

Evidence for an Essential Glutamyl Residue in Yeast Hexokinase[†]

Dang Ba Pho,* Claude Roustan, Agathe Nguyen Thi Tot, and Louise-Anne Pradel

ABSTRACT: Yeast hexokinase is rapidly inactivated by 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate and nitrotyrosyl ethyl ester. Sugar substrates afford a partial protection, which is increased by the addition of ADP. Inactivation of the enzyme takes place concomitantly with the incorporation of 1 mol of nitrotyrosine per mol of 50 000-dalton subunit. Exhaustive proteolytic digestion of the modified protein and isolation of the nitrotyrosyl peptide by affinity chromatography, followed by electrophoresis, lead to the identification of the modified residue as a glutamyl residue.

Yeast hexokinase which catalyzes the first reaction in the glycolytic pathway has prompted numerous studies, since its discovery and extensive binding and kinetic studies have accumulated a considerable amount of data, though their interpretations are not always univocal. In contrast, information about the amino acid residues of the protein involved in its interactions with substrates and products are rather scarce as yet, and data reported are even controversial sometimes. Two thiols per 50 000-dalton subunit have been shown to be essential by means of common or substrate-like thiol reagents (Lazarus et al., 1968; Jones et al., 1975; Otieno et al., 1975). However, their accessibilities vary with the nature of these reagents, and a recent report even excluded any direct involvement of thiol as well as amino or imidazole groups in the catalytic process (Menezes and Pudles, 1976a,b; Grouselle et al., 1973). An active tyrosyl residue has been characterized by reaction with a carbodiimide at alkaline pH and difference spectroscopy (Grouselle and Coffe, 1975). The slow inactivation of the enzyme by continuously regenerated ATP and in the presence of xylose or lyxose reveals the existence of a seryl residue in its active center (Menezes and Pudles, 1976a,b). Nevertheless, the question still remains as to whether the phosphorylation of this residue is part of the catalytic pathway or this phenomenon results only from a secondary rearrangement.

On the other hand, kinetic studies on the pH dependence of the enzyme activity have afforded evidence for the role of a protein residue with $pK_{app} = 6.8$, critical in the binding of free ATP to the enzyme-glucose complex (Bohnensack and Hofmann, 1969). The dissociated form of this residue would favor as a base catalyst the release of the proton from the sugar, concomitantly enhancing the nucleophilic attack of the γ -phosphoryl group of ATP by the hydroxyl group of the sugar. As involvement of a histidyl residue has been shown unlikely, this leaves a dicarboxylic amino acid as the alternative residue. Indeed, particularly hydrophobic microenvironments in proteins can raise considerably the pK s of carboxyl groups which can remain un-ionized at pHs as high as 6.5 (Susi et al., 1959;

This modification of hexokinase occurs without gross conformational changes. The enzyme still binds its substrates, though binding of the nucleotides is perturbed. While the substrates afford a partial protection, they increase the incorporation of nitrotyrosine ethyl ester into the enzyme. This may be attributed to local conformational changes which their binding induces. It is concluded that a glutamyl residue is essential for yeast hexokinase activity and its catalytic function is discussed.

Tanford et al., 1959; Donovan et al., 1960; Lehrer and Fasman, 1966, 1967; Parsons and Raftery, 1970).

Water-soluble carbodiimides have been used successfully for the modification of carboxyl groups in proteins under mild conditions. The addition of the carboxyl group across one of the double bonds of the diimide system gives an *O*-acylurea. Coupled reaction with a suitable nucleophile causes the displacement of the carbodiimide from its adduct with the carboxyl group and results in a stable defined product, the study of which permits to identify the residue involved (Hoare and Koshland, 1967).

We report herein the inactivation and modification of yeast hexokinase by this procedure and evidence for the involvement of a glutamyl residue in this enzyme activity.

Materials and Methods

Materials. ATP[†] (sodium salt) was from Boehringer, ADP (sodium and lithium salts), NADP (sodium salt), and *N*-(2,4-dinitrophenyl)ethylenediamine were from Calbiochem, and sequanal grade urea was from Pierce. 1-Cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (CMC), glucosamine hydrochloride, and tyrosine ethyl ester hydrochloride were obtained from Fluka, glycine ethyl ester hydrochloride was from Merck, taurine was from British Drug Houses, and phenylephrine was from Sigma. Nitrotyrosine ethyl ester hydrochloride was synthesized according to Dall'Asta and Ferrario (1962). Iodoacetic acid was recrystallized three times from ethyl ether-petroleum ether.

