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SPIN LABEL AND LANTHANIDE BINDING SITES ON GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

RAYMOND A. DWEK, H. RICHARD LEVY*, GEORGE K. RADDA and P. JOHN SEELEY
Department of Biochemistry, University of Oxford, Oxford OX1 3QU (U.K.)
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Summary

The electron spin resonance spectrum of rabbit muscle D-glyceraldehyde-3-phosphate dehydrogenase spin-labelled with 4-(2-iodoacetamido)-2,2,6,6-tetramethylpiperidinooxyl has two components. One component is due to a spin label highly immobilized on the enzyme surface and the other to a nitroxyl group able to tumble more rapidly. The spin-labelled enzyme is inactive. Selective modification of the active site cysteine residue (149) and determinations of total sulphydryl content implicate this residue as the site of the immobile spin-label. The mobile spin label is attached to another sulphydryl group.

Crystallographic studies on the human muscle enzyme (Watson, H.C., Duee, E. and Mercer, W.D. (1972) Nat. New Biol., 240, 130) have located a binding site for samarium ion in the active centre. Addition of the paramagnetic gadolinium ion to spin-labelled enzyme reduces the intensity of both the spin label signals (by 72% for the mobile and by 11% for the immobile component). This indicates that the metal ion site (K_d = 0.7 mM) is close to both types of spin label. Measurements of the effect of gadolinium—protein binding on the relaxation rate of solvent water protons enable the enzyme—bound spin label—metal ion distances to be tentatively estimated as 15 Å.

Introduction

The interaction between paramagnetic centres on an enzyme molecule can be quantitatively evaluated by magnetic resonance techniques [1]. Such measurements provide a potentially useful means of defining relations between specific sites on enzymes in solution and of mapping out active site regions and

* Permanent address: Department of Biology, Syracuse University, Syracuse, N.Y. 13210, U.S.A.
Abbreviation: Iodoacetamide spin label, 4-(2-iodoacetamido)-2,2,6,6,-tetramethylpiperidinooxyl.

ligand orientations on enzymes. Paramagnetic centres may be introduced into an enzyme in the form of covalently linked nitroxide radicals (spin labels) and by metal ion substitution [2]. In recent years it has become increasingly clear that there are both experimental and theoretical difficulties in using dipolar interactions for measuring distances in enzymes. It is therefore essential to design experiments where there is a possibility of comparing the structural information derived from magnetic resonance with that obtainable from crystallographic data.

In view of the use of the lanthanide samarium as a heavy atom derivative in crystallographic work on human muscle D-glyceraldehyde-3-phosphate dehydrogenase [3] and the known similarities in the binding properties of different lanthanides we studied the effect of the paramagnetic gadolinium ion on the nitroxide electron spin resonance signal of spin-labelled D-glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12).

Materials and Methods

Glyceraldehyde-3-phosphate dehydrogenase was isolated from rabbit skeletal muscle by a slight modification of the procedure of Ferdinand [4] and had a specific activity of 210 units · mg^{-1} using Ferdinand's assay conditions. The human muscle enzyme was a gift from Dr H.C. Watson, Bristol University, England. Substrates and coenzymes were purchased from the Sigma Chemical Co., St. Louis, Mo., U.S.A. and the Boehringer Corporation, London. The spin labels were obtained from Synvar, Palo Alto, Calif., U.S.A., lanthanides from Koch-Light, Colnbrook, England, iodoacetate and 5,5'-dithiobis-(2-nitrobenzoic acid) from Sigma Chemical Co., tetrathionate from K and K Laboratories Ltd., Plainview, N.Y., U.S.A.

Electron spin resonance (ESR) spectra were recorded on either a Varian E4 or on a JEOL JES-PE-lX X-band spectrometer. Proton relaxation enhancement measurements were carried out at 20 MHz on a spin-echo machine built in the department [5].

