Biochimica et Biophysica Acta, 358 (1974) 275–280

BBA 67282

CONFORMATIONAL CHANGES IN PHOSPHORYLASE *a*, STUDIED BY A SPIN LABEL PROBE

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(Received March 25th, 1974)

SUMMARY

The interaction of the effector AMP with glycogen phosphorylase *a* (EC 2.4.1.1) was studied using a spin-labelled enzyme derivative and by equilibrium dialysis. The extent of the conformational change in the enzyme (monitored by the change in the ESR spectrum) did not parallel its saturation with AMP. These results are consistent with the "two-state" (concerted) model for allosteric transitions, suggesting that all four subunits of the enzyme undergo a conformational change when a single molecule of AMP is bound. A simple purification method for phosphorylase *a* is also described.

INTRODUCTION

Phosphorylase *a*, the active form of glycogen phosphorylase, is further activated by AMP [1]. The enzyme is normally tetrameric and its activation by AMP has been studied by kinetic methods and by equilibrium dialysis [2]. The results of these studies were interpreted in terms of the "two-state" (or concerted) model for allosteric transitions proposed by Monod et al. [3]. However, there have been no detailed studies of the conformational change induced in phosphorylase *a* by AMP, and so the assignment of the "two-state" model to this enzyme must be regarded as tentative.

The spin label method has been successfully used to study the conformational transitions of phosphorylase *b* [4, 5]. It has been shown that fully active phosphorylase *a* can be prepared with a single spin label on each subunit, and that the ESR spectrum of this enzyme undergoes a small change on addition of AMP, indicating that AMP does indeed cause a conformational change in the enzyme [5]. In this paper we describe a method for preparation of spin-labelled phosphorylase *a* free from AMP, and compare the extent of the changes in the ESR spectrum of the enzyme on addition of AMP with the extent of saturation of the AMP sites as determined by equilibrium dialysis. Our results indicate that spin-labelled phosphorylase *a* has the same affinity for AMP as does the unmodified enzyme, and that the "two-state" model of Monod et al. [3] can account for both the ESR and saturation data. The simple "sequential" model for conformational changes [6] is not appropriate for this enzyme.

Ogawa and McConnell [7] have used a similar approach to study the conformational changes in haemoglobin on binding oxygen. They concluded that the "sequential" model was more appropriate in this case. However, the introduction of the spin

label is known to cause perturbations of the haemoglobin structure [8], and thus introduces some uncertainty into the interpretation of these experiments. There have also been a number of studies in which the changes in physical properties such as sedimentation coefficient [9], fluorescence [10] and optical rotation [11] of certain multi-subunit enzymes on addition of ligands have been correlated with the extent of ligand binding. In this paper we describe the use of the spin label method, since it offers a particularly simple and direct way of studying conformational transitions in localised regions of a protein.

MATERIALS AND METHODS

Phosphorylase *b* was isolated from rabbit muscle and reacted with the spin label (*N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl) iodoacetamide) as previously described [4, 5, 12]. Spin-labelled phosphorylase *a* was prepared from spin-labelled phosphorylase *b* using phosphorylase kinase [5]. The phosphorylase *a* was purified by dialysis against 50 mM triethanolamine buffer at pH 7.0, followed by spontaneous recrystallisation at 4 °C from a 30-mg/ml solution. Nucleotides were removed from the spin-labelled phosphorylase *a* by dialysis against 0.4 M imidazole citrate buffer at pH 6.8, followed by dialysis against 50 mM triethanolamine buffer at pH 6.8, containing 0.1 M KCl and 1 mM EDTA. The ratio of absorbancies of the freshly prepared enzyme at 260 and 280 nm was 0.58, indicating the absence of nucleotides [2].

This method of purification of phosphorylase *a* has not been used previously and was found to be much more convenient for preparation of large quantities of the enzyme than the charcoal or Sephadex methods [2]. Its success depends on the fact that AMP binds only weakly to phosphorylase *a* in an imidazole citrate buffer of high ionic strength [2].

The enzyme was assayed by the method of Birkett et al. [12], and its activity was enhanced by 30% when assayed in the presence of 20 μm AMP. The addition of 1 mM AMP gave only a slight (5%) further activation. Since AMP binds very tightly to phosphorylase *a* [2] but much more weakly to phosphorylase *b* ($K_d \approx 100$ μM (12)), the lack of enhancement of activity at the higher AMP concentration implies that there is negligible contamination by phosphorylase *b*. A 30% enhancement of phosphorylase *a* activity by 20 μm AMP was in reasonable accord with previously reported values [1] and this, together with the absorbance data, suggests that there is very little contamination of the spin-labelled phosphorylase *a* by AMP.

The concentrations of solutions of spin-labelled phosphorylase *a* were determined using the method of Lowry et al. [13], with phosphorylase *b* as a standard. The molecular weight of posphorylase *a* is taken as 400.000 and the $E^{1\%}_{280}$ of phosphorylase *b* as 13,2 [1].

