CONFORMATIONAL STATES OF RABBIT MUSCLE PHOSPHOFRUCTOKINASE INVESTIGATED BY A SPIN LABEL PROBE

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1. Introduction

A knowledge of the various conformational states of phosphofructokinase is of central importance to an understanding of the regulatory behaviour of the enzyme. Rabbit skeletal muscle phosphofructokinase has one of the most reactive thiol groups known for any enzyme [1], and its reactivity has already been used to advantage in studying these conformations [2]. To date, however, the experimental approaches have been mainly kinetic, and therefore indirect. Below, we present evidence to show that the reactive thiol can be specifically modified with *N*-(1-oxyl-2, 2,6,6-tetramethyl-4-piperidinyl) iodoacetamide, a spin label which thus acts as a sensitive probe for local conformational changes in the enzyme, without greatly affecting the catalytic properties. The procedure for labelling the enzyme, and some observations of the electron spin resonance spectrum of the modified enzyme and its sensitivity to pH, buffer anion and the binding of ATP and metal–ATP complexes, are described.

2. Materials and methods

ATP, fructose-6-phosphate (F6P) and 5,5′ dithiobis-(2-nitrobenzoic acid) (DTNB) were from Sigma. The spin label was purchased from Synvar Chemical Co. Other reagents were 'Analar' grade. Phosphofructokinase was prepared from rabbit skeletal muscle by the method of Parmeggiani et al. [3] and had 90% of the maximum specific activity observed [3]. It was stored as the ammonium sulphate suspension without crystallisation and used within two months. The assays used were as described previously [4] at both allosteric and non-allosteric pH, except for the omission of 2-mercaptoethanol.

Modification with the spin label was carried out as follows: The stock enzyme was dialysed for several hours at 20° versus 0.05 M Tris-phosphate buffer, 1 mM EDTA, pH 7.2, to a final concentration of approx. 10 mg/ml, and then incubated at 20° in the presence of 1 mM F6P with a 50% molar excess of spin label (with respect to the 90,000 subunit, the smallest well characterised subunit [5]). After 4 hr the reaction was 90% complete. The solution was then dialysed overnight versus Tris-phosphate buffer pH 7.5 without EDTA to remove F6P and excess unreacted label, and then versus Tris-HCl buffer for 2 hr if required.

The modification of the most reactive thiol group was followed by a sampling procedure. The enzyme was diluted into 0.05 M Tris-phosphate buffer, 1 mM EDTA, 1 mM F6P, pH 6.65, to a concentration of approx. 0.5 mg/ml, (5.5 μM) and DTNB added (50 μM). The release of thionitrobenzoate ion (E^{1cm}_{mM} = 11.6) was followed at 420 nm at 25° using a Beckman DB spectrophotometer coupled to a Sargent chart recorder.

Electron spin resonance spectra were recorded at 20° on a Varian E-4 spectrometer operating at X-band. Titrations were performed on 0.5 ml enzyme samples under standardised instrumental conditions. The reproducibility of signals was ± 1%.

3. Results

3.1. *Spin-labelling*

The thiol groups of the enzyme reacted with DTNB according to the scheme shown by Kemp and

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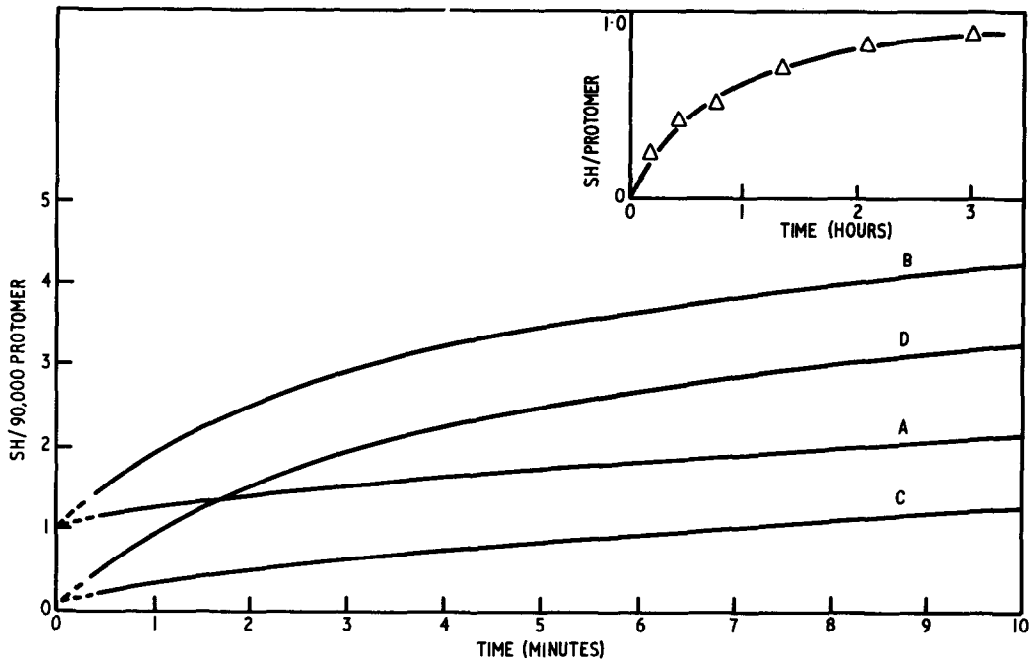