The enzyme was spin-labelled in 100 mM triethanolamine hydrochloride at pH 7.6. A 6 mM solution of spin label 4-(2-iodoacetamido)-2,2,6,6-tetramethyl piperidinooxyl (iodoacetamide spin label) in ethanol—water (10 : 90, v/v) was added to a solution of enzyme in buffer so as to give a mole ratio of spin label to subunits of 1 : 1. The reaction was allowed to run from 6 to 22 h at 0°C. Unreacted spin label was removed by dialysis into 50 mM imidazole hydrochloride, pH 6.0. The final dialysate was checked for unreacted spin label by ESR.

Iodoacetate reactions were carried out in 100 mM triethanolamine hydrochloride, pH 7.6 and involved incubation of enzyme with a stoichiometric amount of iodoacetate for 1 h at 0°C. Tetrathionate experiments involved a 15 min preincubation with the reagent at 0°C before addition of spin label. The inhibition by tetrathionate was reversed by adding 180 mM 2-mercaptoethanol and 2 mM NAD (the 2-mercaptoethanol did not reduce enzyme-bound spin label).

Sulphydryl determinations were carried out according to Habeeb's [6] modification of the Ellman procedure [7].

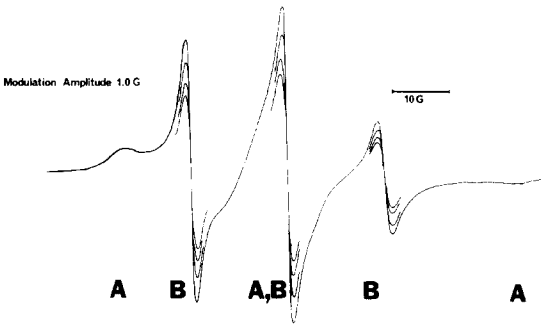

Fig. 1. Spectrum of iodoacetamide spin-labelled rabbit muscle glyceraldehyde-3-phosphate dehydrogenase. The enzyme was spin-labelled in 100 mM triethanolamine—hydrochloride at pH 7.6 by adding a 6 mM solution of iodoacetamide spin label ethanol—water, (10 : 90, v/v) to enzyme in buffer. The mole ratio of spin label to subunits was 1 : 1 and the protein concentration during labelling was 0.77 mM in subunits. Reaction took place over 21 h at 0°C. The enzyme after labelling had 13% of its original activity. Excess iodoacetamide spin label was removed by dialysis into 50 mM imidazole—hydrochloride, pH 6.0. The inner spectra, uncorrected for dilution, represent stages in a Gd^{3+} titration of the spin-labelled enzyme; the final Gd^{3+} concentration was 3 mM.

Results

Fig. 1 depicts the ESR spectrum of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase labelled with iodoacetamide spin label. Two distinct spectral components (A,B) are present, one of which (A) results from a more immobilised spin label than the other (B). Approximately 4 SH-groups per tetramer were modified by the spin label (Table I) (in agreement with studies in this laboratory using a radioactive spin label [8]). The effect of blocking specific SH-groups on the enzyme prior to spin-labelling with iodoacetamide spin label was examined in two experiments. In the first, the enzyme was pretreated with tetrathionate by the procedure of Moore and Fenselau [9]. After reacting this inactivated enzyme with iodoacetamide spin label, the protecting group was removed, giving partial restoration of enzymic activity. In the second experiment, iodoacetate treatment, leading to complete and irreversible inactivation of the enzyme, preceded iodoacetamide spin labelling. Both iodoacetate and tetrathionate are known to react with cysteine 149 in rabbit muscle glyceraldehyde-3-phosphate dehydrogenase [10] and in both experiments sulphydryl titrations showed that approximately four SH-groups per tetramer had reacted. When the enzyme was reacted with iodoacetamide spin label after

TABLE I

SULPHYDRYL DETERMINATIONS BY THE ELLMAN METHOD

Sample	Absorbance at 412 nm	[SH]/[enzyme tetramers]
Native enzyme	0.383 ± 0.003	14.6
+ iodoacetamide spin label	0.287	10.9
+ iodoacetamide	0.278	10.6
+ iodoacetamide + iodoacetamide spin label	0.202	7.7
+ iodoacetamide spin label (after tetrathionate treatment)	0.294	11.2

blocking with either iodoacetamide or tetrathionate in the ESR spectrum the proportion of the immobile component was greatly reduced. In the absence of blocking of the cysteine 149 residue the ratio of immobile to mobile components was also dependent on the amount of spin label used in the reaction mixture. These results, together with parallel studies by Price [8], imply that the immobile component results from reaction with cysteine 149 while the mobile component results from reaction with other SH-groups in agreement with Elek et al. [11].

