Study of the Polarity of the Active Site of Chymotrypsin*

John Kallos and Katy Avatis

ABSTRACT: A new method for measuring the polarity of the environment at the active site of chymotrypsin (CHT) is described. The shift in the difference spectra of *p*-nitrobenzenesulfonylchymotrypsin (nisyl-CHT) *vs.* *p*-toluenesulfonylchymotrypsin (tosyl-CHT) was found to depend on the polarity of the environment at the active site. The spectral shift to shorter wavelength strongly indicates that the nisyl group is buried in a hydrophobic cavity and is inaccessible to water molecules.

The dielectric constant of the microenvironment at the active site was found to correspond to that of cyclohexane. Disruption of the three-dimensional structure of the protein resulted in the displacement of the nisyl group into the aqueous medium. The heat denaturation was found to be reversible, *i.e.*, on cooling the nisyl group returned into its hydrophobic cavity. Measurements of the dielectric constant might shed a new light on the action of enzymes in their native "milieu."

In the study of the correlation of enzyme structure and function, one of the primary questions to be determined is the structure and the properties of the active site. During the past few years, considerable progress has been made in the identification of the amino acid residues at the active site of proteolytic enzymes by the use of chemical labeling techniques (Hartley, 1964). Varied approaches have been utilized in studying the topography of the binding site of proteolytic enzymes. Niemann (1964), using various substrates and competitive inhibitors, suggests a hydrocarbon-like environment at the active site of chymotrypsin[1] (CHT). On the other hand, spectroscopic studies on cinnamoyl-CHT (Bender *et al.*, 1962) indicate a perturbation of the cinnamoyl group in the native enzyme and suggest that the environment of the active site is more polar than the aqueous medium. As an extension of this study, Bernhard *et al.* (1965) investigated the spectra of a variety of arylacryloyl derivatives of CHT in an attempt to identify the acyl-enzyme intermediate. Thus it is apparent that current attempts to correlate spectral and chemical properties of the acyl-enzyme have so far not led to any definite conclusions. Recently, Burr and Koshland (1964) have described a very elegant technique utilizing a "reporter group." This method allows the study of enzyme–substrate interaction, but as yet no results bearing on the above question have been reported.

X-Ray crystallography made it possible to determine the complete three-dimensional structure of myoglobin (Kendrew, 1962, 1963) and hemoglobin. From this study, it has been established that nonpolar hydrophobic residues avoid contact with an aqueous medium by being buried in the interior of the protein molecule where they are in close contiguity, whereas most of the polar groups are located on the protein surface.

It would be of interest to find out if techniques other than X-ray crystallography could yield information as to whether the active site is located on the surface of the protein or buried in the interior in a nonpolar environment. Determination of the "local dielectric constant" and other local effects should yield information about the above problem of the location of the active site and shed new light on the action of enzymes in their native "milieu." To aid in the solution of these problems, a new method has been developed by which the polarity of the environment of the active site can be measured. Briefly stated, it consists in the introduction of a chromophoric group at the active site of the enzyme molecule; as the protein contains a number of polar and nonpolar residues, the interaction between these residues and the chromophoric group can be followed by characteristic spectrals shifts. The principle of the method is as follows: (1) selection of a chromophoric group for which a scale of solvent polarity was established and measured *via* spectral shifts; (2) the introduction of this chromophoric group into the active site of the enzyme by covalent attachment; (3) for compensation of the possible perturbation of the protein surface due to the introduction of the chromophoric group, a parent enzyme derivative was prepared in which the chromophoric group was replaced by a nonchromophoric group of similar shape and size (a similar approach is used for isomorphous replacement in X-ray diffraction studies); and (4) the difference spectrum of the chromophoric enzyme derivative *vs.* the parent enzyme was recorded. The polarity of the microenvironment of the chromophoric group at the active site was

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[1] Abbreviations used: CHT, chymotrypsin; α-CHT, α-chymotrypsin; acyl-CHT, acylchymotrypsin; nisyl-CHT, *p*-nitrobenzenesulfonylchymotrypsin; tosyl-CHT, *p*-toluenesulfonylchymotrypsin; BTEE, benzoyl-L-tyrosine ethyl ester.

