Interaction of Phosphorylase *b* with Eosin

INFLUENCE OF SUBSTRATES AND EFFECTORS ON EOSIN-ENZYME COMPLEXES

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The interaction of rabbit muscle glycogen phosphorylase *b* with Eosin (2',4',5',7'-tetrabromofluorescein) was studied. Eosin was found to be an effective inhibitor of the enzyme. The inhibition constants for the dye were estimated to be approx. 36 and 60 μM with respect to AMP and glucose 1-phosphate respectively. The binding of Eosin to phosphorylase *b* is accompanied by a red-shift of about 12 nm in the dye absorption-spectrum maximum, indicating low-polarity binding sites on the enzyme molecule for the dye. The absorbance in the difference absorption maximum at 537 nm was utilized to follow the conjugation of phosphorylase *b* with Eosin. Scatchard plots of the titration data revealed the existence of at least two classes of binding sites on the protein molecule for Eosin, and the dissociation constants measured in Tris/HCl buffer, pH 7.0 ($I 0.091$), were 7.7 and 41.7 μM respectively. The influence of the substrates and effectors on Eosin-enzyme complexes was used to study the ligand-phosphorylase *b* interactions. IMP displaced the dye completely from the enzyme, indicating that there are two IMP-binding sites per phosphorylase *b* monomer. AMP binding to the enzyme with respect to Eosin concentration is of two types: a non-competitive one for the high-affinity site for AMP and a competitive one for the low-affinity site for the activator. The effects of glucose 6-phosphate, ATP, P_i and glycerol 2-phosphate in the system are in accordance with a partially competitive model. Glucose 1-phosphate and UDP-glucose appear to affect only the high-affinity site for Eosin, whereas glucose and glycogen have no effect on Eosin-phosphorylase *b* complexes. Our results suggest that Eosin can be used as an efficient optical probe for studying the phosphorylase *b* system.

A number of fluorescein derivatives have been used in studying structure-function relationships of enzymes. Some of these studies include covalent modification of particular reactive groups (Heitz & Anderson, 1968; Dimitropoulos *et al.*, 1973; Stallcup & Koshland, 1973; Kalogerakos *et al.*, 1977) and others adsorption of dyes on specific or non-specific binding sites of the enzyme molecule (Wassarman & Lentz, 1971; Jacobsberg *et al.*, 1975; Yip & Rudolph, 1976; Somerville & Quiochio, 1977). Since fluorescein derivatives are very sensitive in probing relatively small changes of the protein structure, their spectroscopic properties have been used in studying ligand-phosphorylase *b* interactions (Oikonomakos *et al.*, 1977). Tetraiodofluorescein has been shown to be an 'adenosine analogue' and to bind effectively to several dehydrogenases and kinases (Somerville & Quiochio, 1977). On the other hand, with aspartate carbamoyltransferase it was found that at low tetraiodofluorescein concentrations the enzyme was activated by the dye to the same extent as that achieved by ATP (Jacobsberg *et al.*, 1973).

Abbreviation used: SDS, sodium dodecyl sulphate.

The activity of glycogen phosphorylase *b* (1,4- α -glucan-orthophosphate α -glucosyltransferase, EC 2.4.1.1) is dependent on the allosteric activator AMP and different inhibitors (Fischer *et al.*, 1971). A number of different probes with characteristic fluorescence (Birkett *et al.*, 1970; Seery & Anderson, 1972) or paramagnetic properties (Birkett *et al.*, 1971; Campbell *et al.*, 1972; Griffiths *et al.*, 1976) have been used to detect ligand-induced conformational changes of the phosphorylase *b* molecule.

In the present work we have studied the interaction of Eosin (2',4',5',7'-tetrabromofluorescein) with glycogen phosphorylase *b*. The spectroscopic changes of the dye due to its displacement from the enzyme were used to characterize the binding sites for substrates and effectors.

Experimental

Phosphorylase *b* was isolated from rabbit skeletal muscles by the method of Fischer & Krebs (1962). 2-Mercaptoethanol was used instead of L-cysteine in all steps of the isolation procedure. The enzyme was recrystallized at least four times and AMP was

removed by passage through a column (2.0cm×38cm) of Sephadex G-25. The protein concentration of phosphorylase *b* was measured spectroscopically by using a specific absorption coefficient, $A_{1\text{cm}}^{1\%}$, at 280nm of 13.2, and calculations of phosphorylase *b* molarity were based on a mol.wt. of 185000 (Buc *et al.*, 1971). In some instances the protein concentration was determined by the method of Lowry *et al.* (1951), with phosphorylase *b* as a reference standard. The purity of the enzyme was always checked by polyacrylamide-gel electrophoresis and analytical ultracentrifugation.

