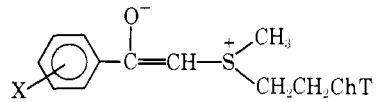
Phenacyl Bromides as Chromophoric Reagents for α-Chymotrypsin*

D. S. Sigman,† D. A. Torchia,‡ and E. R. Blout

ABSTRACT: Several ring-substituted phenacyl bromides were used to alkylate α-chymotrypsin. In each instance alkylation occurred on methionine-192 of the enzyme and the alkylated enzyme exhibited an intense optically active absorption band in the region 290–365 mμ which is not present in the spectra of the native enzyme or the reagent. Two possible explanations for the new band are considered: (1) the formation of a charge-transfer complex between the indole portion of a tryptophan residue of chymotrypsin and the aromatic part of the reagents; (2) the formation of a sulfonium ylide on the enzyme rather than the normal reaction product, the sulfonium salt. To distinguish between these possibilities spectral studies of model charge-transfer systems involving indole and ring-substituted phenacyl moieties were carried out. The results obtained for both inter- and intramolecular complexes are reported. Spectral studies have also been made with model phenacylsulfonium salts and ylides. In addition, the spectral changes observed when the alkylated enzyme was treated with urea or heated at various pH values are discussed. It is concluded from all these data that ylide absorption is the source of the new long-wavelength band. The stability and spectral properties of the ylides are sensitive functions of their environment, and this suggests that phenacyl bromides might be useful reagents for protein modification.

α-Bromo-4-nitroacetophenone[1] alkylates chymotrypsin at methionine-192 to yield an enzyme derivative with a characteristic absorption maximum at 350 mμ (Chou *et al.*, 1963; Sigman and Blout, 1967). The dependence of this new absorption band upon the native conformation of the protein at acidic pH values and the presence of a similar band at 365 mμ in chymotrypsin alkylated with α-bromo-2,4-dinitroacetophenone suggested the anomalous absorption bands arose from a charge-transfer interaction between the nitrophenacyl moiety and a vicinal tryptophan residue (Sigman and Blout, 1967).

In order to study this phenomenon further, the absorption spectra of chymotrypsin modified by a number of other substituted phenacyl bromides were studied. All the enzyme derivatives demonstrated new transitions present neither in the enzyme nor the alkylating agent. However, these new absorptions could not be adequately explained in terms of a charge-transfer interaction. In the present report, evidence is presented that a more likely interpretation of these new bands is that they are due to the stabilization by the enzyme of the dipolar or ylide form (I) of the sulfonium salt produced by alkylation of the methionine residue.



I

Experimental Section

Instrumentation. Ultraviolet and visible absorption spectra were measured on a Cary 15 spectrophotometer, while circular dichroism spectra were measured on a Jasco Model ORD/UV-5 equipped with a circular dichroism attachment and a Perkin-Elmer Model 581 was used for infrared spectra.

Solid-State Spectra. For solid state-ultraviolet absorption measurements, crystals were suspended in a transparent mull composed of PO-1 and dodecane[2] (Kahn and Beychok, 1968). After mixing the crystals into the mull, the mixture was rolled into a ball and pressed between two quartz disks. The disks were kept under constant pressure by placing them

* From the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115 (D. S. S., D. A. T., and E. R. B.), and the Department of Biological Chemistry, U. C. L. A. School of Medicine, Los Angeles, California (D. S. S.). *Received July 17, 1969.* We are pleased to acknowledge the support in part of this work by U. S. Public Health Service Grant AM-07300.

† National Institutes of Health Postdoctoral Fellow at Harvard Medical School, 1965–1967. Present address: Department of Biological Chemistry, U. C. L. A. School of Medicine, Los Angeles, Calif.

‡ National Institutes of Health Postdoctoral Fellow at Harvard Medical School, 1967–1969.

[1] The abbreviations used are: ChT, α-chymotrypsin; Br-4-NAP, α-bromo-4-nitroacetophenone; ChT-4-NAP, ChT alkalated by Br-4-NAP; Br-2,4-DNAP, α-bromo-2,4-dinitroacetophenone; ChT-2,4-DNAP, ChT alkylated by Br-2,4-DNAP; Br-2-NAP, α-bromo-2-nitroacetophenone; ChT-2-NAP, ChT alkylated by Br-2-NAP; Br-3-NAP, α-bromo-3-nitroacetophenone; ChT-3-NAP, ChT alkylated by Br-3-NAP; Br-4-CNAP, α-bromo-4-cyanoacetophenone; ChT-4-CNAP, ChT alkylated by Br-4-CNAP; Br-2-AN, α-bromo-2-acetonaphthone; ChT-2-AN, ChT alkylated by Br-2-AN; DMS-4-NAP, the dimethylsulfonium salt of Br-4-NAP; DMS-2,4-DNAP, the dimethylsulfonium salt of Br-2,4-DNAP; DMS-2-NAP, the dimethylsulfonium salt of Br-2-NAP; DMS-3-NAP, the dimethylsulfonium salt of Br-3-NAP; DMS-4-CNAP, the dimethylsulfonium salt of Br-4-CNAP; DMS-2-AN, the dimethylsulfonium salt of Br-2-AN.

