SPIN LABEL ORIENTATION AT THE ACTIVE SITE OF α-CHYMOTRYPSIN IN SINGLE CRYSTALS

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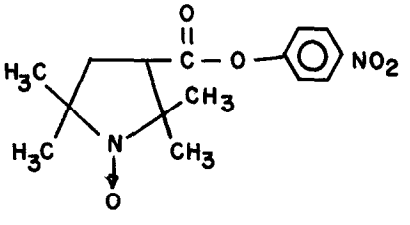
SUMMARY

Single crystals of monoclinic α-chymotrypsin have been spin labeled as the acyl-chymotrypsin intermediate that is formed in the hydrolysis of the substrate (I), (DL)-3-carboxy 2,2,5,5,-tetramethyl pyrrolidine-N-oxyl-p-nitrophenyl ester. The paramagnetic resonance spectra show that each paramagnetic acyl group is held rigidly at the active site in a single unique orientation relative to its host enzyme molecule.

The physical state of a spin label (1) at the active site of an enzyme can be relevant to a number of spectroscopic studies of the structure and function of enzymes, including X-ray diffraction, nuclear magnetic resonance, as well as electron paramagnetic resonance. In previous work (2) we have shown that the α-chymotrypsin "substrate" I



I

reacts with α-chymotrypsin in solution to form, at acid pH, a stable acyl enzyme with the simultaneous release of nitrophenol. The enzyme hydrolyzes only one optical isomer of the substrate (4). It was found that under

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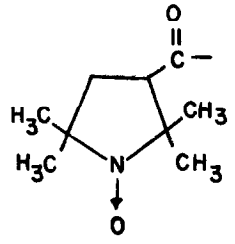
those conditions, in solution, the acyl group II



II

is "immobilized," but not quite "strongly immobilized" (1,2). The present paper summarizes the results of a paramagnetic resonance study of single crystals of α-chymotrypsin having the acyl group II at the active site. This study shows that each spin label group II is "strongly immobilized" and takes on a single unique orientation relative to the host enzyme. This result may be contrasted with spin-labeled hemoglobin crystals where labels have been attached to the reactive sulfhydryl groups at positions cysteine β-93. In this latter case, the labels take on two isomeric orientations relative to the host hemoglobin molecule, and in one of these orientations, the label group is only partially immobilized (5,6,7).

MATERIALS AND METHODS

(i) Spin Label (I) - This was prepared as previously described (2).

(ii) Crystals - Native crystals of α-chymotrypsin (Worthington Biochemical Corp., Lot CD 6150-1) were grown according to the method of Sigler et al.(8). The crystals were spin labeled by soaking them for at least two days in a solution containing 2.1 x 10^{-4}M spin-labeled substrate I dissolved with 2-4% (by volume) acetonitrile in the pH 4.2 "standard supernatant solution" (2.6M $(NH_4)_2SO_4$, 0.1M citrate buffer) described by Sigler (8). The acylated crystals were found to be quite stable for several weeks. Deacylation was negligible compared to the rate in solution at this pH.

(iii) X-ray photographs - Appropriate precession photographs of the labeled crystals showed them to be isomorphous with the native crystals and

the α-chymotrypsin-inhibitor complexes described by Sigler et al.(8).

(iv) ESR measurements - The crystals were mounted in a special teflon/kel-F crystal goniometer (3) which was firmly mounted in the standard Varian X-Band cavity of a V4502 spectrometer with a 12-inch rotating magnet.

RESULTS

The α-chymotrypsin crystal belongs to the space group $P2_1$. There are four enzyme molecules in the unit cell (9). The four molecules in the unit cell may be thought of as consisting of two "dimers." (9). The two dimers are related to one another by the two-fold crystallographic screw axis b. The two molecules within a dimer are related to one another by a non-crystallographic two-fold rotation axis which is approximately parallel to the crystallographic axis a* (9).

