Biochimica et Biophysica Acta, 484 (1977) 322—335

BBA 68240

CHEMICAL REACTIVITY OF THE TYROSYL RESIDUES IN YEAST HEXOKINASE

PROPERTIES OF THE NITROENZYME

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(Received January 14th, 1977)

Summary

Of the 15 tyrosyl residues per subunit of yeast hexokinase A (ATP : D-hexose 6-phosphotransferase, EC 2.7.1.1) two are more accessible to chemical modification with cyanuryl fluoride at pH 9.7 (0°C), or with tetranitromethane at pH 8.0 (0° or 22°C) while only one is modified with *N*-acetylimidazol at pH 7.5 (22°C).

In presence of 0.2 M glucose, only one of the two accessible tyrosyl residues is modified by nitration with tetranitromethane at pH 8.0 (22°C). The nitration of this tyrosyl residue induces dissociation of the protein to its monomeric forms and results in a loss of 90% of enzyme activity. The partially active nitroenzyme has the same pH activity profile as the native enzyme. Furthermore, K_m values for glucose and MgATP are not significatively affected. Moreover, neither the negative cooperativity with respect to MgATP nor the citrate activation effect is affected by the modification of the tyrosyl residue and dissociation of the protein. However the burst-type slow transient in the reaction progress curve is abolished by the nitration process.

These results show that negative cooperativity and the citrate activation effect are not as strictly dependent on the conformational state of the protein as the burst-type slow transient. Furthermore, the large decrease in enzyme activity due to the nitration of the tyrosyl group is related simultaneously to the dissociation of the protein and probably also to the perturbation of the conformation of the enzyme active center region.

Introduction

Yeast hexokinase (ATP : D-hexose 6-phosphotransferase, EC 2.7.1.1) is a key enzyme in the glycolytic chain, which catalyses the transfer of γ-phos-

phoryl group of ATP to the primary hydroxyl group of hexoses.

There are two isoenzymes (A and B) [1,2] which, between pH 5.0 and 7.0 are predominantly dimers of M_r 102 000. These two isoenzymes have been studied in extenso with regard to their kinetic and physical properties [3,4]. In this laboratory a systematic study with functional group reagents has been carried out in order to determine the contribution of the different side chains to the mechanism of action of yeast hexokinase [5—8]. Recently, Grouselle and Coffe [9] have shown that at pH 8.0, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride modifies specifically 1 tyrosine residue out of 15 [5] per subunit of yeast hexokinase A. The modification of this residue induces a loss of 90% of the enzyme activity, which however can be fully reversed after hydroxylamine treatment.

From the remarkable selectivity of the reagent, it was thus important to determine if the same residue also reacts with the usual classical reagents.

This paper deals with results obtained following the modification of tyrosyl residues with cyanuryl fluoride, *N*-acetylimidazol and tetranitromethane. It will be shown that 2 of the tyrosyl residues per subunit are more accessible to chemical modification than the other 13. Of these two residues, one is responsible for the loss of enzyme activity. Moreover, the nitration of this critical residue induces dissociation of the enzyme to its monomeric form. The nitroenzyme is partially active and still displays some of the catalytic and regulatory properties of the native enzyme.

Experimental procedure

Materials

Baker's yeast (Springer) hexokinase A was prepared by a modification of the method of Rustum et al. [10] developed by Grouselle in this laboratory (unpublished). The purified enzyme had a specific activity of 150 μmol of glucose-6-phosphate formed per min/mg protein at 25°C, a fructose/glucose ratio of 3.1, and showed a homogenous peak by ultracentrifugation with $s_{20,w} = 5.8$ in acetate buffer (pH 5.5), $I = 0.1$, and by disc electrophoresis. The following reagents were purchased: pyruvate kinase, glucose-6-phosphate dehydrogenase and phospho*enol*pyruvate dicyclohexyl ammonium salt (Boehringer Mannheim); ATP disodium salt and NADP (PL Biochemical Co); urea, (reagent grade, Prolabo) and was recrystallized from 95% ethanol; 5,5′-dithio-bis (2-nitrobenzoic acid) and β-mercaptoethanol (Aldrich Chem. Co. Inc); 2,4,6-trinitrobenzene sulfonate disodium salt (Nutritional Biochemicals Co); cyanuric fluoride, 2-(*N*-morpholino) ethanesulfonic acid and Tris-(hydroxymethyl)-methyl-2-amino ethane sulfonic acid (Serva), dioxane (ultra pure) and Tris-(hydroxymethyl)aminomethane (Fluka), *N*-acetylimidazol was a generous gift of Dr. C. Ghelis. All other chemicals were of analytical reagent grade. Solutions were prepared in deionized distilled water.