[2] PO-1 is a silicon polyether manufactured by the Pierce Chemical Co. We wish to thank Dr. Sherman Beychok of Columbia University for providing us with helpful advice on preparation of the mull and for supplying us with a sample of PO-1 before the commercial product arrived.

TABLE I: Melting Points[a] and Analyses of Dimethylsulfonium Salts.

Compound		Calcd (%)					Found (%)					Mp (°C)
		C	H	N	Br	S	C	H	N	Br	S	
DMS-4-NAP	$C_{10}H_{12}BrNSO_3$	39.22	3.95	4.57	26.09	10.48	39.28	3.91	4.52	26.08	10.47	114–118
DMS-3-NAP	$C_{10}H_{12}BrNSO_3$	39.22	3.95	4.57	26.09	10.48	39.11	3.85	4.51	26.00	10.48	129–134
DMS-2-NAP	$C_{10}H_{12}BrNSO_3$	39.22	3.95	4.57	26.09	10.48	39.25	3.70	4.53	26.18	10.57	134–138
DMS-2,4-DNAP	$C_{10}H_{11}BrN_2SO_5$	34.20	3.16	7.97	22.77	9.14	34.24	3.08	7.96	22.88	9.11	122–124
DMS-4-CNAP	$C_{11}H_{12}BrNSO$	46.16	4.23	4.89	27.92	11.22	46.15	4.14	4.83	27.95	11.21	143–146
DMS-2-AN	$C_{14}H_{15}BrSO$	54.02	4.85		25.67	10.31	53.91	4.85		25.76	10.27	113–115

[a] All dimethylsulfonium salts melted with decomposition.

TABLE II: Analyses and Melting Points of Intramolecular Charge-Transfer Model Compounds.

	Calcd (%)			Found (%)			Mp (°C)
	C	H	N	C	H	N	
$C_{18}H_{14}N_2O_5$[a]	61.61	4.89	7.56	62.05	4.73	7.75	149–151
$C_{19}H_{16}N_2O_5$	64.70	4.58	7.95	64.07	4.62	8.00	161.5–162.5
$C_{20}H_{18}N_2O_5$	65.50	4.91	7.64	65.56	4.97	7.49	115.5–117.5

[a] One mole of methanol of crystallization per mole of compound assumed in calculation.

in an infrared cell assembly. To check for artifacts in the spectra, the absorbance of a given compound was measured several times using different crystal sizes, and a given sample was scanned a number of times, varying the position of the sample in the beam.

Preparation of Alkylating Agents. Br-4-NAP and Br-2-AN were purchased from Aldrich and recrystallized from chloroform–hexane. Br-4-CNAP was prepared according to Suzuki and Nagawa (1952). Br-3-NAP was prepared by the addition of bromine to the corresponding acetophenone in $CHCl_3$. The reaction flask was illuminated with a 250-W sun lamp and the product was isolated by the addition of hexane. Recrystallization from chloroform–hexane gave the product (mp 95–96°, lit. (Heilbron and Bunbury, 1953) mp 96°), Br-2-NAP was prepared by a literature procedure (Schofield and Simpson, 1948) (mp 54–55°, lit. (Heilbron and Bunbury, 1953) mp 56°).

Br-2,4-DNAP was prepared by the bromination of the acetophenone in acetic acid. The acetophenone (2.66 g) was dissolved in 40 ml of glacial acetic acid and warmed to 50°. Bromine (2.025 g) in 8 ml of acetic acid was then added over a 1-hr period. Stirring was continued for an additional hour and then 175 ml of hexane was added and an oil was obtained which later crystallized. Diamorphic crystals (mp 85–86 and 95–96°) were obtained after recrystallization from chloroform–hexane. *Anal.* Calcd for $C_8H_5BrN_2O_5$: C, 33.2; H, 1.7. Found: C, 33.2; H, 1.7.

The 2,4-dinitroacetophenone was prepared from its corresponding oxime which in turn was prepared from 2,4-dinitroethylbenzene (Schofield and Simpson, 1948; Reich and Nicolaeva, 1919).

Preparation of the Dimethylsulfonium Salts. DMS-4-NAP was prepared by adding 0.755 ml of dimethyl sulfide to a solution of 2.4 g of Br-4-NAP in 16 ml of acetone and 0.7 ml of H_2O. The reaction mixture was stirred for 4 hr at 25° and yielded a white crystalline product which was filtered, washed with acetone, air dried, and recrystallized from MeOH–ether. The syntheses of the other sulfonium salts were performed analogously and Table I contains the melting points and analyses of the resulting compounds.

Syntheses of the Intramolecular Charge-Transfer Model Compounds. The 4-nitrophenacyl esters of indoleacetic, indolepropionic, and indolebutyric acid were synthesized. The ester of the indoleacetic acid was prepared by dissolving 0.55 g of indole-3-acetic acid in 2.95 ml of 1 *N* NaOH. After addition of 0.15 ml of 1 *N* HCl, 0.66 g of Br-4-NAP dissolved in 20 ml of EtOH was added to the solution. The solution was then refluxed for an hour under a nitrogen atmosphere and, after cooling, yellow-orange crystals were collected and recrystallized from MeOH. The propionic and butyric acid derivatives were synthesized in analogous fashion. The infrared spectra of the compounds were consistent with the proposed structures and the analyses and melting points are provided in Table II.