The paramagnetic resonance spectrum of a spin label depends on the direction of the applied magnetic field relative to the principal axis system of the spin Hamiltonian (1). The resonance spectra observed for oriented single crystals of the acyl enzyme consist of the superposition of no more than four single molecule spectra. This result indicates that each spin label acyl group takes up a unique orientation relative to its host enzyme. This conclusion was confirmed by noting that the observed spectra consisted of the superposition of no more than two single molecule spectra when the applied field was perpendicular to b. Finally, the observed spectra were identical to that expected for a single molecular orientation when the applied field was perpendicular to b and also either perpendicular or parallel to a*. Likewise, the observed spectrum was identical to that expected for a single molecular orientation when the applied field was parallel to b (and perpendicular to a*).

Figures 1 and 2 show recorded line positions and resonance spectra for special crystal orientations. From these results we conclude that each spin label acyl group II takes on a single unique orientation relative to its host protein (10). This conclusion could only be invalid if the group

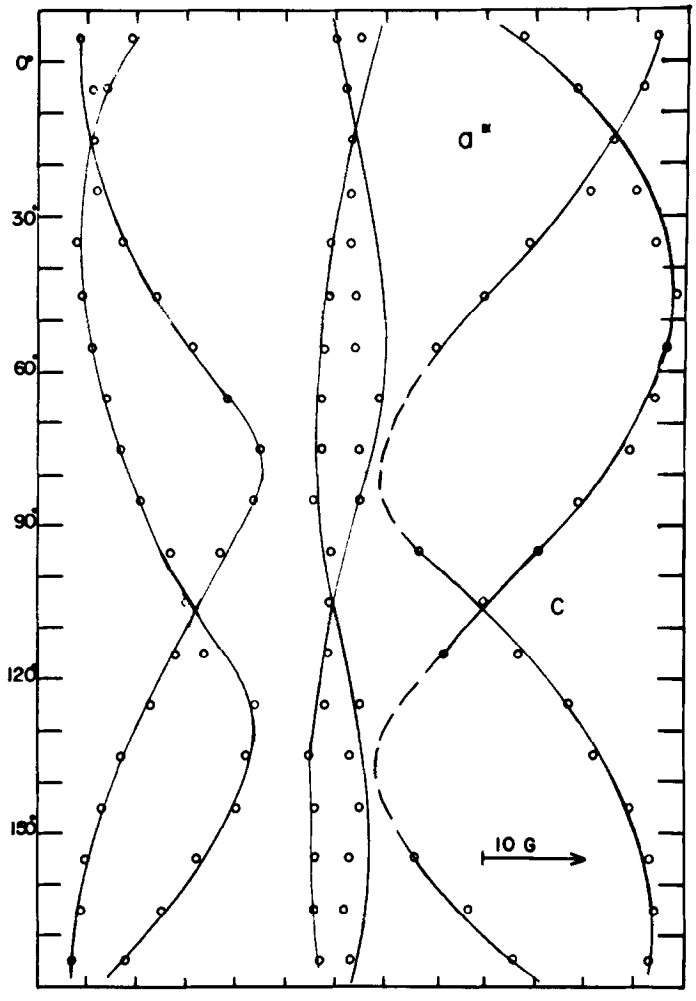


Figure 1. Resonance line positions for a spin-labeled α-chymotrypsin crystal when the applied field is perpendicular to the crystallographic axis b and makes an angle ϑ with a reference axis in the a* c plane. Outer lines are drawn through data points (O). The two center curves and the dashed regions of the outer curves represent computed line positions in regions of severe resonance curve overlap. Estimated uncertainty in ϑ is $\pm$ 2-5°.

II took on two or more orientations (relative to its host enzyme) corresponding to two-fold rotations about one or more of the principal hyperfine axes of the spin Hamiltonian. (In our opinion, this possibility is extremely unlikely. For example, a two-fold rotation about the Π-orbital axis would reverse the direction of the N-O bond.)