Methods

Enzymic assay. The hexokinase activity was measured by the potentiometric titration method described by Hammes and Kochavi [11] on an automatic Titrator Radiometer, model 5 BR 2L and ABU 11. The standard reaction mix-

ture contained a final concentration of 5.7 mM ATP, 20 mM $MgCl_2$ and 40 mM D-glucose, 1—2 μg enzyme. The pH was 8.5, the final volume 1.5 ml, and the temperature 25° C.

For the K_m and activation studies hexokinase activity was followed in the presence of an excess of the coupling enzyme glucose-6-phosphate dehydrogenase and NADP in a Cary 118 C spectrophotometer. Pyruvate kinase and phospho*enol*pyruvate were added to regenerate ATP in the K_m studies, as described by Kosow and Rose [12].

In activation studies of the modified hexokinase and for the burst-type transient kinetics, the amount of enzyme used for assay was such as to compensate for the degree of inactivation and obtain rates comparable to that of the native enzyme.

Protein concentration. Protein concentration was determined by absorption measurement at 280 nm. A value of 0.92 was taken for the absorbance of 1 mg/ml solution of hexokinase in a 1-cm light path according to Lazarus et al. [1]. The protein concentration of the chemically modified enzyme was determined by the Lowry method [13] with hexokinase as the standard reference protein.

Estimation of the sulfhydryl groups. Free sulfhydryl groups were titrated by the Ellmann spectrophotometric method in 4.5 M urea solution in 0.05 M Tris HCl buffer at pH 8.0 [14]. A protein concentration of 0.22—0.25 mg/ml was used throughout.

Amino group determination. Free amino groups were determined with trinitrobenzene sulfonate as described by Habeeb [15].

Thermostability studies. The heat stability of the native and chemically modified yeast hexokinase was carried out in the following conditions: solutions of 2 ml native and modified enzyme (0.25 mg/ml) in 50 mM Tris · HCl buffer (pH 8.0) were incubated in a thermostated bath at 44° C and at different times intervals aliquots were taken and assayed for the residual hexokinase activity. The amount of modified enzyme (nitroenzyme) taken for the assay was 10-fold higher than that of the native enzyme so that the actual rates in the assay were comparable.

Ultracentrifugation. Ultracentrifugation was conducted in a Spinco Model E ultracentrifuge equipped with Schlieren optics. Sedimentation velocity measurements were made at 67770 rev./min. The temperature was 20° C, protein concentration 2 mg/ml in 0.1 M acetate buffer, pH 5.5.

Gel electrophoresis. The method of Weber and Osborn [16] was used for SDS-polyacrylamide disc electrophoresis to determine whether nitration with tetranitromethane induced polymerization of yeast hexokinase.

Reaction with cyanuric fluoride. The reaction with cyanuric fluoride was carried out as described by Gorbunoff [17] with a slight modification. Protein solution 1 ml (5 mg/ml) in 25 mM NaCl solution (pH 7.0) was mixed with 1 ml of 2 M $NaHCO_3$/NaOH buffer of a given pH (9.7 to 13). To this, 50 μl of cyanuryl fluoride of a given concentration (made up by dissolving freshly distilled reagent in dry dioxane) were added and the reaction tube was gently shaken. The final concentration of cyanuryl fluoride was between 10 and 80 mM (408 to 3264 fold molar excess). After standing for 40 s at 25° C (or 0° C), the pH of the reaction mixture was adjusted to 13 with 0.6 ml of 1 M $NaHCO_3$/NaOH

buffer (pH 13.5) and left standing at room temperature for 60 min. An enzyme control was carried out under the same conditions but in the absence of cyanuryl fluoride.

The number of mol of tyrosine residues which had reacted under any given conditions of pH and cyanuryl fluoride concentration was calculated using a molar absorption coefficient for the phenoxide ion of 2300 $M^{-1} \cdot cm^{-1}$ at 295 nm [17]. The mol of reacted tyrosine residues were obtained from experiments where a pH 13 reference was used. From experiments recorded versus a pH 7.0 reference, the number of unreacted residues were obtained.

Throughout this paper, the number of groups modified will be given per enzyme subunit.

Reaction with N-acetylimidazol. Acetylation with *N*-acetylimidazol was performed essentially as described by Riordan et al. [18].

To 1 ml of the protein solution (5 mg/ml), in 25 mM phosphate buffer (pH 7.5), in presence or absence of 1.5 M NaCl, was added between 3.6 and 10.8 mM *N*-acetylimidazol (94 to 282 fold molar excess), and the reaction carried out for 1 h at 22° C. For the determination of the residual enzyme activity at different time intervals, aliquots were taken and diluted at 0° C 41-fold with 25 mM of phosphate buffer containing 0.5 M glycine (pH 7.5), and the residual hexokinase activity determined. At the end of the acetylation reaction, the mixture was treated with 1 M (final concentration) hydroxylamine solution (pH 7.5) and the reversal of the enzyme activity followed for a period of 30 min.

