A SPIN LABEL PROBE FOR THE CONFORMATIONAL CHANGE ON CONVERSION OF PHOSPHORYLASE *b* TO PHOSPHORYLASE *a*

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1. Introduction

Glycogen phosphorylase exists in two forms: phosphorylase *b*, which is normally inactive and is activated by AMP, and the active phosphorylase *a*, which has its activity slightly enhanced by AMP [1]. Phosphorylase *a* is formed from *b* by the phosphorylation of a single seryl residue on each protomer of the enzyme by Mg/ATP, catalyzed by phosphorylase kinase [2].

Phosphorylase *b* can be labelled on a single sulphydryl residue per protomer with the spin label *N*-(1-oxyl-2,2,6,6,-tetramethyl-4-piperidinyl) iodoacetamide [3]. Spin labelled phosphorylase is fully active and, when activated by AMP, displays the usual sigmoid activation curve [3]. The spin label has been shown to be close to the binding sites for AMP, for glucose-1-phosphate (the substrate) and for manganese ion [4].

In the experiments reported in this paper the conformational changes of the enzyme protomer are monitored by the electron spin resonance (ESR) spectrum of the spin label and are used to study the activation of phosphorylase *b* both by AMP and by conversion to phosphorylase *a*.

2. Materials and methods

Phosphorylase *b* was prepared by the method of Fischer and Krebs [5] and phosphorylase kinase by the method of Krebs and Fischer [6] as modified by Brostrom et al. [7].

N-(1-Oxyl-2,2,6,6,-tetramethyl-4-piperidinyl) iodacetamide purchased from Synvar Chemical Co. was used to label a single sulphydryl group per protomer of phosphorylase *b* by a modification of the method of Campbell et al. [3]. AMP was omitted from the reaction mixture and the incubation time was increased to 20 hr. An estimation of the remaining fast-reacting sulphydryl groups [8] with 7-chloro-4-nitro-benzo-2-oxa-1,3-diazole showed that less than 10% remained after spin labelling.

AMP and ATP were obtained from Boehringer and stored frozen in concentrated neutralized solution. Measurements, and the preparations of phosphorylase *a*, were made in 50 mM Tris-HCl, 100 mM KCl buffer at pH 8.5 at room temp. A Varian E4 spectrometer was used.

Spin labelled phosphorylase *a* was prepared from spin labelled phosphorylase *b* by incubating the latter at pH 8.5 with phosphorylase kinase in the presence of 10 mM $MgCl_2$, 5 μM $CaCl_2$ and 3 mM ATP for 6 hr at room temp. The reaction mixture was then dialyzed twice against a 500-fold excess of buffer containing 10 mM Tris-HCl, 1 mM EDTA at pH 7.0 and the modified enzyme was crystallized by addition of 100 mM NaF. The crystals were taken up in Tris-HCl buffer (pH 8.5) and dialyzed against a 1000-fold excess of the same buffer. Phosphorylase assays were carried out by the method of Birkett et al. [8].

3. Results and interpretation

The ESR spectrum of spin labelled phosphorylase *b* is shown in fig. 1A. It displays the characteristics of a partially immobilized nitroxide group: the high field peak (III) is largely collapsed while the middle peak (II) is still sharp and symmetrical and the low

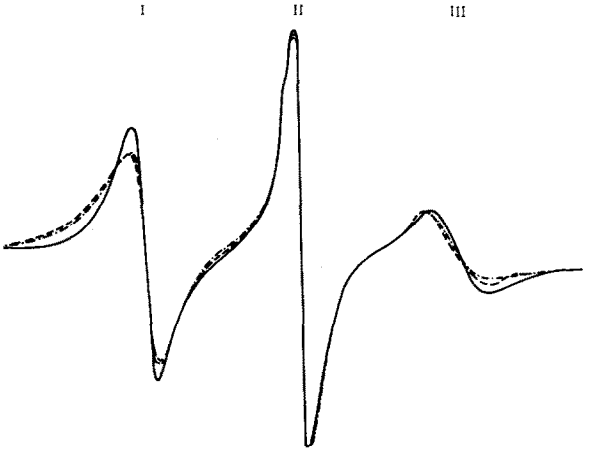

Fig. 1. ESR spectra of spin labelled phosphorylase recorded at 9250 MHz at 22° and pH 8.5. A. (————) 5×10^{-5} M spin labelled phosphorylase *b*. B. (– – – – – –) 5×10^{-5} M spin labelled phosphorylase *b* in 10^{-3} M AMP and 4.7×10^{-5} M spin labelled phosphorylase *a* in 2.2×10^{-3} M AMP. C. (–·–·–·–·–) 4.8×10^{-5} spin labelled phosphorylase *a*.

