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INFLUENCE OF VARIOUS LIGANDS ON THE DESENSITIZATION OF PHOSPHORYLASE *b* BY DINITROPHENYLATION

G. SOMAN AND GEORGE PHILIP
Department of Biochemistry, University of Kerala, Trivandrum-1, Kerala (India)

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SUMMARY

1. The strength of cooperativity of rabbit muscle glycogen phosphorylase *b* (α-1,4-glucan:orthophosphate glucosyltransferase, EC 2.4.1.1) has been investigated with the aid of Hill plots using glucose 1-phosphate as the varying substrate and glucose 6-phosphate as the allosteric inhibitor. The homotropic cooperativity of glucose 1-phosphate sites has been found to be completely abolished on treatment of the enzyme with 1-fluoro-2,4-dinitrobenzene in the presence of AMP (1 mM) and orthophosphate (10 mM) until 20% inactivation occurred.

2. Desensitization does not occur on dinitrophenylation until 50–60% inactivation in the presence of glucose 1-phosphate or AMP or both. Also, the presence of either AMP or orthophosphate in the reaction mixture has been ineffective in desensitising the enzyme. Samples inactivated up to 80% in the absence of any ligand retain partial allosteric character.

3. The desensitization is assignable to modification of one lysyl and one cysteinyl residue. The role of orthophosphate in exposing some specific area (or groups) in the enzyme is discussed.

INTRODUCTION

The catalytic property of rabbit muscle glycogen phosphorylase *b* (α-1,4-glucan: orthophosphate glucosyltransferase, EC 2.4.1.1) has been found to be affected on treatment with 1-fluoro-2,4-dinitrobenzene (FDNB)[1], cyanate[2], 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB)[3] and iodoacetamide[4] among other reagents. Treatment of the enzyme with DTNB has been reported to abolish the homotropic cooperativity of the enzyme for AMP which has been explained as the result of modification of one or probably two cysteinyl residues in the enzyme[3] whereas modification of more than two SH groups with iodoacetamide did not result in loss of cooperativity[5]. Since phosphorylase activity is known to be controlled by various metabolites such as

Abbreviations: FDNB 1-fluoro-2,4-dinitrobenzene, DNP-, 2,4-dinitrophenyl-; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

glucose-1-*P*, AMP, ATP *etc.* characterization of the nature of allosteric control of the enzyme is of great importance.

It has been shown that AMP and glucose-1-*P* protected their respective binding sites against inactivation of phosphorylase *b* by FDNB[1]. Since dinitrophenylation does not cause dissociation of the enzyme unlike other reagents[1] we have investigated the influence of various ligands in protecting or exposing the sites responsible for the maintenance of the allosteric property of the enzyme.

We report in this paper that dinitrophenylation of two residues in the presence of both AMP and inorganic phosphate results in complete loss of cooperativity for glucose-1-*P* apparently due to a specific conformational change induced by these ligands.

MATERIALS AND METHODS

Phosphorylase *b* was prepared from rabbit muscle according to the procedure of Fischer and Krebs[6] but substituting mercaptoethanol for cysteine. Crystallised phosphorylase *b* was dialysed against 3–5 changes of 0.025 M Tris–HCl buffer (pH 7.6) to remove mercaptoethanol as described previously[1]. The enzyme thus obtained has four SH groups less than the native enzyme but its kinetic properties were unchanged[1]. Cysteine·HCl, glycogen and AMP were products of Merck. Glucose-1-*P* (dipotassium salt) was purchased from V.P. Chest Institute, University of Delhi. Glucose-6-*P* and bovine albumin were products of Koch Light Laboratories. FDNB was from B.D.H. Other reagents were of analytical grade.

Enzyme concentration was determined spectrophotometrically using an absorbance index of 13.2 for a 1% solution of protein[7] and also using Folin reagent[8] and Biuret reagent[9]. These reagents were calibrated using bovine serum albumin and crystalline phosphorylase. The molar concentration of phosphorylase *b* was calculated using a molecular weight of 185 000. The dialysed enzyme was treated with charcoal to remove any traces of AMP. Dinitrophenylation was carried out in 0.025 M Tris–HCl buffer (pH 7.6) with a 25-fold molar excess of FDNB in methanol (0.05 ml of 0.05 M FDNB in methanol was added to 5 ml of 4 mg/ml enzyme) and the reaction was stopped at the desired stage of inactivation (using a curve showing time course of inactivation) by the addition of excess 0.03 M cysteine–0.04 M glycerophosphate (pH 6.8). The enzyme was incubated in the buffer for at least 1 h at room temperature prior to rate measurements. Thiolysis does not take place under these conditions[1]. Initial reaction velocities were measured in the direction of glycogen synthesis and the liberated inorganic phosphate was estimated colorimetrically by the method of Fiske and SubbaRow[10]. Substrates containing 2% glycogen, 2 mM AMP and varying concentration of glucose-1-*P* (or 0.032 M for routine estimations) were mixed with an equal volume of suitably diluted enzyme solutions and initial rates measured.

