A Kinetic Method to Establish the Specificity of Spin Labelling of Macromolecules

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A method has been devised for establishing the specificity of spin labelling of a macromolecule. Analysis of the rate of incorporation of spin label under different conditions permits the assignment of reacting groups to classes of differing reactivity. The method is illustrated for the enzyme glycogen phosphorylase *b*.

The spin-labelling technique has been widely used in studies of the structure and function of biological systems (for review see Smith, 1972). In a previous paper (Price, 1973) it was shown how a radioactive spin label could be used to determine the extent of incorporation of spin label into a macromolecule. We now report the further use of the radioactive spin label to establish the specificity of incorporation of a spin label at a given site on a macromolecule. It is important to establish such specificity in order to interpret data on structural changes in a macromolecule which might be detected by the spin label (Campbell *et al.*, 1972) and also if the spin label is used as a paramagnetic reference point in nuclear-magnetic-resonance studies (Taylor *et al.*, 1969).

In principle it should be possible to establish the specificity of modification of a macromolecule by examination of the fragments produced by enzymic cleavage of the macromolecule after reaction. This approach has been used, for instance, in the reaction of aldolase with iodo[2-¹⁴C]acetic acid (Perham & Anderson, 1970). However, in preliminary experiments we have encountered some difficulty in attempting to separate tryptic peptides which carry the bulky non-polar nitroxide moiety by using the normal paper-chromatography systems (Harris & Perham, 1965). Zarkadas *et al.* (1969) found that the cysteine-containing peptides containing the rapidly reacting thiol groups of phosphorylase *b* (α -1,4-glucan-orthophosphate glucosyltransferase, EC 2.4.1.1; from rabbit muscle) labelled with iodo[¹⁴C]-acetamide were unstable. In view of these problems we have chosen to develop a kinetic method to establish specificity, in which the rate of incorporation of spin label is studied, and the results are analysed in terms of the kinetics of multiphase processes. We have

outlined this approach for reaction of macromolecules with chromophoric reagents such as 2,4,6-trinitrobenzenesulphonic acid (Birkett *et al.*, 1970). The present paper describes the application of this method to the reaction of the enzyme phosphorylase *b* with the spin label *N*-(1-oxyl-2,2,6,6-tetramethylpiperidin-4-yl)iodoacetamide, which is known to react with the thiol groups of the enzyme (Campbell *et al.*, 1972).

Materials and methods

Phosphorylase *b* was isolated from rabbit skeletal muscle and assayed as previously described (Birkett *et al.*, 1971). *N*-(1-Oxyl-2,2,6,6-tetramethylpiperidin-4-yl)iodoacetamide (non-radioactive) was purchased from Synvar, Palo Alto, Calif., U.S.A. The radioactive spin label, *N*-(1-oxyl-2,2,6,6-tetramethylpiperidin-4-yl)iodo[2-¹⁴C]acetamide, was prepared as described by Price (1973).

The solution of radioactive spin label for these experiments was prepared by evaporating to dryness a small volume (usually 0.1 ml) of the stock ethanolic solution (20 mM) of *N*-(1-oxyl-2,2,6,6-tetramethylpiperidin-4-yl)iodo[2-¹⁴C]acetamide and dissolving the residue in 1 ml of 15 mM non-radioactive spin label made up in 50 mM-triethanolamine-HCl buffer, pH 7.5, containing 100 mM-KCl and 1 mM-EDTA. Repeated freezing and thawing facilitated the dissolution of the spin label. The total concentration of spin label in the final solution was checked by comparison of the amplitudes of the electron paramagnetic resonance with those of a standard solution of spin label (Price, 1973). Where appropriate a more dilute solution of the spin label was made by addition of the same amount of radioactive material to a diluted (4 mM) solution of the non-radioactive spin label.

The reaction of phosphorylase *b* with spin label was initiated as a routine by addition of 0.1–0.25 ml of the radioactive spin-label solution (prepared as described above) to 0.5 ml of phosphorylase *b* solution (usually about 30 mg/ml) in 50 mM-triethanol-

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Fig. 1. Incorporation of spin label into phosphorylase *b*

