Biochimica et Biophysica Acta, 613 (1980) 370—380

BBA 68965

LOCATION OF THE AROMATIC BINDING SITE AND PREPARATION OF AN AROMATIC DERIVATIVE OF GLYCOGEN PHOSPHORYLASE

THOMAS P. THOMAS and GEORGE PHILIP

Department of Marine Sciences, University of Cochin, Cochin - 682 016 (India)

(Received August 16th, 1979)

Key words: Aromatic binding site; Glycogen phosphorylase; Chemical modification

Summary

Rabbit muscle glycogen phosphorylase *b* (1,4-α-D-glucan:orthophosphate α-D-glucosyltransferase, EC 2.4.1.1) was inactivated by 1,5-difluoro-2,4-dinitrobenzene at pH 7.6 at a rate much faster than by 1-fluoro-2,4-dinitrobenzene. The reaction was very specific at low concentration of 1,5-difluoro-2,4-dinitrobenzene.

Glucose 1-phosphate, glucose 6-phosphate, AMP and ATP afforded some protection against inactivation by 1,5-difluoro-2,4-dinitrobenzene. These results and kinetic analyses of the modified enzyme were used to locate the binding site for aromatic compounds in phosphorylase. The above ligands and aromatic compounds are shown to bind on the enzyme in the same region which is located near the monomer/monomer interface.

An apparently homogeneous dinitrodiphenylene derivative of phosphorylase *b* with only one group per dimeric enzyme and having 50% of the catalytic activity was prepared. This derivative in which the subunits were not cross-linked by the reagent was devoid of the homotropic cooperativity for the substrate or activator sites even in the presence of allosteric inhibitors.

Glucose behaved quite differently from other ligands in its effect on modification and on the kinetics of the modified enzyme. The significance of the glucose site is discussed.

Introduction

Rabbit muscle glycogen phosphorylase *b* (1,4-α-D-glucan:orthophosphate α-D-glucosyltransferase, EC 2.4.1.1) has been subjected to chemical modification studies using a few aromatic reagents, viz. FDNB [1,2], chlorodinitrobenzene [3], 5,5′-dithiobis(2-nitrobenzoic acid) [4] and *p*-chloromercuri-

Abbreviations: F_2DNB, 1,5-difluoro-2,4-dinitrobenzene; FDNB, 1-fluoro-2,4-dinitrobenzene.

benzoate [5]. The dinitrophenyl derivative of phosphorylase *b* prepared under specific conditions has been shown to be partially desensitized of the allosteric properties [6]. Phosphorylase from rabbit muscle has a binding site for aromatic compounds [7] and nitrophenols have been shown to influence the allosteric properties of the enzyme [8]. However, the significance or the location of the aromatic binding site in phosphorylase is not yet known. A suitable chemical derivative of the enzyme is needed to delineate these aspects.

Now that the complete amino acid sequence of phosphorylase is known [9] and the three-dimensional structure is emerging from crystallographic studies [10—13], preparation and analysis of a suitable derivative should locate the aromatic binding site. Since several residues are modified in the reported derivatives [1—5] they are not useful for the purpose. We have selected 1,5-difluoro-2,4-dinitrobenzene which is a bifunctional aromatic reagent and hence can interact with the enzyme at its aromatic binding site and cross-link two regions of the enzyme. Here we report the preparation and some properties of an apparently homogeneous dinitrodiphenylene derivative of rabbit muscle phosphorylase *b* with only one aromatic group covalently incorporated per phosphorylase *b* dimer. The derivative seems to be suitable also for monitoring conformational changes on ligand binding.

Materials and Methods

Phosphorylase *b* was prepared from rabbit skeletal muscle according to Fischer and Krebs [14] but substituting mercaptoethanol for cysteine. Three times crystallised enzyme was used for the investigations. The enzyme was freed from AMP by treating with charcoal in the cold. Glucose-1-*P*, glucose-6-*P*, rabbit liver glycogen, and ATP were purchased from V.P. Chest Institute, New Delhi. AMP and L-cysteine-HCl were products of E. Merck. Trypsin, F_2DNB, FDNB and *p*-chloromercuribenzoate were from Sigma Chemical Company. Sodium β-glycerophosphate was the product of either K. Light or of British Drug House. Other chemicals were of analytical grade.

