Ligand Binding and Self-Association of Phosphorylase b†

R. F. Steiner,* L. Greer, and R. Bhat

ABSTRACT: The mutual influence of ligand binding and self-association has been examined for phosphorylase b in the presence of a series of small ligands. The stepwise equilibrium constants describing the mutual dependence have been evaluated and discussed in terms of possible molecular mechanisms.

The group of proteins which undergo self-association under some conditions is now generally recognized to be very large. It is frequently found that the extent of self-association is dependent upon the reversible binding of a small molecule (Buc & Buc, 1968; Steiner et al., 1972). In order for the ligand to influence the association process, it is formally sufficient that its free energy of binding be different for the monomer and for the associated species. In particular cases it may either favor or inhibit self-association, depending upon the relative magnitudes of the free energies of binding for the two forms.

The mutual influence of ligand binding and self-association may arise in any of several ways. If the ligand combines competitively with a specific site involved in association, it may inhibit self-association or block it entirely. If the ligand is charged, its binding will modify the electrostatic free energy of interaction of two monomer units in contact and thereby affect the association process. If the monomer unit is capable of existing in two or more distinct conformations which differ in their tendency to self-associate, the ligand may combine preferentially with, and stabilize, one of these and in this way influence association. The mutual interaction of ligand binding and self-association is in all probability the prevalent case and independence of the two processes is exceptional.

The enzyme glycogen phosphorylase, which is of central importance in glycogenolysis, exists in the nonactivated form, phosphorylase b, as a dimer of two monomer units of molecular weight 92 500 (Fischer et al., 1971). After conversion by phosphorylation of a serine group to the active form, phosphorylase a, it acquires a strong tendency to self-associate to form the tetramer (Fischer et al., 1971). Phosphorylase b may also be activated noncovalently by the binding of AMP. Under these conditions tetramer formation may occur at low temperatures or in the presence of either of the substrates glucose 1-phosphate or inorganic phosphate, as well as in the presence of the bivalent cations Ca^{2+} or Mn^{2+} (Buc & Buc, 1968; Birkett et al., 1971).

It is the purpose of this paper to analyze quantitatively the linked processes of AMP binding and self-association for phosphorylase b in terms of a set of stepwise equilibrium constants which describe the equilibria between the various possible states of ligation and self-association. Secondary objectives are to obtain binding isotherms unperturbed by self-association and to suggest the relation these observations may have to the allosteric transformations of this enzyme.

Experimental Section

Materials. Glycogen phosphorylase b was isolated from frozen rabbit muscle (Pel-Freeze, Inc.) by the method of Fischer & Krebs (1962) and was recrystallized three times from 0.03 M cysteine (pH 7.0), 1 mM AMP, and 10 mM $Mg(OAc)_2$ at 0 °C. Solutions were prepared by dissolution of the crystals in 0.05 M Tris, 0.1 M KCl, 0.005 M β-mercaptoethanol, pH 8.6, and were freed from AMP by passage first through a 1 × 40 cm Sephadex G-25 column eluted with buffer and then through a 0.5 × 4 cm column of Sephadex G-25 plus activated charcoal. The 260:280-nm absorbance ratio of the solutions employed here was normally in the range 0.50–0.53.

Tris, glucose 1-phosphate, and unlabeled AMP were purchased from Sigma. Tritiated AMP was obtained from the New England Nuclear Corp. All other chemicals were reagent or analytical grade. Glass-redistilled water was used for the preparation of all solutions.

Methods. Determinations of molecular weight by light scattering were made using a Phoenix light-scattering photometer equipped with a hollow cell holder for the circulation of water from a constant-temperature bath. Rectangular 1 × 1 × 4 cm quartz cuvettes were used for the measurement of reduced intensities ($R_{90°}$) at 90° to the incident beam, from which apparent values of the weight-average molecular weight (M_{wa}) were computed. In a separate determination, the correction factor for molecular weight arising from dissymmetry of the scattering envelope was found to be negligible. Apparent weight-average molecular weights were accordingly computed from the relation

$$M_{wa} = KR_{90°}/c \tag{1}$$

where c is the weight concentration (g/mL) and K is given by

$$K = 2\pi^2 n^2 (dn/dc)^2 / N_0 \lambda^4 \tag{2}$$

when n is the solvent refractive index, dn/dc is the refractive index increment of the protein, N_0 is Avogadro's number, and λ is the wavelength. For the measurements reported here, the wavelength was that of the Hg blue line, 436 nm. A value of 0.195 (mL/g) was assumed for dn/dc.

The value of M_{wa} obtained from eq 1 is related to the true weight-average molecular weight M_w by

$$M_{wa} = M_w/(1 + 2BM_wc) \tag{3}$$

where B is the second virial coefficient and c is the weight concentration.