The broken lines are those calculated for the reaction of groups with the given initial concentrations of enzyme and reagent and appropriate second-order rate constants. The solid curves represent the sum of these calculated reaction curves. For other details see the text. (a) Reaction of 312 μM -phosphorylase *b* subunits with a large excess of spin label (8.4 mM). The broken lines correspond to: A, two groups per subunit with rate constant of $0.2\text{M}^{-1}\cdot\text{min}^{-1}$; B, one group with a rate constant of $154\text{M}^{-1}\cdot\text{min}^{-1}$ and one group with a rate constant of $13\text{M}^{-1}\cdot\text{min}^{-1}$. O, Experimental points. Temperature, 22°C . (b) Reaction of 364 μM -phosphorylase *b* subunits with a smaller excess of spin label (3 mM). The broken lines correspond to: A, two groups per subunit with a rate constant of $0.2\text{M}^{-1}\cdot\text{min}^{-1}$; B, one group per subunit with a rate constant of $13\text{M}^{-1}\cdot\text{min}^{-1}$; C, one group per subunit with a rate constant of $154\text{M}^{-1}\cdot\text{min}^{-1}$. O, Experimental points. Temperature, 22°C .

amine-HCl buffer, pH 7.5, containing 100 mM-KCl and 1 mM-EDTA. At known time-intervals 50 μl portions were removed from the reaction mixture and added to 0.5 ml of 1% (v/v) formic acid. Control experiments showed that this stopped the incorporation of spin label. These samples were then dialysed against 1% formic acid for 24 h to remove excess of spin label (i.e. that which had not reacted). After dialysis 0.2 ml samples were taken for determination of radioactivity by liquid-scintillation counting as described previously (Price, 1973). The solution of radioactive spin label was used as a standard; control experiments showed that neither the formic acid nor the protein present affected the measured radioactivity. Concentrations of protein in each sample after dialysis were determined by the method of Lowry *et al.* (1951) by using unmodified phosphorylase *b* as a standard. The subunit molecular weight of phosphorylase *b* was assumed to be 100 000 (Graves & Wang, 1972). In these experiments the incorporation of one equivalent of spin label per phosphorylase subunit corresponded to a radioactivity of approx. 1500 c.p.m. Samples were counted for radioactivity

for 20 min in triplicate and the results averaged. The incorporation measurements have been shown to be accurate to within 2.5% (Price, 1973).

The kinetics of the reaction were analysed by a computer program (kindly written by Mr. P. J. Seeley of this department) which generates progress curves for parallel second-order processes with one reactant in common given the initial concentrations of reactants and the second-order rate constants. The curves are generated as a series of successive tangents by calculating the rates of the various reactions after successive small time-intervals.

Results and discussion

Two types of kinetic experiment were carried out to establish the specificity of reaction of *N*-(1-oxyl-2,2,6,6-tetramethylpiperidin-4-yl)iodoacetamide with phosphorylase *b*.

(a) In the first type of experiment, the enzyme was made to react with a large (30-fold) excess of spin label. The result of this experiment is shown in Fig. 1(a). There is a rapid incorporation of two molecules of spin label per subunit of phosphorylase which is

essentially complete after 30 min reaction. Subsequently a very slow incorporation of further spin label is observed. After 10 h slightly more than three groups per subunit have been incorporated, but further reaction leads to progressive denaturation and precipitation of the enzyme. After 100 min reaction (when approx. 2.2 groups have been incorporated per subunit) the enzyme is essentially fully active.

The simulated curve in Fig. 1(a) assumes that there are two groups reacting in this slow phase, and the fit to the data is good, with a second-order rate constant of $0.2 \text{ M}^{-1} \cdot \text{min}^{-1}$ for these two groups. It should be noted, however, that the reaction of these groups in the slow phase is so slow compared with the groups in the fast phase that the precise values of the rate constant (or even the number of groups reacting) are not particularly important in the overall analysis.

To establish whether the two fast-reacting thiol groups react at the same or at different rates with the spin label a second type of experiment with the spin label was performed.

(b) In this experiment a smaller excess (approx. 8-fold) of the spin label was made to react with phosphorylase *b*. Samples were removed and quenched by addition to formic acid after shorter time-intervals than in the previous type of experiment, so as to focus attention on the initial phase of the reaction. The results (Fig. 1b) show that the two fast-reacting thiol groups do not react at identical rates with the spin label. In Fig. 1(b) the simulated curve which best fits the experimental data assumes second-order rate constants of $154 \text{ M}^{-1} \cdot \text{min}^{-1}$ and $13 \text{ M}^{-1} \cdot \text{min}^{-1}$ for the two groups in the 'fast-reacting' phase. The contribution of the two very slowly reacting groups is negligible in this portion of the reaction under these conditions.