Preparation of Chymotrypsin Derivatives. Worthington three times-crystallized ChT was used. All alkylated chymotrypsins were prepared by allowing a solution of 2–4 $\times 10^{-5}$ M ChT and 2–5 $\times 10^{-4}$ M alkylating agent to stand at room temperature for 10–30 half-lives in 5% acetonitrile–acetate buffer (0.05 M, pH 5.6). The progress of the reaction was monitored spectrophotometrically or by following the rate of loss of activity toward *N*-Ac-L-TyrEt (Schwert and Take-

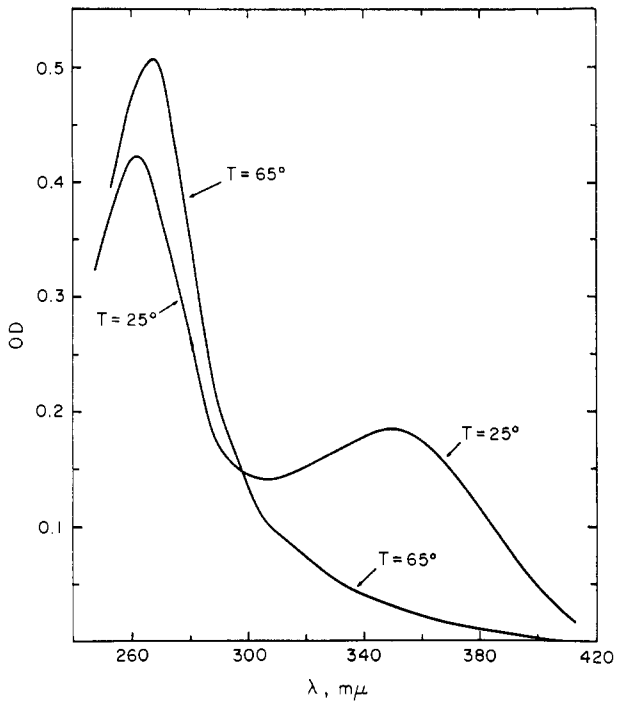


FIGURE 1: Spectral changes of 4-nitroacetophenone on ChT accompanying heat denaturation in 10^{-3} M HCl. Spectra were measured at 25 and 65° and the concentration of ChT-4-NAP was 2.9×10^{-5} M.

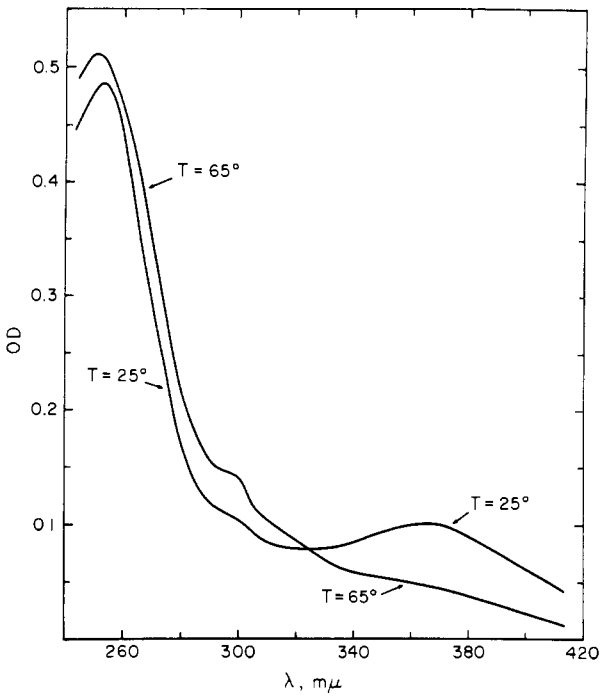


FIGURE 2: Spectral changes of 2,4-dinitroacetophenone on ChT accompanying heat denaturation in 10^{-3} M HCl. Spectra were measured at 25 and 65° and the concentration of ChT-2,4-DNAP was 3.2×10^{-5} M.

naka, 1955). After the reaction was completed, excess alkylating agent was removed by dialysis against 10^{-3} N HCl. The enzyme concentration in milligrams per milliliter was determined by multiplying the optical density at 280 mμ by 0.495 (Wu and Laskowski, 1955) and the molar concentration was obtained using a molecular weight of 25,000. Performic acid oxidations of chymotrypsin and its derivatives (Hirs, 1956) were followed by acid hydrolyses and amino acid analyses which were done on a Beckman Model 120B analyzer.

Results

Alkylation of Chymotrypsin. Chymotrypsin was modified by the following alkylating agents: α-bromo-4-nitroacetophenone, α-bromo-2,4-dinitroacetophenone, α-bromo-2-nitroacetophenone, α-bromo-3-nitroacetophenone, α-bromo-4-cyanoacetophenone, and α-bromo-2-acetonapthone.

