IMPROVEMENT OF THE SPIN-LABELLING TECHNIQUE BY THE USE OF A RADIOACTIVE SPIN LABEL

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1. Introduction

The spin-labelling technique, introduced by McConnell [1], has been extensively used to give information on structural and functional aspects of many biological systems [2–4].

There are two distinct problems in these types of studies. Firstly, it is usually very difficult to determine accurately the number of spin labels bound (either covalently or non-covalently) to a macromolecule. When a spin label is bound to a macromolecule, the sharp 3-line spectrum becomes broadened and asymmetric [1]. In some cases it is possible to stimulate approximately the line shape of the bound label by an appropriate increase in the viscosity of the solution of the free spin label. Fig. 1 shows the effect of increasing viscosity on the EPR spectrum of *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamine (ISL). The decrease in signal amplitude is shown by the change in instrument gain indicated. This type of simulation, which could be used for estimation of the bound label, is, however not always possible, and it becomes progressively more difficult as the spectrum is broadened. There are also many examples where the EPR signal clearly arises from two types of bound spin label (one being more mobile than the other), and this creates further difficulties. Double integration of the EPR spectrum could in principle give the amount of bound label, but this is a difficult and not very exact procedure, especially with broad signals.

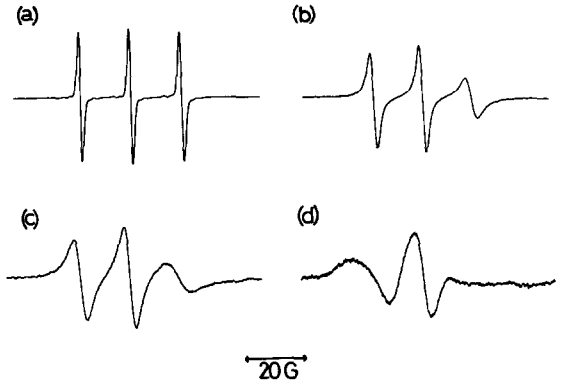

Fig. 1. EPR spectra of ISL (100 μM) as a function of viscosity: (a) in water (23°C); (b) in 95% glycerol (60°C); (c) in 95% glycerol (39°C); (d) in 95% glycerol (23°C). The relative instrument gains were ×1, ×2, ×5, and ×10 respectively.

Secondly, it has only been possible in a few cases to identify the site of reaction of a spin label with a macromolecule. Thus in lysozyme it was shown that His 15 could be labelled, since this is a unique His residue in the enzyme [5]. A more generally applicable method would clearly be desirable.

For these reasons, it was decided to investigate the use of a radioactive spin label. The extent of labelling can be obtained directly from the radioactivity incorporated, and the sites of reaction could be determined by the standard methods of peptide mapping [6]. In this paper the reaction of the spin label with 3 proteins, phosphorylase b, bovine serum albumin (BSA), and rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (G3PDH) is discussed.

Cooke and Morales [7] have referred to the use of a radioactive spin label, but no details were given.

2. Materials and methods

Phosphorylase b was isolated from rabbit muscle

Table 1
Incorporation of spin label into various proteins.

Protein	Labelling conditions	Spin labels incorporated per polypeptide chain	Ratio of peak amplitude to that of free spin label
Phosphorylase b (88 μM)	ISL-C14, 180 μM; 50 mM sodium glycerophosphate; 1 mM EDTA; pH 7.5 (23°C), 17 hr	1.7	0.25
Phosphorylase b (100 μM)	ISL-C14, 180 μM; 50 mM triethanolamine; 100 mM KCl; 1 mM EDTA, pH 7.5 (23°C) 17 hr	1.7	0.26
BSA (130 μM)	ISL-C14, 190 μM; 50 mM Tris–HCl, pH 8.5 (23°C) 17 hr	0.8	0.145
HoloG3PDH (120 μM)	ISL-C14, 190 μM; 100 mM triethanolamine, pH 7.6 (4°C), 17 hr	1.05	0.11
G3PDH previously reacted with iodoacetic acid (120 μM)	As above	0.3	0.18

Protein concentrations are expressed in terms of polypeptide chains.

as previously described [8]. G3PDH was isolated according to the procedure of Ferdinand [9], and was a kind gift of Dr. H.R. Levy and J. Seeley. BSA (crystalline, lyophilised) was purchased from Armour.