Fig. 2 shows the ESR spectrum of human muscle glyceraldehyde-3-phosphate dehydrogenase labelled with iodoacetamide spin label. It is broadly similar to the spectrum in Fig. 1, but the mobile component (B) is now only a very small proportion of the total and the spectrum is that of a highly immobilised nitroxide radical. It is perhaps relevant that the human muscle enzyme has one less SH-group per monomer than the enzyme from rabbit muscle. Spin-labelling of both rabbit muscle and human muscle enzyme leads to inactivation which again suggests that cysteine 149 is reacting.

The addition of gadolinium ion (Gd^{3+}) to spin-labelled enzyme results in a marked decrease in the height of the spectral lines without appreciable broadening, subsequently referred to as 'quenching' (Fig. 1). Measurements of the peak heights in the rabbit muscle enzyme for one of the peaks (+1) are shown in Fig. 3a and the corresponding linear plot of $Gd^{3+}{}_{free}$ vs $Gd^{3+}{}_{free}/Gd^{3+}{}_{bound}$ in Fig. 3b. Values for the limiting quenching at infinite Gd^{3+} concentrations were derived from the weighted double-reciprocal plots ($quenching^{-1}$ vs $(Gd^{3+})_t^{-1}$) from the reciprocal of the intercept on the Q^{-1} axis. These Q_{lim} values were used to calculate the linear plots like Fig. 3b, thus providing a refined estimate of the dissociation constant for the enzyme—Gd^{3+} complex. Derived constants are given in Table II.

In order to assess the extent to which the quenching of the spin signal resulted from dipolar interaction between the nitroxide and the paramagnetic Gd^{3+}, a diamagnetic lanthanide ion (La^{3+}) was substituted for Gd^{3+} in the titrations. As seen in Table II, La^{3+} caused a small change in the height of the mobile component of the spin signal suggesting that binding of lathanides to

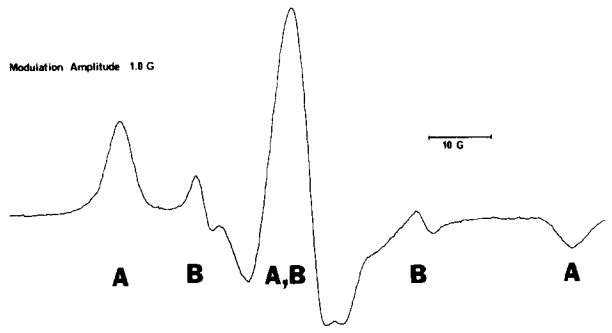

Fig. 2. Spectrum of iodoacetamide spin label-labelled human muscle glyceraldehyde-3-phosphate dehydrogenase. Enzyme was spin-labelled in 100 mM triethanolamine—hydrochloride at pH 7.6 by adding a 6 mM solution of iodoacetamide spin label (in ethanol—water, 10 : 90, v/v) to enzyme in buffer. The mole ratio of spin label to subunits was 1 : 1 and protein concentration during reaction was 0.38 mM in subunits. The reaction ran for 7 h at 0°C. After labeliing the enzyme had 17% of its original activity. Excess spin label was removed by dialysis into 50 mM imidazole—HCl, pH 6.0.

