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ESR STUDY OF FREE AND IMMOBILIZED ELASTASE

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Summary

Porcine pancreatic elastase (EC 3.4.21.11) has been immobilized on polyacrylamide beads using glutaraldehyde ad bridging reagent without important loss of catalytic activity. A nitroxide spin label, 1-oxyl-2,2,5,5-tetramethyl-4-piperidinyl-ethylphosphonofluoridate, reacting covalently with the serine-195 residue of the active centre of free elastase was used as a conformational and dynamical electron spin resonance probe. This signal is quenched by (Cu^{2+}) which bind specifically at the active site at a distance of 7 Å from the nitroxide group. This distance is not significantly affected by the fixation on the solid support.

The electron spin resonance lineshape analysis indicates some mobility of the spin label with respect to the native protein. This restricted motion, which is pH dependent, is not noticeably modified by the immobilization of the enzyme. This immobilization has therefore induced no large conformational change of the protein in the vicinity of the active centre.

Thermal denaturation of elastase in homogeneous solution is irreversible. Immobilization on the polyacrylamide beads results in 70% reversibility, but the temperature of denaturation is not modified.

Introduction

Enzymes immobilized on solid matrices provide stabilized and water insoluble systems which can be used for practical applications [1]. The catalytic properties of such heterogeneous systems compared to those of the corresponding homogeneous solutions may also be an approach to the understanding of the properties of intracellular enzymes, particularly the membrane bound polyenzymatic systems in which each protein is specifically localized

with respect to the others. Measurements of the catalytic activity for specific substrates are not sufficient for a complete description of the effects of immobilization. Other methods providing information about changes of conformation of the protein, its orientation on the matrix, its relative mobility and the accessibility of the active site, must be used in order to understand the modifications of the intrinsic activity. Spectroscopic probes can be used for this purpose. Fluorescence measurements may provide indirect information about conformation changes [2]. The binding of a spin label at the active centre of the enzyme and the investigation of its anisotropic motions by electron spin resonance (ESR) may provide a more direct approach to the nature of its environment within the protein, and the overall orientation and mobility of the labeled system [3]. More information about the topology of the active centre can be obtained if a paramagnetic metal ion having sufficiently long electron relaxation time interacts with the enzyme at a specific position, resulting in changes of intensity of the spin label signal [4]. We report in the present paper the ESR investigation of spin labeled pancreatic elastase in solution and bound to polyacrylamide beads.

Materials and Methods

Binding of elastase on polyacrylamide beads. Glutaraldehyde forms unsaturated oligomers [5,6] which react with the aminogroups of proteins and of polyacrylamide forming the cross links and the bridges necessary for enzyme immobilization [7]. Polyacrylamide beads (Biogel P 300, 100—200 mesh) were hydrated for 24 h at 4°C, then incubated overnight in 10% glutaraldehyde in 0.05 M phosphate buffer (pH 7.5), and finally washed thoroughly with distilled water. Crystalline porcine pancreatic elastase (EC 3.4.21.11) was prepared from pancreatic extracts (Trypsin 1—300 from Nutritional Biochemicals, Cleveland, OH), according to the procedure described by Shotton [8]. Its activity was measured optically at 410 nm with the specific substrate succinyl-trialanine-*p*-anilide [9] purchased from Gipep (France) and its purity was determined by comparison with the protein concentration measured by absorption at 280 nm ($\epsilon_{280} = 5.23 \cdot 10^4\ M^{-1} \cdot cm^{-1}$). It was found to be better than 90%. The pure enzyme was allowed to react with the activated beads for 12 h at 4°C. The preparation was finally washed with 0.2 M glycine buffer (pH 7.5). The extent of the reaction was followed by the measurement of the absorbance of the supernatant at 280 nm and by measurement of the final enzymatic activity of the treated beads.

Enzyme kinetics. The inhibitor action of Cu^{2+} was investigated at 25°C by measuring the effect of increasing amounts of inhibitor upon the activity of a 30 nM solution of elastase in the presence of various concentrations of substrate. The nature and the constant of the inhibition reaction were then analyzed using Dixon plots [10].