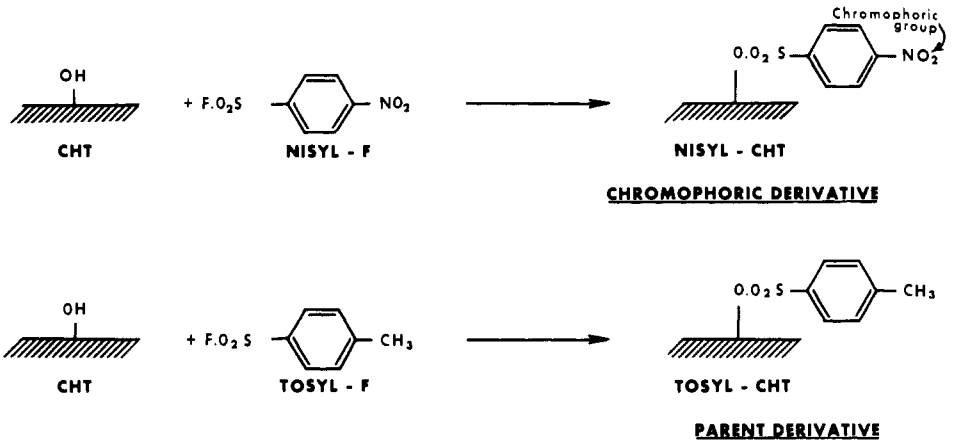

FIGURE 1: Schematic representation of the comparison between chymotrypsin (CHT) containing a chromophoric and a nonchromophoric group.

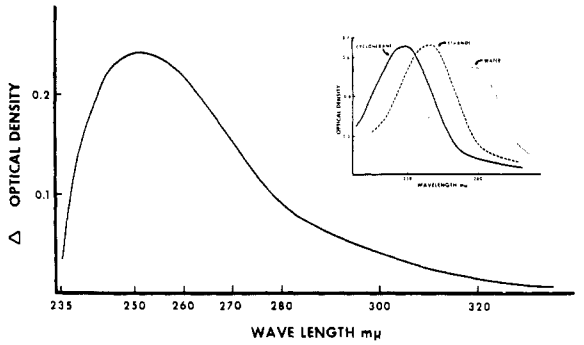

FIGURE 2: Difference spectra of nisyl-CHT *vs.* tosyl-CHT; 0.5% protein solution in 0.02N acetate buffer pH 4.5, 0.05N KCl, 0.02N CaCl. Insert: Solvent effect on the spectra of model compounds of nisyl esters.

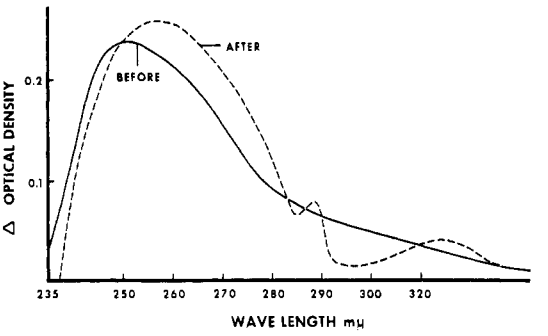

FIGURE 3: Effect of proteolytic digestion on difference spectra of nisyl-CHT *vs.* tosyl-CHT; 0.5% protein solution and 0.01% trypsin solution at pH 7.8 in 0.05 M Tris buffer at 25°; before and after 2 hr of digestion at 38°.

measured by comparing the spectral shift obtained with previously established polarity scales.

The following is the application of this principle to a study of the active site of CHT: a chromophoric group was introduced at the serine residue of the active site of CHT by treating CHT with *p*-nitrobenzenesulfonyl fluoride, called nisyl fluoride. Nisyl fluoride was selected because it was previously shown by us (Kallos and Rizok, 1964) and others (Gold and Fahrney, 1964) that benzenesulfonyl fluoride is a specific inhibitor of CHT and also because the nitrobenzene portion of the molecule undergoes a large spectral shift as the polarity of the medium is varied. For comparative spectral studies a parent enzyme, tosyl CHT, was prepared (Kallos and Rizok, 1964). Figure 1 shows that the two enzyme derivatives are alike in every respect except for the chromophoric nitro group which has been replaced by a methyl group. Thus, difference spectra of nisyl-CHT *vs.* tosyl-CHT should give a measure of the polarity of the active site when compared with the scale of solvent polarity.