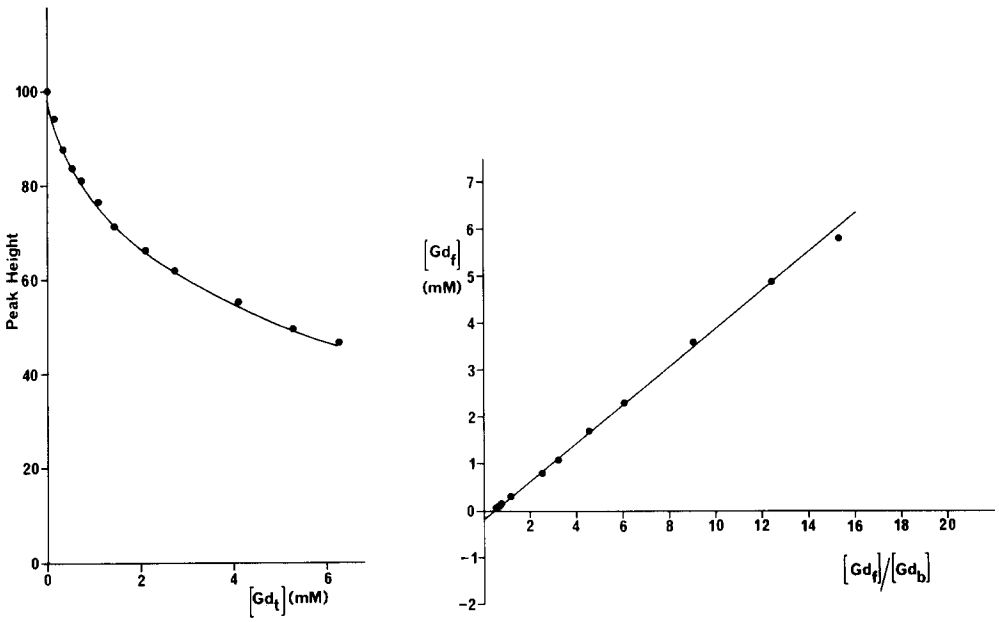

Fig. 3(a). Height of the +1 peak of Fig. 1 vs $|Gd^{3+}|$ total Peak heights have been correlated for dilution using the known proportionality of peak height to concentration of spin-labelled enzyme. The three-line structure of the ESR spectrum of a nitroxide spin label is caused by hyperfine interaction of the unpaired electron with the nuclear spin of the nitrogen-14 nucleus in the N—O bond. Since $I_N14 = 1$ there are three possible components of the nuclear spin along the spectrometer magnetic field: +1, 0, −1, corresponding to the three lines of the spectrum from left to right. The plots are similar for the 0 and −1 peaks. (b) Plot of $|Gd_f|$ vs $|Gd_f|/|Gd_b|$ Ordinate and abscissa calculated from a limiting quenching value (Q_{lim}) obtained from a weighted double reciprocal plot of Q^{-1} vs $|Gd_t|^{-1}$

$$|Gd_b| = (Q\ |enzyme|/Q_{lim})$$

$$|Gd_f| = |Gd_t| - |Gd_b|$$

The dissociation constant is the absolute value of the intercept on the ordinate axis. (b = bound, f = free, t = total)

TABLE II

BINDING CONSTANTS OF La^{3+} AND Gd^{3+} TO SPIN-LABELLED GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE AND THE EXTENT OF QUENCHING OF SPIN LABEL SIGNAL BY La^{3+} AND Gd^{3+}

The numbers in parentheses are the standard errors in the values above them. Data for +1 and immobile peaks. The Q values are limiting quenching values.

	K (mM)		Q_1 (%)		Q_{im} (%)	
	Gd^{3+}	La^{3+}	Gd^{3+}	La^{3+}	Gd^{3+}	La^{3+}
Human muscle	0.5 (0.08)	—	not measured	—	10	—
Rabbit muscle	0.73 (0.04)	1.4 (0.2)	72 (2)	19 (0.8)	11 (0.4)	

glyceraldehyde-3-phosphate dehydrogenase results in some conformational change (at least in the spin-labelled enzyme). Although neither the normal nor esterolytic activities of the enzyme could be studied in the presence of lanthanides (the former because phosphate or arsenate form insoluble precipitates with the metal ions and the latter because the metal ion assisted the non-enzymic hydrolysis of *p*-nitrophenylacetate) the diaphorase activity of the enzyme was unaffected by 15 mM La^{3+}.