Fig. 1. Thiol reactivity with DTNB (50 μM) of native and spin-labelled phosphofructokinase (8 μM) in Tris-phosphate buffer, pH 6.65, at 25°. Curve A) native enzyme plus 1 mM F6P; Curve B) native enzyme, no addition; Curve C) 90% labelled enzyme plus 1 mM F6P; Curve D) 90% labelled enzyme, no addition. Inset: Time course of the spin-labelling reaction, pH 7.2

Forest [1]. Curves A and B, fig.1, show the time course of reaction of the thiol groups of the native enzyme at pH 6.65 in the presence and absence of 1 mM F6P, respectively. A single thiol group per 90,000 subunit (Class I) reacts almost instantaneously, in both cases, and two further thiol groups (Class II) are completely protected by F6P. As the pH is increased to 7.5 the number of rapidly reacting thiol groups increases from approx. 4 to 7.

The optimum pH for reaction with spin label was 7.2. This provided the best compromise between rapidity and specificity for the reaction of spin-label with the class I thiol group. Curves C and D, fig. 1, show the reactivity of the labelled enzyme with DTNB in the presence and absence of F6P. The class I thiol group is 90% blocked but no other reactive thiol groups are affected since pairs A, C and B, D are closely parallel.

The inset to fig. 1 shows the time course of the reaction with spin-label. The complete modification is accompanied by a 40% loss of activity (assayed at pH 8.2) and by minor changes in the allosteric kinetics with respect to F6P; the effects are rather smaller than reported for the DTNB-modified enzyme [6] and suggest that the ligand-binding properties of the enzyme have not been seriously affected.

3.2. *Electron spin resonance spectra*

Fig. 2(a) shows the spectrum of the labelled enzyme in Tris-HCl buffer, pH 7.5 with no additions. The shape is characteristic of a moderately immobilised label, with a rotational correlation time (τ_R) of 3×10^{-9} sec [7]. The label probably has a good deal of segmental mobility relative to the whole enzyme molecule. These conditions of intermediate mobility generally result in a high degree of sensitivity of the label to conformation changes, and the following observations confirm this.

3.3. *Effect of buffer and pH*

In Tris-phosphate buffer, pH 7.5 the spectrum shows that the label is much more 'immobilised' with $\tau_R = 7 \times 10^{-9}$ sec (fig.2b). On titrating to pH 6.0 the spectrum reverts to the more mobile