Sedimentation-velocity experiments were carried out in an MSE Centriscan 75 ultracentrifuge with a 10mm single-sector cell at a rotor speed of 60000 rev./min and a rotor temperature of 20°C. The sedimentation coefficients were obtained from direct measurements of the scanner traces, corrected for viscosity and density of the buffer to water at 20°C.

Assays of phosphorylase *b* activity were performed in the direction of glycogen synthesis (Illingworth & Cori, 1953). In enzyme kinetics experiments, the enzyme (27nm) was incubated at 30°C with various concentrations of AMP, glucose 1-phosphate and glycogen in the absence or presence of different Eosin concentrations in 40mm-glycerol 2-phosphate/30mm-2-mercaptoethanol/1mm-EDTA buffer, pH6.8 (final volume 0.2ml). P_i released in the reaction was measured by the method of Fiske & SubbaRow (1925). Phosphorylase *b* activity was expressed in μmol of P_i formed/min.

Commercial Eosin obtained from BDH Chemicals, Poole, Dorset, U.K. (C.I. 45380), was purified by column chromatography on activated alumina and talc by the procedure of Zwicker & Grossweiner (1963). Dye concentrations were determined spectroscopically at 516nm by using a molar absorption coefficient, ϵ , of 91500 litre·mol⁻¹·cm⁻¹ (Zwicker & Grossweiner, 1963). Oyster glycogen was purchased from BDH Chemicals and it was freed of AMP by dialysis against 0.8mm-sodium acetate buffer, pH4.0, containing Dowex 1 (acetate form) as described by Helmreich *et al.* (1967). Other materials were products of Sigma Chemical Co., St. Louis, MO, U.S.A. (AMP, ATP, glucose 1-phosphate and glycyglycine), BDH Chemicals (IMP, UDP-glucose, glucose 6-phosphate and glucose), E. Merck, Darmstadt, Germany (glycerol 2-phosphate, 2-mercaptoethanol, Tris and EDTA), and Pharmacia, Uppsala, Sweden (Sephadex G-25 fine grade). All other reagents were of the highest commercially available purity.

Absorption and difference spectra of the Eosin-phosphorylase *b* system were recorded in 10mm silica semimicro cells (1.5ml) with a Perkin-Elmer 356 double-beam u.v.-visible recording spectrophotometer.

Measurements of Eosin binding to phosphorylase *b* by the technique of gel filtration were carried out as described by Kastenschmidt *et al.* (1968). A number of Sephadex G-25 columns (0.6cm×40cm) were equilibrated with various Eosin concentrations in the absence or presence of ligands in the buffer used for spectroscopic titrations. Phosphorylase *b* (10–50 μmol) was passed through the column at 23°C and the protein peaks were collected. Bound Eosin was determined by absorbance measurements (at 516nm) of the protein peaks in the presence of 1% SDS after subtraction of the free concentration of the dye. It has been found that SDS at a concentration of about 1% completely displaces Eosin from the enzyme, and that SDS at all concentrations does not affect the absorbance of free Eosin (Oikonomakos *et al.*, 1977).

Results

Binding of Eosin to rabbit muscle glycogen phosphorylase b

In the pH region 4.4–12.0 the absorption spectrum of Eosin in buffered aqueous solutions is constant; in this pH region the dye has an absorption maximum at 516nm (Zwicker & Grossweiner, 1963).

The binding of Eosin to phosphorylase *b* in 100mm-Tris/HCl/10mm-2-mercaptoethanol/1mm-EDTA buffer, pH7.0, is characterized by a bathochromic shift

Fig. 1. Changes in the absorption spectrum of Eosin during its interaction with phosphorylase *b*

The absorption spectrum of the dye was recorded in 100mm-Tris/HCl/10mm-2-mercaptoethanol/1mm-EDTA buffer, pH7.0, at 23°C in the absence (curve 1) and in the presence of 75 μM -phosphorylase *b* (curve 2). The concentration of the dye was 8.4 μM .

in the absorption maximum of the dye from 516 to 528 nm (Fig. 1). The same spectral shift was observed when the dye absorption spectrum was recorded in a mixture consisting of 95% of propan-1-ol and 5% of the above buffer. This result provides strong evidence that the microenvironment of Eosin-binding sites on the phosphorylase *b* molecule is of hydrophobic nature and of polarity that can be roughly equated with that of a solution composed of 95% propan-1-ol and 5% buffer.

Titration of phosphorylase *b* with Eosin were performed by additions of small volumes from a series of concentrated Eosin solutions to both a standard phosphorylase *b* solution in 100 mM-Tris/HCl/10 mM-2-mercaptoethanol/1 mM-EDTA buffer, pH 7.0 (sample cuvette), and to a reference cuvette containing the above buffer alone at 23°C. After thorough mixing of the solutions the difference spectra were then recorded. The spectra were taken 10 min after each Eosin addition. The dilution never exceeded 1% of the starting volume (1.0 ml). Interaction of phosphorylase *b* solutions with Eosin results in a practically instantaneous increase in the difference absorption spectrum of the dye.