For the determination of the number of tyrosyl and amino groups modified per enzyme subunit, the excess of reagent was eliminated by filtration through a Bio-Gel P10 column (2 × 10 cm). In some instances, excess reagent was removed by dialysis. The number of tyrosyl groups modified was determined by the increase of the absorbance at 278 nm after reaction with 1 M hydroxylamine. When no further change in absorbance occurred at approximately 10 min, the number of *O*-acetyltyrosyl residues (N) was determined from the relationship $N = (A_{278} \cdot M_r)/(1160 \cdot C)$ [19], where A is the absorbance, M the molecular weight of the protein, and C its concentration in g/l. The differential absorbance measurements were followed with a Cary 118 C spectrophotometer in tandem silica cells (2 × 0.4375 cm light path) with a protein concentration of 2.2 mg/ml (scale expansion, 0.02 A).

Reaction with tetranitromethane. A protein solution (0.25 mg/ml) in 50 mM Tris · HCl buffer (pH 8.0) containing 1.5 M NaCl in presence or absence of 0.2 M glucose was used. To each ml of this enzyme solution were added 20 µl of a tetranitromethane solution in 95% ethanol. When the reaction was carried out at 22° C, the final concentration of tetranitromethane was 75 and 150 µM (30 and 60 fold molar excess). However the concentration was increased to 650 µM (260 fold molar excess), when the reaction was carried at 0° C. In each case, 1 ml aliquots were taken at the indicated time and the reaction stopped by addition of 100 µl of 60 mM dithiothreitol in 50 mM Tris · HCl buffer (pH 8.0). From this solution aliquots were taken for measurement of the residual enzyme activities.

For the determination of the number of groups modified (tyrosyl and sulfhydryl), the dithiothreitol treated solutions were first thoroughly dialyzed

against 50 mM Tris · HCl buffer (pH 8.0). The number of nitro tyrosyl groups per enzyme subunit was estimated from the absorbance at 428 nm, using a molar absorption coefficient of 4100 $M^{-1} \cdot cm^{-1}$ [20].

Results

The reactivity of the tyrosine residues to cyanuryl fluoride

Cyanuric fluoride modifies the phenolic hydroxyl groups, to form a cyanurate ester which is stable at high pH with an absorbance maximum that is displaced towards the blue. The reaction with proteins is carried out within the pH limits of 9.3 to 12.5 [21]. The accessibility of tyrosine residues in a protein can be classified on the basis of changes in their reactivity with cyanuryl fluoride caused by changes in pH, temperature or both [17,22]. Kurihara et al. [22] suggested that a plateau in the reagent concentration curve indicates that a discrete level of tyrosine reactivity has been reached.

The cyanuration experiments were carried out at pH values in which hexokinase is not stable, and consequently served only to determine the accessibility of the phenolic hydroxyl groups of the tyrosine residues on the assumption that the inactivated enzyme suffered minor dislocation of any of tyrosine groups.

Fig. 1A shows the effect of cyanuration of the tyrosine residues in yeast hexokinase at pH 9.7 and pH 10 at 0°C as a function of reagent concentration. It can be seen that at pH 9.7, a plateau for the chemical reaction was not attained. Under the experimental conditions, we were unable to use more than 80 mM cyanuryl fluoride, since above this reagent concentration we observed the formation of aggregates in the reaction medium.

At pH 10, cyanuration did not attain a plateau. However it can be observed that at 40 mM a large decrease in the number of reactive tyrosyl groups occurs. This suggests that at twice that concentration of the reagent the maximum number of tyrosyl groups in yeast hexokinase can be modified.

In Fig. 1B are shown the results of the cyanuration of the enzyme as a function of pH at a constant reagent concentration of 80 mM both at 0 and 22°C.

At 0°C, it is clear that the accessibility of the phenolic hydroxyl groups is strongly pH-dependent in the zone of reactivity (between pH 9.7 and 11); this contrasts with lack of such dependence with free amino acids [21]. The effect of temperature on the reactivity of these tyrosine residues is noteworthy. Nearly the same number of tyrosyl groups are modified when the reaction is carried at 22°C (pH 9.7) or at 0°C (pH 11). Moreover at 22°C, the reaction attains a plateau at pH 11, as two additional tyrosyl groups are modified. This contrasts with the titration values obtained at 0°C and at the same pH, with two inaccessible residues. Above pH 11, at 22°C, there is a decrease in the extent of reaction, which is due to an increase in the rate of decomposition of cyanuryl fluoride [21].

From these results, we can separate tyrosyl residues in a subunit into three classes based on the accessibility of the hydroxyl group; 2 to 3 are highly accessible, 7 to 8 are probably located in crevices and about 5 must be buried in the protein structure.

