AMP and IMP Binding to Glycogen Phosphorylase b
A CALORIMETRIC AND EQUILIBRIUM DIALYSIS STUDY*

Pedro L. Mateo, Carmen Barton$, Obdulio Lopez-Mayorga, Juan S. Jimenez, and Manuel Cortijo
From the Physical Chemistry Department, Faculty of Sciences, Granada University, Granada, Spain

Reaction microcalorimetry and equilibrium dialysis have been used to study the binding of AMP and IMP to glycogen phosphorylase b (EC 2.4.1.1) at 25 °C and pH 6.9. The combination of both techniques has enabled us to obtain some of the thermodynamic parameters for these binding processes.

Four binding sites were found to be present in the dimeric active enzyme for both AMP and IMP. The binding to two high-affinity sites, which, in our opinion, correspond to the activator sites, seems to be cooperative. The two low-affinity sites, which would then correspond to the inhibitor sites, appear to be independent when the nucleotides bind to the enzyme. The negative ∆Go of binding/site at 25 °C is the result in all cases of a balance between negative enthalpy and entropy changes. The large differences in ∆H and ∆S for the binding of AMP to the activator sites (−27 and −70 kJ mol−1; −32 and −150 J K−1 mol−1) suggest the existence of rather extensive conformational changes taking place in phosphorylase b on binding with the allosteric activator. Whereas the affinity of AMP for the activator sites is about 1 order of magnitude higher than that of IMP, the affinity of both nucleotides, including their ∆H and ∆S values, seems to be the same for the inhibitor sites.

Glycogen phosphorylase b (EC 2.4.1.1) is a key enzyme in the glycogen metabolism which undergoes a distinctive allosteric activation by AMP. Initial studies (1, 2) showed the existence of one AMP site/enzyme protomer. Recent evidence for two AMP-binding sites/monomeric unit of the enzyme has, nevertheless, been provided at 4 °C by equilibrium dialysis (3) and x-ray diffraction studies (4). Support for the presence of these two sites at 25 °C was also supplied by the biphasic thermal titration curves of the enzyme with AMP (5−8). We have shown, however, in a recent communication (9) that these biphasic calorimetric profiles were due, in our case, to the presence of an impurity, AMP aminohydrolase (EC 3.5.4.6), and that monophasic thermal curves are obtained when phosphorylase b is freed from this impurity (9, 10). This result cast doubts on previous experimental evidence for a second AMP site/monomer of phosphorylase b in solution at 25 °C.

In order to perform quantitative thermodynamic analysis of ligand binding to multisubunit proteins displaying site cooperativity, additional equilibrium techniques, besides calorimetry, are required. Even with several techniques, this energetic characterization can often become complicated due to the complexity of the process itself, and not much information of this kind is found in the literature (11−15).

In this context, we have undertaken the study of the binding of AMP, and also IMP, to phosphorylase b by equilibrium dialysis and microcalorimetry at 25 °C and pH 6.9. Under these conditions, four sites have been found per active dimer of the enzyme for both AMP and IMP, which have been assigned to the two nucleotide or activator sites, N, and to the two nucleoside or inhibitor sites, I (4, 16, 17). The binding is, in all cases, enthalpy-driven at 25 °C, overcoming a negative entropy barrier.

MATERIALS AND METHODS

Glycogen phosphorylase b was prepared from rabbit skeletal muscle by the method of Fisher et al. (16, 19) with the modifications described by Krebs et al. (20). The catalytic activity of the enzyme was determined by the assay of Hedrick and Fisher (21). The preparations used had specific activities of 80−90 units/mg. Protein concentration was determined from absorbance measurements at 280 nm using the absorbance coefficient E1%280 = 13,220 (22). The molecular weight of the monomer was taken as 87,400 (23). The enzyme was crystallized at least three times and used within 1 week of the final crystallization.

Phosphorylase b preparations were freed from AMP by passing them through a Sephadex G-25 column equilibrated with 50 mM KCl, pH 7.0, 50 mM EDTA, 0.1 mM β-mercaptoethanol, 0.1 mM EDTA, 50 mM buffer solution (glycylglycine, glycerophosphate, or Tris), adjusted to pH 6.9. The A280/ε ratio for the AMP-free phosphorylase b solutions was always less than 0.53. Traces of AMP aminohydrolase were eliminated by incubation with alumina C₇, as has been described elsewhere (10). [5−14C]AMP and [5−14C]IMP were obtained from the Radiochemical Center, Amersham, England. AMP, IMP, glycylglycine, alumina C₇, Tris, and β-mercaptoethanol were purchased from Sigma; sodium glycophosphate was from Merck, and EDTA was from Fluka. All chemicals used were of the highest available purity. Distilled, deionized water was used throughout.