The weight-average degree of association, α_w, for a particular AMP level is given by eq 4, where M_{w1} is the monomer

$$\alpha_w = M_w/M_{w1} = \frac{M_{wa}(1 + 2BM_wc)}{M'_{wa}(1 + 2B'M_{w1}c')} \tag{4}$$

molecular weight and the quantities M'_{wa}, B', and c' correspond to the absence of AMP. The value of B' was positive and small in each case ($B' = 0.8 \times 10^{-8}$ L/g in buffer). In the presence of 0.1 M glucose, which blocks self-association, no change in B with AMP level was observed. In applying eq 4, the dif-

† From the Department of Chemistry, University of Maryland, Baltimore County, Catonsville, Maryland 21228. *Received August 17, 1978; revised manuscript received December 19, 1978.*

0006-2960/79/0418-1380$01.00/0

Scheme I

$$K_{11} = \frac{[PA]}{[P][A]}; \quad [PA] = K_{11}[P][A]$$

$$K_{12} = \frac{[PA_2]}{[P][A]^2}; \quad [PA_2] = K_{12}[P][A]^2$$

$$K_{21} = \frac{[P_2A]}{[P]^2[A]}; \quad [P_2A] = K_{21}[P]^2[A]$$

$$K_{22} = \frac{[P_2A_2]}{[P]^2[A]^2}; \quad [P_2A_2] = K_{22}[P]^2[A]^2$$

$$\vdots$$

$$K_{2j} = \frac{[P_2A_j]}{[P]^2[A]^j}; \quad [P_2A_j] = K_{2j}/[P]^2[A]^j$$

ference between B and B' was accordingly neglected.

Calibration of the light-scattering apparatus was done using a dilute Ludox suspension whose turbidity ($= {}^{16}/_{3}\pi R_{90°}$) was determined by transmission measurements in a Cary 14 spectrophotometer, using a 10-cm path length cuvette (Doty & Steiner, 1950).

Measurements of the binding of AMP were made by equilibrium dialysis, employing Spectrum dialysis tubing (Spectrum, Los Angeles) of controlled porosity. Tritium-labeled AMP was used: in two parallel series, equivalent amounts of AMP containing [^{3}H]AMP were placed inside or outside the sacs for a set of AMP levels, so that equilibrium was approached from either direction. At the conclusion of dialysis, radioactivities of the internal and of the external solutions were measured and averaged for each AMP level for the two series. The attainment of equilibrium, as judged by the equivalence of radioactivities within the sacs for equilibrium approached from either direction, required 3 h with agitation at 23 °C. Counting was done as described elsewhere (Steiner et al., 1976). The number of molecules of AMP which were bound per phosphorylase dimer was computed from the difference in radioactivities inside and outside the sacs, as described in an earlier publication (Steiner & Greer, 1977).

Data Analysis. The concentrations of the various possible species arising from the mutually dependent processes of the monomer ⇌ dimer self-association of a protein, P, and the binding of a ligand, A, are governed by a set of equilibrium constants of the form shown in Scheme I. It is also possible to define a set of stepwise equilibrium constants corresponding to each stage of the interaction as shown in Scheme II. The set of macroscopic equilibrium constants K_{ij} specifies the values of the various stepwise equilibrium constants since each of the latter may be represented by a ratio of two or more of the K_{ij}'s (eq 5).

$$k_{11\rightarrow12} = K_{12}/K_{11} \tag{5}$$

$$k_{11,11\rightarrow22} = K_{22}/K_{11}^2$$

$$k_{12,11\rightarrow23} = K_{23}/K_{12}K_{11}, \text{ etc.}$$

The molar concentration, m, of all protein species is given by eq 6, and the total molar concentration of all monomer

$$m = \sum K_{ij}[P]^i[A]^j \quad (K_{10} = 1) \tag{6}$$

units, irrespective of the state of liganding or self-association, is given by eq 7, where c is the weight concentration of protein

$$m_t = \sum iK_{ij}[P]^i[A]^j = c/M_{w1} \tag{7}$$

and M_{w1} is the monomer molecular weight. [P] is the concentration of free monomer which is neither self-associated nor complexed with ligand.

Scheme II

$$P + A \rightleftharpoons PA; \quad k_{10\rightarrow11} = \frac{[PA]}{[P][A]} = K_{11}$$

$$PA + A \rightleftharpoons PA_2; \quad k_{11\rightarrow12} = \frac{[PA_2]}{[PA][A]}$$

$$PA + P \rightleftharpoons P_2A; \quad k_{11\rightarrow21} = \frac{[P_2A]}{[PA][P]}$$

$$PA + PA \rightleftharpoons P_2A_2; \quad k_{11,11\rightarrow22} = \frac{[P_2A_2]}{[PA]^2}$$

$$PA_2 + PA \rightleftharpoons P_2A_3; \quad k_{12,11\rightarrow23} = \frac{[P_2A_3]}{[PA_2][PA]}$$

$$\vdots$$

$$PA_i + PA_j \rightleftharpoons P_2A_{i+j}; \quad k_{1i,1j\rightarrow2,i+j} = \frac{[P_2A_{i+j}]}{[PA_i][PA_j]}$$

The quantity $\bar{\gamma}$, which is equal to the average number of molecules of ligand bound per monomer unit, is given by

$$\bar{\gamma} = \frac{\sum_{i,j} jK_{ij}[P]^i[A]^j}{\sum_{i,j} K_{ij}[P]^i[A]^j} \tag{8}$$

It may be shown (see Appendix) that

$$\int \frac{\gamma}{[A]}\, d[A] \equiv \ln \lambda; \quad \lambda = m_t/[P] \tag{9}$$

where the integral on the left-hand side of eq 9 is evaluated at constant m. For a system of the present kind, where the unliganded monomer does not undergo self-association, $1/\lambda$ is equal to the fraction of all monomer species which are unliganded and not involved in self-association.