The enzymatic activity of elastase bound on the polyacrylamide beads was still measured with the same substrate. The time dependence of the reactions at 25°C was followed using aliquots of the beads vigourously shaken in the substrate solution.

Spin labeling reaction. 1-Oxyl-2,2,6,6-tetramethyl-4-piperidinyl-ethylphos-

phonofluoridate (from Syva, Palo Alto, CA) belongs to a class of powerful inhibitors of serine proteases [3,11—14]. It reacts irreversibly and very rapidly with the serine 195 residue at the active centre of elastase in 0.05 M Tris-HCl buffer (pH 8) at 25°C. The spin labeled enzyme was then dialyzed extensively against 0.05 M acetate buffer (pH 5), at 4°C, in order to eliminate all excess of non reacted label. The ESR spectrum of the enzyme at 24°C and pH 5 does not change during several hours. This shows the good stability of the inactivated enzyme. The concentration of the inactivated enzyme as measured by absorbance at 280 nm was not different from that derived from the area under the ESR spectrum at a 20% precision, confirming that the label binds to the enzyme at a single specific site. This eliminates the possibility of minor labeling at another site which could perturb significantly the distance estimation from the copper site (see below). The spin labeled enzyme was then bound to the polyacrylamide beads as described above. Direct spin labeling of the bound elastase was not possible probably because of the lower accessibility of the target serine residue.

ESR measurements. The ESR spectra were recorded using a Varian 4502 spectrometer operating in the X frequency band, with a 100 kHz field modulation. A microwave power of 50 mW could be used without saturation of the spin label bound to the protein. The measurements were carried out using the flat cell for aqueous solutions or, for more reproducible quantitative work, with calibrated capillaries (Corning Micropipettes of 50 μl) mounted in coaxial quartz tubes containing toluene for improving the thermal conduction in the variable temperature experiments (Varian gas flow system).

The measurements of the labeled beads in the presence of Cu^{2+} were carried out by filling first the capillaries with the solution of beads. One end of the capillary was sealed and the sample was sedimented for few seconds in a Beckman 152 Microcentrifuge. The ESR spectra of this metal free sample was then recorded for reference. A given amount of $CuSO_4$ solution was introduced in the capillary with a microsyringe and the second end was sealed before a new sedimentation and ESR measurement. This procedure permits a precise comparison of the ESR signal intensity before and after reaction of Cu^{2+} with a rigourously constant amount of labeled enzyme. In absence of $CuSO_4$ the intensity of the ESR signal was indeed independent of the number of sedimentations.

The characteristics of the ESR signals of the labeled proteins were analyzed by comparison with those of the free label in aqueous solution at room temperature ($g_0 = 2.0066$, $A_0 = 17.2$ G) and frozen at 77°K ($A_{\parallel} = 36.0$ G, $A_{\perp} = 7.8$ G).

Results and Discussion

Binding of Cu^{2+} on native and spin labeled elastase

Inhibition of elastase by Cu^{2+} was observed by Shotton et al. [15] who proposed a binding at the histidine residue of the active centre. We analyzed this inhibition at pH 5 and 25°C in acetate buffer. Dixon plots in which the straight lines corresponding to various concentrations in substrate converge to the x axis. show that the inhibition is non competitive and corresponds to a

constant of 2.6 mM for elastase in solution. Bound elastase is also inhibited by Cu^{2+}. A precise value of the inhibition constant could not be obtained, but in the same buffer $CuSO_4$ at 3 mM reduces the rate of hydrolysis of succinyl-trialanine-*p*-nitroanilide by 60%. Furthermore, the ESR measurements on the labeled enzyme confirmed that in both situations the inhibition is due to a specific interaction of the metal ion with the enzyme.