Protein concentrations were determined by the method of Lowry et al. [15]. The Folin reagent was calibrated using crystalline phosphorylase whose concentration was determined on the basis of an absorbance index of 13.2 for a 1% solution at 280 nm [9]. Molar concentrations were calculated using a molecular weight of 194 000 for phosphorylase dimer [9].

For chemical modification, the crystalline enzyme was first dissolved in 0.01 M sodium β-glycerophosphate, pH 7.0, to obtain a 15—20 mg/ml solution. After removing AMP the enzyme solution was passed through a Sephadex G-15 column equilibrated with 0.05 M Tris-HCl buffer of the required pH. The enzyme was diluted in 0.05 M Tris-HCl buffer of the desired pH so as to obtain an enzyme concentration of 2 mg/ml. For reactions in the presence of ligands, they were included in the enzyme solution. The reaction was started by adding the reagent (F_2DNB or FDNB). In a typical reaction, 0.05 ml of the reagent in CH_3OH was added to 5.0 ml of the enzyme (2 mg/ml) with immediate mixing. CH_3OH at this concentration (1%) had no effect on the enzyme activity. Final concentrations of the reagent were varied by manipulating the concentration of the stock solution so that in every case the volume of the reagent added was

0.05 ml for 5.0 ml of the enzyme solution. Reactions were carried out at 30°C. Aliquots from the reaction mixtures were diluted in 0.03 M cysteine/0.04 M sodium β-glycerophosphate buffer (pH 6.8) for stopping the reaction and for assay.

The enzyme was monomerised by treating it with a 20-fold molar excess of *p*-chloromercuribenzoate. Complete inactivation of the enzyme occurred in about 1 h. Reactivation was achieved by adding excess of neutral cysteine or cysteine/glycerophosphate buffer. Reactivation was between 90 and 100%.

The number of mol of F_2DNB reacted was determined by estimating the unreacted reagent using the benzylamine method of Hill and Davis [16]. The molar absorptivity for the product of the reaction between F_2DNB and benzylamine was $2.78 \cdot 10^4$ (Coleman spectrophotometer).

Ion-exchange chromatography for separating the protein was carried out in 1 × 10 cm columns. Sephadex G-200 gel filtration was performed using a 2.5 × 48 cm column equilibrated with 0.01 M β-glycerophosphate (pH 6.8) containing 0.1 M KCl. When the fractions had to be concentrated KCl was excluded from the buffer and the required fractions were passed through a small DEAE-cellulose column which adsorbed the enzyme completely. The enzyme was eluted from the column with 0.5 M β-glycerophosphate (pH 6.8) to get a 10—15 mg/ml solution.

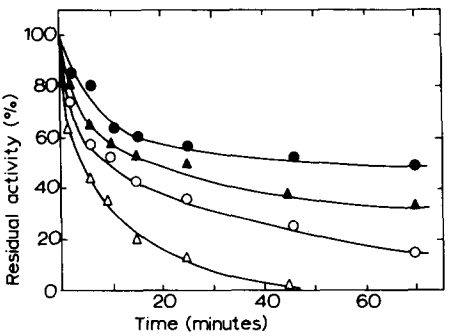
Initial reaction velocities were measured in the direction of glycogen synthesis [17] by estimating the liberated inorganic phosphate according to Fiske and Subbarow [18].

Resilts

Rabbit muscle glycogen phosphorylase *b* was inactivated by F_2DNB. The rate of inactivation was studied in 0.05 M Tris-HCl buffer, pH 7.0, 7.6 and 8.0. The inactivation rates at pH 7.6 and 8.0 were essentially identical and much faster than at pH 7.0. With a 10-fold molar excess of the reagent almost complete inactivation occurred in 30 min at pH 7.6 and 8.0 whereas only 30% inactivation occurred at pH 7.0. Because of the similarity in the rates and extent of inactivation at pH 7.6 and 8.0 and since pH 7.6 was used in some of the reported chemical modification studies with FDNB [1,2] we selected pH 7.6 for further investigations. Moreover, the enzyme was completely stable at this pH for 2 h whereas at pH 8.0 some loss of activity was observed.