An LKB flow microcalorimeter with a water bath at 25 °C was used for the calorimetric measurements. The temperature in the water bath was controlled by a proportional heater with adjustable precision based on a combination threshold detector and zero-crossing trigger (24). The control of the bath temperature was better than 0.01 °C. Electrical and chemical calibrations were made in the same range as that which we obtained in the calorimetric experiments themselves. The chemical calibration was accomplished by the neutralization of Tris with HCl (25). Enzyme and AMP (or IMP) solutions were allowed to flow into the calorimeter at rates of 7 ml h⁻¹ in most experiments, with occasional changes in order to check the completeness of the reaction. The dilution gradient method of Mountcastle et al. (26) was also used in the phosphorylase b/IMP calorimetric titration (see Fig. 5). All appropriate corrections for heats of dilution and mixing were applied. The enzyme activity was routinely checked prior to and after the calorimetric and dialysis experiments. The pH values of the buffer, AMP, IMP, and enzyme solutions were controlled at 25 °C before initiating the binding reaction. The equilibrium dialysis experiments were carried out at 25 °C as described by Helmreich et al. (27).

*This research was supported by a grant from the Comision Asesora from the Spanish government. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡Supported by a fellowship from the Formación de Personal Investigador.
RESULTS

Binding of AMP to Phosphorylase b—The binding of AMP to phosphorylase b was observed as a function of the activator concentration by equilibrium dialysis at 25°C and pH 6.9 (Fig. 1). The results of this binding were presented in a Scatchard plot in Fig. 2, where $Y$ stands for the saturation fraction and $v = nY$ for $n$ (number of sites) equal to 4. The shape of the curve which shows a maximum is of the form expected for a system exhibiting positive cooperativity. Extrapolation of the lower part of the plot leads to a value clearly higher than 2 mol of AMP bound per mol of enzyme at saturation. Similar results were also obtained at 4°C by Johnson et al. (4) in x-ray diffraction studies using an AMP concentration (100 mM).

The shape of the Scatchard plot suggests that AMP starts to bind to the lower-affinity sites when the high-affinity sites are practically saturated. Hence, most of the experimental points at low AMP concentration in Fig. 1 would mainly correspond to the binding of AMP to the high-affinity sites, these being for the most part responsible for the cooperativity shown in the Scatchard plot. Assuming then that the values with $v < 2$ correspond only to the binding to the two high-affinity sites, a straight Hill plot is obtained for these values with a Hill coefficient of 1.4 ± 0.1, which agrees well with those obtained by other authors (28–31) for these sites at similar AMP concentrations.

The reaction of AMP with phosphorylase b can be considered as the binding of AMP to two independent sets of sites. The high-affinity sites show positive cooperativity according to their Hill coefficient, while the low-affinity sites can themselves be considered as independent of each other, an assumption that is justified below. On this basis, the saturation fraction, $Y$, as a function of the free AMP concentration is

$$Y = \frac{1}{2} \frac{K_m[\text{AMP}] + K_n[\text{AMP}]^2}{1 + 2K_m[\text{AMP}] + K_n[\text{AMP}]^2} + \frac{1}{2} \frac{K_m[\text{AMP}]}{1 + K_m[\text{AMP}]}$$

where $K_m$ stands for the microscopic binding constants at the $i$th site, and $K_n = K_m$. The values calculated from this plot, $K_m$, and the values given by Equation 2 (first term in Equation 1), from the experimental points. The resulting difference represents the saturation data for the second class of sites. A Hill plot of these new values gives a straight line with a Hill coefficient equal to 0.97 ± 0.15, which can be taken as evidence of the non-cooperativity of the low-affinity sites.

The results of the calorimetric titration of phosphorylase b with AMP in three different buffer solutions at 25°C and pH 6.9 are shown in Fig. 3. The binding of AMP was exothermic in all cases, giving rise to a well-defined monophasic curve. This curve is the same regardless of the buffer system used, and since the heats of ionization of the three buffers are different (34), no proton uptake or release seems to occur in the activator binding to the enzyme, particularly to the high-affinity sites.

