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ESR study of 5′-nucleotidase from bull seminal plasma

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5′-Nucleotidase of bull seminal plasma has been spin labeled with the sulfhydryl reagent 3-maleimidoproxyl. ESR analysis reveals the presence of two classes of labeled sites. The first is characterized by a long spin label rotational correlation time, from which a protein diameter of about 70 Å can be estimated, under the assumption of a spherical shape. The second class is characterized by a shorter correlation time of the covalently bound spin labels and binding of the substrate sodium thymidine 5′-monophosphate to 5′-nucleotidase results in a reduction of their mobility. Low-temperature ESR analysis shows that no paramagnetic ion is bound to the native protein.

1. Introduction

5′-Nucleotidase (EC 3.1.3.5) is a plasma membrane glycoprotein which hydrolyses nucleoside 5′-monophosphates to their corresponding nucleosides. Immunological evidence [1] and its apparent interaction with cytoskeletal elements attached to the plasma membrane [2,3], as well as with extracellular matrix proteins such as laminin and fibronectin [4], have indicated a transmembrane orientation for 5′-nucleotidase, which has proved to be an ectoenzyme with its active center located toward the exterior of the plasma membrane [1]. Investigations carried out for identification of the nature of the amino acids of the active site by chemical modification of protein side chains have indicated that a cysteine residue is essential for activity of 5′-nucleotidase from the cytoplasmic fraction of bovine brain cortex [5] and rat liver cytosol [6]; in contrast, it was observed that thiol groups were not implicated in the full expression of activity of plasma membrane 5′-nucleotidase from pig lymphocyte [7], rat heart [8] and bovine liver [9,10].

In order to obtain information both on the structure of the protein and on its interaction with some substrates and inhibitors, we have undertaken an ESR study on the dynamical properties of spin-labeled 5′-nucleotidase of bull seminal plasma. This enzyme is a dimeric glycoprotein of 160 kDa and some of its molecular and kinetic properties have already been investigated in our laboratory [11–13]. The spin label used was 3-maleimidoproxyl (3MAL), which, under the experimental conditions adopted, proved to be a satisfactorily specific reagent for spin labeling of the protein-free sulfhydryls. The results obtained indicate that six SH groups per molecule of enzyme are available for covalent binding of the spin label. The ESR spectrum allowed us to measure two very different rotational correlation times (τ_c) for the bound spin label. Analysis of these dynamical parameters, combined with enzyme activity assays, indicates that (i) covalent binding of

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3MAL molecules does not impair the enzymatic activity; (ii) the overall diameter of the dimeric protein is about 70 Å; (iii) TMP binding results in a structural change of the protein.

Finally, low-temperature ESR analysis on 5′-nucleotidase revealed that no paramagnetic ion is bound to the native protein molecule.

2. Materials and methods

2.1. Materials

3MAL was purchased from Syva, Palo Alto. Nucleotides, concanavalin A (Con A) and lectins from *Lens culinaris* (LcH) were obtained from Sigma. Adenosine deaminase (EC 3.5.4.4) was purchased from Boehringer Mannheim. 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) and 4-(chloromercuri)benzoic acid (*p*-HMB) were supplied by Fluka. Amicon type YM30 ultrafiltration membranes were obtained from Grace Italiana. 5′-Nucleotidase of bull seminal plasma was prepared as described previously [11]. Final protein preparations were stored at 4°C in 50 mM Tris-Cl (pH 7.5), containing 0.1 M NaCl, 50 mM sodium cholate and 30% (w/v) glycerol. Protein concentration ranged between 6.5 and 8 mg/ml. The specific activity amounted to 240 U/mg.

2.2. Assay

The 5′-nucleotidase assay was carried out in 50 mM Tris-Cl (pH 7.4), at 25°C, using 5′-AMP as substrate and by continuously recording the absorbance decrease, at 265 nm, due to the conversion of adenosine into inosine, according to Ipata [14]. This assay procedure was used to determine 5′-nucleotidase activity in the absence or presence of 3MAL and the inhibitors ADP, ATP, Con A and LcH. The standard assay mixture contained, in a final volume of 2 ml: 1.5 ml Tris-Cl buffer, 20 ng enzyme, 1 μg adenosine deaminase. The reaction was started by the addition of AMP to a final concentration of 100 μM. Assays in the presence of inhibitors were carried out by adding to the standard assay mixture ADP or ATP to a final concentration of 5 μM, resulting in over 90% inhibition, or Con A or LcH to a final concentration of 0.5 and 5 μM, respectively, giving 90% inhibition. The reaction was initiated by adding the substrate AMP to the reaction mixture, which had been previously incubated for 10 min.