The site of alkylation of all these modification reagents is a methionine residue. Amino acid analysis of the modified enzyme derivatives following performic acid oxidation (Hirs, 1956) demonstrated the presence of 0.25–0.5 mole of methionine/mole of enzyme and 0.8–1.1 moles of methionine sulfone/mole of enzyme. Since alkylated methionines are stable to performic acid oxidation (Neumann *et al.*, 1962) but are only partially regenerated to methionine on acid hydrolysis (Gundlach *et al.*, 1959) these results demonstrate that one of the enzyme's two methionines has been modified. Evidence has previously been presented which shows that Br-4-NAP reacts with ChT at methionine-192 (Sigman and Blout, 1967) and we assume that this methionine residue is the only one that is alkylated by the other bromacetophenones.

Spectra of Alkylated Chymotrypsin. Chymotrypsin modified by all the reagents indicated above exhibits new long-wavelength absorption bands in the 290–365-mμ region which are not present in either the alkylating agent or native enzyme. The new absorption bands of the phenacyl-chymotrypsins are lost when the modified protein is denatured by heat in 10^{-3} M HCl. For example, the spectra[3] of ChT-4-NAP and ChT-2,4-DNAP, at 25 and 65° after subtracting the ChT absorption, are compared in Figures 1 and 2. When the heat denatured alkylated ChT is cooled, the characteristic long wavelength absorption is regained.

When ChT-4-NAP is denatured in 6 M guanidine chloride at pH 5 or in 8 M urea at pH 3, the spectrum of the 4-nitroacetophenone moiety on the enzyme[4] changes in a similar way to that indicated in Figure 1. Removal of the urea by dialysis restores the characteristic absorption maximum at 350 mμ. If the same experiment is performed in 8 M urea at pH 7.0, the long wavelength absorption of ChT-4-NAP is blue shifted by 10 mμ, but is not lost, even after prolonged

[3] The total enzyme concentration determined by the 280-mμ absorption overestimates the concentration of the ylide on the alkylated ChT. Titration of native ChT with *N-trans*-cinnamoylimidazole indicates that 85% of the total ChT concentration is active. Since the sulfonium salt, rather than the ylide, is present at low pH on the denatured enzyme, the ylide concentration is possibly 15% less than that of the total modified ChT concentration at pH 3. We have assumed that the ylide concentration is equal to the total enzyme concentration as a matter of convenience. The values of ε that we have obtained in the long-wavelength region for the absorption of the ylide on the enzyme may be as much as 17% too small for this reason.

[4] Occasionally the absorption spectrum of an alkylating agent *on the enzyme* is spoken of in the text. By this is meant the absorption of the alkylated ChT after subtracting the absorption due to the native ChT.

TABLE III: pK_a's of Dimethylsulfonium Salts of Phenacyl Bromides Used to Alkylate ChT.

Sulfonium Salt[a]	pK_a
DMS-4-NAP	6.8[b]
DMS-3-NAP	7.0
DMS-2-NAP	6.1
DMS-2,4-DNAP	5.0
DMS-4-CNAP	7.0
DMS-2-AN	8.2

[a] Ionic strength of all solutions = 0.15 M. [b] Uncertainty in measurements is ±0.1 pK_a unit.

standing at room temperature. When ChT-2,4-DNAP is denatured in 8 M urea at pH 7.0, the characteristic 365-mμ maximum is lost, but a significant long-wavelength absorption tail remains.

Chymotrypsin modified by every phenacyl bromide studied shows a pronounced positive circular dichroism band at wavelengths greater than 300 mμ. Figure 3 compares the circular dichroism bands of ChT, ChT-4-NAP, and ChT-2,-4-DNAP in the long-wavelength region.

Denaturation of these modified enzymes by 8 M urea at pH 3.0, 4.0, and 7.0 results in the complete disappearance of the long-wavelength circular dichroism bands even though at pH 7 the long-wavelength absorption remains. When the urea is removed by dialysis against 10^{-3} N HCl, the long-wavelength circular dichroism is recovered.

The absorption spectra of the various phenacyl-chymotrypsins studied, change in a complex way as the pH is varied. For example, the long-wavelength absorption of ChT-2,-4-DNAP and ChT-2-NAP are invariant between pH 3 and 10.7. An increase in pH from 3 to 5.5 causes a 15% increase in the 350-mμ maximum of ChT-4-NAP. Increasing the pH from 5.5 to 10.7 does not affect the spectrum. ChT-4-CNAP, ChT-2-AN, and ChT-3-NAP exhibit long-wavelength absorption spectra which vary markedly between pH 3 and 10.7. For example, ChT-4-CNAP has an absorption band at 320 mμ which increases by a factor of almost 2 in going from pH 3.0 to 9.0. One complicating feature of the pH dependence is the observation that at low pH the absorption maxima of ChT-4-CNAP, ChT-3-NAP, and ChT-2-AN are not strictly linear with concentration.