For a monomer ⇌ dimer self-association, the weight-average degree of association, α_w, is related to the weight fraction, χ, of monomer by

$$\alpha_w = M_w/M_{w1} = 2 - \chi \tag{10}$$

or

$$\chi = 2 - \alpha_w$$

The total molar concentration, m_{2t}, of monomer units which are incorporated into dimer species is given by

$$m_{2t} = \sum_{j>0} 2K_{2j}[A]^j[P]^2 = (1 - \chi)m_t = (\alpha_w - 1)m_t \tag{11}$$

We also have

$$m_{2t}/2[P]^2[A] = \sum_{j>0} K_{2j}[A]^{j-1} = K_{21} + K_{22}[A] + K_{23}[A]^2 + \cdots \tag{12}$$

A polynomial regression fit of $m_{2t}/2[P]^2[A]$ as a function of [A] may thus yield values of the set of equilibrium constants K_{2j}.

Also

$$\lambda = m_t/[P] = 1 + K_{11}[A] + K_{12}[A]^2 + \cdots + 2K_{21}[P][A] + 2K_{22}[P][A]^2 + \cdots = 1 + \sum K_{1j}[A]^j + 2\sum K_{2j}[P][A]^j = 1 + \sum K_{1j}[A]^j + m_{2t}/[P] \tag{13}$$

and

$$(\lambda - 1 - m_{2t}/[P])/[A] = (\lambda - 1 - 2\sum K_{2j}(m_t/\lambda) \times [A]^j)/[A] = \sum K_{1j}[A]^{j-1} = K_{11} + K_{12}[A] + \cdots \tag{14}$$

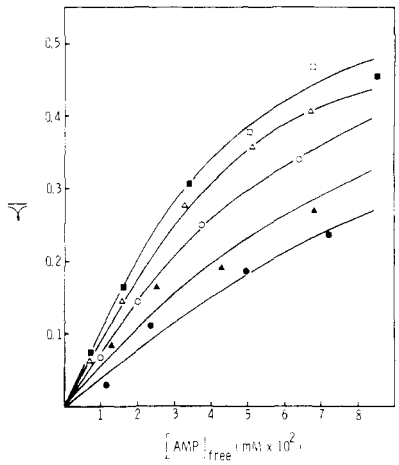

FIGURE 1: The binding of AMP by phosphorylase *b* (6.7 mg/mL) in 0.05 M Tris, 0.1 M KCl, 5 mM β-mercaptoethanol, pH 8.6, 23 °C as a function of Ca^{2+} level. The ordinate is the number of moles of AMP bound per phosphorylase dimer. Ca^{2+} concentration: 11 mM (■, □); 8 mM (△); 4 mM (○); 2 mM (▲); 0 (●).

A polynomial regression fit of the left-hand side of eq 14 as a function of [A] yields the set of equilibrium constants K_{1j}. These describe the binding of ligand by monomer and are unperturbed by the self-association process.

Polynomial regression fits of eq 12 and 14 were done using the polynomial regression program BMDP5R (Health Sciences Computing Facility, UCLA).

From the values of the sets of constants K_{1j} and K_{2j}, it is possible to determine the various stepwise equilibrium constants for ligand binding and self-association as described earlier (Scheme II).

In evaluating λ by the integration of eq 9, the molarity of all protein species ($= \sum K_{ij}[P]^i[A]^j$) should be held constant. The molarity, m, is given by

$$m = m_t\chi + 0.5m_t(1 - \chi) \qquad (15)$$

Evaluating the integral of eq 9 at constant m involves measuring $\bar{\gamma}$ as a function of m and interpolating to a constant value of m. In the present case m decreased by a maximum of 30%, because of self-association, over the range of AMP levels for which binding was measured. At constant [AMP] values, binding measurements in no case revealed any variation in $\bar{\gamma}$ over this range which was outside of experimental error. Accordingly, λ was obtained by integration at constant m_t.

Results

AMP Binding. The binding of AMP by phosphorylase *b* (6.7 mg/mL) in 0.05 M Tris, 0.1 M KCl, 5 mM β-mercaptoethanol, pH 8.6, 23 °C, is shown in Figure 1. These studies were carried out at pH 8.6 in order to avoid a significant degree of time-dependent aggregation, which complicated light-scattering measurements at lower pH. Under these conditions there was no indication of any dimer → tetramer self-association, the sedimentation velocity pattern in the presence of 1.0 mM AMP showing only a single peak with $s_{20,w} = 8.2 \times 10^{-13}$ with no indication of any more rapidly sedimenting material. A plot of $(\lambda - 1)/[AMP]$ vs. [AMP] was linear, with no significant degree of upward curvature (Figure 2). Application of the statistical F test indicated that higher terms in [AMP] were not significant and that binding is confined to only two sites per phosphorylase dimer for this range of AMP levels. The apparent equilibrium constants K_{11}, K_{12}, and $k_{11\to12}$ ($= K_{12}/K_{11}$) may be computed from the intercept and slope of the linear plot of $(\lambda - 1)/[AMP]$ vs. [AMP] and are cited in Table I. It has been demonstrated

