Biochimica et Biophysica Acta, 315 (1973) 195–199
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BBA Report

BBA 61278

An ESR study of the active-site conformations of free and immobilized trypsin

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(Received May 10th, 1973)

SUMMARY

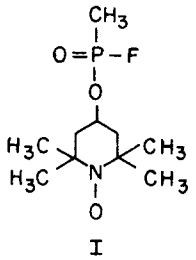
Trypsin covalently bound to porous glass and free trypsin have been spin-labeled with 1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl methylphosphonofluoridate. The active-site conformations are identical whether the enzyme is labeled before or after immobilization. The active-site normality may be determined for immobilized trypsin by the spin-count technique.

Most enzymes *in vitro* are immobilized as a result of their occurrence in multi-enzyme complexes or attachment to membranes. The interest in immobilized enzymes stems not only from biological considerations but also from practical applications in such areas as analysis, synthesis, and therapeutics (for reviews see refs 1–3).

The research approaches to the study of bound enzymes differ from the approaches taken for enzymes which are free in solution. Kinetic studies have shown that diffusion of substrate to the enzyme can considerably complicate the analysis of the rate data[4–7]. The physical–chemical techniques normally used for conformational studies of soluble proteins are not generally applicable to matrix-bound enzymes. One notable exception is the use of a fluorescence technique by Gabel *et al.*[8]. This technique is limited, however, to the observation of protein bound at the surface of the carrier and to systems where the protein concentration is fairly high. We felt that ESR spectroscopy would be useful in the study of bound enzyme conformation since support materials generally are transparent to microwave radiation. Thus, the protein within the matrix could be studied as well as the protein bound at the surface. Also, the protein concentrations required are not high.

*N.S.F. Undergraduate Research Participant, Summer 1972.

We report here an ESR study of the active-site conformations of free trypsin and trypsin bound to porous glass. The active site-directed label used was 1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl methylphosphonofluoridate, I (refs 9, 10).



I

In addition to conformational studies, the normality of active sites may be determined by ESR. The conventional kinetic methods of active site titration are not always applicable to bound enzymes. The "burst" observed may be prolonged by diffusion of the titrant to enzyme which is less accessible to solvent. Formation of a stable spin-labeled enzyme permits the determination of the amount of enzyme which is active after immobilization.

Bovine trypsin (Worthington, lot TRL 2DA) was bound to an arylamine derivative of porous glass by methods previously described[11]. 200 mg of moist immobilized trypsin was suspended in 2 ml of 0.1 M Tris–HCl buffer, pH 7.5 at 25 °C. Spin label I (15 μl of an approx. 0.5 M benzene solution) was added and the reaction mixture was rotated in a baffled tube which was connected to a torque stirrer. At various time intervals, enzyme activity was determined titrimetrically with 1 mM benzoyl-arginine ethyl ester and 25 mM $CaCl_2$ at pH 8. When the reaction was complete, the spin-labeled immobilized trypsin was collected on a sintered-glass filter and washed exhaustively with 1 mM acetic acid. The spin-labeled beads were transferred to sealed-tip disposable Pasteur pipettes for ESR study. In a separate experiment free trypsin was inhibited with spin label I and purified by published methods[12]. One portion of this trypsin sample was immobilized on porous glass by the methods described[11]. Another portion was saturated with solid sucrose in order to approximate conditions of high (infinite) viscosity in solution. All spectra were taken at 26 ± 2 °C on a Varian E-4 spectrometer operating at X-band frequency. In all cases the enzymes were in dilute acetic acid (pH 3.5) which was 0.02 M in $CaCl_2$. The concentration of active sites which were inhibited was determined by hydrolyzing spin label from a fixed weight of beads with a known volume of 0.5 M NaOH and comparing the final free spin-label concentration with that of a standard ("spin count").

Fig. 1a shows the spectrum of (α,β) trypsin inhibited with spin label I at the active-site serine. The mobility of the spin label is of "intermediate immobilization" reflective of a nitroxide group which interacts rather strongly with its (active site) environment. However, the mobility exhibited in this ESR spectrum also contains a component due to macromolecular rotational diffusion of the trypsin molecule. Next are shown the spectra of trypsin which has been immobilized and subsequently labeled