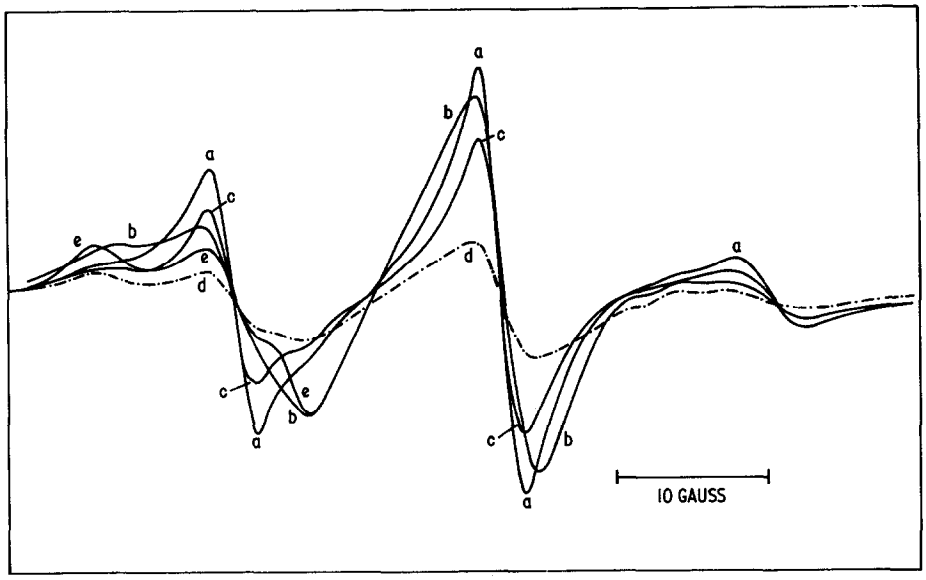

Fig. 2. Electron spin resonance spectra of spin-labelled phosphofructokinase (100 μM approx. with respect to the 90,000 subunit). a) In Tris-HCl buffer, pH 7.5, no additions. b) In Tris-phosphate buffer, pH 7.5, no additions. c) In Tris-HCl buffer, pH 7.5, 10 mM ATP. d) In Tris-HCl buffer, pH 7.5, 5 mM MnATP. e) In Tris-phosphate buffer, pH 7.5, 5 mM MgATP.

type shown in fig. 2(a). The change is centred on pH 6.4 and shows isosbestic points, indicating that the spin label detects two conformations only. The change can be directly correlated with dissociation to the inactive 180,000 subunit, but the spin label is probably detecting a related conformation change rather than the dissociation itself since changes in the slower rotational correlation time of the whole enzyme are not likely to dominate the motion of the label.

3.4. *Effect of binding ATP and its metal complexes*

In Tris-HCl buffer pH 7.5, large spectral changes occur on adding ligands (figs. 2(c), 2(d), and 3). Four points of interest may be noted: i) MgATP produces a spectral change quite distinct from that of free ATP, suggesting that the metal ion plays a vital part in determining the conformation of the ternary complex; ii) The spectrum of the ternary enzyme–ATP–metal complex is quite similar to that of the enzyme in phosphate buffer alone, so that it is difficult to detect other ligand-induced changes in this buffer. This implies that phosphate ion alone causes a conformational change similar to that induced by MgATP. iii) The MgATP spectra show sharp isosbestic points, and the binding curve obtained from these spectra is sigmoidal (fig.4). This shows that only two conformations are detected and that positive cooperativity between MgATP sites is occurring. To our knowledge, this is the first non-kinetic demonstration of cooperative ligand binding to phosphofructokinase. iv) MnATP produces a similar spectral change to MgATP except for a large quenching of the spectrum which is due to the dipolar interaction of the two unpaired spins. From the quenching, which is distance-dependent, in conjunction with other magnetic resonance methods, a preliminary estimate of 12 Å has been made for the distance between the manganous ion and the nitroxide group on the labelled enzyme. Furthermore the spin label causes an appreciable broadening of the high resolution spectra of protons in MgATP bound to the enzyme, and studies of this permit estimates of the ATP proton–nitroxide distances on the enzyme to be made which are compatible with the manganese – nitroxide distance and with the previously determined structure for MnATP in the ternary complex with enzyme [4].