The results of the calorimetric titration of phosphorylase b with AMP at pH 6.9 and 25°C. The heat released per monomer of enzyme is plotted as a function of the free nucleotide concentration. Enzyme concentration was 5 mg/mL. Titration was in 50 mM glycylglycine (O), 50 mM glycophosphate (C), and 50 mM Tris (D). All solutions contained 50 mM KCl, 0.1 mM EDTA, 0.1 mM β-mercaptoethanol. The curve corresponds to Equation 5 using the values shown in Table I.

$$n_m = \frac{2}{1 + (K_m/K_n)^n} \quad \text{and} \quad S_{50} = \frac{1}{(K_m + K_n)^n}$$

The values of $K_m$ can be obtained by subtracting the saturation curve corresponding to the two high-affinity sites, using the $K_m$ and $K_n$ values given by Equation 2 (first term in Equation 1), from the theoretical points. The resulting difference represents the saturation data for the second class of sites. A Hill plot of these new values gives a straight line with a Hill coefficient equal to 0.97 ± 0.15, which can be taken as evidence of the non-cooperativity of the low-affinity sites. The binding constant calculated from this plot, $K_m$, and the $K_m$ and $K_n$ values obtained above were then used as input for the iterative Newton-Gauss method (see Ref. 33) to fit all the experimental data to the theoretical Equation 1. The optimum $K_m$, $K_n$, and $K_m$ values thus obtained are shown in Table I. Curves in Figs. 1 and 2 are the theoretical ones using the calculated binding constants (Table I).

The results of the calorimetric titration of phosphorylase b with AMP in three different buffer solutions at 25°C and pH 6.9 are shown in Fig. 3. The binding of AMP was exothermic in all cases, giving rise to a well-defined monophasic curve. This curve is the same regardless of the buffer system used, and since the heats of ionization of the three buffers are different (34), no proton uptake or release seems to occur in the activator binding to the enzyme, particularly to the high-affinity sites.
clearly extrapolates to tration are shown in Fig. previously obtained. The curve in Fig. to Equation 5 for the calculated rylase b at 25°C. The experimental results for describe similarly to that of AMP and, consequently, with the same equation for the saturation fraction as Equation 1. In this respect, the binding of IMP to the N sites has been reported by several authors (35-37) as cooperative, as is the case for the AMP binding to those sites. In addition, the binding of several ligands (nucleosides, nitrogen bases, FMN) to the I sites has been shown to be non-cooperative (3, 38), which, as we saw before, seems also to be the case for AMP.

Therefore, the nature of the IMP binding to phosphorylase b seems to be qualitatively comparable to that of AMP. In other words, the overall IMP binding process could be described similarly to that of AMP and, consequently, with the same equation for the saturation fraction as Equation 1. In order to fit the experimental data to this equation, an initial value of $K_m = K_{m1} = 600 \text{ M}^{-1}$ and different couples of $K_m$ and $K_n$ values which fulfilled the relation $(K_m, K_n)^n = K_m = 600 \text{ M}^{-1}$ were used. The optimum values obtained for $K_m$ and $K_n$, by a later application of the Newton-Gauss iterative method (see Ref. 33) are included in Table I.

The enthalpy change/mol of dimer would also be described by an equation such as Equation 5, and the corresponding enthalpies/site were obtained as described before for the AMP binding. The thermodynamic parameters for the binding of IMP to phosphorylase b at 25°C and pH 6.9 are also included in Table I.

The different shape of the Scatchard plot for the AMP binding compared to that of IMP could be explained by the
unique valid fit of experimental binding data when several different binding sites are involved. It is only through given assumptions and accepting certain restrictions that a given set of binding parameters can be arrived at. Furthermore, their physical meaning is not only limited by their standard uncertainties (particularly high in our case for the AMP binding to phosphorylase b) but also by the assumptions and, to some extent, the behavior that these parameters are forced to follow.

**DISCUSSION**

In addition to the x-ray studies of Johnson et al. (4) on crystals of phosphorylase b and those of dialysis by Morange et al. (3) and Buc et al. (48), the main evidence for a second AMP-binding site/monomer at 25 °C was the biphasic calorimetric profiles of Wang et al. (5), Ho and Wang (6), and Merino et al. (7, 8). The results of the latter groups showed clear divergences concerning both the AMP saturation range for the second site, I, and the \( \Delta H \) value for this site. We initially obtained biphasic curves for this thermal titration, but, as was shown, it was due to the presence of an enzymic impurity in our phosphorylase preparations, which, once eliminated (10), gave rise to monophasic calorimetric curves (9). It is not possible, however, to obtain information about the number of sites from calorimetric experiments alone when dealing with allosteric enzymes, as it is in the case of phosphorylase b. The combination of equilibrium dialysis and calorimetric studies enables us now to actually obtain the number of binding sites for AMP and IMP in solution at 25 °C, as well as to characterize the thermodynamic parameters associated on binding both nucleotides. The main difference between the binding of these two activators appears to be in the much closer affinity of IMP for the two types of sites, N and I, in the dimer than in the case of AMP.