Spectra of Charge-Transfer Model Systems. The pH independence of the long wavelength absorptions of ChT-2,4-DNAP and ChT-4-NAP suggested that the origin of these transitions was a charge-transfer interaction between the nitrophenacyl group and a nearby tryptophan residue. Solutions of both α-bromo-4-nitroacetophenone and α-bromo-2,4-dinitroacetophenone with indole exhibit anomalous absorption bands in acetonitrile. Assuming that the acetophenones and the indole formed a 1:1 complex, the Benesi-Hildebrand equation (Benesi and Hildebrand, 1949) was used to obtain the association constant and extinction coefficient of the complex. The charge-transfer complex with indole as the donor and Br-4-NAP as the acceptor has an

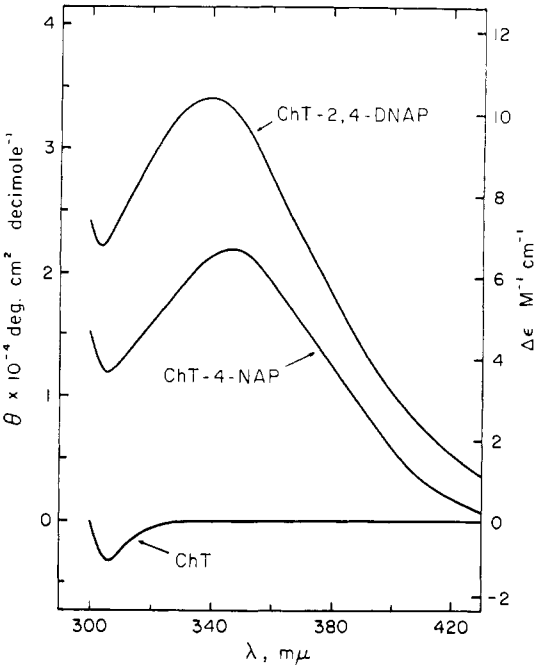
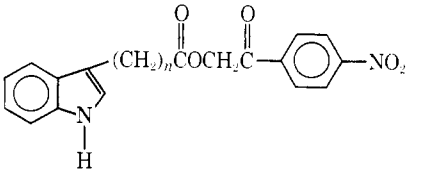


FIGURE 3: Long-wavelength circular dichroism spectra of ChT, ChT-4-NAP, and ChT-2,4-DNAP in 10^{-3} M HCl. Concentration in each case was 2.7×10^{-5} M and $T = 25°$.

extinction coefficient of 1.3×10^3 M^{-1} cm^{-1} at its absorption maximum of 330 mμ at 25°. The charge-transfer complex formed with Br-2,4-DNAP as the acceptor was found to have an extinction coefficient of 6×10^2 M^{-1} cm^{-1} at 345 mμ. The charge-transfer complexes formed with indole as a donor and the dimethylsulfonium salts of Br-4-NAP and Br-2,4-DNAP as the acceptors have absorption maxima at 335 and 360 mμ, respectively, in acetonitrile.

Since none of the absorption spectra of the above intermolecular charge-transfer complexes had extinction coefficients comparable to those observed for the corresponding enzyme derivative, compounds capable of forming intramolecular charge-transfer complexes were synthesized. Such compounds might be better models for the postulated enzyme charge-transfer complex, since the donor and acceptor in both the model compounds and modified ChT are constrained to lie within a certain distance of one another. One type of intramolecular model compound synthesized had the general structure



II

where *n* varied from 1 to 3.

The crystals of these compounds were deeply colored. For example, crystals of the indoleacetic acid derivatives were red-orange. However, the absorption spectra of these compounds in acetonitrile solution failed to exhibit any pro-

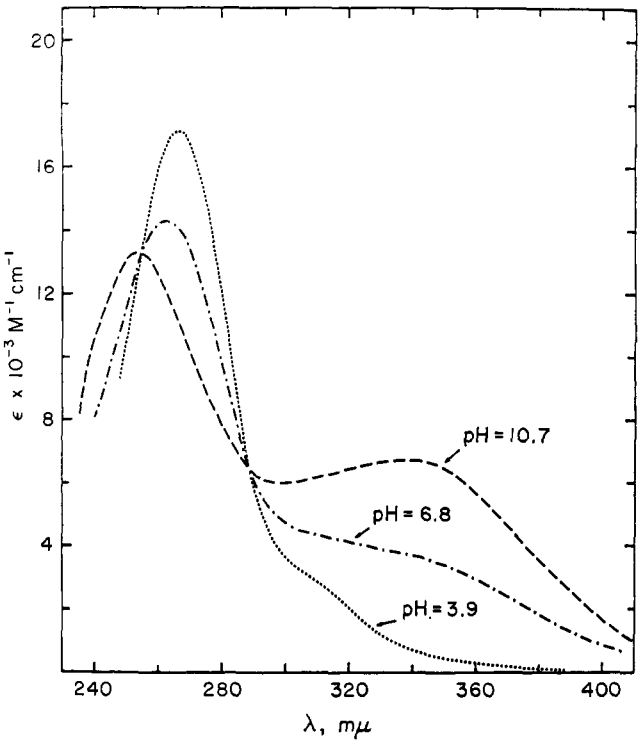


FIGURE 4: Spectrum of DMS-4-NAP in aqueous buffer as a function of pH. DMS-4-NAP at pH 3.9 (------), at pH 6.8 (- · - · -), at pH 10.7 (– – –). Ionic strength = 0.15 M; T = 25°.

