Spin label studies of the essential sulfhydryl group environment in chicken liver fructose-1,6-bisphosphatase

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The local environment of the essential sulfhydryl groups in chicken liver fructose-1,6-bisphosphatase has been investigated by ESR techniques using a series of iodoacetamide spin labels, varying in chain length between the iodoacetate and nitroxide free radical group. The ESR spectrum of spin-labeled chicken liver fructose-1,6-bisphosphatase showed that the sites of labeling were highly immobilized when the enzyme was chemically modified by spin label iodoacetate, suggesting that the sulfhydryl groups of the protein are in a small, confined environment. From the change in the ESR spectra of these nitroxides as a function of chain length, we conclude that the sulfhydryl group is located in a cleft approx. 10.5 Å in depth.

Spin label *ESR* *Essential sulfhydryl group* *Chicken liver* *Fructose-1,6-bisphosphatase*

1. INTRODUCTION

Fru-P_2ase (EC 3.1.3.11) is one of the key enzymes in gluconeogenesis. With a defect in this enzyme system, the process of gluconeogenesis would be impaired. In man, the metabolic disorder known as 'infantile lactic acidosis' was found to be associated with hepatic Fru-P_2ase deficiency [1,2]. During the past 3 decades, Fru-P_2ase has been isolated from different sources. Among these, the enzyme from rabbit liver has been most extensively investigated. Unfortunately, studies on purified rabbit liver Fru-P_2ase prior to 1971 were limited to a modified form of the enzyme which showed an optimum activity at alkaline pH [3]. In 1971, Traniello et al. [4] were the first to report the isolation of the native form of rabbit liver Fru-P_2ase which, like the enzyme in crude extracts, showed an optimum activity at neutral pH. Physicochemical studies have indicated that the native enzyme is a tetramer composed of identical subunits of M_r 35000 [5,6]. Nimmo and Tipton [6] have shown that the modification of the sulfhydryl groups of 'neutral' bovine liver Fru-P_2ase by *p*-chloromercuribenzoate led to 60% stimulation of enzyme activity. From the effect of the pH on the kinetic properties of the 'alkaline' rabbit liver Fru-P_2ase [7], it was concluded that a cysteine residue per monomer in the enzyme with a pK_a of 8.6 is catalytically important in the breakdown of Fru-1,6-P_2. Chatterjee et al. [7] have shown that DTNB, iodoacetamide, and phenylacyl bromide react with highly reactive sulfhydryl groups of pig kidney Fru-P_2ase and cause stimulation of the enzyme when Mn^{2+} is used as an activating cation.

This study was undertaken to clarify the location of the sulfhydryl group by employing a series of spin labels, varying in chain length between the nitroxide and the attaching group, as reporter groups to probe the local environment of the sulfhydryl groups. This 'molecular dipstick' approach takes advantage of the effect that molecular motion has on the ESR spectrum of the nitroxide group.

Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Fru-P_2ase, fructose-1,6-bisphosphatase; Fru-1,6-P_2, fructose 1,6-bisphosphate

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2. MATERIALS AND METHODS

A series of iodoacetate spin labels (see table 1 for chemical structures) were obtained from Aldrich. Highly purified enzyme was prepared by the method of Han and Johnson [8]. Fru-1,6-P_2, glucose-6-phosphate dehydrogenase, phosphoglucoisomerase and other chemicals were purchased from Sigma. The other common reagents used were of high grade quality.

The activity of Fru-P_2ase was measured spectrophotometrically at 25°C by following the rate of reduction of NADP at 340 nm in a coupled reaction. The assay mixture (1 ml) contained 50 mM Tris-HCl buffer (pH 7.5), 2 mM $MgCl_2$, 0.05 mM EDTA, 0.15 mM NADP, 0.1 mM Fru-1,6-P_2, 1 unit each of phosphoglucose isomerase and glucose-6-phosphate dehydrogenase, and an appropriate amount of native or modified Fru-P_2ase. The concentration of purified Fru-P_2ase was determined by its extinction coefficient at 280 nm [8]. Modification of Fru-P_2ase with the spin-labeled iodoacetate series was carried out under the conditions described in the figure legends. The number of reactive sulfhydryl groups per Fru-P_2ase molecule before and after reaction with the iodoacetate spin label series was determined by measuring the absorbance produced at 412 nm by reaction with Ellman's reagent (DTNB); $E = 13600\ M^{-1} \cdot cm^{-1}$ for the reduced form of the native or spin-labeled enzyme.