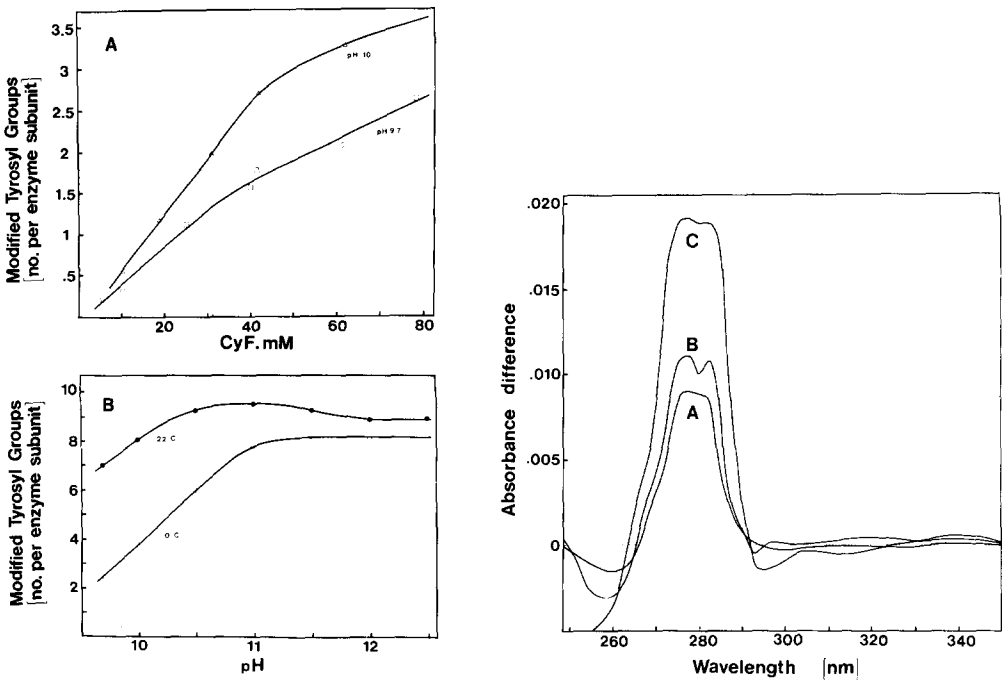

Fig. 1. (A) Dependence of yeast hexokinase tyrosine reactivity upon cyanuric fluoride concentration at 0°C. (B) Dependence of the cyanuration on pH and temperature. The molarity of cyanuryl fluoride is given per enzyme subunit (M_r 102000). The number of groups modified is given per enzyme subunit (M_r 51000). For details see Materials and Methods.

Fig. 2. Differential absorbance of the desacylated enzyme, after treatment with 1 M hydroxylamine versus acetylated enzyme in the reference cell. The molar ratios of *N*-acetylimidazol to protein were: (A) 94; (B) 188; (C) 282. For details, see Materials and Methods.

The effect of N-acetylimidazol

It has been reported that most of the reactive tyrosyl groups in proteins can be acetylated between pH 7.5 and 8.0 with a 60-fold molar excess of *N*-acetylimidazol, and that the reaction is complete within 1 h [19].

Fig. 2 shows the difference absorbance spectra of the deacylated enzyme (after 30 min treatment with 1 M hydroxylamine) versus the acetylated enzyme in the reference cell. It can be inferred that despite in the presence of a large excess of *N*-acetylimidazol, no more than one tyrosyl residue is acetylated per enzyme subunit. In Table I are given the details of these experiments. The acylation of one tyrosyl group per enzyme subunit results in only a 55% loss of activity. This inhibition is not completely reversed after treatment with 1 M hydroxylamine at pH 7.5. It is well known that under these conditions, the *O*-acetyl ester bond is readily hydrolyzed by the nucleophile [18,19]. It must be pointed out that under the very same conditions in which desacylation was carried out, the native enzyme is not affected by the nucleophile. The fact that only partial reversion of the enzyme activity was obtained after hydroxylamine treatment indicates either that groups other than hydroxyls were acetylated or that acetylation of the tyrosyl residue could have induced some measure of

TABLE I

EFFECT OF *N*-ACETYLIMIDAZOL ON YEAST HEXOKINASE

Conditions: protein solution 1 ml (5 mg/ml) in 25 mM phosphate buffer (pH 7.5) in the presence or absence of 1.5 M NaCl. To 1 ml of this solution *N*-acetylimidazol was added. After 1 h at 22°C, aliquots were taken to determine the residual activity and the number of groups acetylated. At the end of the acetylation reaction, the mixture was treated with 1 M NH_2OH at pH 7.5 and the reversal of the activity measured after 30 min. For details, see Materials and Methods and Fig. 2.