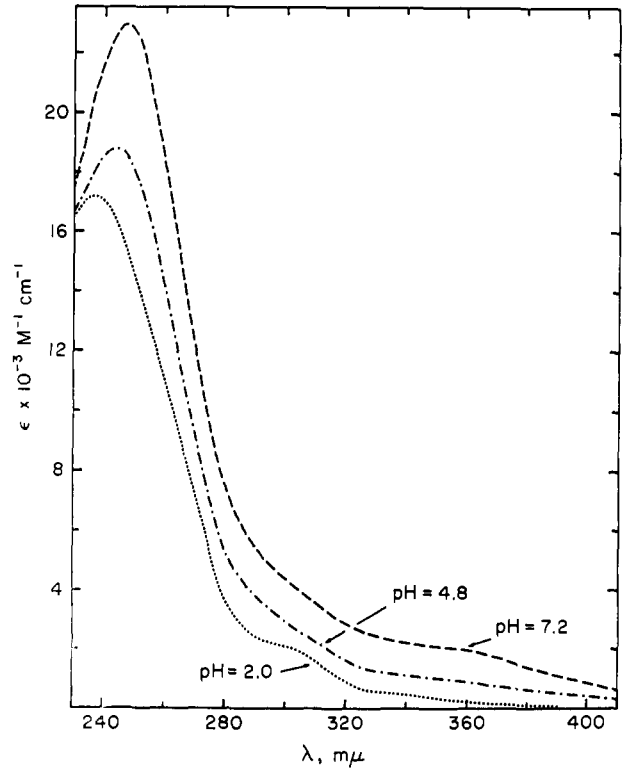
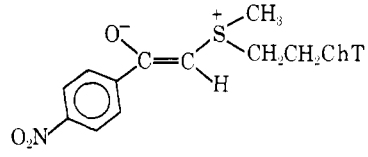


FIGURE 5: Spectrum of DMS-2,4-DNAP in aqueous buffer as a function of pH. DMS-2,4-DNAP at pH 2.0 (------), at pH 4.8 (- · - · -), at pH 7.2 (– – –). Ionic strength 0.15 M; T = 25°.

nounced maxima at 350 mμ at 25 or 0°. The spectra of the crystals in the solid state similarly failed to demonstrate any absorption maxima which corresponded to those of the enzymic derivatives.

Dimethylsulfonium Salts. The inadequacy of the charge-transfer model systems in accounting for the magnitude of the enzymic spectra led to the study of other model systems. It was reported that methionine alkylated with Br-4-NAP had a spectrum in 10^{-2} N NaOH which was very similar to that observed in the enzymic case.[5] This prompted the study of the spectra of model sulfonium salts as a function of solvent and pH. The spectra of the dimethylsulfonium salts of all the phenacyl bromides used to modify chymotrypsin were examined.

The spectra of all the model sulfonium compounds were found to be markedly pH dependent. Examples of this pH dependence are shown in Figures 4 and 5 where the spectra of the dimethylsulfonium salts of 4-nitro- and 2,4-dinitrophenacyl bromide are presented. The molecular species presumably responsible for the absorption at the more alkaline pH values are



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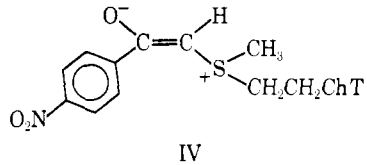

IV

Nuclear magnetic resonance studies on dimethylsulfonium phenacylide suggest III is the more stable species (Trost, 1967). The acidity constants for the dimethylsulfonium salt derivatives were computed from spectral data such as those presented in Figures 4 and 5, and are recorded in Table III. It was found that the 4-nitrophenacyl bromide salt of *N*-acetylmethioninamide had a pK_a of 5.7 as compared to pK_a of 6.8 found for the dimethylsulfonium salt of Br-4-NAP.

The absorption spectra of the dipolar or ylide structure (*i.e.*, III) of the dimethylsulfonium salt derivatives are similar in shape to the spectra of the related phenacyl bromides when they are attached to the enzyme. Corresponding absorption bands are generally within ±15 mμ of one another. The data for the 4-nitroacetophenone derivative are illustrative (Figure 6). The absorption spectra of enzyme derivatives which vary with pH can be analyzed in terms of mixtures of the ylide form and the ketone form of the dimethylsulfonium salts at any given pH. The only sulfonium salt whose ylide spectrum in H_2O did not correspond well with the spectrum of the enzyme derivative was that of Br-2,4-DNAP. The loss of the characteristic 365-mμ absorption maximum of this enzyme derivative in 8 M urea at pH 7.0 is consistent with this observation. This result suggested that the spectrum

[5] Dr. John Kallos had kindly informed us of this result in July of 1967.

