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Modification of α-Chymotrypsin by Bromomethyl Naphthyl Ketones and Behavior of Reporter Groups in the Active Site

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The reaction of α-chymotrypsin (ChT) with bromomethyl 1-naphthyl ketone (1-BNK) or with bromomethyl 2-naphthyl ketone (2-BNK) took place at the active site causing inactivation of the enzyme according to second-order kinetics. In addition to this finding, a comparison of the absorption spectrum of 2-BNK-modified ChT (2-BNK–ChT) with those of native ChT and the model dimethyl(2-naphthoylmethyl)sulfonium bromide (2-NSS) at low pH revealed that the alkylating agent, BNK, reacts with methionine-192 on ChT in a 1 : 1 stoichiometric ratio. Evidence for the existence of BNK-derived reporter groups at the enzyme active site came from the observation of (1) positive induced circular dichroism bands of these groups and of (2) pronounced absorption spectral changes of the 2-BNK-derived pendant on denaturation of the alkylated enzyme with guanidine hydrochloride at pH 7.0. From the pH dependence of the absorption spectra of the modified enzymes and the model sulfonium salts, we determined the pK_a values of 1-BNK–ChT, 1-NSS, 2-BNK–ChT, and 2-NSS to be 6.6, 7.5, 5.2, and 8.4 at 25±1 °C, respectively. The much larger difference in pK_a between 2-BNK–ChT and 2-NSS (ΔpK_a=3.2) compared with that between 1-BNK–ChT and 1-NSS (ΔpK_a=0.9) was explained in terms of stabilization of the 2-BNK-derived ylide pendant due to a configurationally rigid hydrogen-bonding interaction between the ylide carbonyl oxygen atom with a negative charge and the serine hydroxyl group located near the substrate binding site of ChT. Supporting evidence for this interpretation was obtained from solvent effects on the absorption spectra of model compounds as well as pH effects on the remaining activity of the alkylated enzymes.

α-Chymotrypsin (ChT) is one of the most extensively studied proteolytic enzymes. The active-site structure of this enzyme has been clarified by X-ray crystallographic and kinetic studies.[1] Chemical modification has also contributed to the establishment of amino acid residues involved in the binding and catalytic processes of the substrates.[2] The existence of methionine-192 at or near the active site of ChT has been manifested by an analysis of the three-dimensional structure of the active site.[1,3] The modification of ChT with an active-site-directed bifunctional reagent has shown that methionine-192 is spatially located near serine-195.[4]

The finding that alkylating agents which modify methionine-192 inactivate ChT to a different extent suggests that reporter groups attached to this amino acid residue occupy a part of the substrate-binding region in the active site.[4–7] Thus, the attachment of an environmentally-sensitive reporter group to methionine-192 made it possible to explore the microscopic environment near the substrate-binding site.[7] On the other hand, Blout and his co-workers observed a new absorption band, which was not shown by either ChT or the reagent alone, by alkylating methionine-192 with nitro-substituted phenacyl bromides.[6,8] From the pH dependence of the absorption spectra of both the modified enzyme and the model sulfonium salts, they concluded that the formation of sulfonium ylides at the enzyme active site must be responsible for the appearance of this new absorption band.[8] In addition, they showed qualitatively that the substrate-binding region near methionine-192 provides a favorable environment for the sulfonium ylide rather than for the sulfonium salt.

If different but closely-related reporter groups could be introduced into the active site of ChT, we may be able to characterize the interaction between the reporter group and the active-site amino acid residues surrounding it in more detail. To this end, we prepared bromomethyl 1-naphthyl ketone (1-BNK) and its positional isomer, bromomethyl 2-naphthyl ketone (2-BNK), which were reacted with ChT. This paper is concerned with the kinetic analysis of these modification reactions and with the spectroscopic analysis of the behavior of ChT modified by the chromophoric reagents 1-BNK and 2-BNK.

Results

Kinetics of the Inactivation of ChT by BNK. The reaction of ChT (7.4×10^{-6} M, 1 M=1 mol dm^{-3}) with 2-BNK (7.4×10^{-4} M) in 0.1 M phosphate buffer (ionic strength, I=0.25), pH 7.0, at 25±1 °C resulted in more than 90% inactivation of the enzyme within a few hours. A control sample treated in the same way except for the exclusion of 2-BNK lost no activity toward *N*-acetyl-L-tyrosine ethyl ester under the same reaction conditions. As shown in Fig. 1, the activity loss follows first-order kinetics in the presence of excess 2-BNK. In addition, the rate of the enzyme inactivation under the above conditions decreased by a factor of about 3 and 5 in the presence of 3.7×10^{-4} and 7.4×10^{-4} M indole (competitive inhibitor), respectively. This finding suggested the occurrence of a modification reaction near the substrate binding site to result in substantial inactivation of ChT. Similar results were also obtained for the reaction with excess 1-BNK.