The intensity of the ESR spectrum of spin labeled elastase, either in homogeneous solution or bound to the polyacrylamide beads, decreases in the presence of $CuSO_4$ without any other change in the lineshape (Figs. 1 and 2). Such a phenomenon has been analyzed theoretically by Leigh [4]. Following this theory, the residual intensity I_R of the ESR signal of the nitroxide label when the enzyme is saturated with copper can be related to a function of the distance r between the metal ion and the nitroxide label

$$f(r) = \frac{g\beta\mu^2 T_e}{r^6 \hbar \delta H_0} \tag{1}$$

including the magnetic moment μ and the electron relaxation time T_e of the metal ion and the linewidth δH_0 of the nitroxide resonance in absence of metal. A titration of a homogeneous solution of elastase by Cu^{2+} was carried out on the ESR intensity of the central line of the signal of the label. It was analyzed

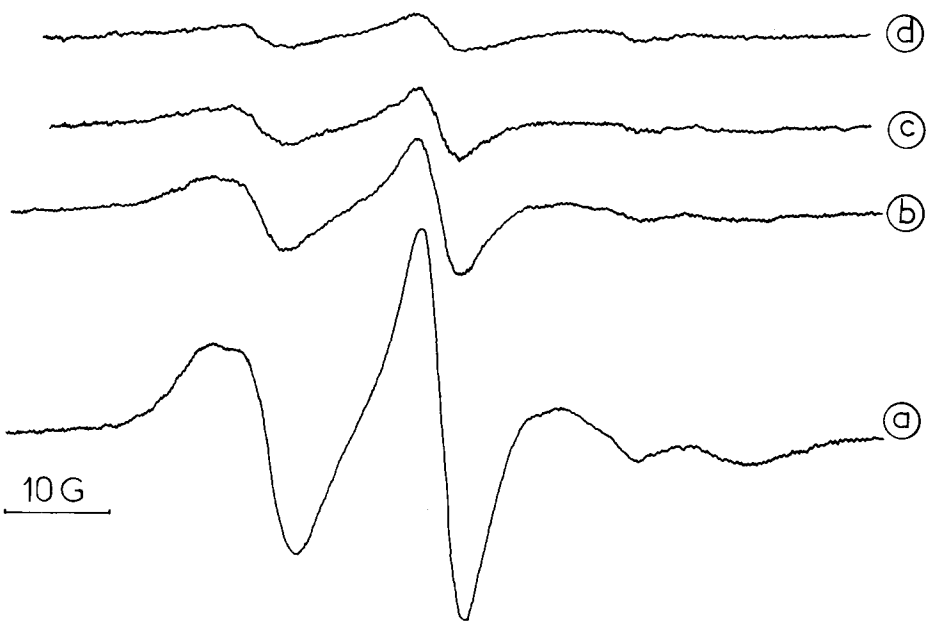

Fig. 1. Effect of Cu^{2+}, (a) no Cu^{2+}, (b) 1.9 mM, (c) 5.6 mM, (d) 13.2 mM, on the ESR spectrum of a 0.14 mM homogeneous solution of spin-labeled elastase, 0.05 M acetate buffer (pH 5.0) at 16°C. A field modulation amplitude of 2.5 G and a non-saturating microwave power of 50 mW were used.