ESR measurements were made on a JEOLCO JES PEI X spectrometer operating at 9.5 GHz, using a modulation amplitude of 2 G and microwave power of 20 mW. The temperature was 18 °C. Small aliquots of a concentrated solution of AMP were added to a 0.2-ml sample of spin-labelled phosphorylase *a*. The ESR spectrum after each addition was recorded at least three times and the results were averaged. There was approximately a 20% dilution of the enzyme during the course of the titration. The ESR spectra were measured by the method used previously [4, 5] in which the ratio of the amplitude of the low-field peak compared with that of the centre

peak is determined. This eliminates errors introduced by variations in the position of the cell in the microwave cavity and by alterations in the microwave tuning.

[U-^{14}C]Adenosine 5′-monophosphate was purchased as the ammonium salt from The Radiochemical Centre, Amersham, Bucks (spec. act. 526 Ci/mole). A stock radioactive solution was made by addition of 1–2 μCi of the radioactive AMP to 20 ml of a solution of non-radioactive AMP (approx. 100 μM) in the 50 mM tri-ethanolamine buffer at pH 6.8, containing 0.1 M KCl and 1 mM EDTA.

The equilibrium dialysis experiments were performed by placing 0.3 ml of samples of a spin-labelled phosphorylase *a* solution (of the same concentration as that used in the ESR measurements) in dialysis sacs, which were then placed in stoppered tubes containing 5.6 ml of radioactive AMP solution. The tubes were rotated gently for 5 h at 18 °C (control experiments showed that equilibration of the AMP across the membrane was complete after this time). 0.05-ml aliquots of the solutions on either side of the dialysis membrane were then taken and the radioactivity determined by scintillation counting as previously described [14]. There was no change in the phosphorylase *a* concentration after the dialysis, and no protein could be detected in the dialysate.

The equilibrium dialysis and ESR titrations were performed simultaneously using aliquots of the same solutions of enzyme and AMP. The buffer was 50 mM triethanolamine at pH 6.8, containing 0.1 M KCl and 1 mM EDTA in all experiments.

RESULTS AND DISCUSSION

The results of the titration of spin-labelled phosphorylase *a* with AMP are shown in Fig. 1, where the ratio (*R*) of the amplitude of the low-field peak to that of

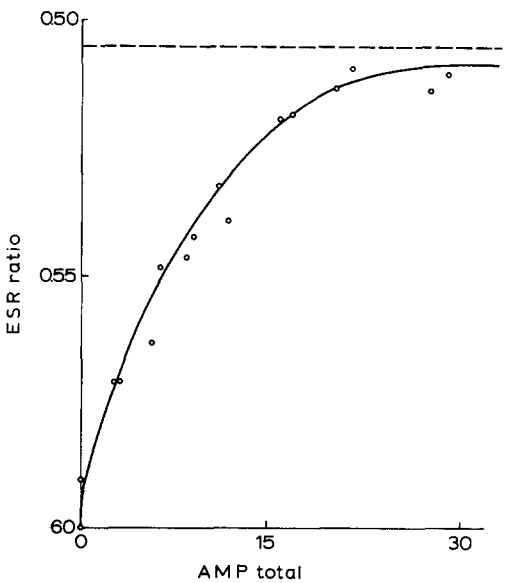

Fig. 1. Titration of spin-labelled phosphorylase *a* with AMP. The ordinate, *R*, represents the ratio the amplitudes of the low-field and centre-field lines, as described in the text. The initial concentration of phosphorylase *a* was 4.3 μM, in 50 mM triethanolamine buffer at pH 6.8, containing 0.1 M KCl and 1 mM EDTA. T = 18 °C. AMP total, in μM concentration.

the centre peak is plotted against the concentration of AMP added. As previously observed, the limiting value of R at saturating amounts of AMP is the same as that observed when spin-labelled phosphorylase *b* is saturated by AMP [5]. It should be noted that the ESR spectra in these experiments were recorded under slightly different conditions from those used in the earlier experiments, and thus the values of R are not strictly comparable with those presented previously [5].

Fig. 2 shows the results of the equilibrium dialysis experiments. The amount of AMP bound to the spin-labelled enzyme is plotted against the total AMP concentration in the dialysis sac. Even without making the small correction for the dilution of the enzyme during the ESR titration, it is clear from Figs 1 and 2 that the amount of binding and extent of conformational change of the enzyme are not equal. This point will be referred to later. The affinity of AMP for the spin-labelled enzyme is very similar to that determined for unlabelled phosphorylase *a* [2].

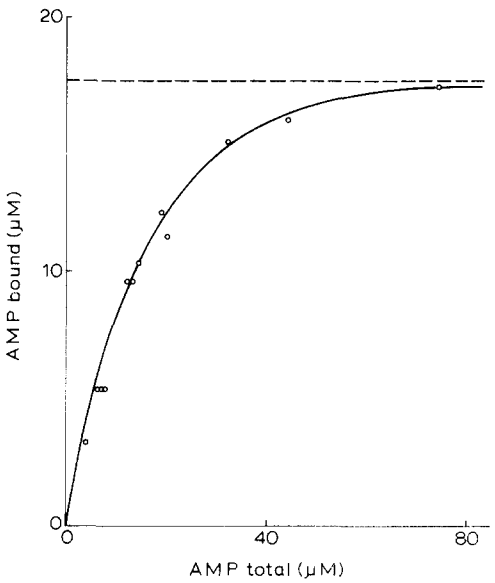

Fig. 2. Binding of AMP to spin-labelled phosphorylase *a*, as studied by equilibrium dialysis. Conditions as for Fig. 1.