Pepsin was a product from Nutritional Biochemical Corp., Pronase P was from Serva, leucine aminopeptidase and lyophilized glucose-6-phosphate dehydrogenase were from Boehringer, and carboxypeptidases A and B were from Worthington. Yeast hexokinase was purchased from Boehringer. Experiments were also performed on this enzyme further purified by column chromatography as described by Roustan et al. (1974). The results were essentially the same

[†] From the Laboratoire de Biochimie Cellulaire, Collège de France, 75231 Paris Cedex 05, France. Received February 28, 1977. This work was supported by a grant from the Centre National de la Recherche Scientifique (G.R. No. 6), Paris, France.

[†] Abbreviations used are: ADP, adenosine 5'-diphosphate; ATP, adenosine 5'-triphosphate; CMC, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate; Nbs₂, 5,5'-dithiobis(2-nitrobenzoic acid); NTEE, nitrotyrosyl ethyl ester; ORD, optical rotatory dispersion; NADP, nicotinamide adenine dinucleotide; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol; ATPase, adenosine triphosphatase.

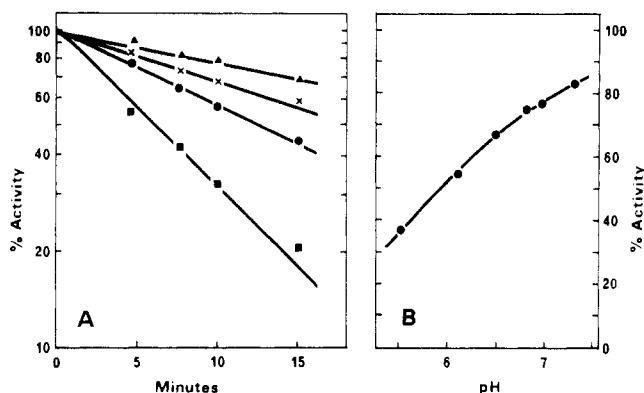


FIGURE 1: Inactivation of hexokinase by CMC. (A) Effect of CMC concentration. Enzyme, 36 μ M; phosphate buffer, 0.01 M, pH 6, 20 $^{\circ}$ C; CMC: (Δ) 0.015 M, (\times) 0.030 M, (\bullet) 0.05 M, (\blacksquare) 0.10 M. (B) Effect of pH. CMC, 0.05 M; buffer, phosphate. Incubation time, 10 min.

with the two enzyme preparations. The enzyme has a low specific activity (230 units/mg at 30 $^{\circ}$ C) and, as shown by Colowick (1973), can be identified with the PI (or A) isozyme.

Protein Concentration and Enzyme Assays. Yeast hexokinase concentration was determined from absorbance at 280 nm using $A_{1\text{cm}}^{1\%} = 9.2$ (Lazarus et al., 1966). The concentration of modified enzyme was determined by the microbiuret method using native enzyme as the standard. A molecular weight of 50 000 was used for the enzyme subunit (Colowick, 1973). Enzyme activity was determined by the spectrophotometric method using a glucose-6-phosphate dehydrogenase as the coupled enzyme (Slein, 1957).

Physical Measurements. Difference spectroscopy (Herkovits and Laskowski, 1962; Theorell and Yonetani, 1964) was performed on a Cary Model 15 spectrophotometer, using matched pairs of quartz cells of 0.437-cm light path.

Optical rotatory dispersion measurements were carried out on a Fica Type Spectropol I spectropolarimeter, in 0.05 M Tris-HCl buffer, pH 7.5, at room temperature.

Reaction of CMC with Nucleophile. Hexokinase (1–5 mg/mL) was incubated with 50 mM CMC and 30 mM nitrotyrosyl ethyl ester (NTEE) in 0.01 M phosphate buffer, pH 6.0. For enzyme assays, the reaction was terminated by a 100-fold dilution of an aliquot of the incubation mixture. For determination of the incorporation of this nucleophile into the protein, concomitant precipitation of another aliquot was performed as described below. Whenever protective effects of ligands were studied, the enzyme was preincubated with them for 5 min before addition of the reagents. Other nucleophiles were also tested: the molarities used will be specified with the results.