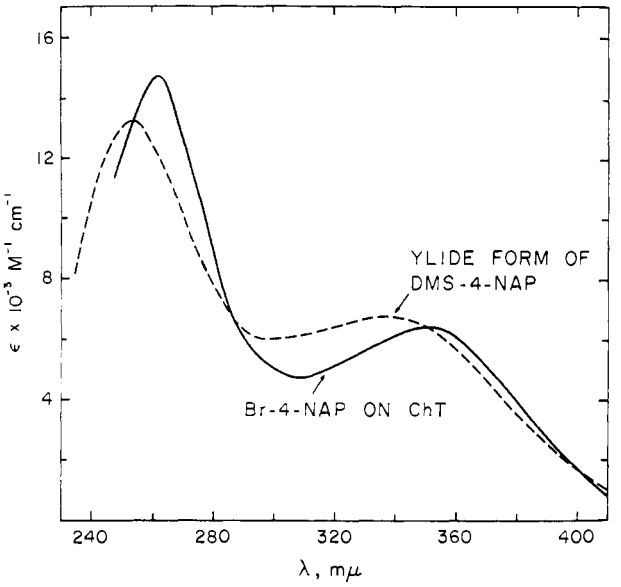

FIGURE 6: Spectrum of the 4-nitroacetophenone moiety on ChT in 10^{-3} M HCl compared with the spectrum of DMS-4-NAP in 10^{-2} M NaOH. Br-4-NAP on ChT at pH 3.0 (—), DMS-4-NAP at pH 12 (– – –).

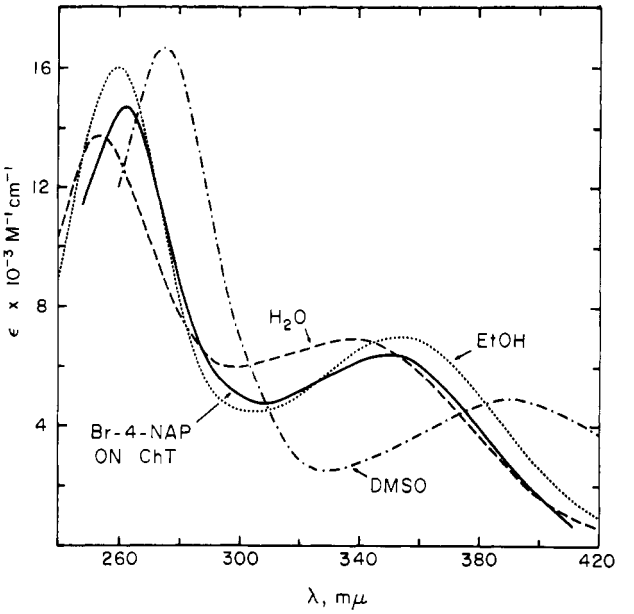

FIGURE 7: Spectrum of the 4-nitroacetophenone moiety on ChT at pH 3.0 compared to the spectra of ylide form of DMS-4-NAP in various solvents. Br-4-NAP on ChT in 10^{-3} M HCl (—), DMS-4-NAP in 99% H_2O, 0.99% EtOH, 0.01% Et_3N (– – –), DMS-4-NAP in 99.99% EtOH, 0.01% Et_3N (· · · ·), DMS-4-NAP in 99% dimethyl sulfoxide, 0.99% EtOH, 0.01% Et_3N (– · – · –). DMS-4-NAP concentration = 1.13 ± 10^{-4} M, $T = 25°$. The 0.01% Et_3N was more than sufficient to convert all DMS-4-NAP to the ylide form, since only an equimolar concentration of the base was required to effect this transformation.

of the ylide in water was different from the spectrum of the postulated ylide in the environment provided by the modified enzyme. By studying the spectra of the dimethylsulfonium salts in organic solvents it was discovered that these solvents markedly affect the ylide spectral properties.

Examples of the solvent dependence of the model ylide spectra are shown in Figures 7 and 8. The strong influence of the solvent on the model ylide absorption seen in these figures was found for all the dimethylsulfonium phenacylides and is probably related to the higher polar structure of the ylides. Generally a blue shift is observed upon increasing the polarity of the solvents. However, a notable exception is the red shift found in the polar solvent dimethyl sulfoxide.

Reducing the temperature does not greatly affect the absorption of the dimethylsulfonium 2,4-dinitrophenacylide in the long-wavelength region. The value of ϵ at 360 mμ in EtOH (0.01% Et_3N) was found to increase monotonically (by a total of 10%) on going from 29 to $-10°$. A further reduction in temperature to $-20°$ led to no additional changes in the measured absorbance.

Discussion

Two possible explanations for the anomalous absorption bands produced by the alkylation of chymotrypsin with a series of substituted phenacyl bromides are that they arise from (1) a charge-transfer interaction or (2) a stabilized, optically active, ylide form of the sulfonium salt of methionine. A number of lines of evidence favor the second interpretation.

The most cogent is that for a variety of phenacyl bromides the spectra of the model ylides in water are in close agreement with the absorption spectra of the corresponding phenacyl bromides on the enzyme. In most cases the spectra of the enzyme-bound phenacyl bromides show shifts in wavelength relative to the model ylides in aqueous solution. Such shifts are not unreasonable since as has been noted, the ylide absorption is strongly solvent dependent, and one would not expect the aqueous solvent and the environment provided by the enzyme to have identical effects on the absorption spectrum of the ylide.