The assay procedure used to determine the enzyme activity with substrates different from AMP measured the P_i released upon their hydrolysis. The assay was carried out in a final volume of 0.6 ml, containing 50 mM Tris-Cl (pH 7.4), 3.5 mU 5′-nucleotidase and varying amounts of substrates to check linearity. After incubation at 37°C for 5 min, the reaction was stopped by the addition of 0.6 ml of 10% (w/v) perchloric acid solution. The mixture was chilled in ice, centrifuged if necessary, and 0.6 ml were added to a solution containing one part 10% ascorbic acid and six parts of 0.42% ammonium molybdate in 0.5 M sulfuric acid. The mixture was incubated at 45°C for 30 min and, after cooling, the absorbance was read at 820 nm against blanks, according to Chen et al. [15].

1 unit of activity corresponds to the formation of 1 μmol of inosine or P_i per min.

Protein concentration was determined by using commercial protein assay kits from Bio-Rad.

2.3. Spin labeling of 5′-nucleotidase

5′-Nucleotidase (4 mg/ml) was incubated overnight at 4°C with a 50-fold molar excess of 3MAL in 50 mM Tris-Cl (pH 7.0), containing 0.1 M NaCl, 50 mM sodium cholate and 30% (w/v) glycerol (buffer A). A control protein sample was incubated in the absence of spin label, under the same conditions, and used for checking enzymatic activity and the extent of spin labeling. Spin-labeled 5′-nucleotidase was separated from excess reagent by ultrafiltration through a YM30 membrane under a stream of nitrogen (at 50 lb/inch^2) in a 10 ml Amicon cell, which was refilled several times with buffer A. The absence of free reagent in the concentrated protein preparations was checked by ESR on the diffusate.

The extent of the reaction of 3MAL with protein sulfhydryls was assessed by titration with either DTNB and *p*-HMB of the free thiols in the protein incubated in the presence or absence of

the spin label. Typically, 50-μl aliquots of authentic or spin-labeled 5'-nucleotidase were mixed in a 1 cm light path cell with 720 μl of 0.1 M Tris-Cl buffer (pH 8.0), containing 0.01 M EDTA and 1% SDS. 30 μl DTNB in 0.05 M sodium phosphate buffer (pH 7.0) were then added to the cell and the absorbance increase at 412 nm was recorded for 45 min. The thiol concentration was obtained by extrapolation to zero time of the downward sloping line by using an ϵ_M of 13600 for the 3-carboxylato-4-nitrothiophenolate, according to Jatanova et al. [16]. Alternatively, the determination of free sulfhydryls in native and 3MAL-derivatized 5'-nucleotidase was performed by using *p*-HMB. The extent of the reaction was determined from the changes in absorbance at 255 nm. Typically, 50 μl of native or 3MAL-labeled 5'-nucleotidase were mixed with 1.9 ml of 0.08 M sodium phosphate buffer (pH 7.0). 10-μl aliquots of stock *p*-HMB solution (0.4 mM) were then added to a reference 1 cm light path cell containing 1.95 ml buffer and to the sample cell. The change in absorbance at 255 nm was recorded until no further change was observed. The equivalence point was determined by plotting the absorbance values against the amount of added *p*-HMB and the protein thiol content was estimated from the known concentrations of the protein and the reagent.

2.4. ESR measurements

To run ESR spectra at controlled room temperature, the aqueous samples were introduced into capillary tubes (1 mm inner diameter). The capillary sample was then placed in a standard ESR tube, which was in turn placed in the ESR variable-temperature Dewar insert filling the central hole of the resonance cavity. ESR spectra were recorded on a Varian E-109 X-band spectrometer. Instrumental settings which gave optimum sensitivity with least distortion of line shape were 1.25 G modulation amplitude, 10 mW incident microwave power, 100 G scanning in 8 min at 0.250 s time constant. Quantitative determinations of spin label species were performed by double integration of the ESR spectra after comparison with calibrated paramagnetic standards. ESR data acquisition was performed on an HP 86A microcomputer, through a home-made interface connected to an IEEE 488 bus. Sample viscosity measurements were performed with an Ostwald-type viscosimeter.