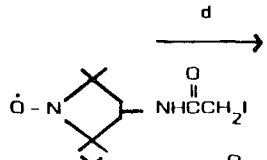
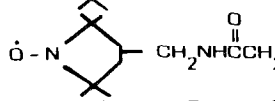
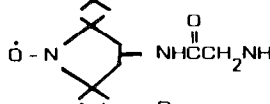
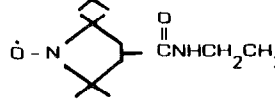
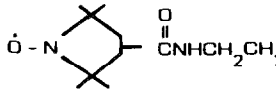
All ESR spectra were recorded with a Varian E-4 operating at 9.1 GHz. The cavity temperature was 25 ± 1°C. The effective rotational correlation time in the slow tumbling region (10^{-9}–10^{-7} s) was estimated by comparing the experimental ESR spectra with stimulated spectra [9]. The effective rotational correlation time in the fast tumbling time region (10^{-11}–10^{-9} s) was determined using the equation given in [9]:

$$1/\tau = 3.6 \times 10^9/\Delta H_0[(H_0/H_{-1})^{1/2} - 1]\ s^{-1}$$

where H is the peak-to-peak width of the central field line (in G), and H_0 and H_{-1} are peak-to-peak heights of the central-field and high-field lines, respectively.

Table 1

Types and length of nitroxide spin labelled sulfhydryl reagents used

Designation	Structure	Systematic name	Length (Å)
I		3-(2-Iodoacetamido)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl	5.1
II		3-[(2-Iodoacetamido)methyl]-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl	6.9
III		3-[2-(2-Iodoacetamido)acetamido]-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl	8.7
IV		3-[[3-(2-Iodoacetamido)propyl]carbamoyl]-tetramethyl-1-pyrrolidinyloxyl	11.2
V		3-[[2-[2(2-Iodoacetamido)ethoxy]ethyl]carbamoyl]-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl	13.5

3. RESULTS AND DISCUSSION

3.1. *Modification of the enzyme by spin-labeled iodoacetamide*

The possible role of sulfhydryl groups in the regulation of fructose bisphosphatase was the subject of earlier work [4–7]. Studies of the properties of several Fru-P_2ases modified at the reactive sulfhydryl group suggest that the cysteine residue is not located at the active or allosteric site of the enzyme [7]. A number of nitroxide-containing spin-labeled sulfhydryl reagents have now been synthesized [10]. In the experiment to be described, chicken liver Fru-P_2ase was reacted with 3-(2-iodoacetamido)-2,2,5,5-tetramethyl-1-pyrolidinyloxyl, as described in section 2, and the spectrum obtained is shown in fig.1. The nitroxide reporter group has a maximum splitting value of 67 G at 25°C which is indicative of cysteine residues buried in a deep pocket. The ESR spectral data confirm that the nitroxide group is in a restricted environment and has a rotational correlation time of 2.0×10^{-8} s [10,11], i.e. the motional freedom of the nitroxide group is no greater than that expected for the entire enzyme molecule [11,12]. Under the experimental conditions only one essential sulfhydryl group per monomer (4 per tetramer) was labeled with spin-labeled iodoacetamide.

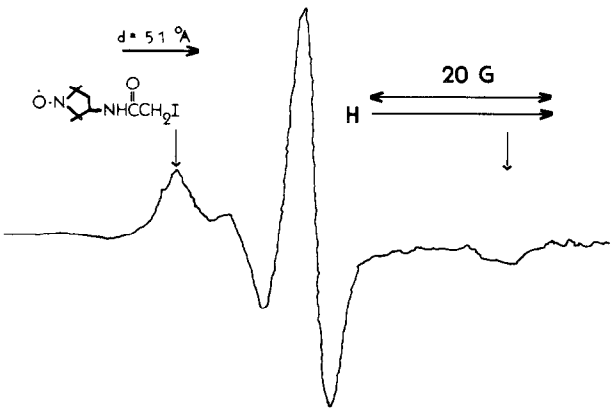


Fig.1. ESR spectrum of chicken liver Fru-P_2ase spin labeled with 3-(2-iodoacetamido)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl. About 1 mg enzyme was incubated with the spin label in 1 ml of 0.05 M potassium phosphate, pH 7.5, and reacted for 18 h at 25°C in a nitrogen atmosphere. The final concentration of spin label added was 30×10^{-4} M. The enzyme solution was exhaustively dialyzed and the ESR spectrum taken as described in section 2. Arrows indicate the location of the low- and high-field peaks that are characteristic of a highly immobilized nitroxide radical.

3.2. *Modification of the enzyme by nitroxide-containing iodoacetamide analogues*

The first requirement in the molecular dipstick technique is that the particular residue of interest be in a restricted environment as indicated by the presence of a highly immobilized spectrum. Fru-P_2ase in which the essential cysteine residue (one per monomer) was reacted with 3-(2-iodoacetamido)-2,2,5,5-tetramethyl-1-pyrolidinyloxyl met this requirement. We have attempted to probe the environment of the essential sulfhydryl group using iodoacetamide derivatives (I–V) (table 1) of varying lengths between the reactive portion and the nitroxide reporter group.

The insertion of a single methylene group between the iodoacetamide and nitroxide group (see spin label II in table 1) decreases the maximum splitting value from 67 to 64 G (fig.2a,b), indicating an increase in motional freedom of the nitroxide group of spin label II bound to chicken liver Fru-1,6-P_2ase. As the chain length progressively increases (spin labels II–IV, figs 2 and 3) the spectrum becomes narrower and more symmetrical, suggesting that the nitroxide group emerges from a restricted environment to one where it rotates relatively freely. The rather gradual increase in mobility along the series (I–IV) suggests that the essential sulfhydryl group of chicken liver Fru-P_2ase is located in a cleft-like environment.