Determination of Incorporated Nitrotyrosyl Groups in Hexokinase. Hexokinase modified by CMC and NTEE was precipitated with rapid addition with stirring of 10 volumes of ethanol. The precipitate collected by centrifugation was washed eight times with ethanol, redissolved in 0.1 M NaOH, and extensively dialyzed against the same solvent. The molar ratio of NTEE to enzyme was determined directly on the protein in this solvent by spectroscopy. Calculations were based on the following absorptivities observed in 0.1 M NaOH: $A_{1\text{cm}}^{1\%} = 10.8$ at 290 nm for hexokinase and $\epsilon_m = 4600$ and 4360 for NTEE at 430 and 290 nm, respectively. Corrections were made for the absorption of incorporated NTEE at 290 nm for the determination of protein concentration. The slight and constant absorption at 430 nm of hexokinase treated by NTEE alone and subjected to the same isolation procedure was

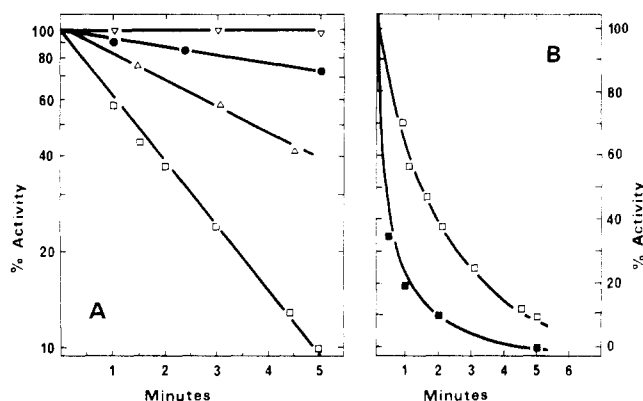


FIGURE 2: Effect of CMC and NTEE on hexokinase. Enzyme, 36 μ M; phosphate buffer, pH 6, 20 $^{\circ}$ C. (A) (∇) 0.03 M NTEE alone, (\bullet) 0.05 M CMC alone, (\square) 0.05 M CMC + 0.03 M NTEE, (Δ) 0.025 M glucose + 0.005 M ADP-Mg + 0.05 M CMC + 0.03 M NTEE. (B) With different concentrations of CMC: (\square) 0.05 M CMC + 0.03 M NTEE, (\blacksquare) 0.1 M CMC + 0.03 M NTEE.

also taken into account.

Proteolytic Digestion of Nitrotyrosyl Hexokinase. Carboxymethylated nitrotyrosyl hexokinase was dissolved in 0.01 N HCl, pH 2.0 (10 mg/mL) and incubated with pepsin (1/10, w/w) at 40 $^{\circ}$ C for 16 h. The incubation mixture was lyophilized, redissolved in 0.1 M ammonium bicarbonate, and incubated with Pronase (1/10, w/w) for 48 h, then with carboxypeptidases A and B (1/10, w/w, each) for another 48 h, and finally with Mg-activated leucine aminopeptidase for the same period of time (1/20, w/w). The preparation was desalted by repeated lyophilization.

Isolation of the Nitrotyrosyl Peptide. Digests of the nitrotyrosyl enzyme were dissolved in 0.1 M ammonium bicarbonate, pH 7.8, and subjected to affinity chromatography on a column of Sepharose 4B coupled with anti-nitrotyrosyl- γ -globulin according to the procedure of Helman and Givol (1971). This column was previously equilibrated with 0.1 M ammonium bicarbonate, pH 7.8. After elution of all contaminant peptides with the same buffer, the nitrotyrosyl peptide was eluted with 1 M ammonia. The fractions of this chromophoric peptide were pooled and lyophilized. The peptide was further purified by high-voltage paper electrophoresis on Whatman 3 MM filter paper, in pyridine-acetate buffer, pH 6.5, for 30 min, at 70 V/cm. Eluted with 1/1 pyridine-water, it was then analyzed for amino acid composition. The peptide yield was 40–45%.

Amino Acid Determinations. Amino acid analysis was done on a TSM 1 Technicon autoanalyzer and acid hydrolysis was carried out in sealed evacuated tubes with constant boiling 6 N HCl at 105 $^{\circ}$ C for 24 h.

The sulfhydryl content was determined with NbS₂ (Ellman, 1959) in 6 M urea, pH 8.0.

