The Nature of the Binding Site for Aromatic Compounds in Glycogen Phosphorylase *b*

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The inhibition of rabbit muscle glycogen phosphorylase *b* (1,4- α -D-glucan-orthophosphate α -glucosyltransferase, EC 2.4.1.1) by aromatic compounds was examined with 15 compounds. The relative effectiveness of the inhibitors correlated well with increasing substituent constant, π , indicating the hydrophobic nature of the binding site. The inhibition was not affected by the ionic-strength variation of the assay mixtures. The results predict that the course of chemical modification of this enzyme and the properties of the derivatives depend on the nature of the reagent and on the incorporated groups. Many of the dissimilar and sometimes contradictory results reported for chemical-modification studies and for chemically modified phosphorylase *b* are explained by the findings presented in the paper.

Rabbit muscle glycogen phosphorylase *b* (1,4- α -D-glucan-orthophosphate α -glucosyltransferase, EC 2.4.1.1) has been subjected to chemical-modification studies using a variety of reagents. Most of the reagents brought about modification of either amino groups or thiol groups or both. The partially active enzyme derivatives obtained showed distinctly different and sometimes contradictory catalytic and allosteric properties even when the modified residues were apparently the same (Philip & Graves, 1968; Johnson *et al.*, 1968; Gold, 1968; Kastenschmidt *et al.*, 1968; Battell *et al.*, 1968; Fasold *et al.*, 1969; Soman & Philip, 1974*b*). One reason for the disparities might be the binding of the reagents to the enzyme before reaction. *p*-Nitrophenol has been shown to increase the homotropic co-operativity of glucose 1-phosphate and AMP sites in phosphorylase *b* (Soman & Philip, 1974*c*). The present study shows the aromatic nature of the binding site and implies that the nature of the reagents and their non-polar interaction before covalent bond formation determine the course of the reaction and the property of the modified enzyme.

Methods

Crystalline phosphorylase *b* was prepared from rabbit skeletal muscle by the procedure of Fischer & Krebs (1962), but substituting 2-mercaptoethanol for cysteine. Concentrations of phosphorylase *b* were determined by using an absorbance index of 13.2 at 280nm for a 1% solution (Seery *et al.*, 1967) and a Folin reagent (Lowry *et al.*, 1951) calibrated against crystalline phosphorylase *b*.

Aromatic compounds were dissolved by adding just sufficient 0.1M-HCl or 0.1M-NaOH solution

and adjusting the pH to 6.8. Stock solutions thus prepared were further diluted in 0.03M-cysteine–0.04M-sodium glycerol β -phosphate buffer, pH6.8. Solutions of the desired concentrations were mixed with an equal volume of suitably diluted enzyme solution in the same buffer, and the enzyme reaction was started by mixing the enzyme solution (or the enzyme–inhibitor solution) with an equal volume of substrate containing 32mM-glucose 1-phosphate, 2mM-AMP and 2% glycogen. Initial reaction velocities were measured in the direction of glycogen synthesis (Illingworth & Cori, 1953) and the liberated P_i was determined colorimetrically by the method of Fiske & SubbaRow (1925). All rate measurements were carried out at 30°C in 0.015M-cysteine–0.02M-sodium glycerol β -phosphate buffer, pH6.8.

Results and discussion

All aromatic compounds tested inhibited phosphorylase *b*. The inhibitions by *p*-nitrophenol and aniline, which were taken as representative compounds, were not time-dependent at least for 30min at 30°C. This is as would be expected for reversible inhibitors. The relative effectiveness of the various inhibitors at three different concentrations is presented in Table 1. There is no correlation between extent of inhibition and Hammett substituent constant or state of ionization of the aromatic compounds. Reasonably good correlation between substituent constant, π , and inhibition is shown.

$$\pi = \log P_X - \log P_H$$

where P_H is the partition coefficient of a parent compound between octanol and water and P_X that of its substituted derivative with the substituent X (Fujita *et al.*, 1964).

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Table 1. Correlation between inhibition of phosphorylase *b* by aromatic compounds and the substituent constant, π

Stock solutions of the compounds were prepared as stated under 'Methods' and initial velocities were measured. The assay mixtures contained 16 mM-glucose 1-phosphate, 1 mM-AMP, 1% glycogen (about 1 mM in glucose units) and the stated concentrations of inhibitors in 0.015 M-cysteine-0.2 M-sodium glycerol β -phosphate, pH 6.8. The specific activity of the enzyme was 45 μ mol of orthophosphate liberated/min per mg of protein at 30°C. Concentration of enzyme in the assay mixtures was 50 μ g/ml. The inhibition results at 5 mM concentration of the inhibitors are the average values from three to four independent determinations and the inhibition results at 2.5 and 7.5 mM concentrations are values from one or two determinations. A partial list of inhibition was published earlier (Soman & Philip, 1974c). However, the correlation with π value was not known then. π values were taken from phenol systems for substituted phenols and from aniline systems for anilines.

