two observations show that the enzyme inactivation results from the monosubstitution of tyrosine.

As judged from the results obtained by ORD and tritium-hydrogen exchange methods, the monoiodotyrosine formation does not induce a significant conformational change. In contrast, an important decrease of $[\alpha]_{233}$ is directly related to diiodotyrosine formation. The variation of λ_c and b_0 parameters vs. inhibition paralleled the results obtained from the variation of Cotton effect at 233 nm and would indicate a loss of helical structure. However, it seems that a complete unfolding does not occur since it may be noticed that the a_0 parameter which is sensitive to the solvent (Fasman, 1963) is not significantly altered. In the same way, tritium-hydrogen measurements show that the number of hydrogens slowly exchanging is the same for the native and labeled enzyme. If we refer to the three-dimensional structure at 3.5 Å resolution of 3-phosphoglycerate kinase given by x-ray diffraction studies (Bryant et al., 1974; Blake and Evans, 1974), this conformational change which seems limited to a loss of helical structure could be explained by the surface location of helical segments and by the relative rigidity of the protein due perhaps to a β-sheet arrangement which forms the central core (Bryant et al., 1974; Blake and Evans, 1974). A gross conformational change is also unlikely since the labeled enzyme still binds nucleotide substrates. Thus, the interaction of ATP or ADP gives spectral effects identical with those observed with the native enzyme. Although the activity is lost, the K_D of ADP remains unchanged. Such results are in contrast to those observed with arginine phosphokinase in which only monoiodotyrosine is formed, a conformational change takes place, and the substrate binding disappears after tyrosine modification (Fattoum et al., 1971).

It is known that the interaction of substrates with 3-phosphoglycerate kinase induces a local conformational change (Markland and Bacharach, 1975; Roustan et al., 1973; Bryant et al., 1974; Blake and Evans, 1974). In particular, 3-phosphoglycerate binding produces a perturbation in the environ-

ment of aromatic residues (Roustan et al., 1973; Markland and Bacharach, 1975). Thus, substrate binding decreases the rate of iodination of the essential tyrosine and modifies the ratio between the constant rate of monoiodotyrosine and diiodotyrosine formation. However, this slight conformational change does not uncover other tyrosines in the protein and does not modify the stoichiometry of the iodination. The fact that all substrates are effective protectors is consistent with tyrosine being located at or near the active site. Furthermore, this location can be supported by substrate binding experiments on the modified enzyme. The spectral effect characteristic of 3-phosphoglycerate binding disappears when the enzyme is inactivated. On this basis one might expect the tyrosine to be situated at the 3-phosphoglycerate site. In addition, although nucleotide substrates still bind normally to the modified enzyme, the dissociation constant of ATP is affected in contrast to that of ADP which remains unchanged. This seems to indicate a proximity between the γ-phosphoryl group of ATP and the essential tyrosine.

In conclusion, tyrosine appears to play a role in substrate binding. Such a role has been already reported in the case of carboxypeptidases A and B (Hartsuk and Lipscomb, 1971; Sokolovsky, 1972), fructose diphosphatase (Horecker et al., 1966), glutamate dehydrogenase (de Prisco, 1971), threonine dehydratase (Vanquickenborne et al., 1969), and creatine phosphokinase (Fattoum et al., 1975a).

Acknowledgment

The authors wish to thank Dr. Nguyen van Thoai for helpful discussions.

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