Ho and Wang (6) gave an average \( \Delta H \) value, -13.2 kcal (mol site)\(^{-1}\), for AMP binding to the two N sites of the phosphorylase b dimer at 25 °C. Twice this value compares well with \( \Delta H_1 \) and \( \Delta H_2 \) obtained in our study for this site (Table I). These authors detected an association of the enzyme to the tetrameric state upon AMP binding at 18 °C, while no association seemed to occur at 25 °C (6). We did calorimetric experiments at different phosphorylase concentrations (Fig. 6), and there was no detectable change in the heat evolved for the concentration range investigated. Thus, no protein association effects seemed to occur in our experiments.

To our knowledge, only Steiner et al. (39) have tried to identify the contribution of each N site to the enthalpy change on the binding of AMP, although they did not investigate the other binding sites because they only used low AMP concentrations (<1 mM). While their values at 23 °C are somewhat different from ours, the total \( \Delta H \) for the N sites coincides with ours within experimental uncertainty. This difference also correlates with the comparison between our \( K_m \) and \( K_m \) values and theirs, in the sense that we obtain a higher homotropic cooperativity as indicated by the Hill coefficient (1.4 in our case, 1.2 in their case). This variation could be attributed to small differences in temperature, pH, and buffer used, although Dreyfus et al. (29) also obtained a Hill coefficient of 1.4 at 20 °C for this binding. An equal value of 1.4 had previously been obtained by Avramovic and Madsen (28) and other authors (30, 31).

The binding of AMP and IMP to the N sites shows positive
homotropic cooperativity. This effect is more pronounced in the AMP binding ($n_H = 1.4$) than that of IMP ($n_H = 1.2$). The difference in cooperativity is also seen in the $\Delta H$ values. Thus, in the case of AMP, the $\Delta H$ values are very different, $-27$ and $-70 \text{ kJ mol}^{-1}$, favoring the entrance of a second AMP molecule into the dimer. The corresponding $\Delta S^0$ values go in an opposite direction, balancing to some extent the enthalpic influence. The binding at $25 ^\circ C$ is clearly of an enthalpic character. The same qualitative situation applies to the IMP binding, although here the $\Delta H$ and $\Delta S^0$ values are comparatively lower than in the AMP binding. The differences in $\Delta H$ and $\Delta S^0$ values between the two N sites for both nucleotides make for a higher affinity and cooperativity in the binding of AMP than in IMP binding to those sites.

A structural interpretation of these thermodynamic parameters is not possible at this point since these values are the joint product of those of the so-called intrinsic binding (40) and those of the conformational change produced in the macromolecule, which is structurally responsible for the cooperative effect. We are currently working out a method to evaluate both contributions to the overall binding process (41).

The corresponding thermodynamic parameters for the binding of AMP and IMP to the I sites are very close for the two nucleotides within experimental uncertainty (Table I). This implies that the I site (nucleoside or inhibitor site) does not apparently discriminate between the two nucleotides. This site seems to be more specific for the inhibitors of the enzyme, such as adenosine or adenosine, although their binding to the I site is also non-cooperative (3,38). It has been suggested that any ligand bound to this site would produce some enzyme inhibition (17), and we have detected in this case a decrease in the enzymic activity at high AMP concentrations (results not shown). In this context, it has also been suggested that the low activation produced by IMP in comparison to that produced by AMP could be assigned to the close affinity of IMP for both the N (activator) and I (inhibitor) sites (17). Despite this possibility, however, we have seen that the thermodynamic parameters for binding AMP and IMP to the activator sites are different enough to expect different conformational changes and, therefore, different final conformations. The differences in these AMP- and IMP-induced conformational changes have been reported by several researchers using various techniques, such as ESR (42), fluorescence (43), and SH reactivity (3).