Materials and Methods

α-CHT, three times crystallized, salt free, lyophilized preparations were obtained from Worthington Biochemical Corp.; trypsin, twice crystallized, lyophilized, was obtained from the same source. Protein concentration of the α-CHT was determined spectrophotometrically at 282 mμ using extinction coefficient $E^{1\%}_{1cm}$ 20.0 (Dixon and Neurath, 1957). The molecular weight of CHT was taken as 25,000 (Wilcox *et al.*, 1957). Enzyme activity was determined by the spectrophotometric method of Hummel (1959) using BTEE as substrate and by the colorimetric hydrazide method (Kallos *et al.*, 1965) using *N*-acetyltyrosine hydrazide as substrate. For spectra, protein solutions were centrifuged at 15,000 rpm to obtain optically clear solutions. For difference spectra, protein samples were usually weighed in triplicate and optical density was checked at 282 mμ. Spectra measurements were performed at 23° using Bausch and Lomb Spectronic 505 or Beckman DK-2, both equipped with a thermostatic cell com-

partment. The nisyl fluoride was prepared from nisyl chloride and the synthesis of *N*-acetyl-*p*-nitrobenzenesulfonylserinamide and other related nisyl esters are described elsewhere (Avatis *et al.*, 1965, and unpublished data). For enzymic hydrolysis, the nisyl and the tosyl-CHT were digested with trypsin (1:50 ratio, enzyme:protein, w/w) at 38° at pH 7.8 for 2 hr.

Preparation of Nisyl-CHT. CHT solution (4.0×10^{-4} M) was prepared by dissolving 720 mg of CHT in 72 ml of 0.05 M phosphate buffer (pH 7.2). Nisyl-F solution (4.5×10^{-3} M) was prepared by dissolving 18.5 mg of nisyl-F in 20 ml of 40% aqueous acetonitrile. The nisyl-F solution was mixed with the CHT solution and stirred gently for 10–15 min. Then the solution was allowed to stand for 1 hr at 4°. After extensive dialysis *vs.* 5×10^{-4} N HCl, the protein solution was lyophilized. The residual enzyme activity was found to be <2%. The inhibited enzyme was crystallized from a 1% protein solution with half-saturated $(NH_4)_2SO_4$. The preparation of tosyl-CHT was previously described.

Results and Discussion

Solvent Effect and Ultraviolet Spectra of Model Compounds. The nitrobenzene group was selected to measure the polarity of the active site since this group undergoes a large spectral shift as the polarity of the medium is varied. The π–π^* transition usually occurs at the longer wavelength as the dielectric constant of the medium is increased (Kasha, 1961; Kosower, 1958). It is apparent from Table I that the absorption maximum of nitrobenzene (Ungnade, 1953) is shifted toward the longer wavelength as the polarity of the solvent increases: 265 mμ in water, 251 mμ in heptane, and 239 mμ in vapor phase.

TABLE I: Solvent Effect on the Spectrum of Nitrobenzene and Nisyl Esters.

		$RO_3SC_6H_4NO_2$	
Solvent	Nitrobenzene (max mμ)	R = Methyl (max mμ)	R = *N*-Acetylserinamide (max mμ)
Water	265.5	260	259.3
Ethanol	259.5	255	255
Ether		252	252.6
Cyclohexane	251.8	250.8	Insol.
Vapor	239.1		

In the nisyl-CHT it has been shown that the nisyl group is attached to the serine residue at the active site *via* an ester linkage (Avatis *et al.*, 1965, and unpublished data). Therefore, various nisyl ester derivatives were prepared and their spectra in different media were examined. It is apparent from Table I that the spectral shift of the nisyl esters is essentially similar to that of nitrobenzene. In a nonpolar medium such as cyclohexane λ_{max} is located at 251 mμ and in water is shifted to 260 mμ. In this way, the dielectric constant of the medium can be measured from the spectral shift.

Difference Spectra of Nisyl-CHT vs. Tosyl-CHT. It was illustrated in Figure 1 that the two enzyme derivatives are alike in every respect except that in the parent enzyme the chromophoric nitro group was replaced by a methyl group. Assuming the similarity in size and shape between the nitro and the methyl groups, we may assume that such replacement is permissible without perturbing the protein structure. Consequently, the difference spectra should reflect only the environment of the nitrobenzene group which is attached to the active site and should be independent of the spectral properties of the protein itself.

The ultraviolet difference spectrum of the nisyl-CHT *vs.* tosyl-CHT at pH 4.5 is shown in Figure 2. This spectrum was found to be pH independent in the range from 3.0 to 8.2. It is seen that the difference spectrum consists of an intense absorption maximum at 251 mμ due to the introduction of the nisyl group. However, the difference spectrum is not symmetrical as are the spectra of other nitrobenzene derivates. It appears that the nisyl group when it is attached to the active site of CHT absorbs as the free nisyl group in a nonpolar medium. These findings suggest that the nisyl group is buried in a *hydrophobic cavity* and is probably inaccessible to water molecules. When the 251-mμ absorption maximum is compared with the scale of solvent polarity, Table I, it seems that the dielectric constant of the microenvironment of the nisyl group at the active site of the enzyme corresponds to that of cyclohexane. Such a hydrocarbon-like environment indicates that the binding site of the enzyme must be built from nonpolar hydrophobic amino acid residues.