The inactivation of phosphorylase *b* by different concentrations of F_2DNB is shown in Fig. 1. The inactivation was nearly 50% when a 1 : 1 molar concentration of the enzyme and the reagent was employed, i.e., at an enzyme monomer to reagent ratio of 1 : 0.5. Double the concentration of the reagent inactivated the enzyme only 65%. Even with a 4-fold excess complete inactivation of the enzyme did not occur. In all these cases the added reagent had completely reacted.

The data of Fig. 1 did not fit either first-order or second-order kinetics satisfactorily although first-order plots showed straight lines up to 10 min of the reaction. In order to find the order of the reaction, the data were analysed by plotting log concentration of the reagent against log inactivation at different fixed time. Curved lines were obtained which could be approximated to two



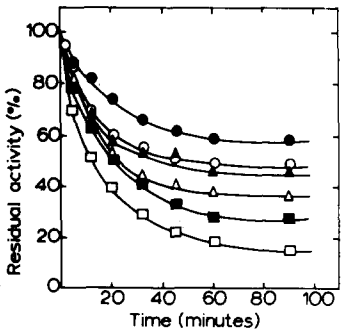


Fig. 1. Time course of inactivation of phosphorylase *b* by F_2DNB. 2 mg/ml phosphorylase *b* freed from AMP and mercaptoethanol was treated with different concentrations of F_2DNB in 0.05 M Tris-HCl buffer, pH 7.6. Aliquots were withdrawn, diluted in cysteine/glycerophosphate buffer (pH 6.8) and assayed. The assay mixtures contained 1% glycogen, 1 mM AMP and 16 mM glucose-1-*P*. F_2DNB in the reaction mixtures was 1-fold (●), 2-fold (▲), 4-fold (○) and 10-fold (△) molar excess over the enzyme dimer. Temperature was 30° C for reaction and assay.

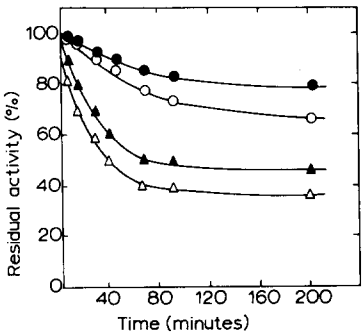
Fig. 2. Influence of AMP on the inactivation of phosphorylase *b* by F_2DNB. Phosphorylase *b* was treated with 1-fold (○, ●) 2-fold (△, ▲) and 4-fold (□, ■) molar excess of F_2DNB in presence (●, ▲, ■) and absence (○, △, □) of 1 mM AMP and inactivation was followed. Other details were as in Fig. 1.

linear portions with slopes (i.e., order of reaction) of 0.8—1.0 and 0.33. Up to about 10 min of the reaction and at lower concentration of the reagent the order was 0.8—1.0 but changed to 0.33 at higher concentration or on continuing the reaction. These results indicated that the reaction was more specific at the initial stages and at lower concentration of the reagent. The fractional order at higher concentration of the reagent and after the initial stages suggested reaction of different types of groups on the enzyme.

Substrate and effectors of phosphorylase *b* were found to afford some protection against inactivation. The protection by AMP at two concentrations of the reagent is shown in Fig. 2. The influence of the following ligands and their combinations on the inactivation of phosphorylase by F_2DNB was examined: (i) 1 mM AMP; (ii) 32 mM glucose-1-*P*; (iii) 32 mM glucose-6-*P*; (iv) 1 mM AMP + 32 mM glucose-1-*P*; (v) 1 mM AMP + 32 mM glucose-6-*P*; (vi) 40 mM glucose; (vii) 1 mM AMP + 40 mM glucose; (viii) 10 mM orthophosphate, and (ix) 1 mM AMP + 10 mM phosphate. The effect in each case was more or less same as that shown in Fig. 2 except in the case of glucose and orthophosphate (vi and viii). In these cases the observed protection was negligible. The pattern of influence of the ligands remained unchanged at 1 : 1, 1 : 2 or 1 : 4 molar concentration of the enzyme dimer to reagent.