Results

Inactivation of Hexokinase by Carbodiimide and Nucleophiles. Upon incubation with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (CMC) at pH 6, hexokinase was progressively inactivated (Figure 1A). Incubation of the enzyme under similar conditions did not alter its activity in the absence of the reagent. The inactivation also increased with CMC concentration (Figure 1A) and its pH profile is given in Figure 1B.

Addition of a nucleophile such as nitrotyrosyl ethyl ester (NTEE) markedly enhanced the inactivation by CMC (Figure 2A), which could lead rapidly to complete inactivation (Figure

TABLE I: Effect of Various Nucleophiles on the Inactivation of Hexokinase by CMC.

Nucleophiles	Concn (mM)	R^a
Glucosamine	210	0.3
Sulfanilic acid	70	0.9
Taurine	50	1.1
Glycine ethyl ester	500	1.0
<i>N</i> -(2,4-Dinitrophenyl)ethylenediamine	7 ^b	1.2
Phenylephrine	400	2.3
Tyrosine ethyl ester	30	3.0
Nitrotyrosine ethyl ester	30	8.0

^a $R = k$ inactivation by (CMC + nucleophile)/ k inactivation by CMC alone. ^b Limited solubility.

TABLE II: Protection of Hexokinase against Inactivation.

Ligands	Protection %
(1) vs. CMC + NTEE	
Fructose (25 mM)	20
Glucose (25 mM)	19
Mannose (25 mM)	21
Glucosamine (190 mM)	17
Xylose (100 mM)	0
ATP-Mg (10 mM)	0
ADP-Mg (2.5 mM)	6
Xylose (100 mM) + ATP-Mg (10 mM)	12
Fructose (25 mM) + ADP-Mg (2.5 mM)	50
Glucose (25 mM) + ADP-Mg (2.5 mM)	42
Mannose (25 mM) + ADP-Mg (2.5 mM)	40
Glucosamine (190 mM) + ADP-Mg (2.5 mM)	50
(2) vs. CMC + tyrosine ethyl ester	
Mannose (25 mM) + ADP-Mg (2.5 mM)	72
Glucosamine (190 mM) + ADP-Mg (2.5 mM)	76

2B). NTEE alone had no effect (Figure 2A). Both inactivations by CMC alone or associated with NTEE appear to follow first-order kinetics.

Tyrosine ethyl ester and phenylephrine also produced significant enhancements, while other compounds with essentially the same nucleophilicity had no effect (Table I). Glucosamine which is a good substrate did not act as a nucleophile but rather as a protector (Tables I and II).

Various substrates or analogues were tested for their ability to protect the enzyme from inactivation. The substrates D-fructose, D-glucose, D-mannose, and D-glucosamine afforded a partial protection (Table II). ADP, which forms an abortive complex with the enzyme and acceptor substrate, increased markedly this protection (Figure 2A and Table II). D-Xylose, a competitive inhibitor vs. glucose, did not protect at all, neither did Mg-ATP alone. Both xylose and ATP binding provided a slight but significant protection.

Incorporation of Nitrotyrosyl Ethyl Ester into Inactivated Enzyme. Nitrotyrosyl ethyl ester has proven the most efficient nucleophile and as a chromophore it has been used to determine the incorporation of nucleophile into the inactivated enzyme. However, NTEE could be strongly adsorbed on the enzyme, and its elimination by simple dialysis was more or less effective, so that preliminary results led to an overestimated value. When isolation of inactivated enzyme, its extensive dialysis, and spectroscopic determination of nitrotyrosine content were performed in denaturing conditions, the results shown in Figure 3 were obtained; 1 mol of nitrotyrosine was incor-

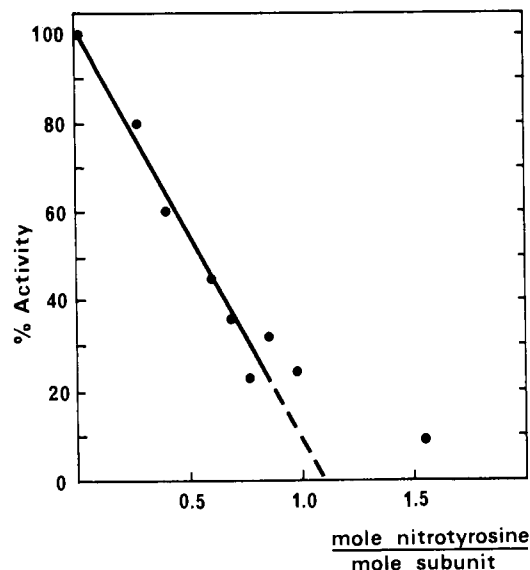


FIGURE 3: Incorporation of nitrotyrosine into inactivated hexokinase.

porated per mol of 50 000-dalton subunit, concomitant with inactivation. The two phenomena occurred at the same rate. Therefore, modification of a single residue may be responsible for the loss of activity.