Additions	Ratio mol *N*-acetylimidazol/ mol enzyme	Tyrosyl groups modified per subunit	Lysine groups modified per subunit	Residual activity * after acetylation	Residual activity * after NH_2OH treatment
1.5 M NaCl	94	0.40 ± 0.05	1.9	108	135
	188	0.50 ± 0.05	3.2	84	110
	282	0.9 ± 0.1	5.2	68	100
None	188	0.35 ± 0.05	1.7	107	129

* μmol/min per mg protein (the specific activity of the enzyme control was of 150 units).

TABLE II

EFFECT OF TETRANITROMETHANE ON YEAST HEXOKINASE

Conditions: protein solution (0.25 mg/ml) in 50 mM Tris · HCl buffer (pH 8.0) containing 1.5 M NaCl in presence or absence of 0.2 M glucose. To 1 ml of this enzyme solution was added 20 μl of a tetranitromethane solution in 95% ethanol, the reaction was carried out either at 22 or 0°C. For details, see Materials and Methods.

Ratio mol tetranitromethane/mol enzyme	Reaction time (min)	Temperature (°C)	Additions	Residual activity *	Tyrosyl groups modified per subunit	SH groups modified per subunit
30	110	22	—	27	0.8 ± 0.1	—
60	90	22	—	15	2.0 ± 0.2	2.7
60	120	22	0.2 M glucose	15	0.9 ± 0.1	0.5
260	150	0	—	15	1.6 ± 0.2	—
260	270	0	—	10	4.0 ± 0.3	0.3

* μmol/min per mg protein (the specific activity of the enzyme control was of 150 units).

irreversible conformational change. In fact, it appears (see Table I) that the major loss of activity is related to the acetylation of the lysine residues, since we have previously shown that modification of these residues induces a loss of the hexokinase activity [7,8]. It may well be that the tyrosyl group which is more accessible to acetylation with *N*-acetylimidazol is of limited importance to enzyme activity and only to the extent of reversal of inhibition by the nucleophile. The partial inactivation must then be related to the modification of the ϵ-amino groups.

Reaction with tetranitromethane

In Table II, there are given the analytical data obtained from the time course of inactivation of yeast hexokinase by tetranitromethane under different experimental conditions (temperature, presence or absence of glucose). Although the rate of inactivation and the number of residues modified are different, loss of the enzyme activity is about the same with the exception of the reaction carried out in presence of 75 μM of tetranitromethane. With 650 μM of tetranitromethane (260 fold molar excess) at 0°C, the rate of inactivation is much slower than that observed at 22°C even with lower concentration of the reagent. At 0°C, 90% of the enzyme activity is lost by a pseudo first-order reaction for 150 min, during which 1.6 tyrosyl residues were modified. By increasing the reaction time to 270 min, an additional 3—5% loss of activity is observed during which 2.4 tyrosine residues are modified along with 0.3 sulfhydryl groups. This suggests that the major loss of the enzyme activity is related to the modification of the tyrosyl groups which are nitrated in the first phase of the chemical process.

When the enzyme is treated with 150 μM of tetranitromethane (60 fold molar excess) at 22°C, a much faster inactivation occurs than is observed at 0°C, and reaches a plateau when 90% of the initial activity is lost. At this point 2.1 tyrosyl and 2.7 sulfhydryl groups were modified. Although the loss of activity in the course of this modification was identical to that observed when the reaction was carried out at 0°C, we could not exclude the possibility that the sulfhydryl groups might be implicated in this loss. This assumption is based on the fact that of the four sulfhydryl groups that are present, the two most reactive are not essential for the enzyme activity whereas the two remaining, which are chemically less reactive, are critical for enzyme activity [8,23].

It has been previously shown that glucose induces a conformational change in hexokinase, which results in a definite decrease in the accessibility of the two essential sulfhydryl groups to chemical modification [7,24]. In order to protect these essential sulfhydryl groups, nitration was carried out with 150 μM tetranitromethane in the presence of 0.2 M glucose at 22°C. Under these conditions, we observed a decrease in the rate of inactivation. However, 90% of the enzyme activity is still lost when 1 tyrosyl group and 0.5 sulfhydryl group are modified. Although a sulfhydryl group was still partially modified, a good correlation between the loss of 90% of the hexokinase activity and the modification of the tyrosyl residue was observed. Both processes followed a first order reaction with a rate constant $k = 0.032\ \text{min}^{-1}$.

These studies indicate the presence of two "accessible" tyrosyl residues, one of which is responsible for the loss of the enzymic activity. In the presence of

glucose, the "non-essential" tyrosyl residue is protected while the "essential" one is not.

Consequently, all the subsequent work which will be described in this paper was performed with the enzyme which was nitrated in the presence of the substrate, thus having one modified tyrosyl group per enzyme subunit and possessing only 10% residual activity. This modified enzyme, referred to henceforth as "nitroenzyme", was thoroughly dialyzed to eliminate glucose and excess reagent. It will be shown below that the 10% residual activity is the intrinsic activity of the nitroenzyme and does not represent a mixture of a fully active 10% with a 90% of a fully inactive modified form.