ISL (non-radioactive) was a product of Synvar. 4-Amino-2,2,6,6-tetramethyl-piperid-1-yloxy was purchased from R.N. Emanuel, Ltd. Iodoacetic acid-2[^{14}C] (specific activity 28 mCi/mmole) was a product of The Radiochemical Centre, Amersham, Bucks, England.

The radioactive spin label, *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodo[2-^{14}C]acetamide (abbreviated as [^{14}C]ISL, was prepared as follows. Fifty μCi of iodo[2-^{14}C]acetic acid was dissolved in 1 ml of dry dichloromethane, and this solution was transferred to a tube containing 20 mg iodoacetic acid. An equimolar amount of 4-amino-2,2,6,6-tetramethyl-piperid-1-yloxy was added, and then dropwise a 5% excess of dicyclohexylcarbodiimide (in dichloromethane). The reaction mixture was stirred for 5 days in the dark at 4°C, and then filtered to remove the urea formed. Excess solvent was removed by evaporation under reduced pressure. The residue was dissolved in ether, and extracted twice with 0.1 M potassium phosphate buffer, pH 7.0, to remove any traces of the starting materials. (Previous experiments had shown that such extractions would remove over 99% of any starting materials into the aqueous layer). The ether layer was evaporated to dryness, and the residue dissolved in ethanol. This stock solution (about 20 mM) was kept at −20°C in the dark. Crystallisation was not attempted on this small scale, but the product migrated as a single spot in TLC (silica gel) in three different solvent systems; diethyl ether, ethanol/water, 50:50 (v/v), and diethyl ether/petroleum ether (40°C–60°C) 50:50 (v/v), with the R_f values 0.48, 0.82, and 0.07 respectively, in each case identical with non-radioactive ISL from Synvar. In addition the concentration of radioactive compound could be estimated by comparison with the initial solution of iodoacetic acid, and the concentration of free radical by comparison of the EPR spectrum with a standard solution of ISL. The two concentrations were identical within 5%, which is the probable experimental error.

The solution of [^{14}C]ISL showed the broad absorption band centred on 450 nm, typical of nitroxide spin labels ($\epsilon_{450} = 11 \times 10^3$ cm^{2}mole^{-1} in ethanol).

Spin labelling of the protein solutions was carried

out by addition of a small aliquot of the stock solution of [^{14}C]ISL to the protein solution (the final concentration of ethanol was $\leqslant 1\%$). The reaction conditions are summarised in table 1. After reaction, the solutions were dialysed against three changes of buffer to remove unreacted [^{14}C]ISL. Preparations of solutions for counting was performed by taking 50 μl aliquots from the solutions, rinsing these in 0.5 ml H_2O, and adding 10 ml of a 3:1 toluene/Triton X-100 solution containing PPO (5 mg/litre) and POPOP (0.1 mg/litre). Scintillations were counted in a Wallac 81000 liquid scintillation counter. Average counts were about 5000 cpm. The presence of protein at the concentrations employed caused no significant quenching of the scintillations.

EPR spectra were recorded on a JEOLCO JES-PE-IX spectrometer, operating at 9.5 GHz at room temperature (23°C). Some earlier spectra were recorded on a Varian E-3 spectrometer, equipped with a variable temperature accessory.

3. Results and discussion

3.1. *Phosphorylase b*

Phosphorylase b was reacted with [^{14}C]ISL under a number of conditions, with the results shown in table 1. In all cases the EPR spectrum of the bound label appeared to arise from a single type of species (in which the label possessed a significantly higher mobility than the whole protein molecule [10]). From a comparison of the amount of radioactivity incorporated into the protein, it was calculated that the amplitude of the centre peak of the spin label was reduced by a factor of 4.0 of being bound to phosphorylase, compared with free [^{14}C]ISL.