Fig. 2. Spectroscopic titration of phosphorylase *b* with Eosin

Difference spectra of Eosin-phosphorylase *b* complexes versus free Eosin. The spectra were recorded 10 min after the addition of Eosin both to the enzyme (5.4 μM) in the sample cuvette and to the reference cuvette to reach the concentration indicated, in 100 mM-Tris/HCl/10 mM-2-mercaptoethanol/1 mM-EDTA buffer, pH 7.0, at 23°C.

The difference spectra of Eosin-phosphorylase *b* complexes versus Eosin show a difference absorption maximum at 537 nm (Fig. 2). A difference absorption minimum also appeared at 511 nm. The extent of Eosin-phosphorylase *b* conjugation was evaluated by following the changes in the difference absorption maximum at 537 nm.

The dissociation constants, *K*, for Eosin-phosphorylase *b* complexes were determined by an analysis of the titration data (difference absorbance measurements at 537 nm) by Scatchard plots by using the equation:

$$r/[Eosin] = (n-r)/K$$

(Klotz, 1974), where *r* is the mol of Eosin bound/mol of enzyme, *n* is the number of Eosin-binding sites on the enzyme molecule, and [Eosin] is the free dye concentration. For each dye concentration, *r* was determined from the relation $r = \Delta A_{537} / \Delta \epsilon \cdot [\text{enzyme}]$, where $\Delta \epsilon$ is the difference molar absorption coefficient at 537 nm for bound Eosin. This coefficient was estimated by measuring ΔA_{537} with small concentrations of Eosin (2–10 μM) at various concentrations of phosphorylase *b*. Extrapolation to infinite protein concentration by means of double-reciprocal plots supplied values that gave $\Delta \epsilon = 50\,000 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$.

Scatchard plots of Eosin binding to phosphorylase *b* are shown in Fig. 3. Curved Scatchard plots were obtained for any particular protein concentration used in the area of $\Delta A_{537} \leq 1$, indicating that the four sites (value obtained approximately by extrapolation) are non-equivalent. It is logical, in view of the dimeric structure of the protein, to assume that there are two pairs of sites. The real values for *K* were derived by taking the reciprocal values of the limiting slopes and treating them as proposed by Klotz & Hunston (1971). The dissociation constants at low ionic strength (*I* 0.013) in 50 mM-glycylglycine/5 mM-2-mercaptoethanol/1 mM-EDTA buffer, adjusted to pH 7.0 with NaOH, were 1.7 and 10.7 μM respectively for each class of sites (Fig. 3a). When the ionic strength of the buffer is increased, the affinity of the dye for phosphorylase *b* is decreased. The *K* values determined in 100 mM-Tris/HCl/10 mM-2-mercaptoethanol/1 mM-EDTA buffer, pH 7.0 (*I* 0.091), were 7.7 and 41.7 μM respectively (Fig. 3b). A noteworthy feature of the spectroscopic titrations of the enzyme with Eosin is that the number of classes of binding sites for the dye is independent of protein concentration, which possibly indicates that in the range of enzyme concentrations tested no changes in the quaternary structure of the enzyme can be detected (Hofer & Radda, 1974). Ultracentrifugation experiments performed with 10.8 μM-phosphorylase *b* in the absence or presence of 20 μM-, 40 μM- and 60 μM-Eosin in the above buffer demonstrated that the

Fig. 3. *Stoichiometry of Eosin binding to phosphorylase b*

(a) Binding of Eosin to phosphorylase *b* was studied in 50mM-glycylglycine/5mM-2-mercaptoethanol/1mM-EDTA buffer, pH 7.0. Various enzyme concentrations (0.81–43.24 μM) were titrated with different Eosin concentrations (5–75 μM), and the data from the difference absorption changes at 537 nm (insert) were arranged in the form of a Scatchard plot. Linear least-squares fits were used in the range of $r < 1.2$ and $r > 2.3$ to obtain, after extrapolation, the values of the limiting slopes. (b) Binding of Eosin to phosphorylase *b* was studied in 100mM-Tris/HCl/10mM-2-mercaptoethanol/1mM-EDTA buffer, pH 7.0. Various enzyme concentrations (1.78–21.62 μM) were titrated with different Eosin concentrations (3–64 μM). The data from the spectroscopic titrations (insert) were arranged in the form of a Scatchard plot. Linear least-squares fits were used in the range of $r < 0.7$ and $r > 1.7$. From the values of the limiting slopes in (a) and (b), the true values of K were then calculated by the procedure of Klotz & Hunston (1971).

sedimentation behaviour of the Eosin–phosphorylase *b* complexes corresponded to that of the native enzyme. A sedimentation coefficient of 8.6S was found in all cases, and this corresponds to the dimeric species (Merino *et al.*, 1976).