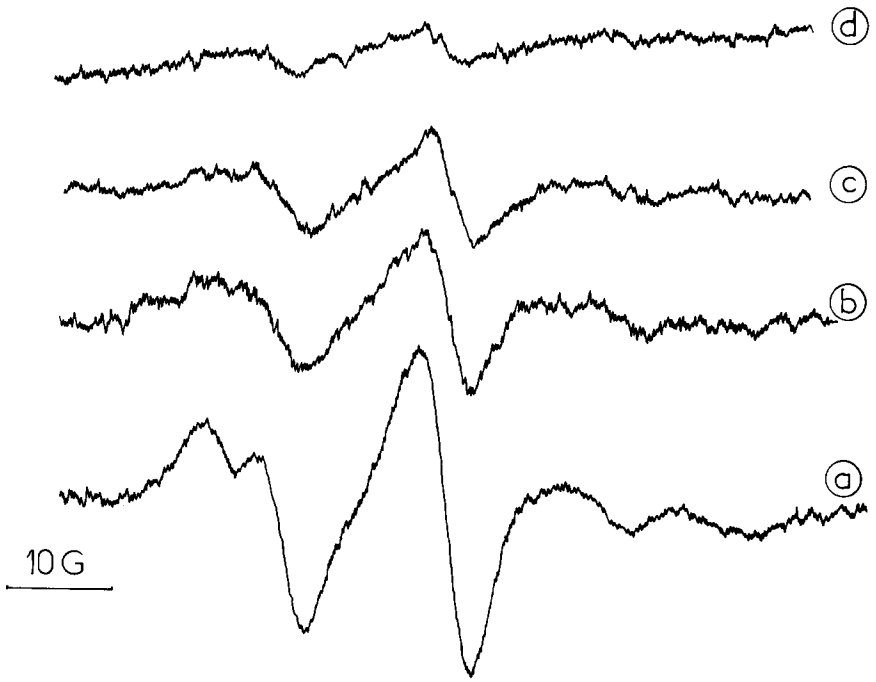

Fig. 2. Effect of Cu^{2+}, (a) no Cu^{2+}, (b) 3.5 mM, (c) 10 mM, (d) 50 mM, on the ESR spectrum of spin-labeled elastase immobilized on polyacrylamide beads in acetate buffer 0.05 M (pH 5.0) at 20°C. The spectra were recorded using different samples having a signal intensity varying by less than 15% before addition of Cu^{2+}, with a field modulation of 4 G of amplitude and 50 mW of microwave power.

using the relations,

$$E + Cu^{2+} \overset{K_d}{\rightleftharpoons} E{-}Cu^{2+} \tag{2}$$

$$I_{obs} = \{I_0[E] + I_R[E{-}Cu^{2+}]\}/[E_0] \tag{3}$$

where $[E_0]$, $[E]$, and $[E\text{-}Cu^{2+}]$ are the concentration in total enzyme, uncomplexed enzyme, and metal complexed enzyme, respectively. I_0 is the initial ESR signal intensity. The best estimates for I_R and K_d were obtained by step variation of I_R from 0 to 0.06 I_0 using a Scatchard plot with

$$\frac{[E{-}Cu^{2+}]}{[E_0][Cu^{2+}]} = \frac{1}{K_d}\left(1 - \frac{[E{-}Cu^{2+}]}{[E_0]}\right) \tag{4}$$

The best fit was obtained for $I_R = 0.03\, I_0$, confirming furthermore the presence of a single site for metal binding with a dissociation constant of 0.9 mM at 16°C. This value compares favorably with the non-competitive inhibition constant of 2.6 mM derived by enzyme kinetics at 25°C. The value of I_R, altogether with an estimate [16,17] of $T_e \approx 10^{-8}$ s and a value of δH_0 of 4.5 G proposed by Leigh [4] for nitroxides bound to proteins of comparable size, gives a value for r of 7 Å using relation (1). However, the resonance lineshape

of the spectrum of the label shows that it is rapidly rotating relative to the protein (see below). In such conditions the estimated distance of the spin label to the copper ion should correspond to its nearest approach, due to the $1/r^6$ dependance of $f(r)$. The exact weighted mean of this distance may thus be somewhat longer than this value. However, this minimal estimate is compatible with Cu^{2+} located in the vicinity of His 57 as proposed by Shotton et al. [15]. The non competitive character of the inhibition and the possibility of copper fixation after substitution of the nitroxide at the serine residue of the active centre agree also with metal binding at another amino acid. Finally, the nearly complete bleaching of the ESR signal at copper saturation eliminates the presence of denatured enzyme since it should correspond to an isotropic residual signal, as observed for thermal denaturation.