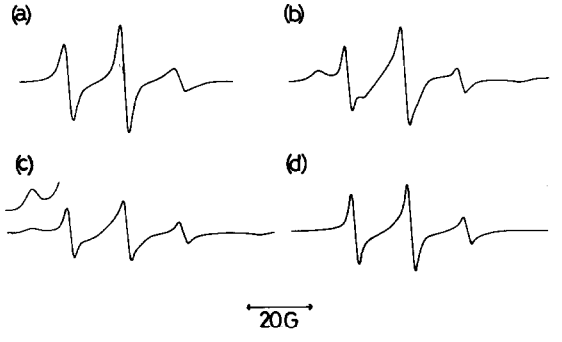

Fig. 2. EPR spectra of spin-labelled proteins: (a) 86 μM spin-labelled phosphorylase b in 50 mM triethanolamine buffer at pH 7.5, containing 100 mM KCl and 1 mM EDTA; (b) 128 μM spin-labelled BSA in 50 mM Tris–HCl buffer at pH 8.5; (c) 120 μM spin-labelled G3PDH in 100 mM triethanolamine buffer at pH 7.6. The inset portion of the spectrum was recorded at 4.4-fold higher gain; (d) 110 μM spin-labelled carboxymethyl-G3PDH in 100 mM triethanolamine buffer at pH 7.6. The extents of spin labelling of these samples are given in table 1. All spectra were recorded at 23°C.

This was confirmed directly in the following way. Equimolar amounts of phosphorylase (subunits) and [^{14}C]ISL were mixed, and the reaction monitored directly in the EPR spectrometer. After 8 hr reaction (at 23°C), the amplitude of the centre peak of the spin label was reduced by a factor of 3.9. This value was also found after dialysis of the solutions, and the incorporation of radioactivity was 100% of that originally added. The shape of the EPR spectrum was identical with that observed in all previous experiments (fig. 2a). This confirms the validity of the radioactive quantitative procedure.

It is of interest that when an approximately 2-fold molar excess of [^{14}C]ISL is added to phosphorylase b more than one spin label with apparently identical EPR spectra can be incorporated. Clearly it would be important to study the rate of incorporation of spin label, and to perform peptide mapping experiments on the products, to check the specificity of labelling at equimolar ratios of [^{14}C]ISL phosphorylase.

3.2. *Bovine serum albumin*

BSA was one of the first proteins to be spin labelled, and the label has been shown to detect the conformational change in the protein which is induced in the pH range 4 to 2 [1]. Under the conditions summarised in table 1, it was found that approx. 0.8 spin labels per polypeptide chain were incorporated. Clearly the spectrum (fig. 2b) contains contributions from a 'mobile' and an 'immobile' spin label. Using the radioactivity as a marker it would be possible to check whether two distinct amino acid residues in the protein were labelled, or whether the two contributions arise from a single labelled residue in two conformational states (as in the case in carboxyhaemoglobin [11]). The amplitude of the centre peak of the EPR spectrum of the bound spin label was 7.0-fold smaller than that of free [^{14}C]ISL, showing that there is substantial contribution of the 'immobile' species to the total signal.

3.3. *G3PDH*

Reaction of holoG3PDH with [^{14}C]ISL gave the results summarised in table 1. Approximately 1.05 groups per subunit were incorporated, and the EPR spectrum (fig. 2c), showed that there were two types of label present as in the case of BSA. The inset to fig. 2c, shows the low field part of the spectrum at higher gain. The centre peak amplitude was reduced by a factor of 9.2 compared with free [^{14}C]ISL, again indicating a significant contribution of the 'immobile' species.

When holoenzyme which had been previously reacted with iodoacetic acid (known to react with the Cys 149 residue [6]), was reacted with [^{14}C]ISL under identical conditions, the extent of incorporation of spin label was 0.3 groups per mole. The resulting spectrum (fig. 2d), shows that the contribution of the 'immobile' component to the total signal has been reduced, and this is supported by the fact that the centre peak amplitude is now a factor of only 5.5 smaller than that of free [^{14}C]ISL. It would appear, therefore, that the Cys 149 residue is one of those that react with the spin label, and that this gives rise to a predominantly 'immobile' signal. The second residue which reacts is also probably a Cys residue, since titrations with Ellman's reagent [12] after denaturation shows a decrease in the SH-content of the enzyme large enough to account for the observed extent of spin labelling.

Peptide mapping experiments to attempt to locate the sites of reaction of the spin label are currently being carried out. A preliminary experiment in which spin-labelled enzyme (prepared as indicated in table 1) was then reacted with excess iodoacetic acid in 8 M urea, showed that most of the radioactivity appeared in a tryptic peptide (or peptides) neutral at pH 6.5.

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