Properties of Inactivated Hexokinase. In an attempt to determine the function of the modified residue, the binding of substrates on the inactivated enzyme was studied by difference spectroscopy. Binding of glucose or glucose 6-phosphate gave the same difference spectra as those obtained with the native enzyme. In contrast, addition of ADP to the modified enzyme-glucose complex fails to induce the characteristic spectrum with a negative peak at 253 nm. However, a spectral effect related to ADP binding is observed and presents a positive peak with a maximum at about 270 nm. ORD spectra of native and modified enzymes were identical, implying that no gross conformational changes occurred.

On the other hand, amino acid analyses showed no change in the histidine and tyrosine contents. Two sulfhydryl groups per subunit were lost, but this took place whatever the inactivation observed (from 9 to 90%). NTEE added to CMC caused no further loss.

Identification of the Modified Residue. Use of NTEE and affinity chromatography on Sepharose 4B coupled with anti-nitrotyrosyl- γ -globulin enabled us to identify the protein residue modified during inactivation.

Inactivated hexokinase was subjected to complete enzymatic hydrolysis, and the nitrotyrosyl peptide obtained was isolated by means of the immunoadsorbent and further purified by high-voltage electrophoresis. Amino acid analysis of its acid hydrolyzate clearly showed that glutamic acid and nitrotyrosine were the major components and present in equal amounts. The position of the nonhydrolyzed peptide in the autoanalyzer chromatogram coincided with that of glutamyl-nitrotyrosine (Desvages, personal communication). This implies that a glutamyl residue of the protein has been modified during inactivation by CMC and NTEE.

Effects of Substrates on Nitrotyrosyl Incorporation. Incorporation of nitrotyrosyl was also determined for hexokinase modified in the presence of its substrates. As it is previously noted, the enzyme was partially protected. Parallel with the decrease in activation, which means a decrease in the modification of the active glutamyl residue, one would expect a decrease in nitrotyrosyl incorporation. However, this incorpo-

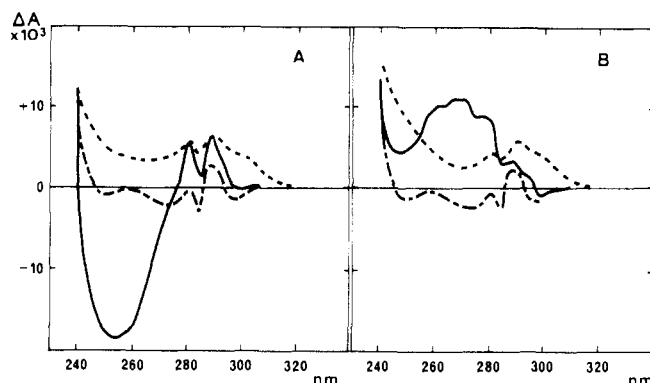


FIGURE 4: Difference spectra between native (A) and 80% inactivated (B) hexokinase and their complexes. Enzyme, 40 μ M; glucose, 1 mM; ADP, 0.1 mM; glucose 6-phosphate, 20 mM. (---) Hexokinase + glucose, (—) hexokinase + glucose + ADP, (- - -) hexokinase + glucose 6-phosphate.

ration is consistently higher in the protected enzyme than in the control, unprotected enzyme. The increase in incorporation observed with sugar substrates or their analogues was further enhanced when ADP was also added to these protectors. If the values are extrapolated to the same degree of inactivation, the incorporation was increased about twofold with the enzyme protected by mannose, for instance, and about threefold with the enzyme protected by mannose and ADP, as compared to the unprotected enzyme.

This suggests that, while the active glutamyl residue was normally protected from modification by the protective substrate (as shown by the decrease in inactivation), other dicarboxylic residues are uncovered to modification by CMC and NTEE (as shown by the increase in nitrotyrosyl incorporation). Kinetic, binding and crystallographic studies have pointed out the important conformational changes induced by the binding of substrates, or some of their analogues, on the enzyme (De La Fuente et al., 1970; Roustan et al., 1974; Anderson and Steitz, 1975). Our results are quite consistent with these data, and nitrotyrosyl incorporation appears to be a good signal for such changes.