Properties of the nitroenzyme

Heat stability. It can be seen in Fig. 3 that the thermoinactivation curve of the native and nitroenzyme are identical (the scales are normalized). This result indicates that the nitration of the "critical" tyrosyl residue does not render the protein heat labile anymore.

Ultracentrifugation studies of the nitroenzyme. Fig. 4, A and B, shows the results of ultracentrifugation of the nitroenzyme and native enzyme (treated under the same conditions but in the absence of reagent) at 2 mg/ml. Both native and nitroenzyme exhibited a single boundary with $s_{20,w}$ values respectively of 5.29 and 3.76. These agree with the values found previously for the dimeric and monomeric forms of the enzyme [7]. Thus the nitration of the essential tyrosyl residue induces a dissociation of the enzyme into its monomeric forms. Moreover, we did not detect the presence of any polymeric forms of the nitrated enzyme in SDS-polyacrylamide electrophoresis.

Kinetic parameters. The loss of the activity due to nitration is not related to a displacement of the pH optimum of the activity (results not shown). On the other hand the K_m values of the native enzyme at pH 8.0 for glucose and Mg ATP were 0.2 mM and 0.16 mM respectively and for the nitroenzyme 0.4 mM and 0.18 mM. Even though the K_m for glucose increased 2-fold, this increase

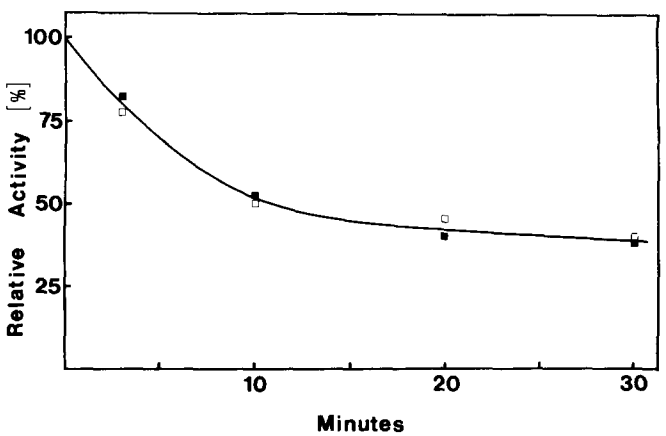

Fig. 3. Thermoinactivation at 44°C of native enzyme (□) and nitroenzyme (■). Protein concentration was 0.25 mg/ml in 50 mM Tris · HCl buffer. At the time intervals indicated, an aliquot of the native or nitroenzyme was taken and immediately assayed at 25°C. For details, see Materials and Methods.

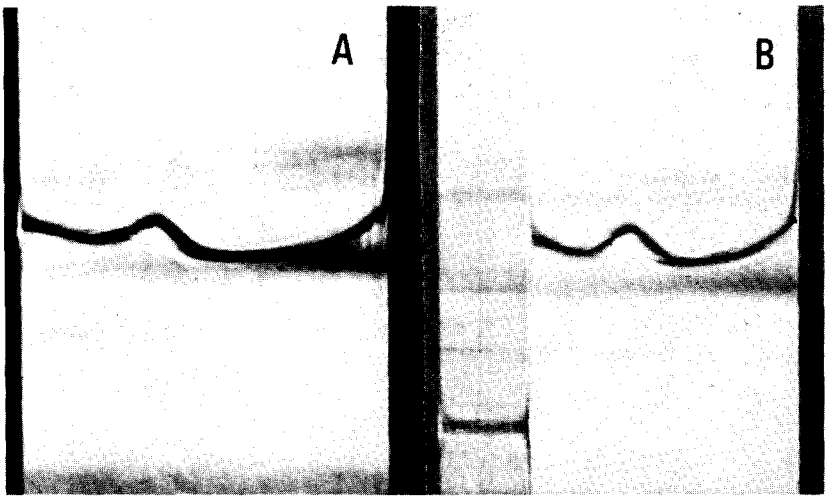

Fig. 4. Sedimentation patterns of the native and nitroenzyme. Pictures were taken at 45 min, native enzyme (A); nitroenzyme (B).

could not explain the 90% loss of the enzyme activity. However, from our results it is evident that the substrates could bind to the nitroenzyme.

The effect of pH on the citrate activation and on MgATP dependence of the nitroenzyme

Kosow and Rose [12] suggested that yeast hexokinase may exist as two conformational isomers, one a less active form, which is favored in the acid range, and the other a more active form which is favored by alkaline pH. They observed also that at pH 6.5, the enzyme can be activated by physiological polyanions (citrate, malate, ATP) but not at pH 8.0. Moreover they noticed a double hyperbolic ATP saturation curve (negative cooperativity) at pH 6.5 and a Michaelian behavior of the enzyme at alkaline pH.