On the other hand, we observed a linear correlation between the pseudo-first-order rate constant for the inactivation (k_{obsd}) and the initial concentration of BNK ($[BNK]_0$, Fig. 2), demonstrating that the reaction is first-order with respect to BNK and ChT. If the inactivation

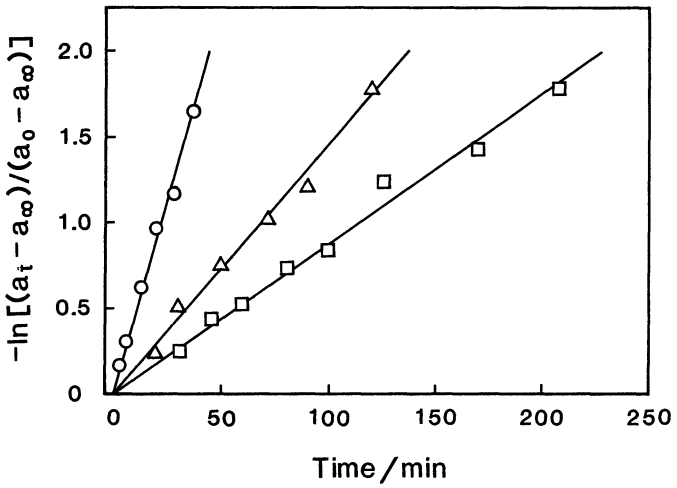

Fig. 1. Inactivation of ChT (7.4×10^{-6} M) on reaction with 2-BNK (7.4×10^{-4} M) in the absence (○) and presence (△,□) of indole in 0.1 M phosphate buffer (I=0.25), pH 7.0 at 25±1°C. Indole concentration (M): ○, 0; △, 3.7×10^{-4}; □, 7.4×10^{-4}. Data are plotted according to Eq. 1.

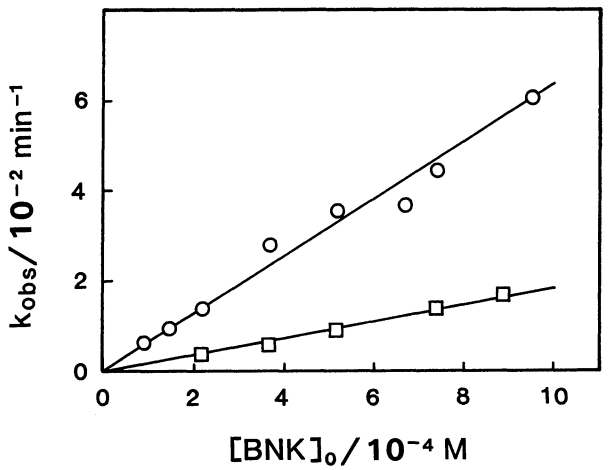

Fig. 2. Pseudo-first-order rate constants (k_{obs}) for the inactivation of ChT (7.4×10^{-6} M) by 2-BNK (○) and 1-BNK (□) at pH 7.0, 25±1°C as a function of BNK concentration.

by BNK takes place through a Michaelis-Menten-type complex, this linear relationship implies that the dissociation constant of the complex, ChT·BNK, must be much larger than the $[BNK]_0$ studied. An analysis of Fig. 2 reveals that 2-BNK reacted with ChT 3.5 times faster than did 1-BNK to inactivate this enzyme.

Effects of pH on the Absorption Spectra of Modified ChT. Fig. 3A shows the absorption spectra of ChT modified by 2-BNK (2-BNK–ChT) at typical pH values. Evidently, alterations in pH exert dramatic effects on the absorption band of a reporter group attached to an amino acid residue present at or near the active site. On increasing the pH of the solution, there appeared a new and intense band from 310 to 380 nm. For comparison,