Low-temperature ESR measurements were conducted as reported in ref. 17.

3. Results

The determination of free sulfhydryls in native and 3MAL-labeled 5'-nucleotidase of bull seminal plasma gave substantially coincident results with the two reagents used, DTNB and *p*-HMB. The number of free sulfhydryl groups per molecule of protein determined by using DTNB was 6 ± 0.5 and 0.4 ± 0.08, respectively, for the control and spin-labeled enzyme. On the other hand, with *p*-HMB the number of SH groups per molecule of enzyme in the control and spin-labeled enzyme was 5.7 ± 0.6 and 0.5 ± 0.06 (values represent means $\pm$ S.D. of 7 determinations). Since we determined, a total of six molecules of bound spin label per molecule of enzyme by ESR analysis, it turns out that 3MAL in our case proved to be a satisfactorily specific reagent of protein free sulfhydryls. The ESR spectra at 20°C of 3MAL in buffer A and of the same spin label covalently bound to 5'-nucleotidase are shown in fig. 1a and b, respectively. The spectrum shown in fig. 1a corresponds to rapidly tumbling spin labels, characterized by a rotational correlation time of $\tau_c < 10^{-11}$ s and displaying isotropic values, g_{iso} and A_{iso}, for the g and A tensors [18]. Spectra of the type depicted in fig. 1b are a composite of two types: one, a three-line isotropic spectrum, is due to a fast moving or weakly immobilized (labeled W) spin label, while the other is an anisotropic signal arising from strongly immobilized spin labels (labeled S) tumbling in a slow motional domain [17,18]. In order to determine the number of spin label molecules bound to one molecule of 5'-nucleotidase, we simulated spectrum b of fig. 1 according to the method reported in ref. 19, using a calibrated concentration of 3MAL. In this way we found that six molecules of spin label were bound per molecule of 5'-nucleotidase, four of