Working under the conditions described by Kosow and Rose [12] we found for our native enzyme preparation an activation factor of 1.7 at pH 6.5 while at pH 7.9 we observed only a slight activation. At pH 6.5 the citrate activation factor for the nitroenzyme is 2.2. Most interesting is that under the effect of the activator, the activity at pH 6.5 was nearly of the same order as that observed at pH 7.9. It is quite possible that, at pH 6.5, more molecular forms of the nitroenzyme are in the less active form and that the citrate may displace the equilibrium towards the more active protonated forms. On the other hand under the conditions described by Kosow and Rose [12], the double reciprocal plots at pH 6.5 and 7.9 as a function of the Mg ATP concentration (Fig. 5) showed that the nitroenzyme behave like the native enzyme [7,8,12]. It can be seen that at pH 6.5, the nitroenzyme presents negative cooperativity at low concentrations of Mg ATP, while at pH 7.9 is has a Michaelian behavior. From the double reciprocal plots, the K_m which corresponds to the low ATP concentration is 52 μM and 56 μM at pH 6.5 for the native and nitroenzyme respectively. It thus appears that nitration of the tyrosyl groups did not affect the K_m adversely.

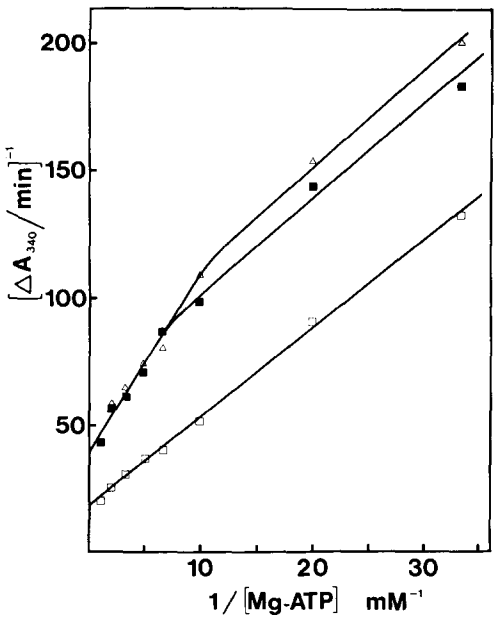

Fig. 5. Double reciprocal plots of velocity of the nitroenzyme as a function of MgATP concentration at pH 6.5 (■) and pH 7.9 (□) and for the native enzyme at pH 6.5 (△). The hexokinase activity was assayed by incubation of the native enzyme (40 ng) or nitroenzyme (170 ng) at 23°C in a final volume of 1 ml with 0.1 M 2-(*N*-morpholino)ethane sulfonic acid (pH 6.5) or 0.1 M Tris(hydroxymethyl)methyl-2-aminomethane sulfonic acid (pH 8.0), 2 mM glucose, 5 mM $MgCl_2$, 0.5 mM NADP, 0.7 unit glucose-6-phosphate dehydrogenase, 1 mM phospho*enol*pyruvate, 1.5 units pyruvate kinase.

Effect of nitration on the burst-type transient

Fig. 6 shows that native hexokinase A undergoes a slow change in activity under assay conditions (pH 6.5) as was observed by Shill and Neet [25]. The

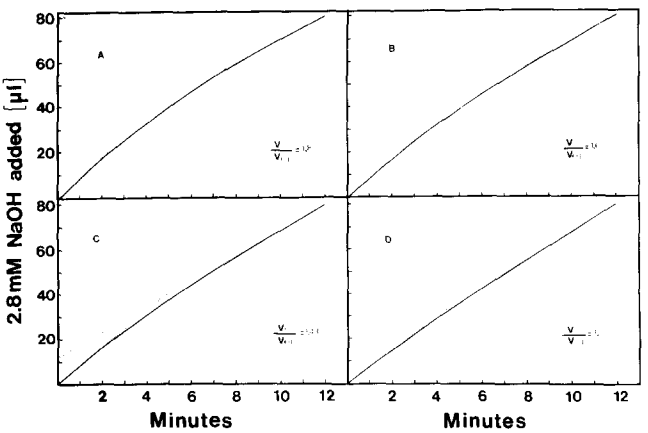

Fig. 6. Effect of nitration on the burst-type transient. Progress curves of the hexokinase reaction at pH 6.5 (with increasing number of tyrosyl group modified). The standard reaction mixture contained a final concentration of 2.8 mM ATP, 10 mM $MgCl_2$ and 20 mM glucose; the volume of this solution used for each test was 1.0 ml. Progress of the hexokinase reaction is indicated by the volume (in µl) of CO_2-free 2.8 mM NaOH added to the reaction under N_2, to maintain constant the pH. Each assay was started by the addition of 0.5 µg of native enzyme (A) and 0.8 to 2.9 µg of nitroenzyme (B, C and D). (A) Native enzyme; (B, C and D), nitroenzyme having 75, 54 and 25% residual activity.

ratio of the initial velocity to the steady state velocity is about 1.8. This ratio decreases in the course of nitration of the enzyme in presence of 0.2 M glucose. It was experimentally more difficult to determine the burst-type transient when the enzyme had lost more than 80% of the initial activity.