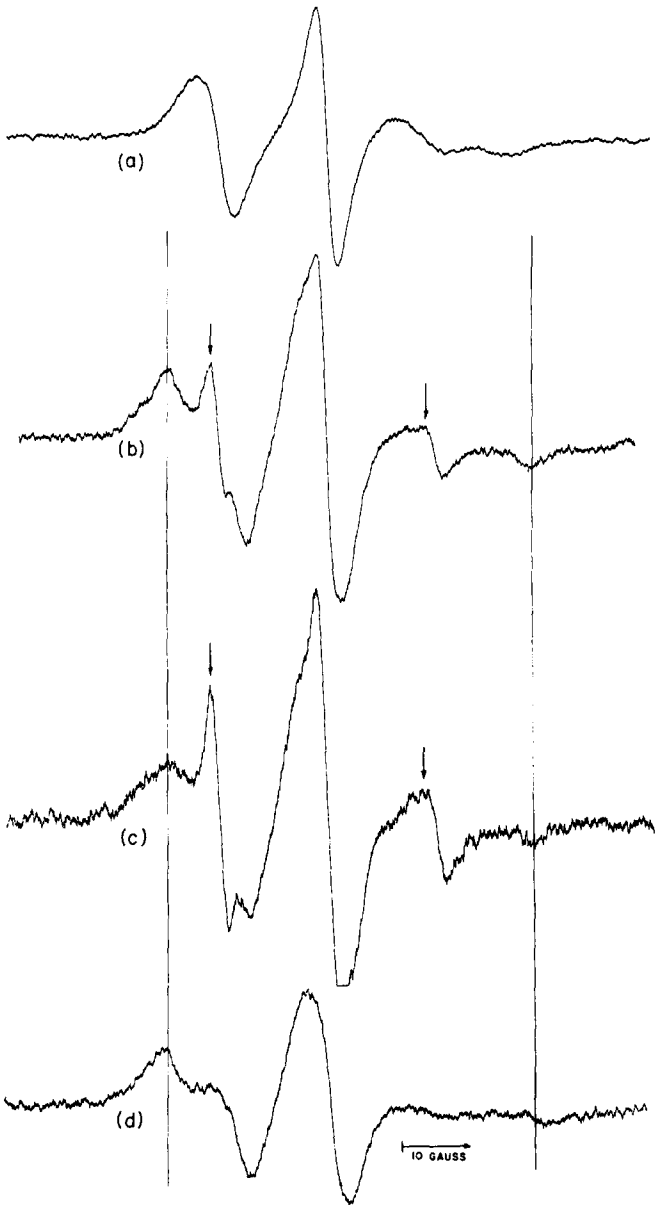


Fig. 1. ESR spectra of solution and immobilized trypsins at pH 3.5 (0.05 M acetic acid, 0.02 M $CaCl_2$) spin labeled with I. (a) Labeled enzyme in aqueous solution. (b) Labeled preimmobilized trypsin. (c) Prelabeled immobilized trypsin. (d) Trypsin from (a) in saturated sucrose solution. The narrow line components which are particularly distinct in (b) and (c) and are denoted with arrows (↓) represent a small fraction of autolyzed trypsin which could not be removed from these preparations. The two vertical lines coincide exactly with the outer hyperfine extrema in (b) and (c).

(labeled preimmobilized trypsin, Fig. 1b) and Fig. 1c where prelabeled solution trypsin from Fig. 1a was subsequently coupled to the beads (prelabeled immobilized trypsin). The small narrow line components denoted with arrows (↓) in Figs 1b and 1c are due to small concentrations of trypsin which had been autolyzed after labeling by the very small amounts of absorbed free trypsin which were in practice impossible to wash completely from the bead preparation. The same problem occurs with the solution labeled enzyme[12]. The spectrum of "solution" trypsin in saturated sucrose is shown in Fig. 1d. While these latter spectra are qualitatively very similar, the sucrose sample had a slightly broader spectrum, the significance of which will be discussed below.

The striking feature in the spectra of Figs 1b and 1c was the superposition of their major broad line components, which represent essentially all of the spin-labeled enzyme (except the autolyzed trypsin lines discussed above). This indicates that the nitroxide mobility was identical in these two states and is a strong argument for the equivalence of their active-site conformations whether the enzyme was labeled before or after the immobilization process. It was, of course, obvious from routine enzyme-activity measurements that the immobilized form of the enzyme was indeed active; however, the more subtle structural changes which occur in enzymes as a change in K_m or V are directly observable only by a few of the 'site-specific' techniques applicable to such systems. A more complete "picture" of the consequences of immobilization process on active site structure may be accomplished through the incorporation of a series of spin labels of differing structural geometry as was recently shown with the free enzyme (Wong, S.S., Quiggle, K., Tripplett, C. and Berliner, L.J., unpublished; Berliner, L.J. and Wong, S.S., unpublished).