Inhibition of glycogen phosphorylase b by Eosin

Extensive kinetic experiments with phosphorylase *b* in the presence of Eosin demonstrated a substantial inhibitory effect of the dye on the enzyme. In the presence of Eosin phosphorylase *b* exhibits a de-

Fig. 4. Kinetics of phosphorylase *b* in the presence of Eosin with respect to AMP

(a) Lineweaver-Burk plots for AMP binding to phosphorylase *b* in the absence or presence of Eosin. Glucose 1-phosphate and glycogen concentrations were held constant at 16 mM and 0.88% respectively. AMP concentration was varied from 0.02 to 1 mM. The concentrations of Eosin were: $\circ$, none; $\blacktriangle$, 20 μM ; $\blacksquare$, 40 μM ; $\bullet$, 60 μM ; $\square$, 80 μM . Assay conditions were as described in the Experimental section. (b) Dixon plots of the reciprocal of the initial velocity versus Eosin concentration in the presence of various AMP concentrations. The concentrations of AMP were: $\square$, 0.035 mM; $\circ$, 0.05 mM; $\blacksquare$, 0.08 mM; $\blacktriangle$, 0.2 mM; $\bullet$, 1 mM.

Fig. 5. Kinetics of phosphorylase *b* in the presence of Eosin with respect to glucose 1-phosphate

(a) Lineweaver-Burk plots for glucose 1-phosphate binding to phosphorylase *b* in the absence and presence of Eosin. AMP and glycogen concentrations were held constant at 1 mM and 0.88% respectively. Glucose 1-phosphate concentration was varied from 4 to 32 mM. The concentrations of Eosin were: $\bullet$, none; $\circ$, 80 μM ; $\blacktriangle$, 120 μM ; $\blacksquare$, 160 μM . Other assay conditions were as described in the Experimental section. (b) Dixon plots of the reciprocal of the initial velocity versus Eosin concentration. The concentrations of glucose 1-phosphate were: $\square$, 4 mM; $\blacksquare$, 8 mM; $\circ$, 12 mM; $\blacktriangle$, 16 mM; $\bullet$, 32 mM.

creased affinity towards the activator AMP. Lineweaver-Burk plots presented in Fig. 4(a) showed the expected co-operative binding of AMP to phosphoryl-

ase *b* (Helmreich & Cori, 1964). When the data were arranged in the form of Hill plots (Fig. 6a) (Madsen & Schechosky, 1967), the Hill coefficient, *h*, with

respect to AMP binding was not significantly affected (Table 1). The K_m value for this nucleotide is dependent on the Eosin concentration (Table 1). The dependence of enzyme activity on Eosin concentration was further analysed by Dixon plots. Assuming a competitive inhibition, extrapolation of the tangent drawn to the Dixon curve obtained in the presence of 1 mM-AMP at low Eosin concentration

(Fig. 4b) yielded a K_i (with respect to AMP) of about $36 \mu\text{M}$ (Dixon & Webb, 1964). At high Eosin concentrations upward curvature is obtained in Dixon plots, suggesting more complex kinetics of the inhibition.

Lineweaver-Burk and Dixon plots illustrating the effect of Eosin on the activity of phosphorylase *b* when glucose 1-phosphate concentration was varied are shown in Fig. 5. The Hill coefficient and the K_m value for this substrate are dependent on the dye concentration (Fig. 6b and Table 1). There is a continual increase in the apparent K_m value and also in the homotropic co-operativity between substrate-binding sites. This result is reminiscent of that obtained by Madsen & Schechosky (1967) with glucose 6-phosphate or ATP. From the Dixon plot illustrated in the presence of 32 mM-glucose 1-phosphate (Fig. 5b), and, assuming Eosin to be a competitive inhibitor of the enzyme, a K_i of approx. $60 \mu\text{M}$ (with respect to glucose 1-phosphate) was estimated, as previously. At high Eosin concentrations phenomena analogous to those observed during enzyme activation by AMP in the presence of Eosin were observed.

Effects of substrates and effectors on Eosin binding to phosphorylase b

Titration of Eosin-phosphorylase *b* complexes with a ligand were performed by additions of small volumes from concentrated solutions of the ligand both to a standard Eosin-phosphorylase *b* complex solution in the usual buffer (sample cuvette) and to a reference cuvette containing Eosin at a concentration equal to that of the sample cuvette.

The influence of glucose 6-phosphate on the dye-enzyme complex is presented in Fig. 7(a). The difference spectra produced by the additions of glucose 6-phosphate were mirror images of those shown in Fig. 2, which indicates that the allosteric inhibitor displaces Eosin from the enzyme. Additional experiments were performed, where the positive difference spectra (above the base-line) of the systems were used. Fig. 7(b) shows the effect of glucose 6-phosphate on the enzyme at a standard concentration ($5.4 \mu\text{M}$) at various dye concentrations.