There is more uncertainty about I_R for immobilized elastase because of technical difficulties for the measurement of K_d due to a poor estimation of the effective cooper concentration and to lower signal to noise ratios. Furthermore, the measured copper inhibition of the hydrolysis of succinyl-trialanine-*p*-anilide by the active immobilized enzyme should combine the effect of the specific metal binding, which is evidenced by the ESR experiment, with that of diffusional limitations [18]. The measured kinetics do not permit separation of these two contributions and it is not possible to use these data to analyze quantitatively the ESR results. I_R is however certainly lower than 0.1 I_0. An error of 0.05 I_0 on the I_R value leads to a change in the value of r of only 2 Å. One may therefore assume that the metal ion binds to fixed elastase at the same site as in free elastase.

ESR lineshape analysis

The analysis of the lineshape of the ESR signal of the nitroxide spin label bound either to free elastase or to the enzyme immobilized on the polyacrylamide beads should permit comparison of the mobility of the label relative to the protein in both situations.

The spectra recorded at various temperatures with the immobilized system (Fig. 3) are similar to the highly anisotropic spectra initially reported by Hubbell and McConnell [19] for random dispersion of phospholipids in aqueous media. This was expected since the motion of the enzyme relative to the magnetic field is strongly restricted by the fixation to the beads. According to these authors, such spectra should correspond to restricted but fast motions of the spin label and very slow motion of the macromolecule. In such conditions, the spectra provide a direct value of $A'_{\parallel}$ and $A'_{\perp}$ which are the components of the hyperfine tensor 'reduced' by the rapid motion of the label. The corresponding isotropic hyperfine constant A'_0 and the order parameter S_0 are defined by the following relations [19]

$$A'_0 = \tfrac{1}{3}(A'_{\parallel} + 2A'_{\perp}) \tag{5}$$

$$S_0 = (A'_{\parallel} - A'_{\perp})/(A_{\parallel} - A_{\perp}) \tag{6}$$

$$= \tfrac{1}{2}(3\overline{\alpha_z^2} - 1) \tag{6'}$$

where $\overline{\alpha_z^2}$ is the time averaged value of the squared cosine of the angle of the