A plot of the effective rotational correlation times of the spectra in fig.3 vs chain length reveals a break at 10.5 Å as shown in fig.4. The distance corresponding to this break has been interpreted as the minimum depth of the cleft [13,14]. The essential sulfhydryl group of chicken liver Fru-P_2ase is therefore most likely located in a cleft-like environment of 10.5 Å in depth. Insertion of spin labels I–V had no effect on both the near- and far-UV CD (not shown). It is concluded that the functional and structural integrity of the enzyme is preserved under the conditions used here.

Chicken liver Fru-P_2ase contains 1–2 sulfhydryl groups per monomer which may be located at dif-

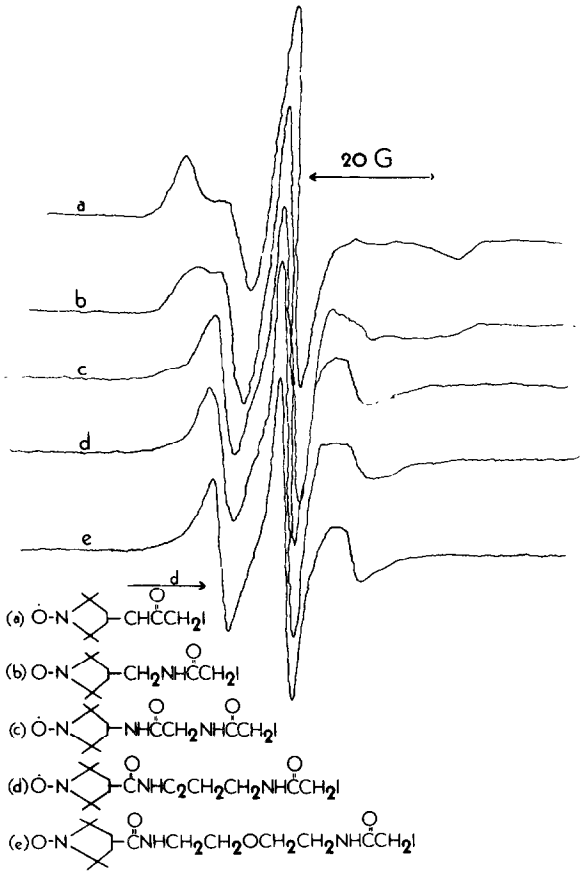


Fig.2. ESR spectrum of iodoacetamide spin-labeled chicken liver Fru-P_2ase (spin labels I–V). The exact conditions are described in the legend to fig.1 and were used to prepare the derivatives in which spin labels I–V were separately reacted with the enzyme.

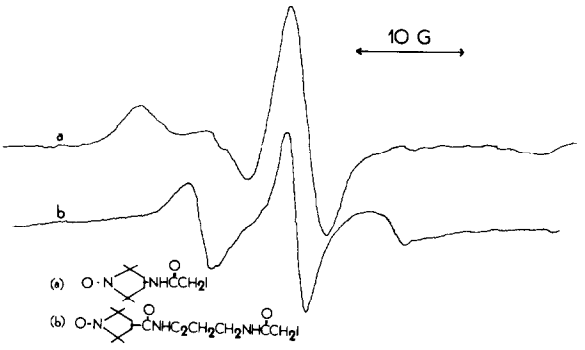


Fig.3. ESR spectrum of chicken liver Fru-P_2ase spin labeled with spin label I (5.1 Å), and spin label IV (11.2 Å).

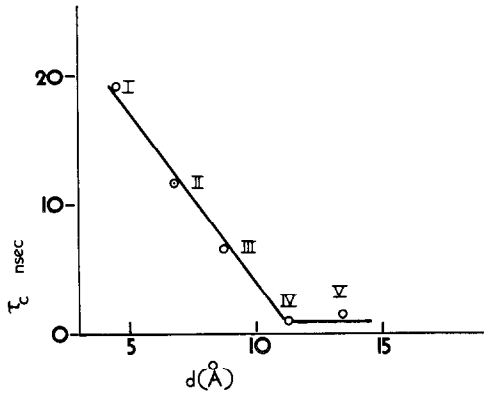


Fig.4. Rotational correlation time (in ns) vs chain lengths of iodoacetamide spin labels.

ferent sites. Modification of these sulfhydryl groups with iodoacetamide spin labels of various chain lengths, namely, spin labels I–V in table 1, revealed only a single-component ESR spectrum (fig.2), arguing that these essential sulfhydryl groups (4 per tetramer) are in similar environments.

The presence of the essential sulfhydryl group in a cleft about 10 Å deep is not unique to chicken liver Fru-P_2ase. The possible role of sulfhydryl groups in the regulation of Fru-P_2ase was the subject of earlier work [4–7]. The sulfhydryl groups in the enzyme have been suggested to be involved as a catalyst in the formation of a disulfide interchange isomer [15]. The exact function of the essential sulfhydryl groups of chicken liver Fru-P_2ase remains a problem for further investigation.

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