The equation for the binding of a single ligand at independent and identical sites on the enzyme is:

$$K_d = \frac{(\text{free ligand})(\text{free sites})}{(\text{occupied sites})} = \frac{L_f(1 - \bar{Y})}{\bar{Y}}$$

where $\bar{Y}$ is the fraction of sites for ligand (L) that are actually occupied, L_f is the free ligand concentration and K_d the dissociation constant of the ligand-enzyme complex. If ΔA , ΔA_0 and ΔA_L are the difference absorbancies at a given point in the spectro-

Fig. 6. Effect of Eosin on the binding of AMP and glucose 1-phosphate to phosphorylase *b*

(a) Hill plots with AMP concentration varied, showing the effects of increasing Eosin concentrations. The concentrations of Eosin were: $\circ$, none; Δ , $20 \mu\text{M}$; $\blacksquare$, $40 \mu\text{M}$; $\bullet$, $60 \mu\text{M}$; $\square$, $80 \mu\text{M}$. Maximum velocity of the enzyme with respect to AMP (V) was estimated from the tangent to the Lineweaver-Burk curve obtained in the absence of Eosin (Fig. 4a). (b) Hill plots for glucose 1-phosphate binding to phosphorylase *b* in the absence or presence of Eosin. The concentrations of Eosin were: $\bullet$, none; Δ , $40 \mu\text{M}$; $\square$, $60 \mu\text{M}$; $\circ$, $80 \mu\text{M}$; $\blacktriangle$, $120 \mu\text{M}$; $\blacksquare$, $160 \mu\text{M}$. V was estimated after extrapolation of the Lineweaver-Burk curve obtained in the absence of Eosin (Fig. 5a).

Table 1. *Effect of Eosin on the Hill coefficients and K_m values for AMP and glucose 1-phosphate binding to phosphorylase *b**
The kinetic data were arranged in the form of Hill plots illustrated in Fig. 6. The K_m values and the Hill coefficients, *h*, were calculated by the procedure of Madsen & Schechosky (1967).

Concn. of Eosin (μ M)	AMP		Glucose 1-phosphate	
	K_m (mM)	<i>h</i>	K_m (mM)	<i>h</i>
0	$0.065 \pm 0.010^*$	1.50	$7.9 \pm 0.6^\dagger$	1.00
20	$0.085 \pm 0.013^*$	1.50		
40	$0.126 \pm 0.004^*$	1.50	10.0	1.08
60	0.195	1.60	12.6	1.10
80	0.302	1.60	15.0	1.10
120			$22.4 \pm 0.9^\dagger$	1.40
160			$30.6 \pm 1.4^\dagger$	1.40

* Average of three determinations with average deviations.
† Average of four determinations with average deviations.

Fig. 7. *Effect of glucose 6-phosphate on the difference absorption spectra of Eosin-phosphorylase *b* complexes*
(a) Difference spectra of Eosin-phosphorylase *b* solution recorded 10 min after each addition of glucose 6-phosphate to a sample cuvette containing the solution of the complex versus the same solution of the complex (reference cuvette). The spectra were taken with 5.4 μ M-enzyme and 20 μ M-Eosin in the presence of glucose 6-phosphate at the final concentrations indicated (see the Figure) in 100 mM-Tris/HCl/10 mM-2-mercaptoethanol/1 mM-EDTA buffer, pH 7.0. (b) Spectroscopic titrations of Eosin-phosphorylase *b* complexes with glucose 6-phosphate. Eosin was present at concentrations of 5 μ M (A), 10 μ M (B), 20 μ M (C), 40 μ M (D) and 60 μ M (E), while enzyme concentration was held constant at 5.4 μ M in the above buffer. Difference spectra were recorded in the absence (top curves) and in the presence of 0.02 mM-, 0.04 mM-, 0.08 mM-, 0.18 mM-, 0.48 mM-, 0.87 mM-, 3.23 mM-, 7.10 mM- and 16.45 mM-glucose 6-phosphate for each dye-enzyme complex.

scopic titration, at zero ligand concentration and at saturation, and assuming that:

$$\bar{Y} = (\Delta A_0 - \Delta A) / (\Delta A_0 - \Delta A_L)$$

then:

$$L_t = L_i - E_t(\Delta A_0 - \Delta A) / (\Delta A_0 - \Delta A_L)$$

where L_t and E_t are the total ligand and enzyme concentrations respectively. Since the enzyme concentration utilized is very low compared with the ligand concentration, $L_t \approx L_i$. Substitution of the

terms in the original equation and rearrangement provides:

$$(\Delta A_0 - \Delta A) / L_i = (\Delta A_0 - \Delta A_L) / K_d - (\Delta A_0 - \Delta A) / K_d$$

which has the form of a Scatchard equation. Multiplying by the factor $100 / \Delta A_0$, the following equation is obtained:

$$\begin{aligned} (\Delta A_0 - \Delta A) 100 / \Delta A_0 L_i \\ = (\Delta A_0 - \Delta A_L) 100 / \Delta A_0 K_d - (\Delta A_0 - \Delta A) 100 / \Delta A_0 K_d \end{aligned}$$