For identification of groups modified, the dinitrophenylated protein was precipitated with a 10% trichloroacetic acid solution, washed repeatedly with 5% cold acid followed by hydrolysing the protein in 6 M HCl in a sealed and evacuated tube (repeatedly flushed with nitrogen) for 24 h at about 110 °C. The hydrolysate was thoroughly extracted with ether and the ether layer which contained no 2,4-dinitrophenyl (DNP) amino acid was rejected .The aqueous layer was evaporated under 60 °C and the residue was dissolved in water and again dried. This process was repeated

several times and the final residue was chromatographed on a Whatman No. 1 paper using the upper layer of *n*-butanol–acetic acid–water (4:1:5, by vol.) as the solvent[11].

The number of DNP groups incorporated into the enzyme was determined by the benzylamine method of Hill and Davis[12] by estimating the unreacted FDNB before and after the reaction with protein. A known volume of FDNB solution (0.5 ml) in Tris–HCl (pH 7.6) was shaken with 20 ml of a 1% solution of benzylamine in toluene for 30 min at room temperature. The toluene layer was filtered through glass wool and the absorbance measured at 343.5 nm against a similarly treated blank. The concentration of FDNB was calculated using an extinction coefficient of 17 300.

RESULTS AND DISCUSSION

The allosteric properties of enzymes can be conveniently analysed with the use of Hill plots[13,14]. The value of the Hill coefficient will indicate the strength of co-operativity. Madsen and Shechosky[15] applied this method to study the allosteric effects of phosphorylase *b*. If kinetic analyses were carried out in the presence of an allosteric inhibitor the value of n will increase and approach the number of binding sites. In the present study we have used this method to assess the strength of co-operativity of phosphorylase *b*. The substrate saturation curve of phosphorylase *b* in the presence of glucose-6-*P* has been shown to be sigmoidal suggesting that it is an allosteric inhibitor of the enzyme[16]. The influence of 10 mM glucose-6-*P* on the activity of phosphorylase *b* with varying concentrations of glucose-1-*P* is shown in Fig. 1 in the form of a Hill plot. In the absence of the inhibitor the value of $n = 0.93$ while in the presence of 10 mM glucose-6-*P* the value is 1.86. Since dinitrophenylation of phosphorylase *b* was shown to alter the binding sites for glucose-1-*P* and AMP[1] we have investigated if such alterations changed the allosteric properties of the en-

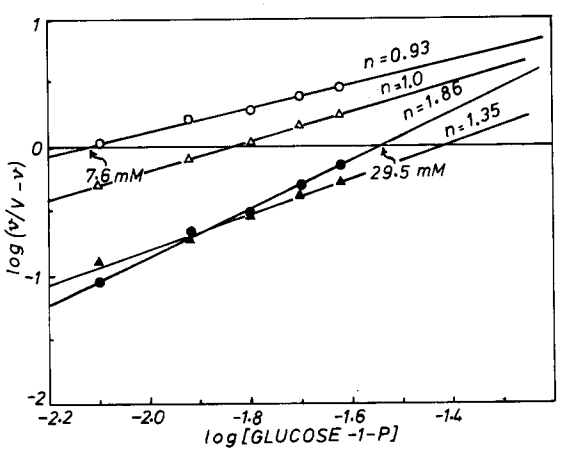

Fig. 1. Hill plot with glucose-1-*P* varied in the absence and presence of glucose-6-*P* for native (unmodified) and DNP–phosphorylase *b*. Phosphorylase *b* was treated with FDNB until inactivation was 55%. The reaction was stopped by the addition of excess cysteine–glycerophosphate (pH 6.8) and the DNP–enzyme assayed (30 °C) in the absence (○) and in the presence of (●) glucose-6-*P* (10 mM). Unmodified enzyme was assayed as above in the absence (△) and in the presence (▲) of glucose-6-*P* (10 mM). Enzyme samples were preincubated in the cysteine–glycerophosphate buffer for 1 h prior to assay. The concentrations of AMP and glycogen in the assay mixtures were 1 mM and 1%, respectively.