F_2DNB was much more effective than FDNB in inactivating the enzyme. The inactivation profiles using the two reagents are presented in Fig. 3. It can be seen that F_2DNB was much more effective than twice the concentration of FDNB, i.e., same concentration of potential reactive centers.

Since the reaction with F_2DNB was specific at lower concentration of the reagent, the effect of adding the same amount of the reagent at one stretch or in portions was studied (Fig. 4). In either case the extent of inactivation was the same. It was also found that when a further molar quantity of the reagent



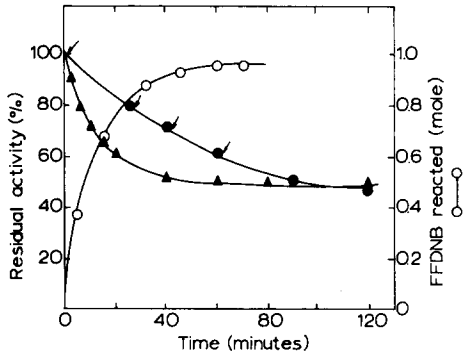


Fig. 3. Comparison of the inactivation profile of phosphorylase *b* by FDNB and F_2DNB. Enzyme concentration in the reaction mixtures was 2 mg/ml. Concentrations of FDNB were 1.3-fold (●) and 2.6-fold (○) and of F_2DNB 1.3-fold (▲) 2.6-fold (△) molar excess over phosphorylase *b*. Experimental details were as in Fig. 1.

Fig. 4. Effect of adding F_2DNB in one stretch and in portions on the inactivation of phosphorylase *b*. 2 mg/ml enzyme was treated with 1.3-fold molar excess of F_2DNB added in one lot at the beginning of the reaction (▲) and the same amount of the reagent under identical conditions added in four portions (●). The arrows indicate the time of addition of the reagent. Also shown is the incorporation of the reagent into the enzyme on treatment with a 1.3-fold molar excess of F_2DNB added in one stretch (○). The result is shown as mol of the reagent incorporated/mol of enzyme dimer.

was added to the 50% inactivated sample there was no further decrease of activity although the added reagent had completely reacted. These observations confirmed the specificity of the reaction at 1 : 1 molar concentrations of the reagent and enzyme dimer and suggested that at higher concentrations the reagent reacted with groups unimportant for the catalytic activity.

Analysis of the extent of reagent incorporation into the enzyme showed that 0.95 and 1.8 mol of groups were incorporated when 1 : 1 and 1 : 2 molar concentrations of phosphorylase *b* and F_2DNB, respectively, were used. The rate of incorporation of the reagent in one case is shown in Fig. 4. The method employed would give the unreacted quantity of the reagent only and would not distinguish between the reaction at one center or both centers of the reagent.

It was found that incorporation of the reagent was exactly 1 mol in 60 min when a 1.3-fold molar excess of the reagent was employed. Moreover, at this concentration the inactivation was repeatedly found to be exactly 50%. Since some hydrolysis of the reagent occurred the slight excess accounted for the hydrolysis and ensured incorporation of 1 mol/mol of the enzyme dimer. Therefore, phosphorylase *b* was treated with a 1.3-fold molar excess of F_2DNB for 60 min at 30°C. Phosphorylase concentration was always maintained at 2 mg/ml in the reaction mixture. The reaction was stopped by precipitating the protein with $(NH_4)_2SO_4$. After removing the salt by passing the enzyme through a Sephadex G-15 column the enzyme was exhaustively dialyzed against distilled water and hydrolysed in 6 N HCl. Chromatography of the hydrolyzate on Whatman No. 3 paper using isobutanol/formic acid/water (75 : 14.5 : 10.5) [19] gave three spots with R_F values 0.235, 0.35 and 0.72. The spot with R_F = 0.72 was the major band and showed a maximum of 342 nm which corresponded to the spectrum of bisdinitrodiphenylene lysine [19,20]. The other

spots gave broad peaks. The results suggested that the derivative contained more than one species. The modified amino acids were not further identified at this stage. Gel electrophoresis of the derivative showed a diffused band.