field peak is intermediate in character. Small changes in the mobility of the spin label (as when the enzyme binds AMP, fig. 1B) cause negligible changes in the amplitude of peak II, (the centre peak) but noticeably affect that of peak I. Thus one may calculate a ratio:

$$R = \frac{\text{peak I}}{\text{peak II}}$$

between the heights of these peaks which will give a measure of the mobility of spin label [3]. As the label is immobilized, II remains constant, I collapses and R therefore decreases. The advantage of using R to measure changes in the spectrum is that any alterations in the amplitude of peak I caused by variations in signal strength or positioning of the cell in the cavity will be accompanied by proportional changes in peak II. It can be shown by careful experiments that the height of the centre peak remains constant on addition of ligands or in the phosphorylase *b* to *a* conversion. Thus peak II effectively provides an internal standard which allows the results from different experiments to be normalized.

The ratio R for spin labelled phosphorylase *b* is between 0.685 and 0.676. This value is unaffected by pH changes between pH 6 and 9 and is only slightly

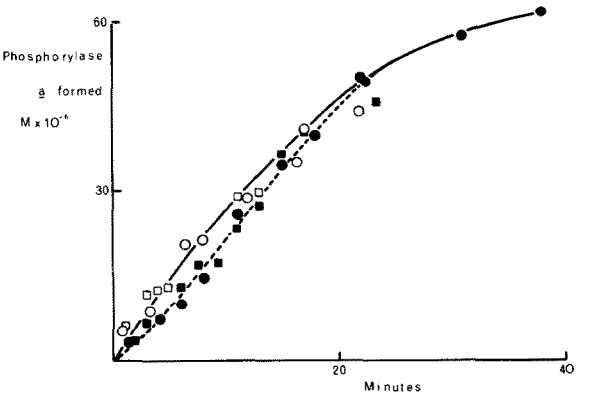

Fig. 2. The rate of phosphorylase *b* to *a* conversion followed by the ESR spectrum of the spin labelled enzyme. Spin labeled phosphorylase *b* (84 μM) incubated with 10 mM $MgCl_2$, 3 mM ATP, 5 μM $CaCl_2$ plus 0.05 ml phosphorylase kinase in 50 mM glycerophosphate, 10% sucrose, 2mM EDTA, pH 6.8, in a final volume of 0.50 ml pH 8.48 Tris-HCl buffer. Incubations A and B (●, ■) were started by adding the kinase, runs C and D (○, □) were started by adding the phosphorylase after the kinase had been preincubated for 10 min with the other reagents.

modified by large changes in enzyme concentration or in the ionic strength of the solution. Values of R for a given preparation were reproducible with an accuracy of between 0.5 and 1%.

The ESR spectrum of spin labelled phosphorylase *b* in the presence of excess AMP is shown in fig. 1B. Peak I is diminished in amplitude while II is almost unaffected and R has become 0.510.

When spin labelled phosphorylase *b* is activated by conversion to phosphorylase *a* under the conditions described in the legend to fig. 2, R becomes 0.53. The spectrum of purified spin labelled phosphorylase *a* is shown as fig. 1C and the value of R is now 0.56. Although the value of R increases slowly as the preparation ages it is never lower than 0.55, even with freshly prepared enzyme. If an excess of AMP is now added to the purified enzyme its ESR spectrum becomes indistinguishable from that of AMP activated phosphorylase *b* (fig. 1B).

