Organophosphate Spin-Label Studies of Inhibited Esterases, α-Chymotrypsin and Cholinesterase

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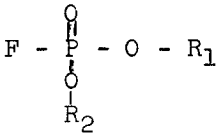
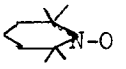
ABSTRACT

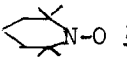
A new ESR spin-label has been synthesized which parallels the action of the standard DIFP (diisopropylfluorophosphate) inhibitors for esterase enzymes. The labels differ from DIFP in that one or more of the ester linkages is an organic nitroxide molecule. Preliminary inhibition studies of α-chymotrypsin, trypsin, choline-esterase, and thrombin show strikingly different spectra for the spin-labelled inhibited enzymes indicating a variation in the structure of the active site in the vicinity of the active serine. It is suggested that such labels should be of a very general use in studying the nature of the active site under catalytic conditions.

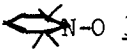
Spin labelling as a general tool for probing the structure of macromolecules has been well established.[1,2,6] The usefulness of the technique is somewhat dependent upon one's ability to design labels which will specifically attach themselves, via a covalent or hydrophobic linkage, to that particular part of the macromolecule of interest. Thus spin labels which are sulfhydryl reagents, ε-amino reagents, substrates, haptens or long chain fatty acids have found extensive use in probing biomolecules. We report here a series of organo-phosphate spin labels which parallel the well known DIFP esterase enzyme inhibitor and which should expand considerably the application of this technique in the area of enzyme active site structure and function.

The following spin labels have been synthesized and used in preliminary studies of α-chymotrypsin, trypsin, cholinesterase, and thrombin.



where $R_1 = R_2 =$  1

R_1 = isopropyl, $R_2 =$  2

R_1 = cyclohexyl, $R_2 =$  3

The synthetic routes followed were those illustrated in (3) & (4) with a few modifications.

The spin labels react identically to their DIFP counterparts and therefore serve as excellent probes of the structural differ-

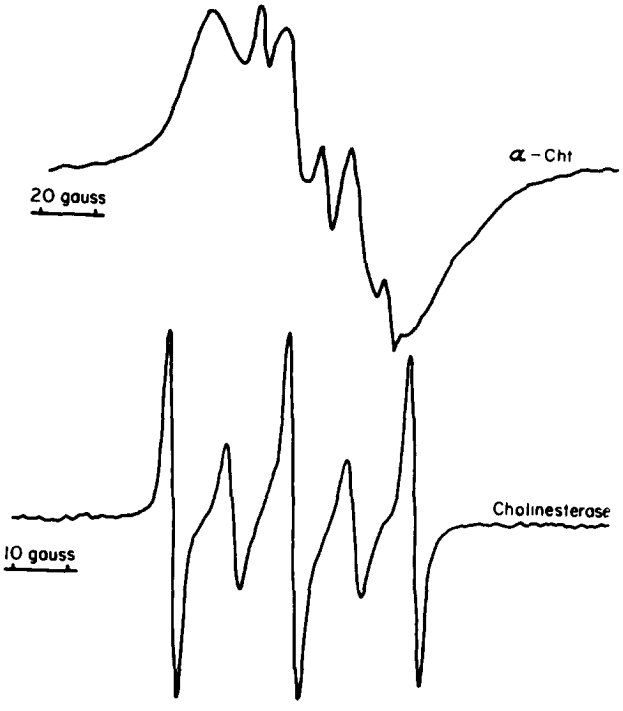


Fig. 1 a.) ESR spectrum of inhibited α-chymotrypsin using the double spin label phosphate ester 1. Spectrum shows a high degree of immobilization of the spin and a retardation in electron - electron exchange. pH = 8.0

1 b.) Illustrates ESR spectrum for inhibited choline esterase (horse serum) using some spin label phosphate ester as 1a. Spectrum shows only partial immobilization as reflected in the decrease of electron- electron exchange.

ences in the active sites of the serine esterases. This is exemplified by Figures 1 a and b, which reproduce the spectra of label 1 attached to α-ChT and cholinesterase (horse serum) respectively. It can be seen from these figures that the spin label spectra for the two enzymes are quite different, one showing a greater degree of immobilization of the spin label than the other, suggesting that the active site of α-chymotrypsin is in a more confined region of the protein whereas that of cholinesterase is less confining and probably on or near the surface of the protein. This label is affected by the other esterases studied in equally different and dramatic ways. For this comparative study, the double label 1 is more useful, but not the only

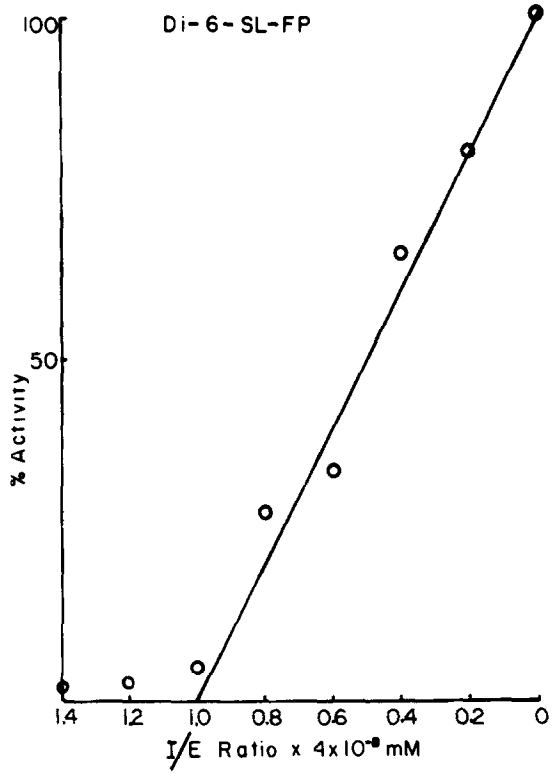


Fig. 2 Plot α-chymotrypsin activity as a function of the ratio of inhibitor spin label 1 concentration to enzyme. Curve extrapolates to a stoichiometry of 1 mole inhibitor per mole enzyme at zero activity.

one to show the differences, for not only does the overall rotational freedom of the label affect the observed spectrum, but the orientational requirements for electron exchange adds an intramolecular indicator of subtle structural differences.

Figure 2 illustrates the stoichiometry of the inhibition reaction of α-ChT with 1 which is identical to that for inhibition by DIFP. Furthermore, previously inhibited α-ChT(DIFP) does not incorporate any label, substantiating the identical behavior of these two organophosphates. The pH - rate profile of inhibition for α-chymotrypsin with spin label 1 is nearly identical to that of DIFP, yielding a $K_{I\ max} = 1.2 \times 10^5\ M^{-1}\ sec^{-1}$ at pH 8.5. Importantly, the rate of inhibition of trypsin and cholinesterase is substantially slower. We are currently designing specific inhibitors for these anionic enzymes (cation acceptors) and in the case of cholinesterase labelling it in vivo in cell membranes and nerve tissue. The details of all phases of these investigations will be published later.

Acknowledgement

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