Calorimetric Titration of Phosphorylase b with AMP

ANOMALOUS THERMAL LIGAND-BINDING PROFILES INDUCED BY AN ENZYMIC IMPURITY*

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Calorimetric titrations of glycogen phosphorylase b (EC 2.4.1.1) from rabbit muscle with its allosteric activator, AMP, have been carried out at 25 °C and pH 6.9 in three different buffer systems (glycylglycine, glycerophosphate, and tris(hydroxymethyl)aminomethane). Calorimetric curves of biphasic nature were originally obtained. This biphasic behavior was always concomitant with: (a) a dependency of the saturation heat on the flow rate of the solutions entering the calorimetric unit; (b) an alteration in the spectrophotometric properties of AMP after being mixed with phosphorylase b. After some time the nucleotide displayed spectral properties resembling those of IMP; (c) an anomalous increase in the pH of the AMP-phosphorylase complex in the outflow from the calorimeter. These observations led to the conclusion that glycogen phosphorylase b seems to be contaminated by traces of AMP amino hydrolase (EC 3.5.4.6) which catalyzes the conversion of AMP into IMP with the liberation of ammonia. Calorimetric titrations of phosphorylase b freed from this impurity yield monophasic titration curves, which are practically identical for the three buffer solutions used, thus indicating that no proton uptake or release seems to take place on the AMP binding to phosphorylase b. The heat of the reaction at saturating concentration of AMP for these monophasic curves was totally independent of the calorimetric flow rates. Likewise, the phosphorylase preparations displaying monophasic titration curves did not show any change in pH or UV spectrum of AMP on adding this nucleotide. Hence, we concluded that the monophasic curve is the one precisely corresponding to the calorimetric titration of phosphorylase b with AMP. These results cast doubt on previous experimental evidence for a second AMP site per monomer of phosphorylase b at 25 °C.

The importance of purity in the study of the physical properties of molecules is self-evident. In the case of proteins, however, the criteria for purity must be vastly more rigorous if the contaminating materials themselves possess enzymic activity, which might modify any constituent of the system. We show here how the interpretation of thermodynamic ligand-binding data on a well-characterized protein can be rendered confusing by a contaminating enzyme, present at the level of a few parts/million. Such problems are a particular hazard in relation to techniques that require high protein concentrations for measurements of satisfactory precision.

Glycogen phosphorylase b (EC 2.4.1.1) has served as an archetype for allosteric enzymes because of its characteristic activation by AMP. Equilibrium binding studies had traditionally shown that there is 1 binding site for this nucleotide/monomeric unit of the enzyme (for reviews, see Refs. 1 and 2). Recently, however, Johnson et al. (3) have shown, by x-ray diffraction, the existence of 2 different AMP binding sites/monomer of glycogen phosphorylase b. A similar finding has also been reported for the form of the enzyme by Kasvinsky et al. (4). Evidence for a second AMP binding site for phosphorylase b in solution, at 25 °C, has been provided by the biphasic calorimetric titration curves of the enzyme with AMP, reported by Wang and collaborators (5, 6) and Merino et al. (7, 8), although there are clear divergences between the results of these two groups concerning both the AMP saturation range for the second site and the enthalpy values for this site. We have undertaken a similar calorimetric study, and the second "plateau" observed at AMP saturation has proved to be an artifact arising from traces of a contaminating enzyme. A monophasic titration curve showing a single plateau for saturation with AMP was obtained once the impurity had been eliminated. These results cast doubts on the experimental evidence for a second AMP site in phosphorylase b in solution at physiologically relevant temperatures.

MATERIALS AND METHODS

Glycogen phosphorylase b was prepared from rabbit skeletal muscle by the method of Fischer et al. (9, 10) with the modifications described by Krebs et al. (11). The catalytic activity of the enzyme was determined by the assay of Hedrick and Fischer (12). The preparations used had specific activities of 80 to 90 units/mg. Protein concentration was determined from absorbance measurements at 280 nm using the absorbance coefficient $E_{280}^{1\text{cm}} = 13.2$ (13). The molecular weight of the monomer was taken as 97,400 (14). 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 G-25 Sephadex column equilibrated with 50 mM KCl, 0.1 mM $\beta$-mercaptoethanol, 0.1 mM EDTA, 50 mM of buffer solution (glycylglycine, glycerophosphate, or tris(hydroxymethyl)aminomethane), adjusted to pH 6.9. The $A_{280}/A_{400}$ ratio for the AMP-free phosphorylase b solutions was always less than 0.53.