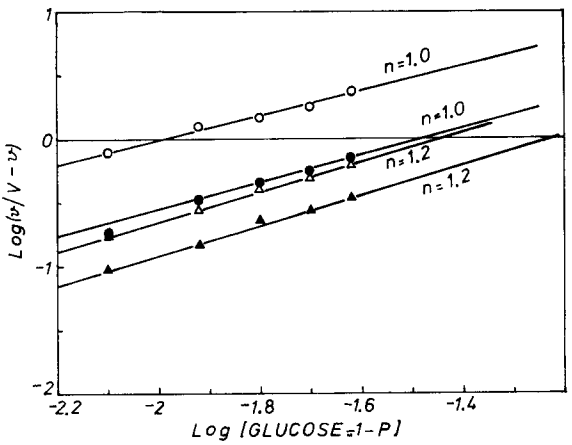

Fig. 2. Hill plot with glucose-1-*P* varied in the absence and presence of glucose-6-*P* (10 mM) for DNP-derivatives of phosphorylase *b*. Phosphorylase *b* was treated with FDNB in the presence of AMP or orthophosphate or both until inactivation was 50–60% and the DNP-derivatives were assayed in the presence or absence of glucose-6-*P*. ○, DNP–phosphorylase *b* prepared in the presence of AMP (1 mM) and orthophosphate (10 mM) and assayed in the absence of glucose-6-*P*; ●, the above derivative assayed in the presence of glucose-6-*P* (10 mM); △, DNP–enzyme prepared in the presence of orthophosphate (10 mM) alone and assayed in the presence of glucose-6-*P* (10 mM); ▲, DNP–enzyme prepared in the presence of AMP (1 mM) alone and assayed in the presence of glucose-6-*P* (10 mM). Other details are as in Fig. 1.

zyme. The enzyme was dinitrophenylated with FDNB until about 50–60% inactivation occurred and the sample examined kinetically. These results also are included in Fig. 1. The results show that there is a decrease in the value of n but the allosteric nature of the enzyme is not abolished.

In order to delineate the nature of involvement of various ligands phosphorylase *b* was dinitrophenylated in the presence of these ligands and the kinetics of the DNP-derivatives investigated in detail. When dinitrophenylation was carried out in the presence of AMP and P_i the allosteric site for glucose-1-*P* was found to be completely desensitized. The results are presented in Fig. 2. The figure also shows the Hill plots with DNP–enzyme prepared in the presence of either AMP or P_i. It can be seen that the value of n approached unity when dinitrophenylation was carried out in the presence of both AMP and orthophosphate indicating the involvement of these ligands in exposing certain specific areas or groups in the enzyme.

Since phosphorylase *b* was found to be desensitized with respect to the glucose-1-*P* binding sites on dinitrophenylation in the presence of AMP and orthophosphate, it was of interest to compare the influence of various ligands. Thus glucose-1-*P*, AMP and inorganic phosphate were tested separately and in combination. The results were analysed with the use of Hill plot and also reciprocal plot. The results are given in Table I along with the K_m values for glucose-1-*P* in the presence and in the absence of glucose-6-*P*. Since dinitrophenylation was allowed until activity was 50–60%, V values were not comparable and hence omitted in the table. The results show that desensitization is only partial in the absence of orthophosphate. Complete desensitization occurred only when AMP and orthophosphate were present in the reaction mixture. Since alterations in the binding sites are reflected in the K_m values for the respective substrates, these values can be used for comparing the relative modi-

TABLE I

INFLUENCE OF DINITROPHENYLATION ON THE HILL COEFFICIENT AND K_m VALUES OF PHOSPHORYLASE *b*

Phosphorylase *b* was dinitrophenylated with FDNB as detailed in Materials and Methods until inactivation was 50–60%. The reaction was stopped by the addition of excess cysteine–glycerophosphate buffer (pH 6.8). The DNP-enzyme was preincubated in the same buffer for 1 h at room temperature prior to rate measurements. Initial rates were measured with glucose 1- phosphate as the varying substrate. The concentration of AMP and glycogen in the assay mixtures were 1 mM and 1%, respectively.

Dinitrophenylated in the presence of	*n values for glucose-1-P*		*K_m values (mM)*	
	No ligand	*With glucose-6-P (10 mM)*	*No ligand*	*With glucose-6-P (10 mM)*
None	1.0	1.3	14.6	39.8
AMP (1 mM)	1.0	1.2	24.5	37.6
Orthophosphate (10 mM)	1.0	1.2	16.6	60.3
AMP (1 mM) and orthophosphate (10 mM)	0.93	1.0	11.2	36.3
Glucose-1-*P* (40 mM)	1.0	1.3	7.8	39.8
AMP (1 mM) and glucose-1-*P* (40 mM)	0.9	1.3	7.2	22.4
Unmodified (native) enzyme	0.93	1.8	7.4	29.5

fication of binding sites. Such a comparison of the K_m values for various DNP–enzymes indicate that while glucose-1-*P* protected its binding site, AMP or orthophosphate only increased K_m for glucose-1-*P*. The influence of orthophosphate on dinitrophenylation was more dramatic in the apparent K_m for glucose-1-*P* in the presence of glucose-6-*P*.