Discussion

Several arguments favor the involvement of a dicarboxylic residue in the action of yeast hexokinase. Enzymes catalyzing transphosphorylation from ATP or its cleavage coupled with group transfers or syntheses have been shown to possess an active histidyl (Colomb et al., 1972; Wälinder, 1969; Pradel and Kassab, 1968; Roustan et al., 1970) or dicarboxylic residue (Suzuki et al., 1969; Anthony and Spector, 1970, 1971; Todhunter and Purich, 1974; Degani and Boyer, 1973; Brevet et al., 1973). By analogy, either of these two types of residues might conceivably play a role in the action of hexokinase. Furthermore, this enzyme must catalyze the abstraction of one proton from the sugar substrate for its phosphorylation to take place. The presence of a critical residue with pK_{app} 6.8 (Bohensack and Hofmann, 1969) is also suggestive of either a histidyl or a dicarboxylic residue in a hydrophobic microenvironment. Histidine has been shown to play no direct role in the action of yeast hexokinase. Therefore, it was interesting to investigate a possible role for a dicarboxylic residue in the enzyme.

Indeed, the results here presented provide evidence for an involvement of such a residue. Hexokinase is quite rapidly inactivated by CMC and NTEE concomitantly with the stoichiometric incorporation of the nucleophile into the protein.

Apart from carboxyl, some other functional groups of the protein might react with CMC on its own (Khorana, 1953; Kurzer and Douraghi-Zadeh, 1967). Two SH groups were lost on reaction with CMC, whatever the inactivation might be. No enhancement of this effect was observed when NTEE was added to CMC. Thus, the loss of these two thiols is not related to the loss of activity and this is in keeping with the existence of two nonessential sulfhydryls in yeast hexokinase.

On the other hand, alcohols such as serine or threonine are inert toward CMC in the absence of catalysts (Khorana, 1953). Lysine modification is also improbable, as amines react only in their unprotonated form with carbodiimides. Similarly, the pH dependence of the inactivation, shown in Figure 1B, is opposite to that expected for histidine modification. Besides, amino acid analyses show no change in the histidine and tyrosine contents of the inactivated enzyme. Modification of tyrosine is unlikely also on the account of the marked enhancement by NTEE of the effect of CMC on enzyme activity and the rate constant of the inactivation by these two reagents ($k = 0.47 \text{ min}^{-1}$ with 0.05 M CMC and 0.03 M NTEE), much larger than that of tyrosine modification observed in yeast hexokinase by Grouselle and Coffe (1975). The concomitant stoichiometric incorporation of nitrotyrosine as well as the characterization of glutamyl nitrotyrosine in the inactivated enzyme finally provide strong evidence for modification at the γ -carboxyl group of a glutamyl residue with covalent binding of the nucleophile.

The possibility of rapid and complete inactivation of hexokinase by CMC and NTEE and its occurrence at the same rate as the incorporation of 1 mol of nitrotyrosine per mol of active subunit imply that this inactivation correlates with the modification of a single active glutamyl residue over the 110 dicarboxylic residues and that this residue possesses an enhanced reactivity. The protection afforded by substrates also suggests that this glutamyl residue is located in the active center of the enzyme.

Thus, the data presented argue in favor of the existence of an essential glutamyl residue in yeast hexokinase.

Its modification takes place without gross conformational changes, as judged by ORD measurements. Difference spectra (Figure 4) show that the sugar substrate still binds normally. Nucleotide substrate binding is also observed, but its microenvironment appears to be different in the modified enzyme. This ability to bind the substrates suggests that the modified residue does not function as a binding site but may rather be involved in the catalysis. This idea is reinforced by the characteristics of the protection afforded by the substrates added to the enzyme before inactivation. While substrates protect the active carboxyl group from modification, they simultaneously uncover other carboxyl groups to this modification. This reflects a conformational change their binding has induced and which modifies the microenvironment of the active carboxyl group.

Such changes have been reported (De La Fuente et al., 1970; Roustan et al., 1974; Anderson and Steitz, 1975) and our data confirm these findings. Together with the preservation of substrate binding capacities, they imply that the protective action of substrates is not to be related to competitive interactions with binding sites but to a local conformational change which limits the susceptibility of the active glutamyl residue to the attack by CMC and NTEE.