Gd^{3+} binding to the enzyme could also be followed by studying the longitudinal relaxation rate of water protons as there was a significant enhancement (with a limiting value, ϵ_b of 6 [1]) of the relaxation rates on addition of enzyme to a solution of Gd^{3+}. Comparison of Gd^{3+} binding in the presence and absence of La^{3+} indicated that these two lanthanides were competing for the same binding sites on the enzyme.

Titrations with Gd^{3+} for the spin-labelled human muscle enzyme gave 10% limiting quenching of the immobile component (i.e. similar to that observed for the same component in the rabbit muscle enzyme where La^{3+} had no effect on this signal) and also gave a similar dissociation constant for the enzyme—metal complex.

Discussion

Our results, together with those of Elek et al. [11] and Price [8], show that even for glyceraldehyde-3-phosphate dehydrogenase, where cysteine 149 is very reactive towards iodoacetamide, spin-labelling does not result in a uniquely modified enzyme. In such situations one can only use the dipolar interactions between the spin label and another paramagnetic centre for mapping out relationships if the ESR signal of the label in different positions is clearly resolvable. Because of the large differences in mobilities of the iodoacetamide spin label label in different positions of the glyceraldehyde-3-phosphate dehydrogenase molecule two different signals are clearly seen. Labelling of the human muscle enzyme is more specific than for the rabbit muscle enzyme.

The paramagnetic Gd^{3+} which bind to the enzyme diminish the line intensities of both types of signal but to different extents. The quenching is inversely related to the separation of the paramagnetic centres on the enzyme [12]. Assuming that the Gd^{3+} ion is rigidly fixed with respect to the nitroxide, and further that any spin label is only relaxed by a single Gd^{3+}, the separation (r) between Gd^{3+} and nitroxide N—O· is given by

$$r = [g\beta^3\mu^2\tau_{1s}/C]^{1/6} \quad (1)$$

where τ_{1s} is the longitudinal relaxation time of the Gd^{3+} electrons, C is a measure of the dipolar Gd—N—O coupling and the other symbols represent the usual constants. In determining an r value it is important to separate out that part of the quenching which is purely dipolar. One advantage in using lanthanides is that the appropriate diamagnetic blank is readily available in the form of La^{3+}. In our case La^{3+} itself quenches the nitroxide signal (for the mobile label) and therefore the limiting dipolar quenching is the difference between the Gd^{3+} and La^{3+} values for a given sample. The diamagnetic La^{3+} effect is very likely the result of a metal ion induced structural perturbation in the

enzyme. It is not possible at present to say to what extent this occurs in the unlabelled enzyme or in the crystalline state (where a samarium derivative is being employed in X-ray structure determination). It is likely, however, that the structural change is not very large since the enzyme catalysed reduction of 1,6-dichlorophenolindophenol by NADH (diaphorase activity) is unaffected by 15 mM La^{3+}. Although partial 'overlap' of the active sites for diaphorase action and oxidative phosphorylation is suggested by the inhibition of diaphorase activity by substrates of oxidative phosphorylation it cannot be inferred that La^{3+} does not interfere with the glycolytic catalysis.

The limiting dipolar quenching for the +1 peak of the label on the rabbit muscle enzyme is 53 ± 3%, which using Leigh's data gives a dipolar coupling constant C of 6.8 ± 0.4 G. The dipolar quenching of the immobilised signal is 11% in the rabbit muscle and 10% in the human muscle enzyme respectively giving the corresponding C values of 0.40 G (± 0.05). The remaining unknown in equation (1) is τ_{1s}, which may be estimated from the limiting proton relaxation enhancement value (ϵ_b) of 6 from the relation:

$$\epsilon_b = q^* f(\tau_c^*)/q f(\tau_c) \qquad (2)$$

where q^* and q are the hydration numbers for Gd^{3+} bound to the enzyme and free in solution respectively, τ_c^* and τ_c are the correlation times for longitudinal water relaxation in the presence and absence of enzyme, $f(\tau_c^*)$ is the correlation function for the frequency of the proton relaxation determinations. Taking the values as $\tau_c = 4.5 \cdot 10^{-11}$ s, $q = 10$, $q^* = 7$ and using a graph of $f(\tau_c)$ vs τ_c [1], $\tau_c^* = 5.3 \cdot 10^{-10}$ s. Assuming that the relaxation is τ_s dominated and thus that τ_c^* is a valid estimate of τ_{1s}, substitution into equation (1) gives r = 14.0 ± 0.2 Å for the mobile nitroxide to Gd^{3+} distance and r = 22.9 Å for the immobile component. Since $\tau_{1s} \geqslant \tau_{2s}$, a lower limit for the distance can be taken from a substitution of τ_{2s} into equation (1). τ_{2s} is obtained from the width of the gadolinium ESR line, which to a first approximation is dominated by a single ± ½ transition [1]. The value of $\tau_{2s} = 1.5 \cdot 10^{-10}$ s. The corresponding distances are 11.3 and 18.5 Å for the mobile and immobile components respectively. The experimental error in these numbers is due to the error in the limiting quenching. The major problem is not this but the uncertainty in the τ_{1s} values and the assumption that the dipoles are fixed rigidly with respect to one another.

The assumption of fixed dipoles is likely to be valid for the immobilised label and therefore we would expect that the active site spin label on glyceraldehyde-3-phosphate dehydrogenase is in the range of 18—23 Å from the lanthanide binding site. The mobile spin label appears to be closer and if the theory applies to the mobile component the range of Gd^{3+} to label distance is 11—14 Å.

In summary, the observed quenching by Gd^{3+} of the spin signal from a nitroxide proximately bound to it on a macromolecule not only extends the range of techniques for monitoring solution conformation and topography but also, in the case of glyceraldehyde-3-phosphate dehydrogenase, should provide a method for comparing the results from the magnetic resonance experiments in solution with those from crystallographic studies.

In this connection it should be noted that comparison of the heavy atom

sites of human and lobster glyceraldehyde-3-phosphate debydrogenase [13] shows that the lobster enzyme site for K_2HgI_4 is within 0.8 Å of the human enzyme samarium site. It has been concluded by Buehner et al [13] that this heavy atom site is "closely related to the cysteine residues 149 and 153 in the active centre".

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References

1 Dwek, R.A. (1973) in NMR in Biochemistry — Some Applications to Enzyme Systems, pp. 395, Clarendon Press, Oxford
2 Mildvan, A.S. and Cohn, M. (1970) Adv. Enzymol. 33, 1—70
3 Watson, H.C., Duée, E. and Mercer, W.D. (1972) Nat. New Biol. 240, 130—133
4 Ferdinand, W. (1964) Biochem. J., 92, 578—585
5 Bell, J.D. (1967) D. Phil. Thesis, Oxford University
6 Habeeb, A.F.S.A. (1972) Methods Enzymol. 25, 457—464
7 Ellman, G.L. (1959) Arch. Biochem. Biophys. 82, 70—77
8 Price, N.C. (1973) FEBS Lett. 36, 351—354
9 Moore, J. and Fenselau, A. (1972) Biochemistry 11, 3762—3770
10 Harris, J.I. and Perham, R.N. (1968) Nature 219, 1025—1028
11 Elek, G., Sajgo, M., Grigorian, G.L., Chibrikin, V.M. and Keleti, T. (1972) Acta Biochim. Biophys. Acad. Sci. Hung. 7, 119
12 Leigh, J.S. (1970) J. Chem. Phys. 52, 2608—2612
13 Buehner, M., Ford, G.C., Moras, D., Olsen, K.W. and Rossmann, M.G. (1974) J. Mol. Biol. 82, 563—585