The spectrum of spin-labeled trypsin in the presence of saturated sucrose, where the macroscopic viscosity was several thousand cP at 26 °C, is shown in Fig. 1d. The outer hyperfine extrema are separated by approximately 56–57 G compared with 54–55 G for the immobilized enzymes in Figs 1b and 1c*. A splitting of this magnitude is characteristic of a spin label with significantly more immobilization than as in Fig. 1a, and is in the extremely sensitive tumbling range on the ESR time scale; however, it is not that of a rigidly bound nitroxide group such as found in the case of other spin-labeled proteins[14,15]. The small difference between the immobilized and sucrose-exposed enzyme may be accounted for by three possible explanations at this time: (1) the free solution enzyme experienced a subtle, yet not crucial change in active-site conformation caused by the high sucrose concentrations; (2) in fact, a small but less consequential change occurred upon immobilization; or, (3) the less complete averaging of anisotropic motion of the spin label on the rigidly immobilized-enzyme matrix relative to that in solution. Further refinement, as well as a quantitative analysis of the specific residues involved in the enzyme–matrix support complex are in progress (Royer, G.P. and Uy, R., unpublished).

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*The estimated uncertainty in this measurement arises from the quite difficult resolution of the very broad high-field line.

TABLE I

DETERMINATION OF THE ACTIVE SITE NORMALITY OF IMMOBILIZED TRYPSIN BEADS BY THE SPIN COUNT METHOD

The data shown are for a typical determination. The average of several determinations yielded a percent active enzyme per total protein of 45 ± 7.

mmoles I / *mg beads*	*mg protein** / *mg beads*	*% labeled trypsin* / *total protein (w/w)*	*max. inhibition by I (in 3.5 h)*	*% active enzyme* / *total protein (w/w)*
$3.3 \cdot 10^{-7}$	0.023	36	87	42

*By amino acid analysis according to ref. 11.

The stoichiometry of inhibition and calculation of active enzyme per g of bead is shown in Table I. The results indicate that in the order of 40% of the coupled protein was active enzyme. This correlates well with approximate calculations from activity assays. It is also not surprising to have so low a percentage since commercial trypsin alone may contain as low as 30–50% of the active α- and β-forms. The use of a spin label here instead of a radiochemical label offers the double advantage of being a qualitative marker of the specific active site environment whose concentration is determined. Thus the "specificity" of the label can be verified before obtaining its percent incorporation.

The spin-label method offers distinct advantages in the probing of protein structure The sensitivity to investigations of conformation and the ease of active-site 'titration' have been shown in this study of matrix-fixed enzymes. The future possibilities for such an approach in the immobilized-enzyme field appear to be optimistic.

This investigation was supported by grants from the National Institutes of Health (GM-19364 and GM-19507) and the National Science Foundation (GI-32141).

REFERENCES

1 Goldman, R., Goldstein, L. and Katchalski, E. (1971) in *Biochemical Aspects of Reactions on Solid Supports* (Stark, G.R., ed.), pp. 1–78, Academic Press, New York
2 Melrose, G.J.H. (1971) *Rev. Pure Appl. Chem.* 21, 83–118
3 Mosbach, K. (1971) *Sci. Am.* 224, 26–33
4 Lilly, M.E., Hornby, W.E. and Crook, E.M. (1966) *Biochem. J.* 100, 718–723
5 Sundaram, P.V., Tweedale, A. and Laidler, K.J. (1969) *Can. J. Chem.* 48, 1498–1504
6 Goldman, R., Kedem, O. and Katchalski, E. (1968) *Biochemistry* 7, 4518–4531
7 Moo-Yound, M. and Kobayashi, T. (1972) *Can. J. Chem. Eng.* 50, 162–167
8 Gabel, D., Steinberg, D.Z. and Katchalski, E. (1971) *Biochemistry* 10, 4661–4669
9 Morrisett, J.D., Broomfield, C.A. and Hackley, Jr, B.E. (1969) *J. Biol. Chem.* 244, 5758–5761
10 Morrisett, J.A. and Broomfield, C.A. (1972) *J. Biol. Chem.* 247, 7224–7231
11 Royer, G.P. and Uy, R. (1973) *J. Biol. Chem.* 248, 2627–2629
12 Berliner, L.J. and Wong, S.S. (1973) *J. Biol. Chem.* 248, 1118–1120
13 Schroeder, D.D. and Shaw, E. (1968) *J. Biol. Chem.* 243, 2943–2949
14 Smith, I.C.P. (1972) in *Biological Applications of Electron Spin Resonance* (Swartz, H.M., Bolton, J.R. and Borg, D.C., eds), pp. 483–539, Wiley-Interscience, New York
15 Berliner, L.J. (1973) in *Progress in Bioorganic Chemistry*, Vol. III (Kaiser, E.T. and Kexdy, F., eds), Wiley-Interscience, New York, in the press