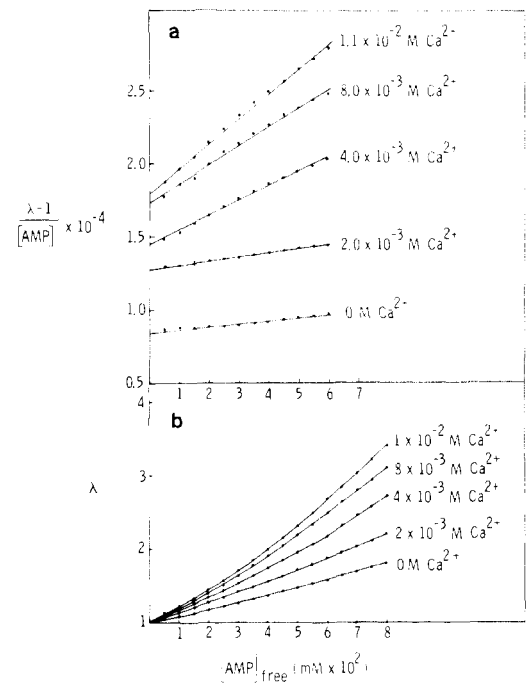

FIGURE 2: (a) Computed values of $(\lambda - 1)/[AMP]$ as a function of [AMP] for the data of Figure 1. (b) Computed values of λ as a function of [AMP] for the data of Figure 1.

Table I: Apparent Values of Binding Constants for AMP[a]

additive	$k_{11} \times 10^{-4}$	$k_{12} \times 10^{-8}$	$k_{11\to12} \times 10^{-4}$
none	0.83 ± 0.04	0.23 ± 0.01	0.28 ± 0.03
0.1 M glucose	0.64 ± 0.03	0.21 ± 0.01	0.33 ± 0.03
0.3 M KCl	0.53 ± 0.03	0.17 ± 0.01	0.32 ± 0.03
2.0 mM Ca^{2+}	1.30 ± 0.06	0.28 ± 0.01	0.22 ± 0.02
4.0 mM Ca^{2+}	1.50 ± 0.07	0.85 ± 0.05	0.57 ± 0.06
8.0 mM Ca^{2+}	1.80 ± 0.10	1.10 ± 0.05	0.61 ± 0.06
11.0 mM Ca^{2+}	2.00 ± 0.10	1.50 ± 0.07	0.75 ± 0.08
11.0 mM Ca^{2+} + 0.1 M glucose	0.77 ± 0.04	0.17 ± 0.01	0.22 ± 0.02
0.1 M glucose-1-P	2.70 ± 0.14	2.40 ± 0.10	0.89 ± 0.09
0.1 M glucose-1-P + 0.1 M glucose	0.36 ± 0.10	<0.1	
0.1 M PO_4^{3-}	1.50 ± 0.07	1.10 ± 0.05	0.73 ± 0.07
0.1 M PO_4^{3-} + 0.1 M glucose	<0.1		
0.1 M SO_4^{2-}	0.21 ± 0.01	0.31 ± 0.015	1.50 ± 0.15
0.1 M SO_4^{2-} + 0.1 M glucose	<0.1		

[a] Measurements were carried out in 0.05 M Tris, 0.1 M KCl, 5 mM β-mercaptoethanol, pH 8.6, 23 °C, plus the indicated additive. Glucose-1,-P, glucose 1-phosphate in this and the following tables.

by Ho & Wang (1973) and by Kasvinsky et al. (1978) that two classes of AMP binding site occur in phosphorylase. However, one of these is of low affinity and is occupied to a significant extent only at AMP levels greater than 1 mM (Ho & Wang, 1973). Binding to this low affinity site should thus not be measurable at the AMP levels employed here. Merino et al. (1976) have reported that phosphorylase *b*, under different conditions from those employed here, forms tetramers at concentrations above ~2 mg/mL in the presence of 10 mM AMP, but not at lower concentrations, in the absence of a second ligand. Under our conditions no tetramer formation was observed in the presence of AMP alone for AMP levels of 1 mM or less at any protein concentration.