The conversion of phosphorylase *b* to phosphorylase *a* is a time-dependent process which may be monitored, in the case of spin labelled phosphorylase, by the change in R from 0.680 to 0.562. If one assumes that all the partially phosphorylated intermediates formed during the reaction will have the same

spectrum as phosphorylase *a* one may calculate the quantity of phosphorylase *b* remaining at any time from the relationship:

$$[\mathrm{Ppb}] = [\mathrm{Ppb}]_0 \frac{R - R_a}{R_b - R_a}$$

where [Ppb] and $[\mathrm{Ppb}]_0$ are the remaining and original concentrations of phosphorylase *b* respectively, R is the value of *R* observed at any point in the reaction and R_a and R_b are the values of *R* observed for phosphorylase *a* and *b*, respectively.

Under the conditions described in the legend to fig. 2 the reaction rate will be pseudo-first order, depending only on the concentration of phosphorylase *b*, the substrate. Four incubations are shown in which identical aliquots of non-activated phosphorylase kinase, stored at pH 6.8, were added to spin labelled phosphorylase *b* in the presence of excess Mg/ATP and Ca^{2+} ion at pH 8.4. Incubations A and B, which were started by adding the non-activated kinase to the other reagents, did not reach their maximal velocity for 10 min. Incubations C and D were started by adding the phosphorylase *b* to kinase which had been preincubated with the other reagents for 10 min at 22°, and they show normal first order kinetics. After 20 min the two curves are indistinguishable within experimental error.

The lag phase observed in incubations A and B is probably caused by activation of the kinase limiting the rate at the early stages of the reaction. This activation, a phosphorylation by Mg/ATP, may be catalyzed either by a cylcic AMP dependent protein kinase or by phosphorylase kinase itself [9]. Since the protein kinase could only be present as a trace impurity and no cyclic AMP was added to the system, autocatalysis by phosphorylase kinase is probably the predominant mechanism. Autocatalytic activation of phosphorylase kinase, at pH 8.4, though known to occur [10], has less marked effect on the activity of the kinase than at pH 6.8. This is because the nonactivated kinase has an appreciable activity at high pH but is almost totally inactive at pH 6.8.

A similar lag was observed in the series of incubations reported in table 1. Varying concentrations of spin labelled phosphorylase *b* were used and the reaction was started by adding the kinase, which was kept

Table 1

Pseudo-first order rate constants for the conversion of phosphorylase *b* to phosphorylase *a*.

Concentrations of phosphorylase *b* (μM)	pH	k_1 (sec^{-1})
137.3	8.44	6.0×10^{-4}
68.6	8.45	5.4×10^{-4}
68.6	8.50	5.3×10^{-4}
34.3	8.42	6.2×10^{-4}
34.3	8.45	5.2×10^{-4}

Identical aliquots of kinase were incubated with varying concentrations of spin labelled phosphorylase *b* under the same conditions as fig. 2.

constant in all these runs. The first order plot of these reactions became linear only after 7–8 min. The first order rate constants reported in the table were obtained from the straight line plot corresponding to the period from 10 to 20 min. The rate constant is essentially unchanged between 30 and 140 μM substrate concentration, suggesting that the K_m for the reaction was not greatly exceeded. This is not consistent with the K_m of 33 μM obtained by Krebs et al. [10] with native phosphorylase *b* and non-activated kinase, using slightly different conditions, at pH 8.5. The system is markedly dependent on pH and on the concentration of calcium ions however, and it may be that under our conditions the K_m is nearer to that of 250 μM which Krebs et al. found at pH 7.6 [10].

4. Conclusions

The change in the spin label spectrum coincides with the appearance of activity, thus establishing a relation between the conformational change and activity. The spin label also indicates that the conformation of active phosphorylase *a* is similar to but not identical with the conformation of phosphorylase *b* saturated with AMP. Thus again the additional activation of the *a* form by AMP can be related to a small but observable structural change caused by the ligand. Because the spin label method is relatively rapid and sensitive the lag phase in the activation of phosphorylase by phosphorylase kinase, which cannot normally be observed by conventional activity measurements,

is easily studied. In principle the method should also be capable of relating the behaviour of mixed hybrids formed in this reaction (postulated from kinetic experiments) to the overall conformation of the oligomer.

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