The specific influence of inorganic phosphate on desensitization by dinitro-

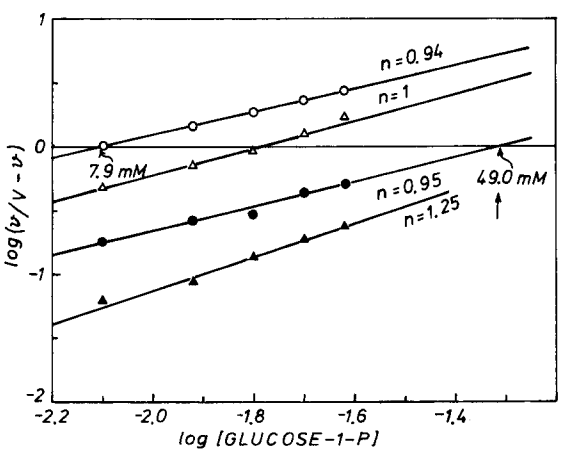

Fig. 3. Hill plot with glucose-1-*P* varied in the absence and presence of glucose-6-*P* (10 mM) for DNP-derivatives of phosphorylase *b*. Phosphorylase *b* was treated with FDNB in the presence of AMP (1 mM) and orthophosphate (10 mM) until inactivation was 20% and the DNP-derivative assayed as in Fig. 1, in the absence (○) and in the presence (●) of glucose-6-*P*. An enzyme sample inactivated 80% in the absence of any ligand was assayed similarly in the absence (△) and presence (▲) of glucose-6-*P*.

phenylation was further assertained with samples inactivated to lesser extent. A sample inactivated only 20% in the presence of AMP and orthophosphate was found to exhibit an n value of unity where as enzyme samples inactivated up to 80% in the absence of any ligand still retained partial allosteric character (Fig. 3). These results lend further support to the unique role of P_i in the desensitization process by FDNB treatment.

It was shown previously that FDNB treatment of phosphorylase under conditions similar to those used in this study resulted in modification of cysteinyl and lysyl residues[1]. The same may be expected here as well. However, three different samples of DNP-enzyme were analyzed for the groups modified: (a) enzyme inactivated 50–60% in the presence of glucose-1-*P* and AMP, (b) enzyme inactivated 50% in the absence of any ligand and (c) enzyme inactivated 20% in the presence of AMP (1 mM) and orthophosphate (10 mM). In all these cases only two colored spots corresponding to DNP-cysteine and DNP-lysine could be detected.

The number of groups incorporated into the enzyme which was inactivated 20% in the presence of AMP and phosphate was estimated using the benzylamine method[12]. It may be mentioned here that good agreement of results obtained by this method and by using radioactive FDNB was reported earlier[1]. In the present case an average of about two groups were found to be modified, showing that modification of one cysteinyl and one lysyl residue leads to complete desensitization of the glucose-1-*P* sites of phosphorylase *b*. Attempts to assign the desensitization to any single residue were not successful. Therefore, any conclusion as to the relative importance of these residues can not be made from these studies.

Several authors have reported faster reaction of a few SH groups of phosphorylase *b* with chemical reagents[17–19]. Battell *et al.*[4] classified SH groups of phosphorylase *b* and concluded that two groups reacted faster with iodoacetamide without loss of activity. Gold[19] observed reaction of four SH groups of the enzyme with dinitrochlorobenzene without loss of activity. Kastenschmidt *et al.*[3] showed that modification of two or three SH groups of phosphorylase *b* with DTNB resulted in the loss of the homotropic cooperativity of the enzyme for AMP whereas Battel *et al.*[5] observed no such loss of cooperativity with the alkylated enzyme. As was previously suggested by Johnson *et al.*[20] the nature of the reagent and possible noncovalent interaction prior to covalent bond formation determine the course of chemical modification.

Since modification of more residues in the absence of any ligand or in the presence of some ligands does not cause desensitization as reported here, the observed influence of AMP and inorganic phosphate must be due to a certain specifically induced conformational change in the enzyme rather than shielding of any particular area from attack by the reagent.

Preliminary experiments indicate that the desensitization observed is specific for the glucose-1-*P* binding sites rather than the allosteric property of the enzyme in general. This would mean clear differences in conformations between the desensitized enzyme reported here and that reported by Kastenschmidt *et al.*[3]. Work is in progress in this direction using other reagents and proteolytic enzymes.

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