Rotational Correlation Time of Spin-Labeled α-Chymotrypsin

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Summary

α-Chymotrypsin has been spin labeled with (I), 3-carboxy 2, 2, 5, 5-tetramethyl pyrrolidine-N-oxyl-p-nitrophenyl ester, and (II), the N-oxyl-4', 4'-dimethyl oxazolidine of 5-α-androstan-3-one-17-β-methyl phosphonofluoridate. The positions of the high field hyperfine components in the paramagnetic resonance spectra of the spin-labeled enzymes have been measured as a function of solvent viscosity and have been used to determine a rotational correlation time of 12 nanoseconds for α-chymotrypsin in water at 20° C.

The paramagnetic resonance spectrum of a nitroxide spin label depends on its rotational motion.[1] In principle this dependence of resonance spectra on rotational motion offers the possibility of determining the rotational correlation times, τ_2, of proteins in solution. There are two obstacles to the determination of such rotational correlation times using spin labels. The first obstacle involves the quantitative analysis of paramagnetic resonance line shapes in the case of slow motion. The second obstacle involves the selection of an appropriate spin label which undergoes little or no rotational motion relative to the tertiary structure of the protein. Both of these obstacles have been largely overcome in the present work, which describes a general method for determining rotational correlation times using α-chymotrypsin as an example. A detailed description of the theoretical analysis of magnetic resonance line shapes used in the present work will be given elsewhere.[2]

The paramagnetic resonance spectra of α-chymotrypsin spin-labeled at the active site in single crystals and in solution have been reported previously.[3] The separations of the outer hyperfine extrema in these two cases are not identical. This difference can be attributed to rotational motion of the α-chymotrypsin in solution.

The quantitative dependence of the resonance positions of the outer hyperfine extrema on rotational motion was determined theoretically by solving a set of Bloch equations coupled by the addition of rotational diffusion terms. The spin Hamiltonian for the appropriate nitroxide spin label was assumed to be axially symmetric since small deviations from axial symmetry have no significant effect on the shapes and positions of the outer hyperfine extrema. The calculated shift, $\Delta H(\tau_2)$, of the high field hyperfine signal relative to its position at a correlation time equal to infinity is plotted as a function of τ_2 in Figure 1. The use of the high

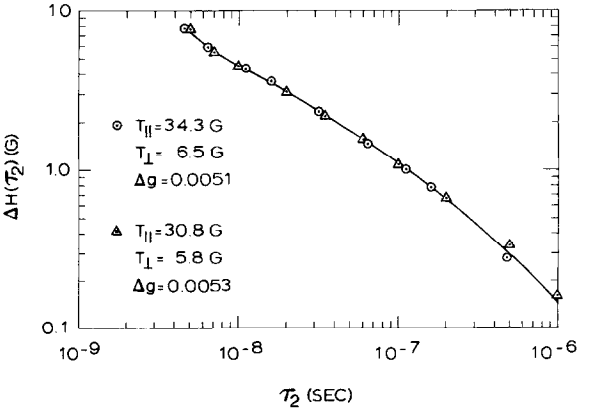
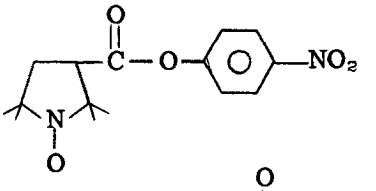


Figure 1. The theoretical shift $\Delta H(\tau_2)$ of the highfield hyperfine signal vs. τ_2 calculated for two sets of parameters for an axially symmetric Hamiltonian.

field hyperfine line for these measurements is more appropriate than the use of low field hyperfine line because of the larger shift and because of the absence of overlap from the central portion of the spectrum. We have measured $\Delta H(\tau_2)$ and have determined the rotational correlation times of two spin labels bound to α-chymotrypsin.

I



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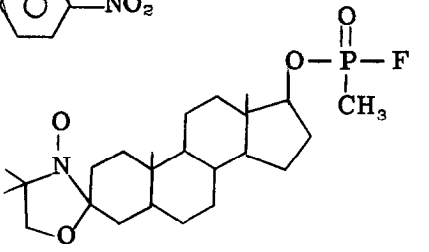

Experimental

α-Chymotrypsin (Calbiochem) reacts with label I at acid pH with the release of nitrophenol to form a stable acyl enzyme.[4] This was dialyzed against 0.1M NaCl, 0.001M HCl, at pH 3.15 to remove unreacted label I. Label II was used to inhibit α-chymotrypsin by the method of Morrisett and Broomfield.[5] After the enzyme activity had fallen to approximately 5% of its original value, the reaction mixture was passed through a Sephadex G-25 column equilibrated with 0.1M NaCl, 0.001M HCl, at pH 3.0 to separate the phosphonyl enzyme from the unbound label. The chymotrypsin concentration was determined by absorbance at 280 nm using $E^{1\%}_{280} = 20.4$.[6]

All spectra were run at 20.0 ± 0.3° C at 9.5 GHz on a Varian E-12 spectrometer with an acyl chymotrypsin concentration of 3.8×10^{-4} M and a phosphonyl chymotrypsin concentration of 1.0×10^{-4} M.