A comparison of the magnitudes of K_{11} ($= k_{10\to11}$) and $k_{11\to12}$ (Table I) indicates that the value of the latter is elevated significantly above that of $k_{10\to11}/4$, which is the value pre-

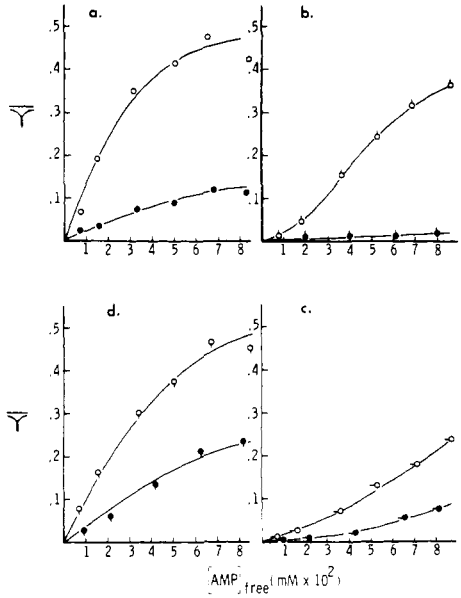

FIGURE 3: (a) The binding of AMP by phosphorylase *b* (5.3 mg/mL) in 0.05 M Tris, 0.1 M KCl, 5 mM β-mercaptoethanol, 0.1 M glucose 1-phosphate, pH 8.6, 23 °C in the absence (○) and presence (●) of 0.1 M glucose. (b) Same, except with 0.1 M PO_4^{3-} instead of 0.1 glucose 1-phosphate (◇, ◆). (c) Same, except with 0.1 M SO_4^{2-} instead of 0.1 glucose 1-phosphate (⊖,-●). (d) Same, except with 11 mM Ca^{2+} instead of 0.1 glucose 1-phosphate (♀, ♦).

dicted from purely statistical considerations if binding by the two sites were completely independent and noncooperative (Van Holde, 1970). The implication is that a significant degree of cooperativity is present.

The presence of 0.1 M glucose reduces the magnitude of the stepwise binding constants to a minor degree and has no significant effect upon the cooperativity (Table I).

The presence of increasing levels of Ca^{2+} at phosphorylase concentrations of 6.2 mg/mL results in a progressive elevation in the extent of binding of AMP and in the magnitudes of the apparent stepwise binding constants computed from the intercept and slope of a plot of $(\lambda - 1)/[AMP]$ vs. [AMP]. Moreover the ratio of $k_{11\rightarrow12}$ to $k_{10\rightarrow11}$ remains significantly elevated, indicating a persistance of some degree of positive cooperativity (Table I).

In the presence of 11 mM Ca^{2+}, the effect of 0.1 M glucose is substantially greater than in the absence of Ca^{2+} (Table I). The apparent values of both $k_{10\rightarrow11}$ and $k_{11\rightarrow12}$ are significantly increased, with no important alteration in their ratio.

In the presence of 0.1 M PO_4^{3-} or 0.1 M glucose 1-phosphate, the apparent values of both $k_{10\rightarrow11}$ and $k_{11\rightarrow12}$ are substantially increased, as is also, in the former case, the ratio of $k_{11\rightarrow12}$ to $k_{10\rightarrow11}$ (Figure 3 and Table I). In both cases the effect of 0.1 M glucose is particularly dramatic; for 0.1 M glucose 1-phosphate a major reduction in binding occurs, while in 0.1 M PO_4^{3-} binding is almost abolished (Figure 3).

The presence of 0.1 M SO_4^{2-} or 0.3 M KCl significantly reduces the magnitude of $k_{10\rightarrow11}$, while substantially elevating the ratio of $k_{11\rightarrow12}$ to $k_{10\rightarrow11}$, and hence the positive cooperativity (Figure 3 and Table I). The addition of 0.1 M glucose in the presence of 0.1 M SO_4^{2-} almost eliminates binding (Figure 3 and Table I).

Self-Association. In the presence of buffer alone, sedimentation velocity measurements indicated a single peak with $s_{20,w} = 8.2 \times 10^{-13}$, corresponding to the native phosphorylase dimer. The addition of AMP to a level of 1 mM does not alter either the shape of the sedimentation profile or the sedimentation coefficient. However, in the presence of 1 mM AMP, plus 0.01 M Ca^{2+}, 0.1 M PO_4^{3-} or 0.1 M glucose 1-phosphate, the enzyme (6 mg/mL) sedimented as a single component with $s_{20,w} = 13.6 \times 10^{-13}$, corresponding to the tetramer, in agreement with earlier studies (Birkett et al., 1971).

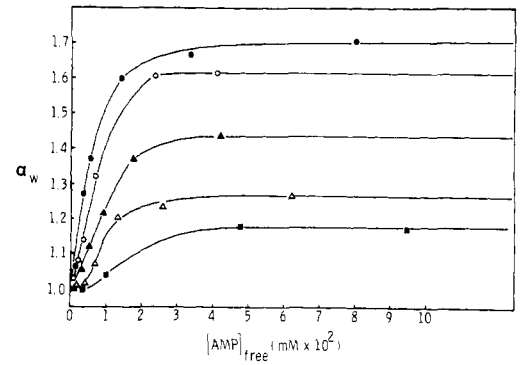

FIGURE 4: The variation of the apparent degree of association with the level of AMP for phosphorylase *b* (6.7 mg/mL) in 0.05 M Tris, 0.1 M KCl, 5 mM β-mercaptoethanol, pH 8.6, 23 °C, for a series of Ca^{2+} concentrations. The solid lines drawn for $[Ca^{2+}] = 4$, 8, and 11 mM are computed from eq 9-11, employing the values of $K_{21}...K_{23}$ which are cited in Table II. Ca^{2+} concentration: 11 mM (●); 8 mM (○); 4 mM (▲); 2 mM (△); 0 (■).