Fig. 8. Effect of IMP on the difference absorption spectra of Eosin-phosphorylase *b* complexes

(a) In the spectroscopic titrations Eosin was present at concentrations of $5\text{ }\mu\text{M}$ (A), $10\text{ }\mu\text{M}$ (B), $20\text{ }\mu\text{M}$ (C), $40\text{ }\mu\text{M}$ (D) and $60\text{ }\mu\text{M}$ (E), while enzyme concentration was held constant at $5.4\text{ }\mu\text{M}$ in the usual buffer. Difference spectra were recorded in the absence (top curves) and in the presence of 0.4 mM -, 0.8 mM -, 1.4 mM -, 2.4 mM -, 3.9 mM -, 5.8 mM -, 9.5 mM - and 14.8 mM -IMP for each dye-enzyme complex. (b) The data from the above titrations were arranged in the form of Scatchard plots according to the equation derived in the text. The curves correspond to $5\text{ }\mu\text{M}$ - (A), $10\text{ }\mu\text{M}$ - (B), $20\text{ }\mu\text{M}$ - (C), $40\text{ }\mu\text{M}$ - (D) and $60\text{ }\mu\text{M}$ -Eosin (E). The values of the apparent dissociation constants obtained by the procedure of Klotz & Hunston (1971) treatment are shown in Table 2.

Fig. 9. Effect of AMP on the difference absorption spectra of Eosin-phosphorylase *b* complexes

In the spectroscopic titrations Eosin was present at concentrations of $5\text{ }\mu\text{M}$ (A), $10\text{ }\mu\text{M}$ (B), $20\text{ }\mu\text{M}$ (C), $40\text{ }\mu\text{M}$ (D) and $60\text{ }\mu\text{M}$ (E), while enzyme concentration was held constant at $5.4\text{ }\mu\text{M}$ in the Tris/HCl buffer. Difference spectra were recorded in the absence (top curves) and in the presence of 0.02 mM -, 0.04 mM -, 0.08 mM -, 0.18 mM -, 0.48 mM -, 2.1 mM -, 4.7 mM - and 8.7 mM -AMP for each dye-enzyme complex.

In this equation, used also by Klungsøyr (1974), r is represented by the percentage of the difference absorption change at 537 nm .

The apparent dissociation constants, K_d , calculated from the slopes of the Scatchard plots for a number of substrate- and effector-enzyme complexes (with respect to Eosin binding) are shown in Table 2. When the K_d for a given ligand had approximately the same value at all Eosin concentrations tested, the behaviour of this ligand was characterized as non-competitive.

Where linear and non-linear dependency of K_d against Eosin concentration was found, the behaviour of the ligand was characterized as competitive and partially competitive respectively.

The spectroscopic titrations of Eosin-phosphorylase *b* complexes by various ligands provided the following results.

(i) IMP completely displaced the dye from the enzyme (Fig. 8), indicating that IMP-binding sites are related to the Eosin-binding sites on the enzyme.

Since IMP-binding sites have been characterized by crystallographic studies (Kasvinsky *et al.*, 1978), it appears that Eosin-binding sites are related to the nucleotide- and nucleoside-binding sites of phosphorylase *b*. A competition of the dye displacement by IMP can be demonstrated at least for the nucleoside-binding site (Table 2).

(ii) AMP binding to the enzyme with respect to Eosin is characterized by two different modes: a non-competitive one with an apparent dissociation constant of about $90\mu\text{M}$, and a competitive one where AMP binding depends on Eosin concentration (Fig. 10 and Table 2). However, the AMP effect differs from that of IMP. Thus (Fig. 9) as Eosin concentration increases in the system the percentage of Eosin displacement from the enzyme decreases much more with AMP than with IMP.

(iii) The effects of glucose 6-phosphate, ATP, P_i and glycerol 2-phosphate on Eosin-enzyme complexes are in accordance with a partially competitive model (Table 2). None of these ligands completely abolished the dye-enzyme difference absorption spectrum. To see how these ligands affect Eosin binding to phosphorylase *b*, Scatchard plots were drawn (not shown), and these yielded approximately four sites for Eosin in the presence of various con-

centrations of ligands. From such linear Scatchard plots the calculated K values for Eosin-enzyme complex were found to be approx. 50 , 100 and $200\mu\text{M}$ with respect to glucose 6-phosphate (0.18 , 0.87 and 16.45mM respectively), $70\mu\text{M}$ with respect to ATP (5.4mM), $70\mu\text{M}$ with respect to P_i (30mM) and $60\mu\text{M}$ with respect to glycerol 2-phosphate (30mM). These values indicate that the previous ligands apparently decrease Eosin affinity for both classes of its binding sites.