We have assumed other rate constants for the two rapidly reacting groups in the fast phase of the reaction in order to check the uniqueness of the fit shown in Fig. 1(b). The results of such simulations are shown in Fig. 2 for the cases where (i) the ratio of the two rate constants is significantly greater than in Fig. 1(b) (curve A), and (ii) the ratio of rate constants is significantly less than in Fig. 1(b) (curve B). From the results of a number of such attempted simulations it is clear that the analysis shown in Fig. 1(b) represents the best fit to the experimental data.

The conclusion from the analysis of the reaction is that the subunit of phosphorylase *b* contains three different types of group which react with the spin label: (i) one fast-reacting group which reacts with a second-order rate constant of approx. $150 \text{ M}^{-1} \cdot \text{min}^{-1}$; (ii) one slower-reacting group (rate constant approx. $13 \text{ M}^{-1} \cdot \text{min}^{-1}$); (iii) two very slowly reacting groups (rate constant approx. $0.2 \text{ M}^{-1} \cdot \text{min}^{-1}$).

We can use these data to calculate the distribution

Fig. 2. Curves to show the incorporation of spin label into phosphorylase *b* under the conditions as for Fig. 1(b)

Curve A is calculated assuming that the reaction consists of one group with a rate constant of $154 \text{ M}^{-1} \cdot \text{min}^{-1}$, one group with a rate constant of $10 \text{ M}^{-1} \cdot \text{min}^{-1}$ and two groups with a rate constant of $0.2 \text{ M}^{-1} \cdot \text{min}^{-1}$. Curve B is calculated assuming that the reaction consists of one group with a rate constant of $130 \text{ M}^{-1} \cdot \text{min}^{-1}$, one group with a rate constant of $20 \text{ M}^{-1} \cdot \text{min}^{-1}$ and two groups with constants of $0.2 \text{ M}^{-1} \cdot \text{min}^{-1}$. $\circ$, Experimental points.

of spin-labelled products formed by reaction of equimolar concentrations of spin label and phosphorylase *b* (subunits). The calculation shows that 85% of the enzyme is spin-labelled at the fast-reacting thiol group and 14% at the slower-reacting thiol group. (There are very small amounts of reaction at the very slowly reacting groups and of doubly labelled enzyme.) This demonstration of a high specificity of reaction of the spin label at a single site on the enzyme puts the interpretation of data on ligand-induced conformational changes in the enzyme (detected by the spin label) on a firmer basis.

Our results are comparable with those previously obtained from a study of a reaction of the enzyme with iodoacetamide (Zarkadas *et al.*, 1969), but we have been able to demonstrate by kinetic analysis that the two fast-reacting groups of phosphorylase *b* (Fig. 1a) do indeed react at markedly different rates with the spin label. Recently it has been shown that a similar pattern is observed in the reaction of iodoacetamide with the enzyme, although there is a smaller difference between the rates of reaction of the two fast groups in

this case (O. Avramovic-Zicic, D. J. Brooks, S. J. W. Busby & G. K. Radda, unpublished work).

This type of kinetic analysis of the spin-labelling reaction could be extended to other enzymes, such as rabbit muscle glyceraldehyde 3-phosphate dehydrogenase, which can incorporate more than one equivalent of spin label. This should help to establish the specificity of labelling and put the results of spin-labelling studies on a firmer basis.

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Birkett, D. J., Freedman, R. B., Price, N. C. & Radda, G. K. (1970) *Biochem. Soc. Symp.* **31**, 147-155

- Birkett, D. J., Dwek, R. A., Radda, G. K., Richards, R. E. & Salmon, A. G. (1971) *Eur. J. Biochem.* **20**, 494-508
- Campbell, I. D., Dwek, R. A., Price, N. C. & Radda, G. K. (1972) *Eur. J. Biochem.* **30**, 339-347
- Graves, D. J. & Wang, J. H. (1972) *Enzymes*, 3rd edn., **7**, 435-482
- Harris, J. I. & Perham, R. N. (1965) *J. Mol. Biol.* **13**, 876-893
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- Perham, R. N. & Anderson, P. J. (1970) *Biochem. Soc. Symp.* **31**, 49-58
- Price, N. C. (1973) *FEBS Lett.* **36**, 351-354
- Smith, I. C. P. (1972) in *Biological Applications of Electron Spin Resonance* (Swartz, H. M., Bolton, J. R. & Borg, D. C., eds.), pp. 483-539, Wiley-Interscience, New York
- Taylor, J. S., Leigh, J. S., Jr. & Cohn, M. (1969) *Proc. Natl. Acad. Sci. U.S.A.* **64**, 219-226
- Zarkadas, C. G., Smillie, L. B. & Madsen, N. B. (1969) *Can. J. Biochem.* **48**, 763-776