We determine the correlation times by measuring the position of

the high field hyperfine component as a function of viscosity, η, and extrapolating to infinite viscosity. The difference between its position for a solution of infinite viscosity and that for an aqueous solution at 20° C is used to determine the rotational correlation time.

The addition of sucrose to an aqueous solution of the enzyme was used to vary the viscosity. The position of the high field component was measured with respect to the high field isotropic line of a standard, which was either the spin-labeled acid formed upon the deacylation of the acyl enzyme or a dilute solution of 2, 2, 6, 6-tetramethyl-4-oxopiperidine-N-oxyl fixed to the outside of the sample cell in the case of the phosphonyl enzyme.

Results and Discussion

Figure 2 illustrates the change in the spectrum of phosphonyl chymotrypsin for three values of viscosity.

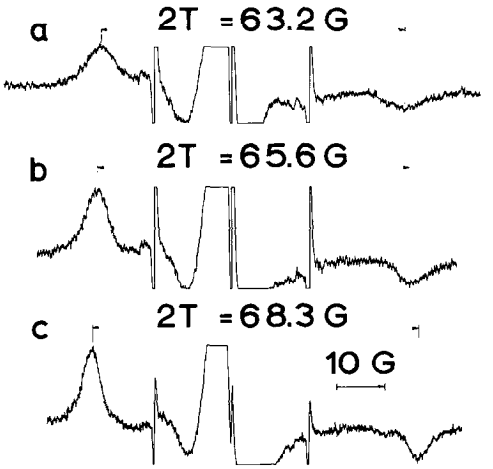

Figure 2. The paramagnetic resonance spectrum of phosphonyl chymotrypsin for η/T in poise/°K equal to a) 3.4×10^{-5}, b) 6.7×10^{-5}, and c) 2.2×10^{-4} illustrating the separation between the outer hyperfine components, 2T, which is dependent upon τ_2.

Theoretically, $\Delta H(\tau_2)$ is proportional to $\tau_2^{-2/3}$ for a wide range of correlation times. As a result, we have plotted in Figure 3 the difference between the position of the high field component and that of our standard, $H_h - H_o$, as a function of $(\frac{T}{\eta})^{2/3}$ for each of the two spin label-enzyme complexes.

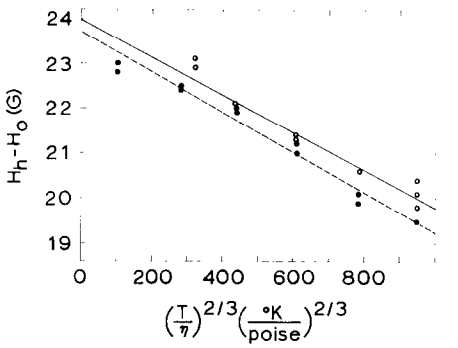

Figure 3. The shift of the high field hyperfine signal relative to a standard vs. $(T/\eta)^{2/3}$ for O 1.0 x 10^{-4}M phosphonyl chymotrypsin (————); and ● 3.8 x 10^{-4} M acyl chymotrypsin (---------).

From a least squares fit of the data, we find that the extrapolated shift, $\Delta H(\tau_2)$, is 4.25 gauss for acyl chymotrypsin and 4.0 gauss for phosphonyl chymotrypsin. These values correspond to $\tau_2 = 11.5$ and 13.0×10^{-9} sec, respectively. If we estimate an error of $\pm$ 0.3 gauss in both the measurements and the extrapolation procedure, then the average value of the rotational correlation time of spin-labeled α-chymotrypsin is $12 \pm 2 \times 10^{-9}$ sec in aqueous solution at 20.0° C.

It is known that label I is bound with a unique orientation to α-chymotrypsin in the crystalline state;[3] and it is probable that label II, by reason of its bulkiness, is rigidly bound also. Moreover, our observed value of τ_2 is greater by a factor of 1.6 than the value predicted for a rigid sphere of the same mass and density as α-chymotrypsin. We may

therefore conclude that our measured value of the rotational correlation time reflects the rigid body motion of the enzyme. This time can be compared with a correlation time of 16 nanoseconds obtained from fluorescence depolarization measurements on anthraniloyl chymotrypsin.[7] A possible source of disagreement between these times is a rotational diffusion of α-chymotrypsin that is not exactly isotropic. This would lead to two different averagings of the rotational motions for a nonspherical protein and, consequently, to two different correlation times.

Measurements of the rotational correlation times of other proteins and enzymes with this method are in progress.

Acknowledgements

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