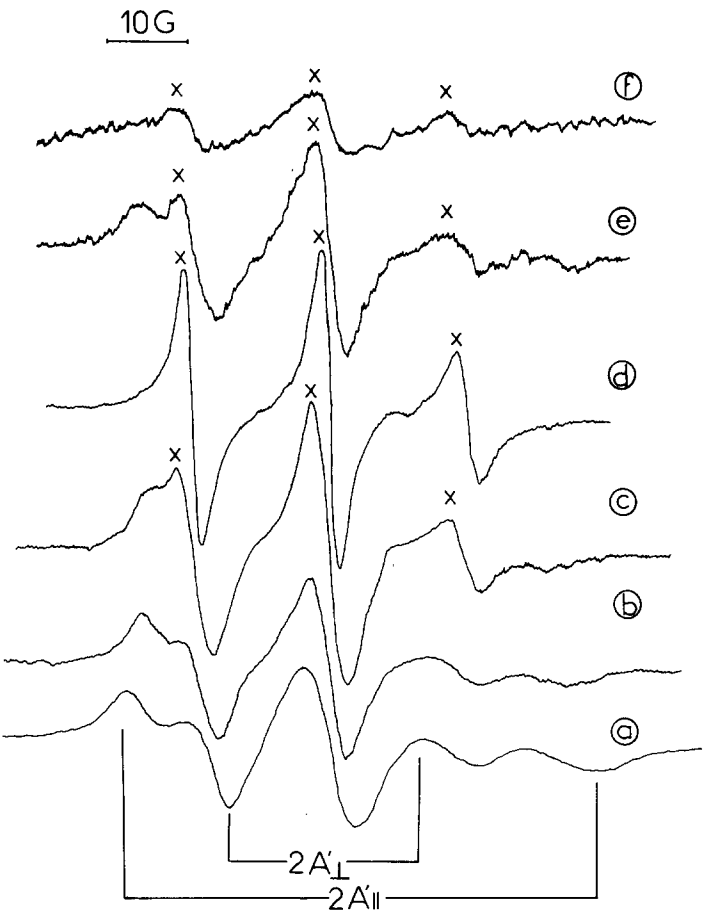

Fig. 3. Effect of temperature on the ESR spectrum of spin labeled elastase immobilized on polyacrylamide beads in acetate buffer (0.05 M (pH 5.0). Accumulation of 10 scans was used for the spectrum recorded at 4°C. A single scan was used at the other temperatures with a field modulation of 3.2 G anf 50 mW of microwave power. The narrow lines, marked by a cross, are due to some denaturation of the bound enzyme. These spectra show: 1, the alterations of the spectrum of the native enzyme when the temperature is increased, (a) 4°C, (b) 39°C; 2, the progressive denaturation of the enzyme, (c) 51°C, (d) 60°C; 3), the partial reversibility of the thermal denaturation, (e) the sample warmed at 60°C is cooled back to 20°C during 24 h; (f) copper sulfate is then added to this sample resulting in the extinction of the anisotropic component of the spectrum.

axis of the $2p_z$ orbital of the nitrogen atom of the nitroxide group with the axis of rotation of the spin label. As a function of decreasing temperature (Table I), A'_0 remains nearly equal to the isotropic hyperfine constant of the free label (17.2 G) but $A'_{\parallel}$ increases progessively up to $A_{\parallel}$ (36.0 G) and S_0 increases up to a value of 1. This could be interpreted as a progessive diminution of the amplitude of the motion of the label ($\overline{\alpha_z^2} \rightarrow 1$).

The lineshape evolution with temperature should better correspond to the theory developed by Mason et al. [20] in which the label motion is considered sufficiently slow to have an effect on the ESR lineshape. Then, lowering the temperature results in a slower motion of the label and to the corresponding

TABLE I

CHARACTERISTICS OF THE ANISOTROPIC ESR SIGNAL OF SPIN-LABELLED ELASTASE IMMOBILIZED ON POLYACRYLAMIDE BEADS, AS A FUNCTION OF TEMPERATURE

$A'_{//}$ and $A'_{\perp}$ str are apparent anisotropic hyperfine constants (see text) and S_0 the corresponding order parameter [14].

T (°C)	$A'_{//}$ (G)	$A'_{\perp}$ (G)	$A'_{//}-A'_{\perp}$	A'_0 (G)	S_0
44.5	25.6	12.3	13.3	16.7	0.47
34.0	26.3	11.7	14.6	16.6	0.52
31.5	26.8	11.9	14.9	16.9	0.53
23.0	26.9	12.5	14.4	17.3	0.51
18.0	27.4	12.3	15.1	17.3	0.54
12.0	27.5	11.9	15.6	17.1	0.55
4.0	29.0	11.8	17.2	17.5	0.61

observed evolution of $A'_{||}$ and S_0 without introducing the hypothesis of a change of conformation of the environment of the label responsible for the diminution of the amplitude of the motion. A comparison with the computer simulated spectra of these authors [20] indicates that the motion of the label on the immobilized enzyme is properly described by a time of rotation $\tau_{||}$ varying from 1 ns at 4°C to 0.4 ns at 45°C. A more complete lineshape analysis [21] should be necessary in order to derive the energy of activation of that rotation from the observed spectra. Anyhow, this interpretation corresponds to a relatively high mobility of the spin label with respect to the protein.

In contrast with these dynamical interpretations, a static representation in which the observed spectra should correspond to two equilibrium related conformations is less probable. Although only simulations would distinguish between these two interpretations, it is unlikely that both components could be similarly affected by copper ions and that the thermal equilibrium could be comparable in the bound and in the soluble enzyme (Fig. 3 and 4).