AMP, IMP, glycylglycine, tris(hydroxymethyl)aminomethane, and mercaptoethanol were purchased from Sigma; sodium glycerophosphate 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 (15). The control of the bath temperature was better than 0.01 °C. Electrical and chemical calibrations were made in the same heat range as that which we obtained in the calorimetric experiments themselves. The chemical calibration was accomplished by the neu-
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The calorimetric titration of phosphorylase b with AMP is shown in Fig. 1A. The heat values obtained at a high level of AMP concentration depended on the particular enzyme batch used. Many curves somewhere between the two limiting cases shown here were obtained with different preparations of phosphorylase b, all of them showing a biphasic contour, which might suggest a two-step saturation of the enzyme by the nucleotide. The heat evolved by any particular sample, at 10 mM AMP, depended on the flow rate (Fig. 1B). This seems to imply that the binding reaction does not attain equilibrium inside the calorimeter cell at the flow rates used here. It has been reported, however, that the AMP binding process is faster by several orders of magnitude than the highest flow rates used in these experiments (17). This fact, together with the variability observed in the absolute values for the second plateau of Fig. 1A led us to suspect the purity of the enzyme. All the enzyme preparations, however, showed a single band by gel electrophoresis and had specific activities within the range of 80-90 units/mg.

The AMP-phosphorylase complex in the outflow from the calorimeter was monitored for pH, and showed minor, although systematic increases in pH despite the fact that the medium was buffered. These changes were proportional to the amount of heat released by a particular preparation, i.e. the greater the heat evolved, the greater the pH increase. Therefore, binding experiments were carried out in the pH-stat, in the presence and absence of buffers, in order to study the kinetics of the process and to calculate the total number of free protons removed from the solution during the reaction.

We were surprised to find that this number was in some cases 200 times larger than the number of phosphorylase molecules present in the solution, i.e. approximately corresponding to all the AMP molecules available. The UV spectrum of phosphorylase b-AMP solutions (under the same conditions used for calorimetry) was registered as a function of time in separate experiments. It was then observed that the characteristic AMP absorption (maximum at 259 nm) shifted to shorter wavelengths and displayed, after some time, a spectrum almost identical with that of the IMP from a commercial source (maximum at 250 nm). We observed mixtures of both spectra at intermediate times and we were able, in fact, to follow the kinetics of the spectral changes by monitoring the decrease in the 259 nm absorption.

We were finally able to obtain reproducible data by controlling certain aspects of the enzyme purification. During the second step of purification (10), the pH value of the extract was very carefully adjusted to 5.2. We repeatedly observed that slight increases in this value correlated with the anomalous effects described above. The subsequent centrifugation was carried out at 5000 x g for 45 min at 3 °C, which also improved the purification. In the final purification step, successive crystallizations also seemed to decrease the level of what we assume to be an impurity in the phosphorylase b preparation. At any rate, a small number of protein preparations still showed some minor, although significant changes in pH and UV spectrum on the addition of AMP. At present we are working out a new complementary purification step based on the use of adsorbents to eliminate the impurity, and the full experimental procedure will be described in a forthcoming paper. At this point, we wish to report some results of AMP binding with the purified enzyme. These new preparations did not show any pH or spectrophotometric changes on the addition of AMP. Furthermore, a reproducible monophasic curve was observed in the calorimetric titration (Fig. 2, and also included in Fig. 1A as open circles for comparative purposes). Likewise, the heat of the reaction at saturation concentrations of AMP was totally independent of the calorimetric flow rate (Fig. 1B, open circles). This contrasts markedly with the dependence on flow rate shown by those enzyme preparations that displayed biphasic titration curves (Fig. 1B, closed circles). Hence we concluded that the monophasic curve is the one precisely corresponding to the calorimetric titration of phosphorylase b with AMP.

It is worth noting that the two groups of workers who have reported biphasic titration curves markedly disagree over the magnitude of the total heat evolved at saturation concentrations of AMP. This discrepancy has been attributed by Merino et al. (7, 8) to the different buffers used (glycylglycine and glycerophosphate). As can be seen in Fig. 2, however, we obtained the same monophasic calorimetric curve regardless of the buffer system used; thus, no characteristic effect on the heat of binding appears to be attributable to any of the particular buffers employed. On the other hand, given the different heats of ionization of the three buffers used in our experiments (18), no proton uptake or release seems to take place in the AMP binding to phosphorylase b. In this context, it is interesting to note, too, that the heat of binding at saturation per mol of monomer in our results (Fig. 2) compares well with the van't Hoff enthalpy of binding reported by Katenschmidt et al. (19) for this system (16.9 kcal/mol).