Earlier studies showed that FDNB reaction of phosphorylase resulted in the modification of lysyl and cysteinyl residues [1,2]. Four SH-groups on the surface of the enzyme reacted with chlorodinitrobenzene [3] and iodoacetamide [21] faster than other groups. Prolonged dialysis of the enzyme in Tris-HCl buffer was shown to destroy these surface-exposed groups [1]. Therefore, in the present study the enzyme was exhaustively dialyzed and treated with F_2DNB. The rate and extent of inactivation of the dialyzed enzyme was found to be identical with those of the undialyzed enzyme showing that the surface-exposed SH-groups either did not react or if reacted did not influence the activity. The result also supported the specific nature of the reaction. The specific activity of the enzyme remained unchanged in the presence of 1 mM 2-mercaptoethanol indicating that there was no interaction of thiol groups leading to activity change.

Kinetic studies also suggested that the derivative consisted of more than one population of molecules. Chromatography on DEAE-cellulose, CM-cellulose and Amberlite IRC 50 were not successful in purifying. With the cellulose derivatives the total amount of the protein fed was eluted in a single peak on elution using concentration or pH gradient. With the Amberlite, the protein could be eluted only under conditions that led to partial inactivation.

If the F_2DNB-reacted phosphorylase *b* consisted of tetrameric forms formed as a result of intermolecular cross-linking they could be detected by gel filtration on Sephdex G-200. Fig. 5 shows gel filtration profiles of the modified and native enzymes. The figure shows that some cross-linking had occurred and that the tetramer (or oligomers, if any) was eluted out in the void volume of the column. The major portion of the derivative was eluted with the same elution

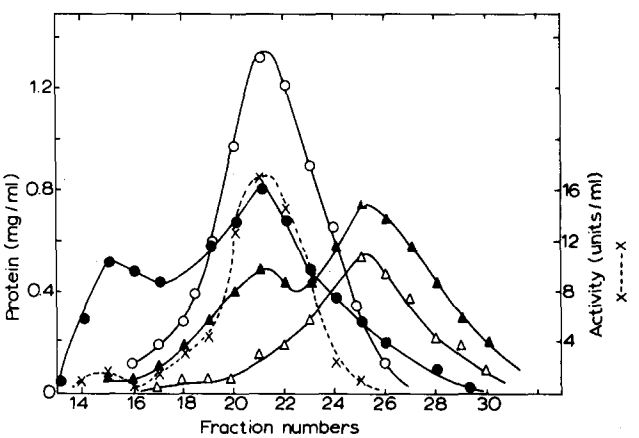


Fig. 5. Gel filtration on Sephadex G-200 of native and F_2DNB-reacted phosphorylase *b* before and after reaction with *p*-chloromercuribenzoate. ●, F_2DNB-reacted phosphorylase *b* prepared by reacting with a 1.3-fold excess of F_2DNB (35 mg); ○, 40 mg native phosphorylase *b*; ▲, F_2DNB-reacted phosphorylase *b* after treatment with 35 mg *p*-chloromercuribenzoate; △, native phosphorylase *b* after treatment with 18 mg *p*-chloromercuribenzoate; X- - - - -X, enzyme activity profile of the F_2DNB-reacted phosphorylase *b*. Fraction volume in all cases was 5 ml. For other details see text.

volume as that of the native enzyme dimer. Since reaction with *p*-chloromercuribenzoate would convert phosphorylase completely into monomers we used this reaction to find the extent of intersubunit cross-linking in the derivative. These results are also given in Fig. 5. The major portion of the derivative was eluted with the same elution volume of the native enzyme monomers. The minor band with lower elution volume appeared to represent cross-linked protein which probably came from the protein eluted out in the void volume.