The binding of these nucleotides to the I site is enthalpy-driven at $25 ^\circ C$, overcoming the negative entropy barrier (Table I). It seems that the enthalpic contribution would mainly come from the interaction between Tyr-612 and/or Phe-285 with the nitrogen bases in the nucleotides (44). The $\Delta S^0$ values cannot be easily accounted for since the site structure is not well defined at present. The binding of FMN to this site in phosphorylase a shows a negative $\Delta C_p$ value which is itself a function of temperature (38). If it were also the case for our nucleotides, one would expect a change in the sign of $\Delta S^0$ at temperatures somewhat lower than $25 ^\circ C$. This FMN binding shows the same enthalpy value as AMP and IMP, $-37 \text{ kJ mol}^{-1}$, but a positive change in the entropy is responsible for the higher affinity of this compound for the I site at $25 ^\circ C$ (38).

Finally, the binding of AMP to the I site (which, it has been suggested, may play a possible role in vivo in controlling the enzyme activity (7)) does not seem to have much physiological role in muscle given the levels of free AMP in either resting muscle or under extreme fatigue (45,46) and the affinity of AMP for this site, although our affinity values were obtained in vitro, in the absence of any other physiological effectors and/or substrates.

REFERENCES

1. Fisher, E. H., Pocker, A., and Saari, J. C. (1970) in Essays in Biochemistry (Campbell, P. W, and Dickens, F., eds) Vol 6, pp. 23-68, Academic Press Inc., Ltd., London.
2. Graves, D. J., and Wang, J. H. (1972) in The Enzymes (Boyer, P. D., ed) 3rd Ed., Vol 7, pp. 435-482, Academic Press, New York.
3. Morange, M., Garcia-Blanco, F., Vandenbunder, B., and Buc, H. (1976) Eur. J. Biochem. 69,553-563.
4. Johnson, L. N., Sture, E. A., Wilson, K. S., Sansom, M. S. P., and Weber, I. T. (1979) J. Mol. Biol. 134, 639-652.
5. Wang, J. H., Kwok, S.-C., Wierch, E., and Suzuki, I. (1970) Biochem. Biophys. Res. Commun. 40,1340-1347.
6. Ho, H. C., and Wang, J. H. (1973) Biochemistry 12, 4790-4795.
7. Morino, C. G., Garcia-Blanco, F., and Laynez, J. (1977) FEBS Lett. 73,97-100.
8. Merino, C. G., Garcia-Blanco, F., Pocovini, M., Menendez, M., and Laynez, J. (1980) J. Biochem. (Tokyo) 87,1483-1490.
9. Cortijo, M., Baron, C., Jimenez, J. S., and Mateo, P. L. (1982) J. Biol. Chem. 257,1121-1124.
10. Baron, C., Mateo, P. L., Cortijo, M., and Jimenez, J. S. (1982) Anal. Biochem. 124,84-87.
11. Velick, S. F., Baggett, J. P., and Sturtevant, J. M. (1971) Biochemistry 10,779-786.
12. Alleva, N. M., Friedland, J., and Niekamp, K. (1975) Biochemistry 14, 224-230.
13. Niekamp, C. W., Sturtevant, J. M., and Velick, S. F. (1977) Biochemistry 16,436-445.
14. Aha, D. H., and Ackers, G. K. (1974) Biochemistry 13,2376-2382.
15. Gaud, H. T., Barisas, B. G., and Gill, S. J. (1974) Biochem. Biophys. Res. Commun. 59,1389-1394.
16. Fletterick, R. J., and Madsen, N. B. (1980) Annu. Rev. Biochem. 59,31-61.
17. Dombradi, V. (1981) Int. J. Biochem. 11,125-139.
18. Fisher, E. H., Krebs, G. E., and Kent, A. B. (1968) Biochem. Prep. 6,68-72.
19. Fisher, E. H., and Krebs, G. E. (1962) Methods Enzymol. 5,369-372.
20. Krebs, E. G., Love, D. S., Bratvoed G. E., Trayser, K. A., Meyer, W. L., and Fischer, E. H. (1964) Biochemistry 3,1022-1033.
21. Hedrick, L. J., and Fischer, E. H. (1965) Biochemistry 4,1337-1343.
22. Buc, M. H., and Buc, H. (1968) in FEBS Symposium: Regulation of Protein Structure and Function, Dombradi, V.
AMP and IMP binding to glycogen phosphorylase b. A calorimetric and equilibrium dialysis study.

P L Mateo, C Baron, O Lopez-Mayorga, J S Jimenez and M Cortijo

J. Biol. Chem. 1984, 259:9384-9389.

Access the most updated version of this article at http://www.jbc.org/content/259/15/9384

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/259/15/9384.full.html#ref-list-1