If the nisyl group is located in a hydrophobic cavity surrounding the active site, then the disruption of the three-dimensional structure of the protein should result in the transfer of the nisyl group into the aqueous medium. This expectation was fulfilled when the protein structure was disrupted by partial enzymic hydrolysis or by heat denaturation. When nisyl-CHT and tosyl-CHT are digested with trypsin, the difference spectrum is displaced from 251 to 260 mμ as shown in Figure 3. The maximum at 260 mμ corresponds to the absorption of the nisyl group in aqueous medium as was already shown in Table I. These results confirm that by breaking some of the covalent bonds in the protein by enzymic hydrolysis, the nisyl group becomes transferred to the aqueous medium.

The heat denaturation of nisyl-CHT and tosyl-CHT was followed by difference spectra in the temperature range from 30 to 50° and the results are shown in Table II. It is evident from Table II that there is a direct relationship between the increasing heat effect and the successive displacement of the absorption

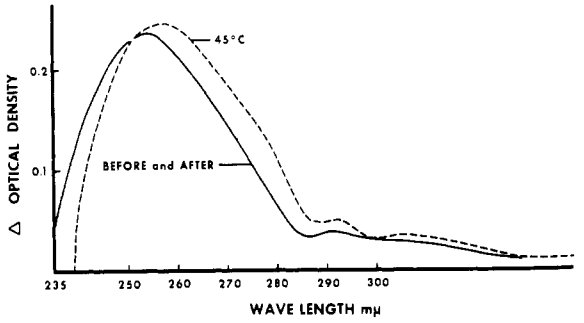

FIGURE 4: Effect of heat denaturation on difference spectra of nisyl-CHT *vs.* tosyl-CHT; 0.5% protein solution in 0.02 N acetate buffer, pH 3.6, 0.05 N KCl, 0.02 N $CaCl_2$.

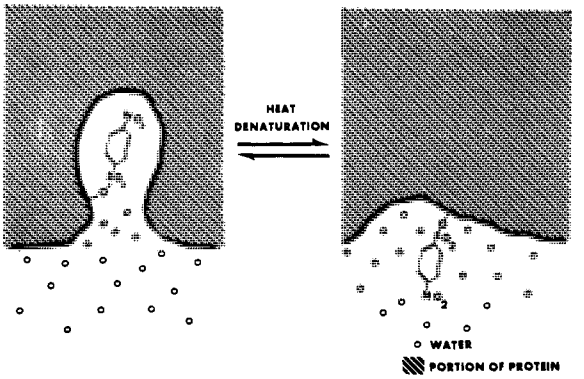

FIGURE 5: Schematic representation of the nisyl group location inside or outside of the protein molecule.

TABLE II: Increasing Heat Effect on the Difference Spectra of Nisyl-CHT *vs.* Tosyl-CHT.[a]

Temp (°C)	λ_{max} (mμ)
37	251
42	254
45	256
48	259

[a] Protein solution (0.5%) in 0.02 N acetate buffer, pH 3.6, 0.05 N KCl, 0.02 N $CaCl_2$, after 15 min of heating.

maximum to longer wavelength. Finally, heat denaturation was found to be reversible when it was carried out at 45°. When the two enzyme solutions are heated to 45° at pH 3.5, the difference spectrum shifts from 251 to 256 mμ as is illustrated in Figure 4. After standing at 4° for 2 days, the maximum returned to the original position at 251 mμ. The likely interpretation of this reversible heat denaturation as illustrated in Figures 4 and 5 is that the nisyl group is located in a hydrophobic cavity in the native protein. Heat denaturation partly disrupts the protein structure and this results in the displacement of the nisyl group into the aqueous medium. On cooling the process is reversed, and the nisyl group is returned to its hydrophobic cavity. This experiment indicates the flexibility of the nisyl group which is able to move from the inside to the outside and *vice versa*. We have indicated that the dielectric constant of the microenvironment of the active site corresponds to that of cyclohexane. No numerical values have been given to the dielectric constant as these measurements are based on several assumptions and are best used as a comparative scale rather than as absolute values.