(iv) Glycogen and glucose have no effect on Eosin-phosphorylase *b* complexes. Since glucose binds to the active site of the enzyme (Kasvinsky *et al.*, 1978), it appears that this site is not affected by Eosin.

(v) A non-competitive scheme was observed at all Eosin concentrations, when glucose 1-phosphate and UDP-glucose were added in the system. This finding suggests that the above ligands are not directly related to the binding of the dye on phosphorylase *b* (Table 2). However, these ligands affect Eosin binding to its high-affinity site, since in the presence of 30mM -glucose 1-phosphate and 8.0mM -UDP-glucose the dissociation constants for Eosin-enzyme complex were found to be approx. $30\mu\text{M}$ and $40\mu\text{M}$ respectively.

To test whether or not the observed spectroscopic

Fig. 10. AMP binding to phosphorylase *b* in the presence of Eosin

(a) Spectroscopic titrations of Eosin-phosphorylase *b* complexes were carried out as described in the legend to Fig. 9. The data were arranged in the form of Scatchard plots according to the equation derived in the text to calculate the K_d for AMP-enzyme complex. The experimental points correspond to 0.02mM -, 0.04mM -, 0.08mM -, 0.18mM -, 0.28mM -, 0.48mM -, 1.0mM - and 2.1mM -AMP. Titrations were carried out in the presence of $5\mu\text{M}$ - ($\bullet$), $10\mu\text{M}$ - ($\blacktriangle$), $20\mu\text{M}$ - ($\circ$), $40\mu\text{M}$ - ($\triangle$) and $60\mu\text{M}$ -Eosin ($\blacksquare$). (b) Scatchard plots drawn for higher concentrations of AMP. The points correspond to AMP concentrations of 2.1 , 4.7 , 8.7 and 20.0mM . The values of the slopes of the straight lines obtained in (a) and (b) were treated by the procedure of Klotz & Hunston (1971) to calculate the true values of K_d (shown in Table 2).

Table 2. *Apparent dissociation constants of ligand-phosphorylase b complexes*

The spectroscopic titrations of the Eosin-phosphorylase *b* complexes were performed essentially as described in the legend of Fig. 7(b). Enzyme concentration was kept constant in all titrations ($5.4\mu\text{M}$) in 100mM-Tris/HCl/10mM-2-mercaptoethanol/1 mM-EDTA buffer, pH 7.0 (23°C). The values of the dissociation constants, K_d , were estimated from the slopes of the Scatchard plots drawn according to the equation derived in the text and are given below. For AMP and IMP, where curved Scatchard plots were obtained, the true values of K_d were calculated after treatment of the Scatchard plots as proposed by Klotz & Hunston (1971).

Concn. of Eosin (μM)	K_d (mM)									
	AMP		IMP		Glucose 6-phosphate	P_i	Glycerol 2-phosphate	ATP	Glucose 1-phosphate	UDP-glucose
5	0.084	1.0		1.4	0.15	12.1	13.9	2.4	23.8	9.1
10	0.086	1.3	0.9		3.9	12.9	13.3	2.4	25.8	9.5
20	0.087	2.2	1.0		5.1	11.6	15.4	2.7	20.6	9.4
40	0.090	3.6	1.4		11.5	17.3	19.2	3.8	23.2	10.3
60	0.086	5.5	1.7		18.3	21.0	21.6	4.6	19.2	10.8

changes at 537 nm during ligand addition were really due to the Eosin dissociation from the enzyme, a series of gel-filtration experiments were performed (see the Experimental section). These experiments demonstrated that the observed decrease of ΔA_{537} was due to the actual displacement of Eosin from its complexes with the enzyme. On the other hand, all ligands tested have no effect on the absorption spectrum of Eosin alone.

Discussion

Eosin-phosphorylase *b* interaction is expressed with a red-shift in the spectrum of the dye (Fig. 1), as in the tetraiodofluorescein-aspartate carbamoyl-transferase interaction (Jacobsberg *et al.*, 1975). The observed spectral changes were similar to those recorded when Eosin was dissolved in solvents of low polarity. From fluorimetric titrations of phosphorylase *b* with 8-anilino-naphthalene-1-sulphonate Seery & Anderson (1972) have demonstrated the existence of at least two classes of sites for that dye with a dissociation constant of 50–70 μM for the high-affinity site at low ionic strength (I 0.03). Eosin-binding sites (two per subunit of phosphorylase *b*) have stronger affinities for the dye, as can be concluded from their dissociation constants (Fig. 3). Also, the high-affinity site for Eosin binds this dye about 10 times more strongly than it does 8-anilino-naphthalene-1-sulphonate.