In fact, lowering the temperature of the homogeneous solution of labeled elastase from 25 to 4°C produces large changes in the ESR lineshape (Fig. 4). The spectrum recorded at 4°C is nearly superimposable to that of the immobilized system at 39°C. These observation can still be interpreted by the theoretical calculations of Mason et al. [20]. They have shown that only rotation times of the macromolecules lower than $5 \cdot 10^{-8}$ s have no significant effects of the lineshape. Elastase has a molecular weight of 25 000 which should correspond to a rotational time of 2 to $5 \cdot 10^{-8}$ s in the temperature range of 25 to 4°C. This is the right order of magnitude to explain the observed lineshape evolution.

This interpretation of the ESR spectra suggests strongly that the motion of the label relative to the protein in homogeneous solution has the same nature, with perhaps a slightly slower correlation time, as that in the system immobilized on the polyacrylamide beads. The absence of large change upon binding of the environment of the spin label is further demonstrated by a similar pH dependence of the ESR spectra in both system (Fig. 5). The $A'_{||}$ splitting observed at the same temperature increases in both cases when the pH value is raised from 5 to 7.5. A small conformational change of the protein with the pH is therefore still possible after immobilization.

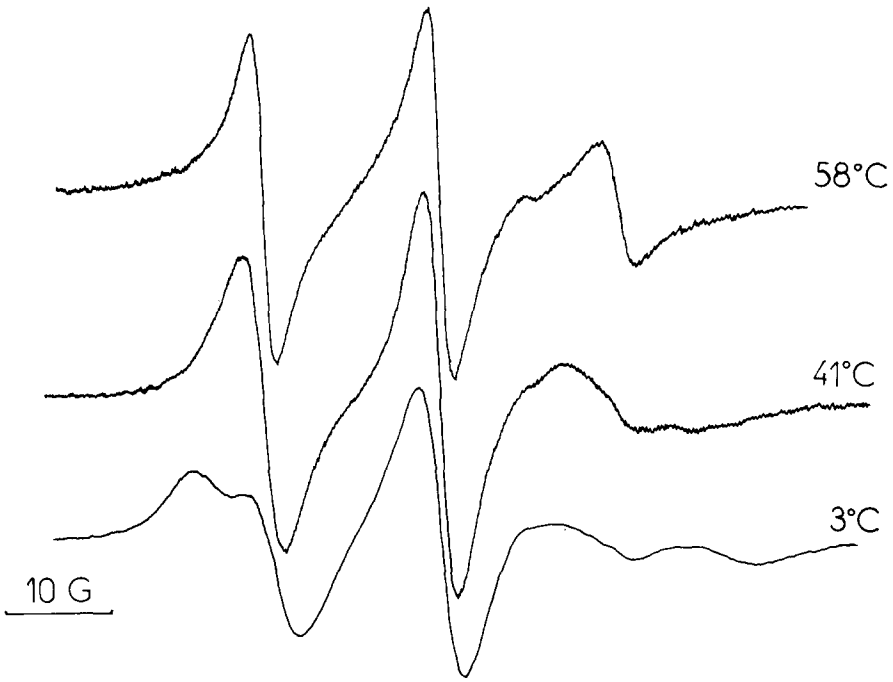

Fig. 4. Effect of temperature on the ESR spectrum of spin labeled elastase in 0.14 mM homogeneous solution in acetate buffer 0.05 M (pH 5.0). The spectra were recorded in the same conditions as in Fig. 3, with a 2.5 G modulation amplitude. Some precipitation occurred between 41 and 58°C resulting in some loss of signal intensity.

Stability of the immobilized protein

Irreversible thermal denaturation of elastase in homogeneous solution is observed at 50°C. It may be evidenced by various spectroscopic techniques, such as optical absorption and proton magnetic resonance spectroscopy, and results in rapid precipitation of the enzyme. The ESR spectrum of spin labeled elastase just exhibits a narrowing of the hyperfine lines upon raising the temperature from 25 to 60°C. This narrowing must be due, at least in part, to the decrease of the rotational time of the whole protein. Precipitation after denaturation corresponds to a severe broadening and disappearance of the ESR signal.