Discussion

The chemical modification of the tyrosine residues of several proteins with cyanuryl fluoride, *N*-acetylimidazol and tetranitromethane has been performed in order to discriminate between the availability of the phenolic OH group and the benzene ring [26]. However, since these reagents react at different positions of the same residue, even if it is assumed that the reaction is non-random, the results are not necessarily the same. In fact, we did not observe any agreement in the results obtained by the modification of the phenolic OH group between cyanuryl fluoride and *N*-acetylimidazol. By contrast, in some experimental conditions, the results with cyanuryl fluoride may be comparable to those obtained with tetranitromethane revealing the presence of two tyrosine residues per enzyme subunit which are more reactive than the thirteen others. In addition, the acetylation by *N*-acetylimidazol of the single tyrosyl group per enzyme subunit, does not seem to be significant. The major effect is due to the acetylation of the amino groups. These latter results support previous observations in which it was shown that succinylation [7] or trinitrophenylation [8] of the amino groups induces a partial loss of the enzyme activity. It is quite possible that the tyrosyl group which is more accessible to *N*-acetylimidazol is the one of two more available for reaction with cyanuryl fluoride and tetranitromethane.

This assumption is supported by the data obtained with nitration of the enzyme at 22°C in the presence and absence of glucose. In presence of the substrate, one tyrosyl group was modified and the loss of the enzyme was 90%, while in the absence of substrate two tyrosyl groups were modified for the same loss of the enzyme activity. This indicates that the non-essential tyrosyl group is protected by the conformational change induced by glucose, leaving only the essential tyrosyl group accessible to the reagent. It must be kept in mind that our conclusion that this tyrosine is essential for activity is a purely operational definition.

Judging by the apparent similarities of the effects of carbodiimide and tetranitromethane on hexokinase (partial loss of the activity without affecting the substrate binding sites), we regard it as quite possible that the same tyrosyl residues are modified by both reagents. However contrary to the effect of carbodiimide, nitration induces the dissociation of the protein into monomers.

Shill and Neet [25] observed that the monomeric form of the enzyme has about 50% of the specific activity of the dimeric form. Since neither the pH optimal of activity nor the K_m for the substrates were significantly affected by the nitration of the tyrosyl residue, it follows that the 90% decrease of the enzyme activity is due to two effects: the dissociation of the protein into monomers, along with that resulting from possible perturbation of the conformation at the active center region.

It is interesting to remark that in both cases in which yeast hexokinase was

dissociated into its monomeric forms, either by nitration of the tyrosyl residue or by succinylation of the lysine residues [7], the negative cooperativity with respect to ATP is not affected. However, in contrast to succinylation, nitration does not abolish the activation effect exerted by citrate. This result seems to support our previous suggestion [7] that the disappearance of the activation effect induced by the polyanions in the succinyl enzyme is related to the introduction of the additional negative charge. The negative charge would prevent the binding of the anionic activator by simple electrostatic repulsion.

Shill and Neet [25] working at a protein concentration that yields mostly the monomeric form, still observed the burst type slow transient, while nitration or succinylation [7] abolished this kinetic property which apparently is a characteristic of both monomeric and dimeric forms. Neet and Ainslie [27] have suggested the existence of a slow isomerization process in monomeric enzymes is probably due to the presence of two conformational forms of the protein, with different kinetic properties. It is then possible that the disappearance of the burst-type slow transient in the course of nitration or succinylation [7] could result from a shift in the conformational equilibrium which in turn alters the rate constants predicted by the model of Ainslie et al. [28]. This change in the rate constants would result in a transient which is either too rapid or too slow to be observed in our experimental conditions.

In some aspects, our results may possibly relate to those obtained by Meunier et al. [29] with wheat germ hexokinase (a monomeric enzyme). This enzyme exhibits negative cooperativity towards glucose and this property can be suppressed after treatment of the protein with low concentrations of urea or dodecyl sulfate, and the enzyme now obeys Michaelis kinetics.

The burst-type slow transient with yeast hexokinase is highly sensitive to perturbation of enzyme conformation. This is not the case for the negative cooperativity with respect to ATP or to the polyanion activation. The last two properties are not affected even after extensive chemical modification of the lysyl, histidyl or sulfhydryl groups [8].

Acknowledgments

This work was supported by grants from the Centre National de la Recherche Scientifique (E.R. 142), Délégation Générale à le Recherche Scientifique et Technique (contract number 75.7.0188). We are grateful to Professor V. Najjar for revising this manuscript, and to M. Grouselle and G. Foucault for valuable discussions.

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