When fractions 19—23 from the F_2DNB-reacted enzyme (Fig. 5) were pooled, concentrated, treated with *p*-mercuribenzoate and rechromatographed on the same column, the total amount of protein was eluted with an elution volume of the monomer.

Polyacrylamide gel electrophoresis of the derivative (fractions 19—23, Fig. 5) showed a single band similar to that obtained with the native enzyme. It was presumed that the fractions contained only one species. Identical results were obtained on repeating the reaction and the gel filtration several times. The dinitrophenylenephosphorylase *b* thus obtained showed a specific activity of 20 (μmol of inorganic phosphate liberated/min per mg of protein) when assayed in the presence of 1% glycogen, 16 mM glucose-1-*P* and 1 mM AMP at 30°C. Under these conditions the specific activity of the native unmodified enzyme was 40.

The purified enzyme derivative (dinitrophenylenephosphorylase *b*) was used for further studies. It was found to be considerably less stable in the presence of 1 M urea as compared to the native enzyme. The stability of the enzyme derivative was also less at elevated temperatures (45, 50, and 60°C). The deriva-

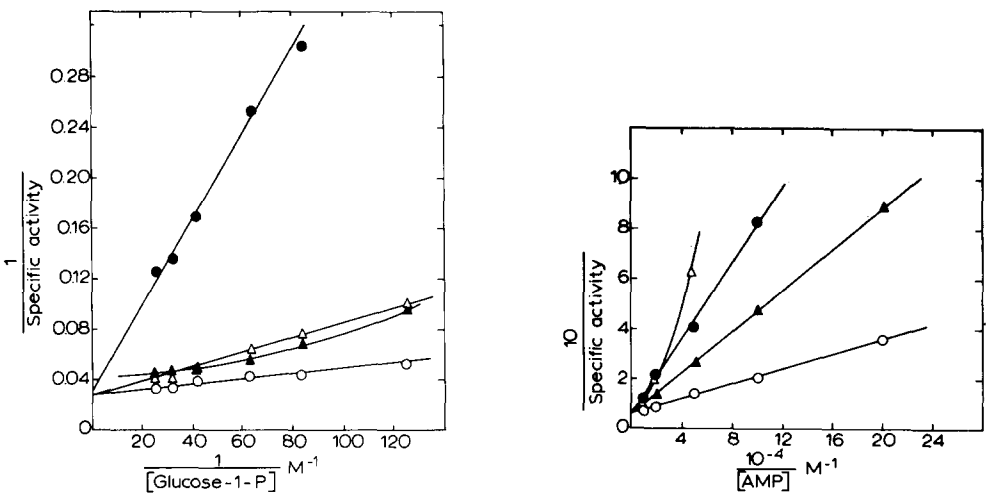


Fig. 6. Lineweaver-Burk plots for glucose-1-*P* in the absence and presence of glucose, glucose-6-*P* and ATP for dinitrophenylenephosphorylase *b*. The assay mixtures contained 1% glycogen, 1 mM AMP and varying concentrations of glucose-1-*P* in 15 mM cysteine/20 mM glycerophosphate buffer, pH 6.8. ○, no inhibitor; ●, 10 mM glucose-6-*P*; △, 10 mM ATP; ▲, 10 mM glucose. Assay temperature was 30°C.

Fig. 7. Lineweaver-Burk plots for AMP in the absence and presence of glucose, glucose-6-*P* and ATP for dinitrophenylenephosphorylase *b*. The assay mixtures contained 1% glycogen, 16 mM glucose-1-*P* and varying concentrations of AMP. ○, no inhibitor; ●, 10 mM glucose-6-*P*; ▲, 10 mM ATP; △, 10 mM glucose. Other details were as in Fig. 6.

TABLE I

INFLUENCE OF GLUCOSE, GLUCOSE-6-*P* AND ATP ON THE K_m VALUES AND HILL COEFFICIENTS FOR GLUCOSE-1-*P* AND AMP FOR THE NATIVE AND DINITROPHENYLENEPHOSPHORYLASE *b*

Conditions of experiments were the same is in Figs. 6 and 7. The K_m and *n* values were taken from Hill plots.