Carboxyl groups have been shown to be essential in several enzymes, such as carboxypeptidase (Riordan and Hayashida, 1970), lysozyme (Imoto et al., 1972), triosephosphate isomerase (Hartman, 1971; Miller and Waley, 1971), isocitrate dehydrogenase (Colman, 1973), kinases (Anthony and

Spector, 1970, 1971; Todhunter and Purich, 1974; Degani and Boyer, 1973; Brevet et al., 1973), and ATP:citrate lyase (Suzuki et al., 1969). In the absence of an essential histidyl residue in yeast hexokinase, the pK_{app} of 6.8 found for a critical residue (Bohnensack and Hofmann, 1969) may be attributed to a carboxyl group located in a hydrophobic microenvironment. Lysozyme active Glu-35 has a nonpolar environment and a pK of 6–6.5 (Imoto et al., 1972). Triosephosphate isomerase essential Glu-165 has a pK of 6 (Parsons and Raftery, 1970) and is surrounded by very hydrophobic amino acid residues (Corran and Waley, 1973). Yeast hexokinase affinity for aromatic nucleophiles, such as tyrosine ethyl ester, NTEE, and phenylephrine, is quite compatible with a nonpolar environment of the active glutamyl residue.

The role of this residue in the enzyme remains to be elucidated. Formation of an acyl-phosphate bond has been reported for acetate kinase (Anthony and Spector, 1970, 1971; Todhunter and Purich, 1974), ATP citrate lyase (Suzuki et al., 1969), and reticulum sarcoplasmic ATPase (Degani and Boyer, 1973). This implies a transfer of an ATP γ -phosphoryl group via phosphorylation of a glutamyl or aspartyl residue of the enzymes, leading to more or less stable phosphoenzymes. Such a mechanism requires careful investigation before its validity could be asserted (Johnson et al., 1976; Switzer and Simcox, 1974). Carboxyl groups can also act as an acid-base catalyst in a "charge-relay system", as in serine proteases (Blow et al., 1969), or an acid catalyst, as in lysozyme (Imoto et al., 1972). A similar mechanism has been suggested by Bohnensack and Hofmann (1969): a dissociated group with $pK_{app} = 6.8$ in the enzyme would favor as a base catalyst the release of a proton from the sugar substrate, increasing in the same time the nucleophilic attack of the γ -phosphoryl of ATP by the hydroxyl group of the sugar. This hypothesis is also compatible with the existence of an active glutamyl residue in yeast hexokinase. Further investigation is necessary to the elucidation of its function.

Acknowledgments

We thank A. Vaysse for the generous supply of anti-nitrotyrosyl antiserum, and G. Desvages for the preparation of the immunoadsorbent, as well as the amino acid analyses.