From crystallographic studies it has been shown that IMP binds at two sites on phosphorylase *a* monomer (Kasvinsky *et al.*, 1978). These sites have been characterized as the nucleotide (or activator)-binding site and the nucleoside-binding site of the enzyme. IMP-binding sites of phosphorylase *b* were easily detected by the substantial spectroscopic changes of Eosin-enzyme complexes observed during the titration of the systems by IMP (Fig. 8). These

changes resulted from the decrease of Eosin binding to the enzyme, possibly because of direct displacement (nucleoside-binding site) or indirect displacement (nucleotide-binding site) of Eosin. At 10 μM -Eosin, two IMP-binding sites were found, with K_d values 0.9 and 3.9 mM. Previous studies have indicated K_m about 2.5 mM for IMP binding to phosphorylase *b* from kinetic experiments over a wide range of glucose 1-phosphate concentrations (Black & Wang, 1968). Also, from calorimetric studies Ho & Wang (1973) have found K_d 1.2 mM for this nucleotide. However, these studies do not discriminate between the two sites of IMP binding.

Phosphorylase *b* has been shown by several groups of workers to bind two molecules of AMP per dimer with K_d about 100 μM (Seery & Anderson, 1972; Brooks *et al.*, 1974; Griffiths *et al.*, 1976). Wang *et al.* (1970) also found that the enzyme binds additional molecules of AMP with K_d about 3 mM. The latter binding sites were also confirmed by equilibrium studies (Morange *et al.*, 1976). AMP-binding sites were demonstrated by the spectroscopic titrations of Eosin-enzyme complexes with AMP (Figs. 9 and 10 and Table 2). The observed spectroscopic changes suggest that AMP can displace Eosin competitively from its low-affinity site and non-competitively from its high-affinity site (Table 2). The differences in the behaviour between AMP and IMP (Figs. 8 and 9) might be explained by the suggestion of Black & Wang (1968) that AMP induces on phosphorylase *b* a different conformation from that due to IMP.

The allosteric inhibitors glucose 6-phosphate and ATP decrease enzyme affinity for Eosin in a partially competitive scheme (Table 2). These ligands appear to decrease Eosin binding to phosphorylase *b* at both classes of binding sites. Analogous effects were observed for P_i and glycerol 2-phosphate. Merino *et al.* (1976) suggested from calorimetric studies that glycerol 2-phosphate at 25°C drives the phosphorylase

b molecule to a conformation analogous to that induced by P_i . The spectroscopic changes observed during the addition of P_i and glycerol 2-phosphate on Eosin-enzyme complexes as well as the calculated K_d (Table 2) demonstrate the similarity of the structural changes of phosphorylase *b* induced by these two ligands.

Glycogen and glucose have no effect on Eosin-phosphorylase *b* complexes. This possibly indicates that these ligands bind at different binding sites on the enzyme molecule from those for Eosin. In fact, the glucose-binding site has been characterized (Kasvinsky *et al.*, 1978) to be the active site, which also binds the substrates glucose 1-phosphate and P_i and the inhibitor UDP-glucose. UDP-glucose behaves like the substrate, glucose 1-phosphate (Table 2), indicating that this inhibitor binds at the glucose 1-phosphate site on the enzyme, as proposed by Madsen & Schechosky (1967) from enzyme inhibition experiments. Since these ligands have been found to bind not only to the active site of the enzyme but also to the nucleotide-binding site (Sygusch *et al.*, 1977), the results obtained with Eosin might reflect the binding of these ligands to the nucleotide-binding region of the enzyme.

Kinetic experiments demonstrated that Eosin is an effective inhibitor of phosphorylase *b*. The dye appears to inhibit the enzyme in a competitive manner with respect to both AMP and glucose 1-phosphate (Figs. 4 and 5). A homotropic co-operativity was also revealed between substrate-binding sites in the presence of Eosin, suggesting that the dye induces the expression of homotropic co-operativity of the glucose 1-phosphate-binding sites in conditions when it does not normally occur. Kasvinsky *et al.* (1978) have demonstrated that binding of a ligand to the nucleoside-binding site inhibits phosphorylase activity via allosteric competition with glucose 1-phosphate. In our work we have shown that the low-affinity site for Eosin associated with the nucleoside-binding site of the enzyme. The possibility remains that the presence of Eosin affects AMP binding and that this influences glucose 1-phosphate binding and co-operativity (Black & Wang, 1968). The contradiction between spectroscopic and catalytic data (Figs. 5 and 6 and Table 2) might be due to the absence from the spectroscopic titrations of the constituents used in catalytic experiments. Preliminary experiments involving Eosin-enzyme complexes, AMP and glycogen showed that AMP can completely displace the dye from the enzyme. This behaviour of AMP in the presence of glycogen, which is analogous to that found for IMP, might explain these inconsistencies between kinetic and spectroscopic experiments.

In conclusion, the observed spectroscopic alterations during the interaction of Eosin with phosphorylase *b* revealed that this fluorescein derivative

can be used successfully to follow ligand-enzyme interaction in the visible-absorption region, where neither phosphorylase *b* nor its substrates or effectors absorb.

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