On the contrary, the ESR signal of the immobilized system remains characteristic of highly anisotropic motion up to 52°C (Fig. 3). Above this temperature, this anisotropic spectrum decreases rapidly in intensity and disappears at 60°C. It is progessively replaced by an isotropic signal similar to that of the free label in solution. The superimposition of these two signals corresponding to native and denatured elastase indicates that the exchange between these two forms is slow (less than 10^8 s^{-1}) on the ESR time scale. When the temperature is subsequently lowered down to 20°C about 70% of the initial intensity of the anisotropic signal is recovered. The reversibility of the denaturation of the bound protein is further confirmed by the specific bleaching of the anisotropic part of the signal by Cu^{2+}. When a solution of immobilized protein at 20°C

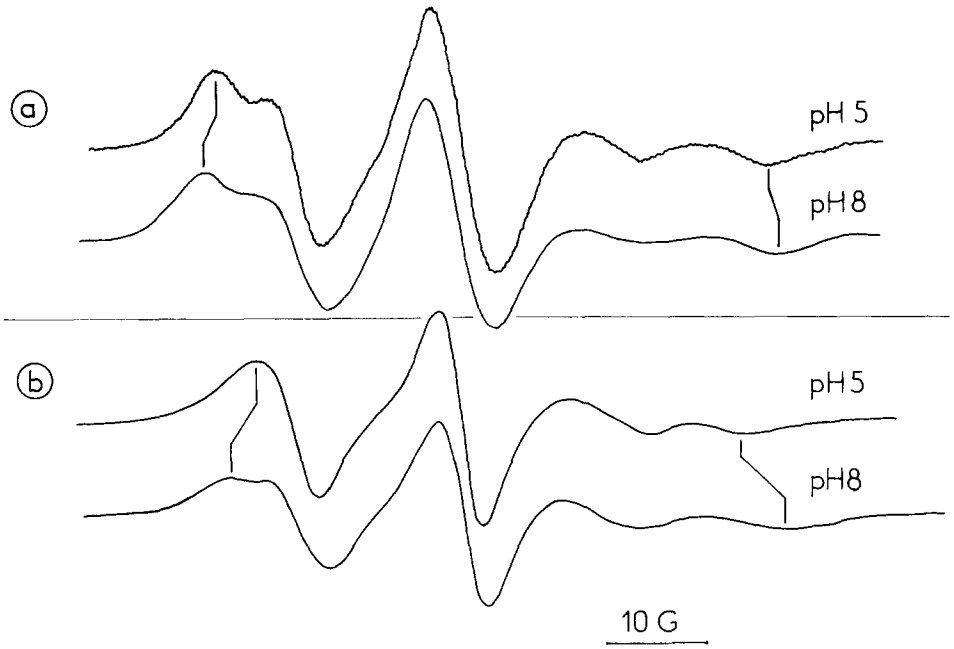

Fig. 5. Effect of pH at 20°C on the ESR spectrum of spin labeled elastase, immobilized on polyacrylamide beads (a), and in homogeneous solution (b).

contains enough copper for maximum bleaching of the spin label signal, the isotropic signal of the label reappears upon heating over 50°C because of the denaturation of the protein. The intensity of this isotropic signal after complete denaturation at 60°C can be compared to that of the signal of a solution of free label at the same temperature to measure the concentration of immobilized elastase in the sedimented samples. Values of 0.02 to 0.025 mM were confirmed directly by radioactive labeling of the active centre by [^{3}H]diisopropylfluorophosphate.

ESR has thus shown the reversibility of thermal denaturation of elastase after immobilization on a solid matrix. This functional stabilization of the system does not correspond however to an increased intrinsic stability of the protein since denaturation occurs at the same temperature as in the homogeneous solution. Fixation on the beads at relatively low concentration just prevents the intermolecular interactions responsible for the precipitation observed after denaturation in homogeneous solution.

Acknowledgements

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