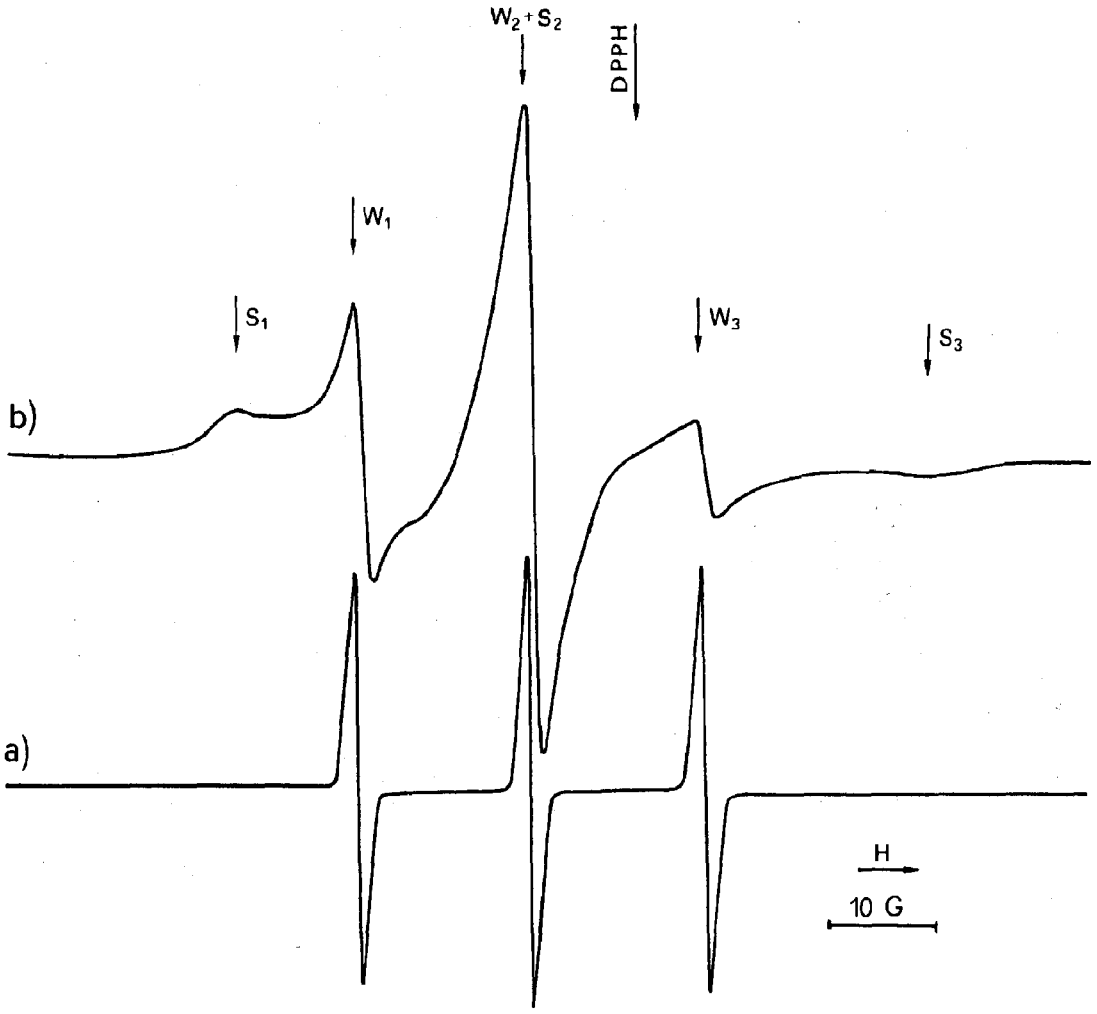

Fig. 1. Room temperature (20 ° C) ESR spectrum of (a) 3MAL spin label and (b) 3MAL spin-labeled 5'-nucleotidase.

which turned out be weakly immobilized, the remaining two being strongly immobilized. In this respect, since the enzyme is probably composed of two identical subunits of about 80 kDa [11], it is likely that each subunit might contain, respectively, two sites of weak and one site of strong immobilization of the spin label. The spectral components referring to the strongly immobilized spin label reflect a situation in which the spin label tumbles with the same rotational correlation time as that of the overall protein [17–19]. In this case and under the hypothesis that the protein can be approximated by a sphere of radius R rotating in a medium of viscosity η, the Stokes-Debye-Einstein relationship:

$$\tau_c = \frac{4\pi\eta R^3}{3k_B T} \quad (1)$$

connects this radius with the above-mentioned rotational correlation time, which can be extracted from the ESR spectrum.

To estimate τ_c we used the approximate expression provided by Freed [21]:

$$\tau_c = a\left(1 - \frac{S_z'}{S_z}\right)^b \quad (2)$$

Where S_z' is one half of the separation between the outer hyperfine extrema (S_1 and S_3) measurable in fig. 1b); S_z is equal to this separation in the rigid limit value; a and b are constants depending on the specific reorientation model assumed. In our case, using $a = 5.4 \times 10^{-10}$ and $b = -1.36$, we obtained $\tau_c = 1.6 \times 10^{-7}$ s, which, when inserted into eq. 1, gives $R = 35$ Å, thus providing an estimate for the overall size of 5'-nucleotidase. On the other hand, analysis of experimental spectral features (W_1 and W_3) due to the weakly immobilized spin labels bound to 5'-nucleotidase may lead to an estimate of their rotational correlation time in agreement with the expression given by Likhtenshtein [22]:

$$\frac{1}{\tau_c} = \frac{1.2 \times 10^9}{\left(\sqrt{\frac{h(+1)}{h(-1)}} - 1\right)\Delta H(+1)} \ \mathrm{s}^{-1} \qquad (3)$$

where $\Delta H(+1)$ is the width (in G) of the line furthest downfield (W_1 of fig. 1b), and $h(+1)$ and $h(-1)$ denote the intensities of the lower and upper field weakly immobilized lines (W_1 and W_3, respectively).

We obtained a value of $\tau_c = 1.5 \times 10^{-9}$ s for the 3MAL-5'-nucleotidase system. This value indicates that the spin labels are bound to 5'-nucleotidase molecules at sites which allow the labels to retain a large degree of rotational freedom with respect to the tertiary structure of the protein. Moreover, since the g and A values measured for the isotropic component of the 3MAL spectrum are consistent with a spin label situated in a polar medium [18], it can be inferred that these SH groups are located at the surface of the molecule and that they are freely exposed to the solvent. It transpires that these kinds of bound spin labels are very sensitive reporters of the microenvironment around their binding sites. In other words, even a slight change in their degree of rotational freedom, such as that induced, for example, by a conformational modification of the macromolecule, or subsequent to a molecular interaction with a substrate or inhibitor, would immediately be registered as a change in the width and height of ESR lines, and hence in the correlation time. In this connection, we submitted our system to interaction with the substrates AMP, CMP, GMP, TMP and UMP, and the inhibitors ADP, ATP, Con A and LcH. The results reported in table 1 show that the addition of TMP produced the greatest change (about 20%) in rotational correlation time of the weakly immobilized spin labels. However, the possibility cannot be excluded that a non-reported interaction could also occur with the other substrates tested.