	Dinitrophenylenephosphorylase *b*		Native phosphorylase *b*	
	K_m (M)	*n*	K_m (M)	*n*
Glucose-1-*P* kinetics				
No inhibitor	$7.1 \cdot 10^{-3}$	1.1	$7.5 \cdot 10^{-3}$	1.0
10 mM glucose-6-*P*	$1.0 \cdot 10^{-1}$	1.0	$1.5 \cdot 10^{-2}$	1.6
10 mM ATP	$1.7 \cdot 10^{-2}$	1.1	$1.3 \cdot 10^{-2}$	1.5
10 mM glucose	$1.1 \cdot 10^{-2}$	1.5	$1.8 \cdot 10^{-2}$	1.5
AMP kinetics				
No inhibitor	$2.5 \cdot 10^{-5}$	1.0	$5.0 \cdot 10^{-4}$	1.0
10 mM glucose-6-*P*	$1.0 \cdot 10^{-4}$	1.0	$2.5 \cdot 10^{-4}$	1.6
10 mM ATP	$6.3 \cdot 10^{-5}$	1.1	$1.0 \cdot 10^{-4}$	1.5
10 mM glucose	$8.3 \cdot 10^{-5}$	1.6	$7.7 \cdot 10^{-5}$	1.8

tive was inactivated by trypsin faster than the native enzyme. The dinitrophenylenephosphorylase *b* could be converted to the *a* form with phosphorylase kinase in the presence of ATP and Mg^{2+}. Pyridoxal-5'-phosphate could be resolved from the derivative. However, the apoenzyme derivative could not be reconstituted with added pyridoxal-5'-phosphate to a catalytically active form. The results of resolution and reconstitution with the cofactor and analogues and spectral characterization will be the subject of another communication.

The kinetics for glucose-1-*P* and AMP of the dinitrophenylenephosphorylase *b* in the presence and absence of glucose-6-*P*, ATP and glucose are presented in Figs. 6 and 7. The results obtained with the derivative are compared with those of the native enzyme in Table I. From Figs. 6 and 7 and from the table the following observations could be made: (i) The dinitrophenylenephosphorylase *b* did not show any cooperativity between substrate sites and between activator sites in the presence of the allosteric inhibitors unlike the native enzyme which showed strong cooperativity between sites; (ii) the inhibition of the derivative by ATP and glucose-6-*P* was competitive in nature with respect to the kinetics for AMP and glucose-1-*P*; (iii) glucose, unlike the other ligands, induced cooperativity of sites like in the native enzyme.

Discussion

The inactivation of phosphorylase *b* by F_2DNB is specific at lower concentration of the reagent and at the initial stages of the reaction as evidenced from the following: (i) at a 1 : 1 molar quantity of the reagent and the enzyme dimer, i.e., 1 : 0.5 of monomer and the reagent, the inactivation is nearly 50% whereas at double the reagent concentration the inactivation is only 65%; (ii) addition of a molar equivalent of the reagent to the 50% inactivated sample has not resulted in further inactivation although the added reagent has reacted; (iii) the addition of a molar quantity of the reagent in one lot or in portions has

brought about the same extent of inactivation and (iv) the changing order of the inactivation process.

Since phosphorylase has a binding site for aromatic compounds [7] F_2DNB at lower concentration will interact non-covalently before reacting with amino acid residues. Protection against inactivation observed with the ligands AMP, glucose-1-*P*, glucose-6-*P* or their combinations has been more or less similar at different molar concentrations of the reagent suggesting that the protection is likely to be of the specific inactivation process. The concentration of the ligands is much more than the concentration of the reagent. Therefore, the aromatic site is not completely hindered by the ligands. It is possible that the conformation induced by the ligands has resisted inactivation to some extent. However, it seems unlikely that the activator, the substrate and the allosteric inhibitor and their combinations would bring about the same conformational change. Therefore, partial hindrance to the binding of the reagent is more likely to be the reason for the observed protection. We have earlier pointed out from kinetic studies that the sites for the above ligands are situated very near to each other [6]. Recent X-ray crystallographic studies [13] show that all these ligands bind on the same location in phosphorylase *b*.