Recently, considerable attention has been focused on the role of water in the structure of macromolecules and on the noncovalent attractive forces which stabilize a protein. Based on thermodynamic properties of liquid water and aqueous hydrocarbons, Némethy and Scheraga (1962) made an estimate of the free energy change for hydrophobic bond formation. According to this model system the driving force for the transfer of nonpolar side chains to the interior of the protein molecule arises from the gain in entropy resulting when these side chains leave the aqueous phase and the water becomes more disordered.

The hydrophobic bond undoubtedly also plays an important role in enzyme–substrate interaction. The binding site might be visualized as a hydrophobic cavity of proper shape and size into which the aromatic group of the CHT substrate must fit. As for the binding energy, perhaps the simplest explanation involves the transfer of the aromatic substrate from the aqueous medium into the microorganic cavity of the enzyme with the exclusion of water.

The binding site though buried in a hydrophobic cavity must be in close proximity to the functional groups at the catalytic site consisting of histidine-40, histidine-57, and serine-196. The amino acid residues that constitute the binding site have yet to be established. Although the catalytic process of the enzyme requires a high water concentration around the histidine and serine at the catalytic site, our findings strongly suggest that the binding site is hydrophobic and inaccessible to water.

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Hydrolysis of Neutral Substrates by Acetylcholinesterase*

R. M. Krupka

ABSTRACT: Evidence is presented to show that the same active center in a purified preparation of bovine erythrocyte acetylcholinesterase acts upon cationic and uncharged substrates. The pH dependence of V/K_m for hydrolysis of the two types of substrate differs. Experiments with acetylcholine show that the enzyme becomes inactive when an ionizing group of pK = 6.3 in the free enzyme is protonated, while experiments with phenyl acetate and isoamyl acetate indicate that another functional ionizing group in the free enzyme has a pK of 5.5. Thus two enzyme groups that ionize on the acid side of the pH optimum affect substrate hydrolysis. One (pK = 6.3) plays no essential role in substrate binding or in reaction of the enzyme–substrate complex, but when protonated, prevents cationic substrates from becoming bound to the active center. The other ionizing group (pK = 5.5) is an essential component of the catalytic mechanism.

Variations in the rates of enzymic reactions with pH may be due to ionizations of groups in the free enzyme which function in catalysis, but when the substrate bears a positive or negative charge, they may also result from ionizations that simply affect substrate binding. The latter groups may be revealed by comparison of the hydrolysis of neutral and charged substrates at different pH values. Before this comparison can be made, however, the same active center must be shown to act upon both types of substrate. This may be done in several ways. (1) Mixtures of the two substrates may be shown to compete for a single active site. (2) The dissociation constants of specific inhibitors may be shown to be the same with both types of substrate. (3) The same pH dependence for inhibition may be demonstrated with charged and neutral substrates.

Acetylcholine (AcCh),[1] the natural substrate of acetylcholinesterase (AChE), was chosen as a charged substrate, and phenyl acetate and isoamyl acetate as neutral substrates. Phenyl acetate is rapidly split by AChE, the rate-limiting step probably being deacetylation, as in the case of AcCh (Krupka, 1964). Isoamyl acetate is more slowly hydrolyzed, but is one of the best uncharged acetyl ester substrates derived from an aliphatic alcohol, since it sterically resembles AcCh (Mounter and Whittaker, 1950; Mounter and Cheatham, 1963).

Experimental Methods

The enzyme, supplied by Sigma Chemical Co. or Nutritional Biochemicals Corp., was a purified preparation from bovine erythrocytes. The two preparations had essentially the same kinetic properties. Spectrophotometric determinations of phenyl acetate hydrolysis were carried out in a Beckman DK2 recording spectrophotometer fitted with a thermostated cell holder, through which water at 26° was circulated. The reaction mixture was prepared as follows: to 5 ml of sodium phosphate buffer, 0.1 M, pH 7.5, were added various volumes (0.05–0.2 ml) of 0.517 M phenyl acetate in methanol[2] together with corresponding volumes of pure methanol, so that the total of methanol and phenyl acetate was 0.2 ml in all cases. In inhibited reactions 0.3 ml of 2.01×10^{-2} M AcCh was added. The volume

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[1] Abbreviations: AcCh, acetylcholine; AChE, acetylcholinesterase.

[2] Methanol (1–2%) caused only a small decrease (*ca.* 2–3%) in the maximum rate of AcCh hydrolysis and was accordingly used to solubilize the neutral substrates.