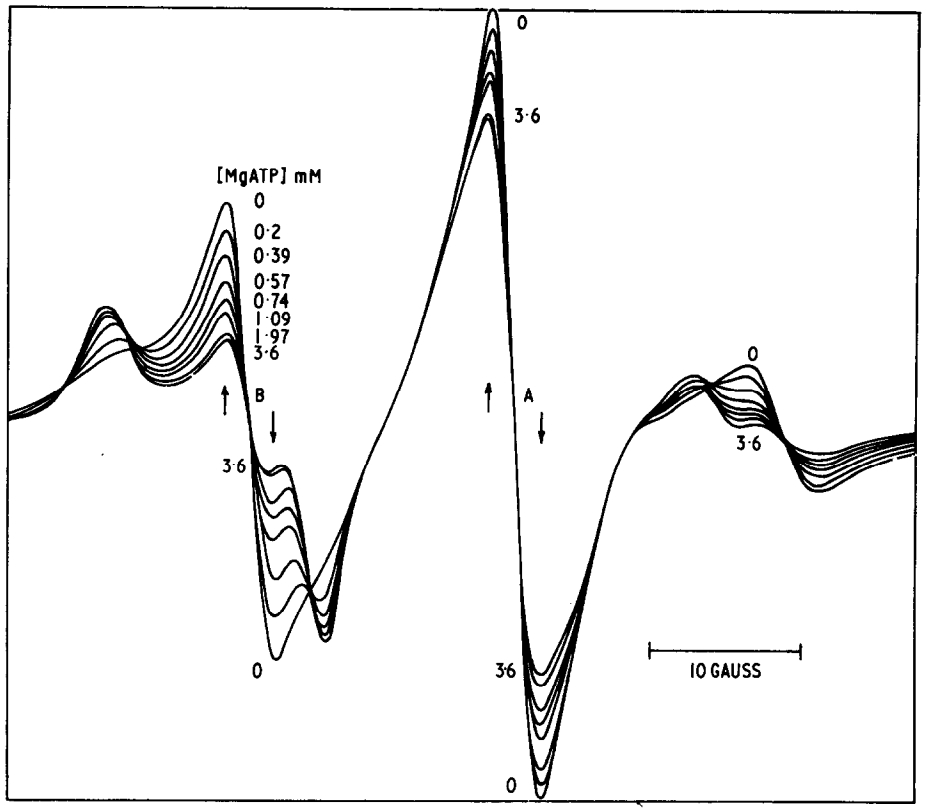

Fig. 3. Electron spin resonance spectra of spin-labelled phosphofructokinase (120 μM) in Tris-HCl buffer, pH 7.5, titrated with MgATP. The concentration of MgATP is given at the curves, which are not corrected for a dilution factor of 5–10%.

4. Discussion

In an earlier communication [4] we described the use of magnetic resonance techniques to investigate the conformation at the MnATP binding sites of phosphofructokinase; the methods suffered from complications common to the use of any dissociable probe. By contrast, the use of a spin label has provided a more direct method for the study of conformational changes in phosphofructokinase as in other enzyme systems such as creatine kinase [8].

The reactive thiol group of phosphofructokinase which we have labelled in this study has not been directly implicated in either inhibitory or catalytic site binding of MgATP and its relationship to these sites is as yet unclear; thus we have no prior way of knowing whether the metal–ATP sites detected by the spin label are inhibitory or catalytic. The observation [9] that inhibitory binding of MgATP reduces the reactivity of the class I thiol group suggests that it becomes immobilised or buried and thus favours the former alternative since we detect binding of MgATP as an immobilisation of the spin label. The similarity of this spectrum to that in inorganic phosphate favours the second alternative because phosphate is an activator and never an inhibitor and would not be expected to induce a similar conformational change to that produced by inhibitory MgATP binding. However, the spectra are not identical and may represent two discrete conformational states of the enzyme. At present the only generalisation possible concerning the spectra is that agents

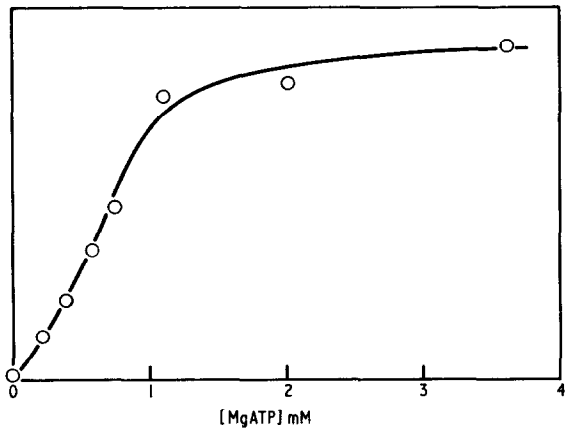

Fig. 4. Binding curve for phosphofructokinase (120 μM) with MgATP in Tris-HCl buffer, pH 7.5. The ordinate is a parameter of spectral shape: in this case the ratio of peak-to-peak heights at A and B in fig.3.

known to stabilise the activity of the enzyme in solution (high pH, phosphate buffers, MgATP) also produce immobile conformations at the labelling site. Nevertheless, the spin label detects cooperativity between MgATP sites on the enzyme. Kinetic evidence for cooperativity at both catalytic and regulatory sites for MgATP has already been obtained [10].

The complex shapes of the spectra of the enzyme–label complex are unusual and cannot be fully interpreted at the present time. However, we are reasonably certain that the labelling is specific for a single thiol group, and identical spectra have been obtained as a result of several labelling experiments with slightly varied conditions. Two possible explanations for the unusual shape of the spectra are as follows:
i) A single conformation could give rise to complex spectra if there were appreciable rotational anistropy or g-value anisotropy in the enzyme-bound spin label.
ii) There might be two discrete orientation states for the spin label at the same site on the enzyme which are present in equilibrium in all conformations of the enzyme and which have different spectra. This kind of explanation has been put forward to explain the complex spectra of spin-labelled haemoglobin crystals [11].

Despite these uncertainties, it is clear from the preliminary results presented here that the incorporation of a spin-label into phosphofructokinase provides a potentially powerful probe for investigating ligand-induced conformational changes and for mapping the distances between atoms of the various bound substrates. These experiments will be reported in a later communication.

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