The spectral changes which take place upon subjecting the alkylated enzymes to denaturating conditions are readily interpreted in terms of the stabilization of an ylide by the enzyme. Thus, if a modified enzyme is denatured at pH where the keto rather than the ylide form of the sulfonium salt is stable, pronounced changes in the absorption of the enzyme derivatives are observed. Further, these spectral changes are understandable in terms of the transformation from an ylide-type spectrum to one characteristic of the keto form of the sulfonium salt. On the other hand, if the denaturation is carried out at pH values where the ylide form of the sulfonium salt is stable, it is observed that the long-wavelength absorptions of the alkylated enzymes shift to closely resemble the spectra of the model ylides in water. This result is expected, since on denaturation, the environment provided by the enzyme is destroyed and the ylide is exposed to an aqueous environment.

The spectrum of the enzyme derivative prepared from Br-2-AN particularly supports the ylide interpretation. Acetonapthone is unlikely to serve as a charge-transfer acceptor; yet when attached to the enzyme, significant spectral perturbations are observed. These special effects can readily be interpreted in terms of ylide formation but not on the basis of charge-transfer interaction. These results demonstrate that chymotrypsin is able to stabilize the formation

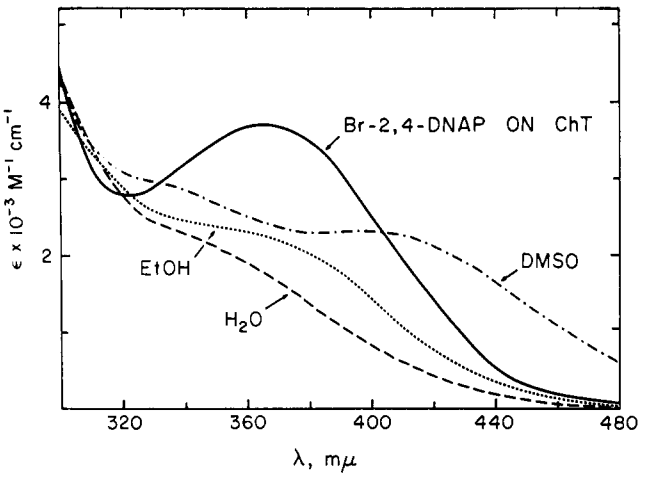

FIGURE 8: Spectrum of the 2,4-dinitroacetophenone moiety on ChT at pH 3.0 compared with the spectra of the ylide form of DMS-2,4-DNAP in various solvents. Br-2,4-DNAP on ChT in 10^{-3} M HCl (———), DMS-2,4-DNAP in 99% H_2O, 0.99% EtOH, 0.01% Et_3N (– – –), DMS-2,4-DNAP in 99.99% EtOH, 0.01% Et_3N (· · · · · ·), DMS-2,4-DNAP in 99% dimethyl sulfoxide, 0.99% EtOH, 0.01% Et_3N (–· – ·–). DMS-2,4-DNAP concentration = 1.13×10^{-4} M, T = 25°. The 0.01% Et_3N was more than sufficient to convert all DMS 2,4-DNAP to the ylide form, since only an equimolar concentration of the base was required to effect this transformation.

of an ylide even when the phenacyl bromide does not possess electron-withdrawing substituents.

The good correspondence of the ylide models with the enzymic spectra is contrasted to the lack of congruity of the model charge-transfer complexes with the enzymic spectra. None of the model charge-transfer complexes exhibit long-wavelength transitions of comparable intensity to that observed with the enzyme derivatives. In addition, the hypothesis of ylide formation accounts readily for the pH dependence of the absorption spectra observed with some of the enzyme derivatives and the spectral changes which take place upon denaturation of the enzyme derivatives over a wide pH range.

One of the most notable features of the new long-wavelength absorption bands of the enzyme derivatives is their strong circular dichroism bands. There are two possible explanations for the optical activity. The first is that the enzyme stabilizes one of the optical isomers of the asymmetric sulfur atom of the ylide. The second is that the enzyme stabilizes both optical isomers of the ylide and induces optical activity by providing an asymmetric environment for the racemized ylide. Both explanations are consistent with the loss of the circular dichroism band when the modified enzyme is denatured. However, the former interpretation better explains the large ellipticity observed for these circular dichroism bands; the magnitude of the long-wavelength circular dichroism band for all the phenacyl enzyme derivatives is from 20 to 50 times larger than the ellipticity of a single aromatic residue of the native enzyme.

The circular dichroism data suggest that there is region near methionine-192 of chymotrypsin which binds an aromatic ring in a unique and rigid configuration. Studies of the effect of solvents on the absorption spectra of model ylides suggest the environment of this site is less polar than water. One environmental factor that may contribute to decreasing the pK_a of the sulfonium salt on the derivatized enzyme 2–3 pK_a units relative to appropriate model compounds could be the presence of a center of positive charge near methionine-192 (Hille and Koshland, 1967).

In summary, the sulfonium salts produced by alkylation of chymotrypsin at methionine-192 with phenacyl bromides are stabilized by the enzyme in an optically active, ylide form. The new absorption bands found in these enzyme derivatives reflect ylide formation and not a charge-transfer interaction as had been postulated. The stability and spectral properties of the ylides are sensitive functions of their environment and this suggests that phenacyl bromides with electron-withdrawing substituents might be useful reagents for protein modification.

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