The data from Fig. 2 is shown in the form of a Scatchard plot in Fig. 3. From the intercept on the abscissa, it is seen that there is one AMP binding site per subunit of phosphorylase *a*. It is noteworthy that the Scatchard plot shows no significant deviation from linearity above half saturation, indicating that any cooperative interactions between the subunits of the enzyme must be relatively weak. Helmreich et al. [2] have also presented linear Scatchard plots of the binding of AMP to phosphorylase *a*, but their binding experiments were conducted in the presence of glycogen which may well alter the allosteric properties of the enzyme [1].

We have computed theoretical Scatchard plots using the equations of Monod et al. [3], and assuming that there are four binding sites for AMP on the tetrameric

enzyme. Our analysis indicates that a Scatchard plot of the form shown in Fig. 3 (i.e. linear above half saturation) can only be obtained if the value of L (the ratio of T to R forms of the enzyme in the absence of ligand) is 5 or less and the value of C (the ratio of the dissociation constants of AMP from the R and T forms) is 0.1 or greater. If values for these parameters outside these ranges are assumed, the Scatchard plots are markedly curved below 75% saturation of the binding sites. Helmreich et al. [2] obtained comparable values of L and C from an analysis of their data on the binding of AMP to phosphorylase a after correction for the effects of glycogen [2]. In Fig. 3 the computed curve uses values of 3 and 0.2 for L and C, respectively ($K_R = 2\ \mu M$; $K_T = 10\ \mu M$).

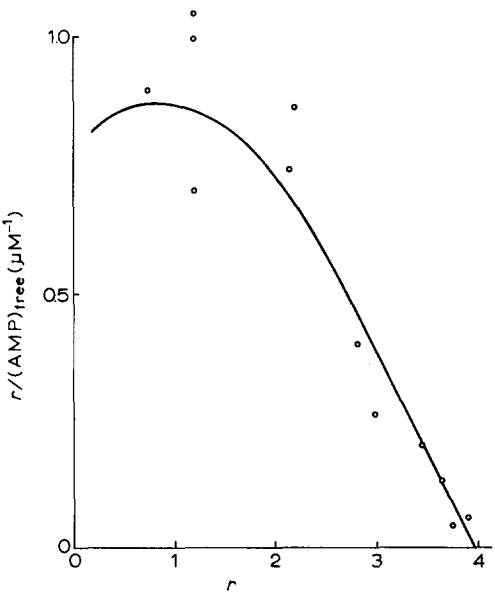

Fig. 3. Scatchard plot of data from Fig. 2. r is the number of moles AMP bound per mole phosphorylase a. The solid curve is computed from the equations of the "two-state" model, as described in the text, using the following parameters. $L = 3$, $C = 0.2$, $K_R = 2\ \mu M$, $n = 4$. ○, experimental points.

The equations of Monod et al. [3] can also be used to calculate the fraction of the enzyme in the R conformation (and hence the percentage conformation change) as a function of the extent of saturation of the ligand sites. In Fig. 4 the solid curve is calculated using the values of L and C from Fig. 3, and the open circles represent the experimental data from the ESR titration after correction for the dilution of the enzyme during the titration. While these values of L and C are clearly not unique, the very good agreement between the calculated curve and the experimental points suggests that the simple "two-state" model of Monod et al. [3] can successfully account for both the binding and conformational change data. The broken line in Fig. 4 is that predicted by the simple "sequential" model of Koshland et al. [6]. Evidently the "two-state" (concerted) model is more appropriate for phosphorylase a.

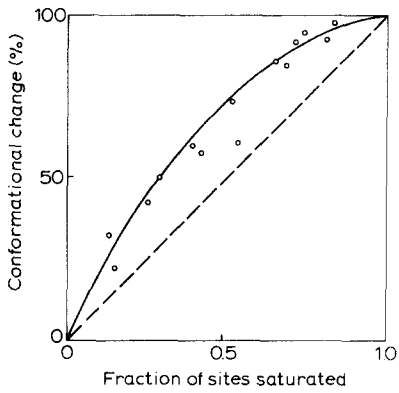

Fig. 4. Plot of the extent of the conformational change of spin-labelled phosphorylase *a* (determined from the ESR data) against the fraction of AMP sites saturated (determined by equilibrium dialysis). The solid line is computed using the same parameters as for Fig. 3, and the dashed line is that expected from the simple "sequential" model. ○, experimental points.

ACKNOWLEDGMENTS

We thank the S.R.C. for financial support. J.R.G. is a Junior Research Fellow of the Medical Research Council. We are grateful to JEOLCO for making ESR facilities available.

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