References

- Anderson, W. F., and Steitz, T. A. (1975), *J. Mol. Biol.* 92, 279–287.
- Anthony, R. S., and Spector, L. B. (1970), *J. Biol. Chem.* 245, 6739–6741.
- Anthony, R. S., and Spector, L. B. (1971), *J. Biol. Chem.* 246, 6129–6135.
- Blow, D. M., Birktoft, J. J., and Hartley, B. S. (1969), *Nature (London)* 221, 337–340.
- Bohnensack, R., and Hofmann, E. (1969), *Eur. J. Biochem.* 9, 534–541.
- Brevet, A., Roustan, C., Desvages, G., Pradel, L.-A., and van Thoai, N. (1973), *Eur. J. Biochem.* 39, 141–147.
- Colman, R. F. (1973), *J. Biol. Chem.* 248, 8137–8143.
- Colomb, M. G., Chérut, A., and Vignais, P. (1972), *Biochemistry* 11, 3378–3386.
- Colowick, S. P. (1973), *Enzymes*, 3rd Ed. 9, 1–47.
- Corran, P. H., and Waley, S. G. (1973), *FEBS Lett.* 30, 97–99.
- Dall'Asta, L., and Ferrario, P. (1962), *Helv. Chim. Acta* 45, 1065–1071.
- Degani, C., and Boyer, P. D. (1973), *J. Biol. Chem.* 248, 8222–8226.
- De La Fuente, G., Laguras, R., and Sols, A. (1970), *Eur. J. Biochem.* 16, 226–233.
- Donovan, J. W., Laskowski, M., Jr., and Scheraga, H. A. (1960), *J. Am. Chem. Soc.* 82, 2154–2163.
- Ellman, G. L. (1959), *Arch. Biochem. Biophys.* 82, 70–77.
- Grouselle, M., and Coffe, G. (1975), *Fed. Eur. Biochem. Soc. Meet., Proc.* 10, 619, abstract.
- Grouselle, M., Thiam, A., and Pudles, J. (1973), *Eur. J. Biochem.* 39, 431–441.
- Hartman, F. C. (1971), *Biochemistry* 10, 146–154.
- Helman, M., and Givol, D. (1971), *Biochem. J.* 125, 971–974.
- Herskovits, T. T., and Laskowski, M. (1962), *J. Biol. Chem.* 237, 2481–2492.
- Hoare, D. G., and Koshland, D. E., Jr. (1967), *J. Biol. Chem.* 242, 2447–2453.
- Imoto, T., Johnson, L. N., North, A. C. I., Philipps, D. C., and Rupley, J. A. (1972), *Enzymes*, 3rd Ed. 7, 665–686.
- Johnson, P. E., Abbott, S. J., Orr, G. A., Sémériva, M., and Knowles, J. R. (1976), *Biochemistry* 15, 2893–2901.
- Jones, J. G., Otieno, S., Bernard, E. A., and Bhargava, A. K. (1975), *Biochemistry* 14, 2396–2403.
- Khorana, H. G. (1953), *Chem. Rev.* 53, 145–166.
- Kurzer, F., and Douraghi-Zadeh (1967), *Chem. Rev.* 67, 107–152.
- Lazarus, N. R., Derechin, M., and Barnard, E. A. (1968), *Biochemistry* 7, 2390–2400.
- Lazarus, N. R., Ramel, A. H., Rustum, Y. M., and Barnard, E. A. (1966), *Biochemistry* 5, 4003–4016.
- Lehrer, S. S., and Fasman, G. D. (1966), *Biochem. Biophys. Res. Commun.* 23, 133–138.
- Lehrer, S. S., and Fasman, G. D. (1967), *J. Biol. Chem.* 242, 4644–4651.
- Menezes, L. C., and Pudles, J. (1976a), *Biochimie* 58, 51–59.
- Menezes, L. C., and Pudles, J. (1976b), *Eur. J. Biochem.* 65, 41–47.
- Miller, J. C., and Waley, S. G. (1971), *Biochem. J.* 123, 163–170.
- Otieno, S., Bhargava, A. K., Barnard, E. A., and Ramel, A. H. (1975), *Biochemistry* 14, 2403–2410.
- Parsons, S. M., and Raftery, M. A. (1970), *Biochem. Biophys. Res. Commun.* 41, 45–56.
- Pradel, L.-A., and Kassab, R. (1968), *Biochim. Biophys. Acta* 167, 317–325.
- Riordan, J. F., and Hayashida, H. (1970), *Biochem. Biophys. Res. Commun.* 41, 122–127.
- Roustan, C., Brevet, A., Pradel, L.-A., and van Thoai, N. (1974), *Eur. J. Biochem.* 44, 353–358.
- Roustan, C., Pradel, L.-A., Kassab, R., Fattoum, A., and van Thoai, N. (1970), *Biochim. Biophys. Acta* 206, 369–379.
- Slein, M. W. (1957), *Methods Enzymol.* 3, 154–157.
- Susi, H., Zell, T., and Timasheff, S. N. (1959), *Arch. Biochem. Biophys.* 85, 437–443.
- Suzuki, F., Furunishi, K., and Takeda, Y. (1969), *J. Biochem. (Tokyo)* 66, 767–774.
- Switzer, R. L., and Simcox, P. D. (1974), *J. Biol. Chem.* 249, 5304–5307.
- Tanford, C., Bunville, L. G., and Nozaki, Y. (1959), *J. Am. Chem. Soc.* 81, 4032–4036.
- Theorell, H., and Yonetani, T. (1964), *Arch. Biochem. Biophys.* 106, 252–258.
- Todhunter, J. A., and Purich, D. L. (1974), *Biochem. Biophys. Res. Commun.* 60, 273–280.
- Waley, S. G. (1972), *Biochem. J.* 126, 255–256.
- Wälinder, O. (1969), *J. Biol. Chem.* 244, 1065–1069.