Table II: Values of Combined Equilibrium Constants for Binding and Self-Association[a]

additive	$K_{21} \times 10^{-8}$	$K_{22} \times 10^{-13}$	$K_{23} \times 10^{-17}$
4.0 mM Ca^{2+}	0.29 ± 0.02	0.17 ± 0.03	0.130 ± 0.012
8.0 mM Ca^{2+}	0.98 ± 0.08	0.31 ± 0.05	0.61 ± 0.07
11.0 mM Ca^{2+}	2.70 ± 0.06	0.45 ± 0.08	0.80 ± 0.03
0.1 M glucose-1-P	1.50 ± 0.15	2.40 ± 0.09	1.50 ± 0.12
0.1 M PO_4^{3-}	0.42 ± 0.01	0.200 ± 0.007	0.055 ± 0.008
0.1 M SO_4^{2-}	0.38 ± 0.02	0.015 ± 0.004	
0.3 M KCl	0.0018 ± 0.0001	0.062 ± 0.015	0.086 ± 0.042

[a] Measurements were carried out in 0.05 M Tris, 0.1 M KCl, 5 mM β-mercaptoethanol, pH 8.6, 23 °C, plus the indicated additive.

Light-scattering studies of the dependence of molecular weight upon AMP concentration in the presence of varying levels of Ca^{2+} indicate a progressive increase with increasing Ca^{2+} level of the degree of self-association produced by a given concentration of AMP (Figure 4). At a concentration of Ca^{2+} of 0.01 M and AMP concentrations greater than 0.1 M, the value of α_w approaches 2, indicating that the enzyme exists largely as a tetramer.

From polynomial regression analysis of $(\alpha_w - 1)m_t/2[P]^2[A]$ as a function of [A], where [A] is the concentration of free AMP [AMP], according to eq 11 and 12, values of $K_{21}...K_{23}$ were computed (Table II). Since the precision of binding measurements decreased for free AMP levels greater than 0.2 mM, values of λ were computed for concentrations below this value. The polynomial regression fit was extended until the 95% confidence limit was reached, as indicated by application of the statistical *F* test (Graybill, 1961), or until termination of the series was compelled by the appearance of negative terms. In most cases three terms were adequate. The curves drawn in Figure 4 were computed from the relationship

$$\alpha_w = 1 + \frac{2}{m_t}\sum_{j=1}^{j=4} K_{2j}[AMP]^j\left(\frac{m_t}{\lambda}\right)^2 \qquad (16)$$

The values of K_{2j} listed in Table II were substituted in this equation. The standard errors in the values of K_{2j} are cited in Table II.

Table III: Binding Constants Corrected for the Effects of Self-Association[a]

additive	$k_{10\rightarrow11} \times 10^{-4}$	$K_{12} \times 10^{-8}$	$k_{11\rightarrow12} \times 10^{-4}$
4.0 mM Ca^{2+}	1.3 ± 0.2	0.41 ± 0.1	0.32 ± 0.1
8.0 mM Ca^{2+}	1.4 ± 0.2	0.77 ± 0.2	0.55 ± 0.1
11.0 mM Ca^{2+}	1.0 ± 0.2	1.30 ± 0.3	1.30 ± 0.6
0.1 M PO_4^{3-}	<0.1		
0.1 M glucose-1-P	1.7 ± 0.30	0.55 ± 0.1	0.32 ± 0.1
0.1 M SO_4^{2-}	<0.1		

[a] Measurements were carried out in 0.05 M Tris, 0.1 M KCl, 5 mM β-mercaptoethanol, pH 8.6, 23 °C, plus the indicated additive.

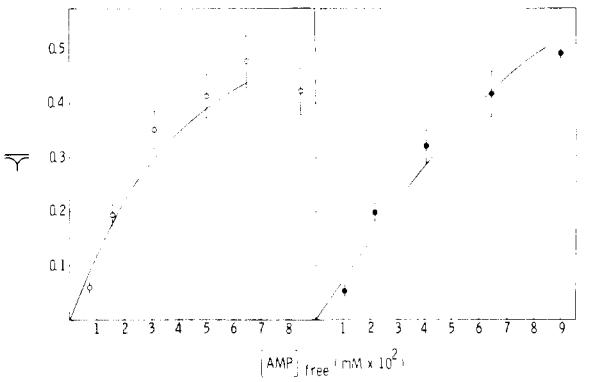

FIGURE 5: (Left) A comparison of computed binding isotherms and experimental values for phosphorylase *b* in 0.05 M Tris, 0.1 M KCl, 5 mM β-mercaptoethanol, pH 8.6, 23 °C, plus 0.1 M glucose 1-phosphate. The computed curves (see text) were calculated using the values of K_{11}, and K_{12}, and $K_{21}...K_{23}$ cited in Tables II and III. (Right) Same, except 11 mM Ca^{2+} instead of 0.1 M glucose 1-phosphate.