The effects of glucose and orthophosphate differ from those of the other ligands. The kinetic behavior of the modified enzyme to glucose also is different (see below). These results suggest that the binding site of glucose is distinctly different from that of glucose-1-*P* or glucose-6-*P*.

Does the dinitrophenylenephosphorylase (fractions 19—23 of Fig. 5) consist of an equimolar mixture of completely inactive and fully active dimeric enzyme molecules or of a homogeneous population of 50% inactive dimeric molecules? Kinetics with modified enzyme does not show major changes in K_m for AMP or glucose-1-*P* ruling out the possibility of affinity changes as the reason for the decreased activity of the derivative. Electrophoresis of the derivative gives only one band. If it were a mixture further addition of the reagent would have inactivated the enzyme completely. In fact the added reagent has reacted without influencing the activity. Therefore, the dinitrophenylenephosphorylase *b* obtained by Sephadex gel filtration seems to represent a homogeneous population of dimeric molecules with only 50% of the activity of the native enzyme and with 1 mol of the aromatic group incorporated/mol of the dimeric phosphorylase *b*.

The dinitrophenylenephosphorylase can be monomerized completely and reconstituted like native enzyme showing that the subunits are not cross-linked in the derivative. The specificity of the reaction, the results of Fig. 4 and the fact that there is 1 mol of group incorporated/mol of dimeric enzyme suggest that phosphorylase *b* dimer has only one exposed site where the reagent binds and reacts resulting in inactivation. The subunits of phosphorylase are identical and are arranged with center of symmetry [11—13]. Hence the incorporation of the group is likely to be at the subunit interface.

It is possible that one subunit in the phosphorylase *b* dimer is completely inactive and the other subunit is potentially active. It has been reported from hybridisation experiments that an inactive monomer can induce activity in a potentially active monomer of phosphorylase so that an active monomer-inactive monomer hybrid has 50% activity of the native enzyme [22].

There is considerable similarity in the inhibition pattern of phosphorylase *b* by aromatic compounds [7] and AMP analogues [23,24]. Phosphorylase from *Escherichia coli* which is not affected by AMP is not inhibited by phenol or nitrophenol at concentration where the rabbit enzyme is 50% inhibited (unpublished observation). Therefore it seems likely that the binding site for aromatic compounds and AMP is the same in phosphorylase *b*. Crystallographic studies [11—13] show that AMP, glucose-1-*P*, glucose-6-*P* and ATP bind to the same region near the monomer/monomer interface. This site, the nucleotide binding domain, is shared between the monomers [11,12]. This seems to explain the specific reaction of F_2DNB reported in this article. The fact that aromatic compounds influence the allosteric property of the enzyme in a similar manner as glucose-6-*P* or ATP supports the above view.

There is a second site in phosphorylase *a* which binds nucleosides and purines preferentially and is located towards the center of the monomer near the pyridoxal-5′-phosphate [11,12]. Even if such a site exists in phosphorylase *b* the results of this paper are not in agreement with assigning the aromatic binding site in this nucleoside site.

Some of the recent studies assign the glucose site to the active site where glucose-1-*P* binds weakly in addition to its stronger binding on the nucleotide domain [12,25]. The lack of protection shown here against inactivation suggests that it does not bind at the aromatic site (nucleotide binding domain). The influence of glucose on the kinetics is clearly different from that of other ligands. This paper thus shows that glucose not only does not bind on the aromatic site but its binding elsewhere causes allosteric transition. The glucose effect appears to be very significant in the characterization of the structural modulations of phosphorylase.

Acknowledgements

The authors wish to thank Prof. C.V. Kurian, Head of the Department for facilities and Prof. Dr. D. Palm (University of Wuerzburg) for critically going through the manuscript. T.P.T. is grateful to the D.G.H.S., Government of India, for a Fellowship.

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