For nitroxide spin labels, the orientation of the principal axis of

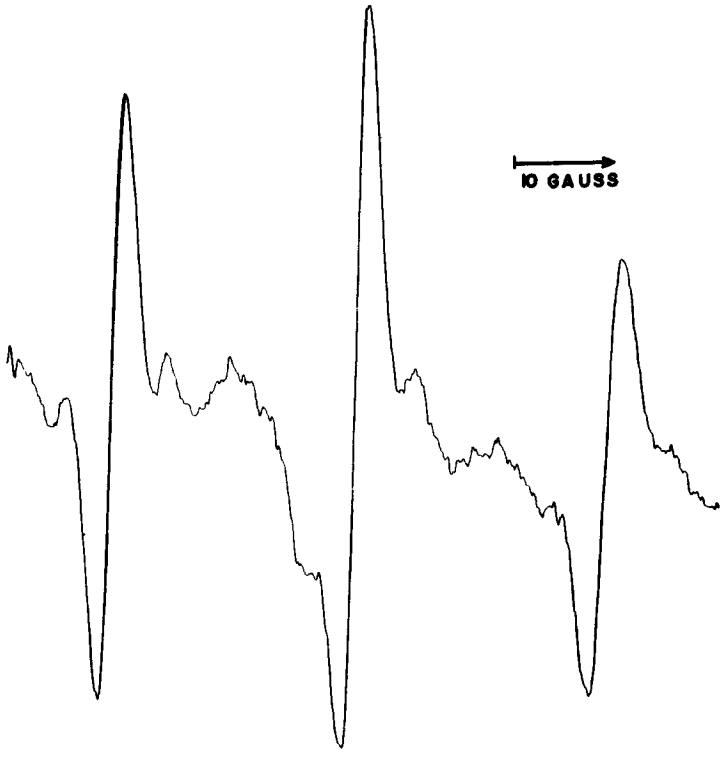


Figure 2. Resonance spectrum of acyl-chymotrypsin when the applied field is perpendicular to the crystallographic two-fold screw axis b and parallel to the two-fold "dimer" axis a*. Only a single hyperfine triplet is seen for this orientation, since all four spin label acyl groups give identical spectra for this special orientation.

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largest hyperfine splitting $\pm \underset{\sim}{\Pi}$ can be determined easily and accurately from resonance spectra taken at X-band (9500 Mc and 3,200 gauss). The vector $\underset{\sim}{\Pi}$ is essentially the direction of the Π-orbital axis, and is perpendicular to the plane of the pyrrolidine ring (11). In the present work, one Π principal hyperfine axis was found to be parallel to the vector that makes angles of 36°, 65°, and 63° with the unit cell directions a*, b and c. The other three directions are generated by two-fold rotations parallel to a* and b. The assignment of these axis directions to particular molecules cannot be deduced from the resonance experiment. An exact placement of the pyrrolidine ring in the α-chymotrypsin molecule might be achieved through testing each of the possible symmetry related orientations

with an atomic model of the protein. Precise determination of the orientations of the principal hyperfine axes perpendicular to $\underset{\sim}{\Pi}$ can doubtless be achieved with resonance spectra taken at higher magnetic fields (e.g., Q-band).

DISCUSSION

The paramagnetic resonance spectra of the spin label acyl group II at the active site of α-chymotrypsin in solution and in single crystals are consistent with one another in that in both cases, the acyl group is "immobilized." The degree of immobilization does not appear to be identical in the two cases, however. In the single crystal spectra, orientations could be found where the outer lines of the hyperfine triplet were separated by $2T_{||}$ = 64 gauss, whereas the outer hyperfine extrema in the solution spectra are separated by $2T_{||}$ = 60 gauss. Order of magnitude estimates suggest that this reduction of hyperfine splitting in the solution case could easily arise from rigid body diffusional rotation of the protein molecule. A quantitative discussion of this effect of "slow" protein motion on spin label resonance spectra will be given elsewhere.

The physical state of the acyl group II at the active site of α-chymotrypsin found in the present work - it has a single unique orientation that is strongly immobilized in crystals and probably strongly immobilized relative to the protein in solution - suggests that this acyl derivative may warrant further study with X-ray diffraction, nuclear magnetic resonance, and electron paramagnetic resonance.

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