Table 1

Rotational correlation time of 5'-nucleotidase 3MAL immobilized sites in the presence of substrates and inhibitors

Values represent means ± SD of five determinations. (i) Inhibitor.

Effector	τ_c (s) ($\times 10^9$)	% variation
None	0.83 ± 0.08	–
TMP	1.02 ± 0.11	19
GMP	0.96 ± 0.09	13
IMP	0.93 ± 0.09	10
AMP	0.87 ± 0.09	4
ADP (i)	0.84 ± 0.07	1
ATP (i)	0.82 ± 0.08	1
Con A (i)	0.85 ± 0.08	2
LcH (i)	0.83 ± 0.08	–

Finally, the kinetic parameters K_m and V_{max} of the spin-labeled protein, determined in the presence of the above-mentioned substrates, were not significantly different from those of the unlabeled enzyme, thus indicating that at least the spin-labeled thiol groups are not essential for 5'-nucleotidase activity.

4. Discussion

The use of the spin-labeling approach in studying the molecular dynamics of 5'-nucleotidase has provided us with a number of interesting pieces of information. First of all, the results obtained indicate that, under the conditions adopted, six sulfhydryl groups per molecule of enzyme are available for the binding of 3MAL, without any significant impairment of the enzymatic activity. Moreover, the differing rotational dynamics of the SH groups which are able to bind 3MAL reflect an inequivalence in the constraints imposed by folding of the polypeptide chain on the motion of the

spin label. The rotational correlation time of the strongly immobilized label measured can, to a good approximation, reflect the overall correlation time of the molecule and, under the assumption of a spherical shape, a diameter of about 70 Å can be attributed to the molecule. A similar value can also be obtained by using the expression:

$$D = 2(3M\bar{v}/4\pi N)^{1/3} \quad (4)$$

where a molecular mass of 160 kDa and a partial specific volume of 0.722 cm^3/g were assumed for the dimeric globular protein of spherical shape. It thus appears that the diameter of the 5′-nucleotidase molecule is of the same order as the thickness of the membrane bilayer, thus supporting the possibility of a transmembrane orientation of the protein, as was previously reported for 5′-nucleotidase from different sources [1–4].

The weakly immobilized spin labels, very likely located at sites exposed to polar solvent, provide useful information on the interaction of 5′-nucleotidase with TMP. This substrate is able to modify the microenvironment of these labels, as can be observed from the significant modification of the rotational correlation time. We consider the τ_c variations obtained with the other 5′-nucleotidase effectors as being within experimental error and are not commented upon further. We have previously reported [11] that TMP shows a K_m value lower than that of AMP (5–9 μM vs. 11–18 μM) and the ratio V_{max}/K_m (23 for AMP and 5 for TMP), which is often assumed as a parameter for evaluation of substrate specificity, indicates, however, that even if TMP has a higher affinity for 5′-nucleotidase (lower K_m value) AMP is the better substrate (V_{max}/K_m ratio). The significant increase in τ_c observed in the presence of TMP does not rule out the possibility that the interaction of TMP with 5′-nucleotidase could also occur at sites different from those involved with other substrates.

ESR analysis carried out at low temperature has shown that no paramagnetic metal ion is bound to 5′-nucleotidase of bull seminal plasma. Preliminary results from atomic absorption spectroscopy indicate that two zinc atoms are bound to one molecule of protein (unpublished results). Zinc was also found to be bound to 5′-nucleotidase of human lymphoblast [23], while magnesium or manganese were proposed for 5′-nucleotidase of bovine liver plasma [9] and it was also proposed that the bound metal might play a role in the catalytic process [10]. Following our procedure of ESR analysis, we are now investigating whether zinc could actually be involved in the active site of the seminal enzyme and, for this purpose, the substitution for this diamagnetic metal ion with a paramagnetic one, for example, Mn^{2+} or Cu^{2+} will provide information both on the role of metal and on the geometry of the active site.

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