Compound	Concn. of compound (mM)	Inhibition (%)			π
		2.5	5.0	7.5	
3,5-Dinitrosalicylic acid	—	50	82	92	0.95
<i>p</i> -Chlorophenol	—	48	73	84	0.93
2,4-Dinitrophenol	—	—	67	—	0.83
<i>o</i> -Cresol	—	40	63	70	0.67*
<i>m</i> -Cresol	—	57	67	75	0.56
<i>m</i> -Nitrophenol	—	60	82	92	0.54
<i>p</i> -Nitrophenol	—	34	53	63	0.50
<i>p</i> -Cresol	—	37	61	63	0.48
Phenol	—	28	42	45	0
Aniline	—	25	41	44	0
3,5-Dinitrobenzoic acid	—	22	40	40	-0.10†
<i>m</i> -Aminophenol	—	—	20	—	-1.29‡
Hippuric acid	—	—	22	25	-1.49§
Benzyl alcohol	—	—	15	—	-1.70
Benzylamine	—	10	—	16	—
Cyclohexylamine	—	0	0	4	—
Cyclohexanol	—	—	0	0	—

* π value taken from phenoxyacetic acid system.

† π value taken from benzoic acid system.

‡ π value taken from phenol system.

§ π value taken from benzene system for $-\text{CONH}_2$ group.

Therefore the higher the value of π for a compound the higher its hydrophobicity (Anderson & Graves, 1973). 3,5-Dinitrosalicylic acid and *p*-chlorophenol, which have the highest π values, are the most effective inhibitors, and *m*-aminophenol and benzyl alcohol, which have low π values, are poor inhibitors. One exception is *m*-nitrophenol; although its π value is slightly higher than that of *p*-nitrophenol, its very large inhibitory effect cannot be accounted for by this relationship.

Correlation of π values would be more appropriate with inhibitor constants, but, since the compounds tested (*m*-nitrophenol, *p*-nitrophenol and aniline) yielded non-linear reciprocal and Dixon plots (Dixon, 1953) (both curved upwards) it was not possible to calculate the inhibitor constants. The upwardly curved Dixon plots indicate more than one locus of inhibitor-binding in non-allosteric systems. To test whether the non-linear Dixon plot with phosphorylase *b* was due to its allosteric nature or to the presence of multiple binding sites, a desensitized 2,4-dinitrophenyl derivative of phosphorylase *b* (Soman & Philip, 1974b) and a naturally occurring desensitized form, namely

phosphorylase *b* from *Indocibium guttattam* (Soman & Philip, 1974a), were analysed for their inhibition behaviour. Both these enzymes gave linear reciprocal and Dixon plots with the nitrophenols and aniline as inhibitors. Therefore it appears that the non-linear Dixon plot is attributable to the allosteric property of glycogen phosphorylase *b*. Also the desensitized enzyme showed mixed inhibition. The Dixon plots have not so far been analysed for allosteric enzymes.

Since the influence of ionic strength on inhibition is useful in the understanding of the nature of binding, the enzyme activities at five different concentrations of the substrate were measured in the presence of NaCl alone and in the presence of NaCl and *p*-nitrophenol. No change of activity could be found by varying the NaCl concentration up to 0.2 M. Thus charge-transfer or other interactions which are weakened by increased ionic strength are not the predominant force in the enzyme-inhibitor complex-formation.

The good agreement between inhibition and increasing hydrophobicity of the aromatic compounds shows clearly that the major contribution to

binding is from hydrophobic interactions. The observation that the aromatic compounds examined effected allosteric transitions indicates conformational changes caused by binding (Soman & Philip, 1974c). The binding, however, does not alter the dimeric state of phosphorylase *b* (Soman & Philip, 1974c).

The results predict clear differences in the course of reactions of aliphatic and aromatic reagents. Since the binding by aromatic compounds causes conformational change in the enzyme, the groups modified will, to a very large extent, depend on the nature and concentration of the reagents, and the catalytic and allosteric properties will be influenced by the incorporated groups. This paper thus explains the following observations reported in chemical-modification studies with phosphorylase *b*. (1) Modification of two to three SH groups with 5,5'-dithiobis-(2-nitrobenzoic acid) resulted in partial desensitization of the allosteric property (Kastenschmidt *et al.*, 1968; Soman & Philip, 1974b) whereas modification of even more SH groups with iodoacetamide had no effect on the allosteric property of the enzyme (Battell *et al.*, 1968). (2) Inactivation of phosphorylase *b* required modification of about five lysine residues on treatment with an 8-fold molar excess of 1-fluoro-2,4-dinitrobenzene (Philip & Graves, 1968) whereas complete inactivation occurred on modification of just two lysine residues when the enzyme was treated with a 180-fold molar excess of the same reagent (Fasold *et al.*, 1969). Under different conditions Gold (1968) observed modification of mostly SH groups by chlorodinitrobenzene with little effect on the activity of the enzyme. (3) Complete inactivation of the enzyme required carbamoylation of 18 lysine residues with cyanate (Huang & Madsen, 1966). (4) Modification of up to ten amino groups with

aliphatic aldehydes did not affect either the structure or the activity of phosphorylase *b* (Wang & Tu, 1969).

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