Corrected Binding Constants. Values of the equilibrium constants K_{11} (or $k_{10\rightarrow11}$), K_{12}, and $k_{11\rightarrow12}$, which describe the binding of AMP by the phosphorylase dimer, may be computed, using eq 14, from linear least-squares fits of $(\lambda - 1 - 2\sum K_{2j}(m_t/\lambda)[AMP]^j)/[AMP]$ vs. [AMP] (Table III). These values are unperturbed by the effects of self-association and correspond to the binding of AMP by the phosphorylase dimer for the given conditions. The calculations were performed in each case for binding data at two concentrations of phosphorylase differing by a factor of three and the results averaged. The uncertainties in these constants (Table III) are somewhat larger than for the K_{2j}'s, since the calculation involves the difference between two numbers of comparable magnitude. With the use of eq 14 and the values of the equilibrium constants cited in Tables II and III, it is possible to compute values of $\bar{\nu}$ as a function of [AMP]. Figure 5 compares computed curves with the experimental points.

A comparison of the corrected and apparent equilibrium constants indicates that the corrected values are in general significantly smaller. The corrected values also show a somewhat diminished dependence upon Ca^{2+} level. However, the degree of cooperativity, as reflected by the ratio of $k_{11\rightarrow12}$ to $k_{10\rightarrow11}$, is not in general greatly altered by correction.

Computation of $k_{10\rightarrow11}$, K_{12}, and $k_{11\rightarrow12}$ by the above procedure for 0.1 M PO_4^{3-} leads to values too small for accurate measurement. The implication is that this elevated level of phosphate effectively blocks the binding of AMP by the phosphorylase dimer and that the observed binding under these conditions is largely correlated with the linked formation of tetramer. This result is qualitatively in harmony with the finding that AMP binding is almost nil in the presence of 0.1 M glucose, where self-association does not occur (Figure 3), and with the conclusion that inorganic phosphate binds at the nucleotide site (Kasvinsky et al., 1978).

Table IV: Stepwise Association Constants[a]

medium	$k_{10,11\rightarrow21} \times 10^{-4}$	$k_{11,11\rightarrow22} \times 10^{-4}$	$k_{10,12\rightarrow22} \times 10^{-4}$	$k_{11,12\rightarrow23} \times 10^{-4}$
4 mM Ca^{2+}	0.22 ± 0.04	1.0 ± 0.3	4.1 ± 1.5	2.5 ± 1.0
8 mM Ca^{2+}	0.72 ± 0.15	1.7 ± 0.3	4.1 ± 1.0	5.8 ± 1.0
11 mM Ca^{2+}	2.70 ± 1.00	4.4 ± 2.0	3.6 ± 1.0	6.5 ± 2.5
0.1 M glucose-1-P	0.89 ± 0.20	7.9 ± 2.0	42.9 ± 9.0	16.1 ± 4.0

[a] Measurements were made in 0.05 M Tris, 0.1 M KCl, 5 mM β-mercaptoethanol, pH 8.6, 23 °C, plus the indicated additive.

Table V: Stepwise Binding Constants for the Binding of AMP by the Tetramer

medium	$k_{21\rightarrow22} \times 10^{-4}$	$k_{22\rightarrow23} \times 10^{-4}$
4 mM Ca^{2+}	5.9 ± 1.0	0.79 ± 0.20
8 mM Ca^{2+}	3.2 ± 0.7	1.90 ± 0.30
11 mM Ca^{2+}	1.7 ± 0.2	1.80 ± 0.30
0.1 M glucose-1-P	15.3 ± 3.0	0.65 ± 0.12
0.1 M PO_4^{3-}	4.7 ± 0.5	0.28 ± 0.10
0.3 M KCl	33.8 ± 2.0	1.40 ± 0.70

A similar result is obtained for the case of 0.1 M SO_4^{2-}. When analyzed without regard for self-association, the apparent binding constants are of significant magnitude and show a high degree of cooperativity. The corrected values of $k_{10\rightarrow11}$, K_{12}, and $k_{11\rightarrow12}$ are too small for accurate determination.

This behavior in 0.1 M glucose 1-phosphate is somewhat different. In this case the corrected values of $k_{10\rightarrow11}$, K_{12}, and $k_{11\rightarrow12}$ remain significant and are substantially larger than the values observed in 0.1 M glucose. In this case glucose appears to have an additional effect beyond the blocking of self-association.

Dependence of Tetramer Formation upon AMP Binding. From the sets of equilibrium constants K_{1j} and K_{2j}, it is possible to compute from eq 5 the stepwise equilibrium constants corresponding to the dimer → tetramer reaction for each of the possible states of ligation. In this manner $k_{11,10\rightarrow21}$ (= K_{21}/K_{11}), $k_{11,11\rightarrow22}$ (= K_{22}/K_{11}^2), $k_{12,10\rightarrow22}$ (= K_{22}/K_{12}), and $k_{12,11\rightarrow23}$ (= $K_{23}/K_{12}K_{11}$) were computed and are cited in Table IV.

There are several aspects of these equilibrium constants which deserve comment. The values of $k_{11,10\rightarrow21}$ are of significant magnitude in each case, suggesting that the binding of a single molecule of AMP per tetramer unit suffices to stabilize the tetramer. The values of $k_{11,11\rightarrow22}$ are invariably significantly larger than those of $k_{11,10\rightarrow21}$, suggesting that two singly liganded dimers have a higher mutual affinity than a singly liganded and an unliganded dimer.