Our explanation for these results is that glycogen phosphorylase b might generally be contaminated with traces of AMP aminohydrolase (EC 3.5.4.6) which catalyzes the hydrolysis of AMP to IMP. The known properties of this enzyme (20) would explain why the pH and UV absorption changes...
Fig. 2. Calorimetric titration curve of purified phosphorylase b with AMP at pH 6.9, 25 °C, protein concentration, 5 mg/ml. The heat released per monomer of enzyme, corrected for dilution heats, when the nucleotide is bound is plotted as a function of the total nucleotide concentration. O, titration in 50 mM glycylglycine. C, titration in 50 mM glycophosphate. X, titration in 50 mM tris(hydroxymethyl)aminomethane. All solutions contained 50 mM KCl, 0.1 mM EDTA, 0.1 mM β-mercaptoethanol.

are reduced or even absent in the presence of inorganic phosphate, a known hydrolyase inhibitor, and would also explain why, on addition of IMP to phosphorylase, a monophasic calorimetric curve is always observed, whatever the enzyme batch used, with no change in either the pH or in the UV spectrum (data not shown). Enzymatic assays for AMP aminohydrolase (21) allowed us to estimate that the proportion of this enzyme in phosphorylase preparations lay in the range of parts/million. Although this impurity level is not usually relevant, it leads, in the type of experiments described here, to dramatically erroneous results given the high phosphorylase and nucleotide concentrations used and the catalytic nature of the impurity. The biphasic calorimetric curves could then be the result of a catalytic process in addition to the AMP binding, and the so-called second plateau would correspond to the heat effects due to the catalyzed reaction of the transformation of AMP into IMP and the concurrent buffer deprotonation, since ammonia is liberated during this reaction. It should be stressed here that precisely because of the high level of AMP concentrations used in these experiments a very small fraction of AMP to IMP conversion might be undetectable when “looking at” the AMP but may substantially alter the results of the heat of binding related to the phosphorylase, the concentration of which is about two orders of magnitude lower than that of AMP. In this respect, it is interesting to note that, in fact, Johnson et al. (22) suggested in 1974 that AMP was converted into IMP by a contamination in some glycogen phosphorylase preparations, very probably AMP aminohydrolase, although the presence of this enzymic impurity has not received any subsequent consideration from the groups concerned with the study of phosphorylase b. AMP aminohydrolase has also been reported as an impurity in myosin preparations (23) and Harris and Suelter (24) found that it could be reduced to levels of a few parts/million by chromatography on a phosphocellulose column. This residual impurity did not significantly affect the results with myosin on account of the low adenine nucleotide concentrations used in those experiments.

CONCLUSION

The only experimental data concerning the relative affinity of the two binding sites for AMP in solution, at 25 °C, came from the calorimetric studies of Wang and collaborators (5, 6) and Merino et al. (7, 8). The biphasic nature of the calorimetric titration curve of phosphorylase b with AMP led these authors to suggest the existence of a second site for AMP on the monomer of the enzyme, the affinity of which would be about 10 times less than that of the stronger affinity site. As we have described above, the purification of phosphorylase b leads to a monophasic titration curve which, to all appearances, could be interpreted as indicating a single AMP site in phosphorylase. However, it must be admitted that, for oligomeric proteins displaying cooperative binding of a ligand, no information regarding the number of ligand binding sites and equilibrium constants is obtainable from only calorimetric titration curves. In fact, additional studies would have to be carried out with AMP and phosphorylase b in solution, at 25 °C, to permit an appropriate quantitative analysis of the calorimetric curve. It is worth noting here that Morange et al. (25), using equilibrium dialysis, made a claim for the existence of 2 AMP binding sites/monomer of enzyme, although experimental data were reported only at 4 °C. Additional evidence for these two AMP sites comes from x-ray diffraction studies with crystals incubated with AMP at low temperatures (3, 4). It should be noted here that the experimental conditions for the dialysis and the x-ray studies were precisely the optimum ones for the enzymic activity of the impurity described in this work, i.e. large concentrations of phosphorylase and nucleotide and a long time for the experiments. Moreover, the x-ray studies, at the resolution carried out with phosphorylase, cannot distinguish between AMP and IMP molecules bound to the enzyme. The possible presence of impurities could then lead to a mixture of AMP- and IMP-enzyme systems in those experiments.

Nevertheless, even without the assumption of the impurity in those experiments, the binding of AMP to the nucleoside site (second site) would only have become apparent at low temperatures. Hence, we believe that, at present and considering our own results, there is no experimental evidence for a second AMP binding site per monomer of phosphorylase b in solution at physiological temperatures.

Finally, we would like to reiterate what has already been illustrated in this report, that otherwise irrelevant impurity traces, when displaying catalytic activity, might well play an undesirable role, particularly when using experimental techniques that demand rather high concentrations of biological material.

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