The values of $k_{12,10\rightarrow22}$ are also consistently higher than those of $k_{11,10\rightarrow21}$, although, in the case of 11 mM Ca^{2+}, the difference is within experimental uncertainty. In the case of 0.1 M glucose 1-phosphate, the difference is exceptionally large, amounting to over an order or magnitude. This suggests that the binding of a second molecule of AMP by a dimer results in a further enhancement of its ability to associate with unliganded dimer. Similarly, the values of $k_{11,12\rightarrow23}$ are consistently higher than those of $k_{11,11\rightarrow22}$, suggesting that the binding of a second molecule of AMP by a dimer also enhances its tendency to associate with a singly liganded dimer. However, the relatively large experimental uncertainty in the values of $k_{11,12\rightarrow23}$ renders this result somewhat tentative.

Dependence of Binding Affinity upon State of Association. Table V cites values of $k_{21\rightarrow22}$ and $k_{22\rightarrow23}$, which correspond to the stepwise constants for the combination of tetramer with a second and a third molecule of AMP, respectively. (The value of $k_{20\rightarrow21}$ cannot be obtained, since the unliganded

tetramer does not occur under our conditions.) The magnitudes of both quantities are greatly elevated over the corresponding quantities for the dimer and presumably reflect the progressive stabilization of the tetrameric structure by the binding of successive molecules of AMP.

Discussion

It is clear from the preceding that the presence of a ligand-dependent self-association process can influence considerably the form of the binding isotherm for the ligand and that, in order to obtain binding constants which are characteristic of the monomer and unperturbed by the association process, it is necessary to correct for the effects of self-association. This may be done either by elaborate computational procedures (Colosimo et al., 1974, 1976) or by a method of the type described here, which has been shown to yield self-consistent results for the present system.

Qualitatively, the present results confirm earlier findings that the binding of AMP in the presence of excess Ca^{2+}, SO_4^{2-}, PO_4^{3-}, or glucose 1-phosphate induces a dimer → tetramer association of phosphorylase *b* and that glucose stabilizes the dimeric form in each case. The mechanism of how glucose blocks tetramer formation remains uncertain. The alternative explanations include a conformational change induced by glucose binding or the blockage by glucose occupancy of a site involved in the mutual contact of two phosphorylase dimers.

It is of interest that the presence of sufficiently high levels of PO_4^{3-} or SO_4^{2-} largely blocks AMP binding by the phosphorylase dimer and that most of the binding is associated with tetramer formation. One explanation is that, at high concentrations of either anion, competitive binding may occur at the AMP site but that the additional free energy decrease associated with tetramer formation by the conformation induced by AMP binding compensates for this. Buc & Buc (1968) have also observed that, at high levels of PO_4^{3-}, AMP binding is greatly reduced when self-association is blocked by the presence of glucose.

It is also of interest that the binding of a second AMP molecule by a dimer appears to enhance its tendency to combine with either unliganded or singly liganded dimer. This result is somewhat counter to the strict symmetry model for the interaction of phosphorylase with AMP (Monod et al., 1965), which predicts that the binding of a single AMP by a dimer brings both subunits to the same conformation.

Appendix

The number of moles of ligand bound per monomer unit is given by

$$\bar{\gamma} = \frac{\sum_{ij} jK_{ij}[P]^i[A]^j}{\sum_{ij} iK_{ij}[P]^i[A]^j} \quad (A-1)$$

Also

$$m = \sum_{ij} K_{ij}[P]^i[A]^j \quad (A-2)$$

If m = constant, then

$$dm = 0 = \sum_{ij} jK_{ij}[P]^i[A]^{j-1}d[A] + \sum_{ij} iK_{ij}[P]^{i-1}[A]^j d[P] \quad (A-3)$$

And

$$\sum_{ij} iK_{ij}[P]^{i-1}[A]^j d[P] = -\sum_{ij} jK_{ij}[P]^i[A]^{j-1}d[A] \quad (A-4)$$

We also have, for constant m

$$\int \frac{\bar{\gamma}}{[A]}\, d[A] = \int \frac{\sum_{ij} jK_{ij}[P]^i[A]^{j-1}d[A]}{\sum_{ij} iK_{ij}[P]^i[A]^j} = -\int \frac{\sum_{ij} iK_{ij}[P]^{i-1}[A]^j d[P]}{\sum_{ij} iK_{ij}[P]^i[A]^j} = -\int \frac{d[P]}{[P]} = -\int d \ln [P] \quad (A-5)$$

Finally, if unliganded monomer does not self-associate

$$\int_0^{[A]} \frac{\bar{\gamma}}{[A]}\, d[A] = \ln \frac{\{[P]\}_{[A]=0}}{[P]} = \ln m_t/[P] = \ln \lambda \quad (A-6)$$

where

$$\lambda = 1 + \sum iK_{ij}[P]^{i-1}[A]^j$$

and m_t refers to the value in the absence of added ligand.

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