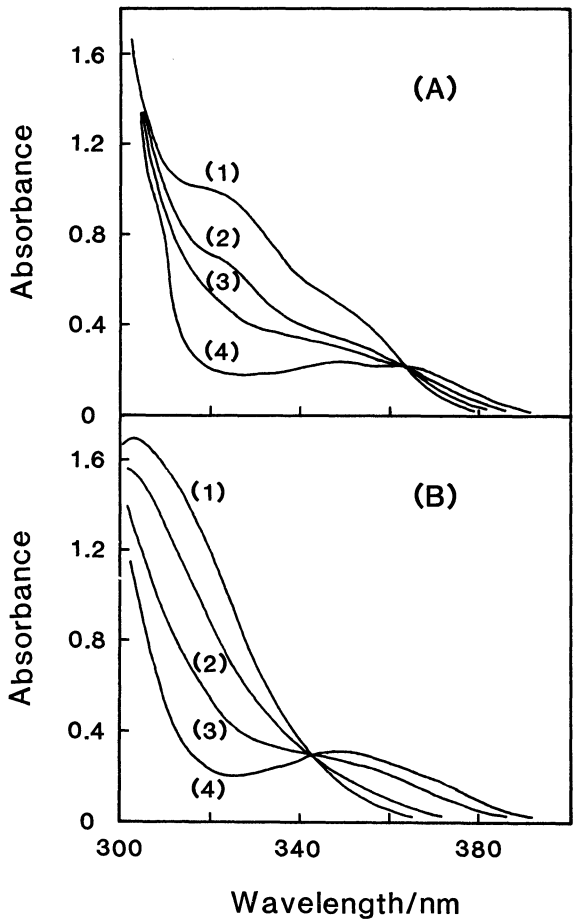

Fig. 3. pH Dependence of the absorption spectra of 2-BNK–ChT (A, 4.6×10^{-5} M) and 2-NSS (B, 6.0×10^{-5} M) at 25±1°C, I=0.25. pH (A): (1), 8.0; (2), 5.5; (3), 5.0; (4), 2.5. pH (B): (1), 10.0; (2), 9.0; (3), 8.0; (4), 5.0.

we prepared the sulfonium salt (2-NSS), dimethyl(2-naphthoylmethyl)sulfonium bromide, and its ylide (2-NSY), dimethyl(2-naphthoylmethyl)sulfonium ylide, as model compounds and examined the pH dependence of their absorption spectra. Both model compounds exhibited exactly the same spectral behavior in the pH range 2.5 to 10.0 (Fig. 3B), indicating that these spectral changes were due to interconversion between the sulfonium salt, 2-NSS, and the sulfonium ylide, 2-NSY. The finding that a chromophoric pendant on the enzyme gave a pH dependence of its absorption band similar to that of the model compound suggested the occurrence of sulfonium salt-ylide interconversion in a modified enzyme molecule. The 2-BNK-derived ylide pendant on the enzyme showed an absorption maximum around 320 nm (curve 1 in Fig. 3A), though we could not determine the accurate absorption-maximum wavelength owing to an overlap with the strong absorption of ChT itself. This ylide band appeared at a shorter wavelength than did the *p*-nitrophenacyl bromide (NPB)-derived ylide band with a maximum at 350 nm.[8] The nitro group with a strong electron-withdrawing ability in NPB should be able to stabilize the ylide structure causing a

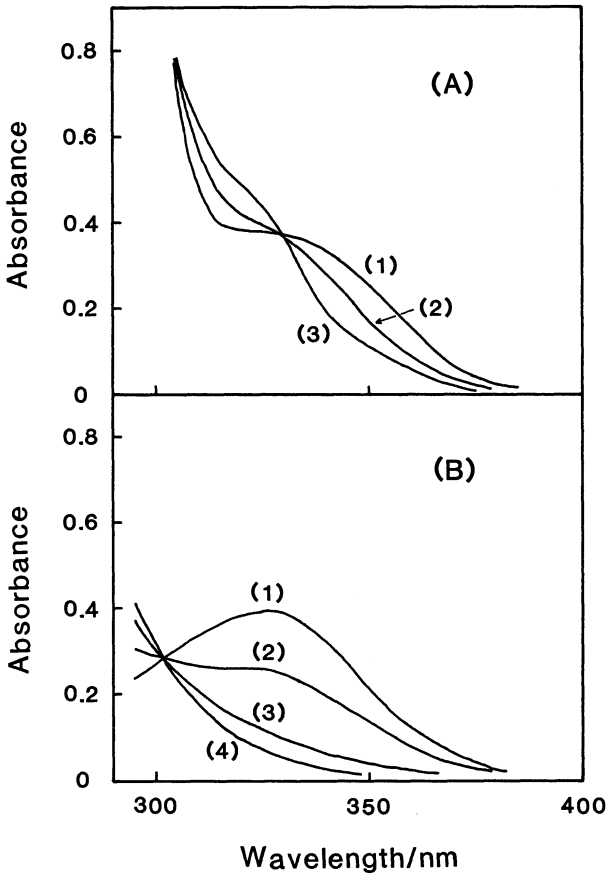

Fig. 4. pH Dependence of the absorption spectra of 1-BNK-ChT (A, 5.2×10^{-5} M) and 1-NSS (B, 5.5×10^{-5} M) at 25±1°C, I=0.25. pH (A): (1), 4.0; (2), 7.0; (3), 10.0. pH (B): (1), 2.5; (2), 7.0; (3), 8.0; (4), 10.0.

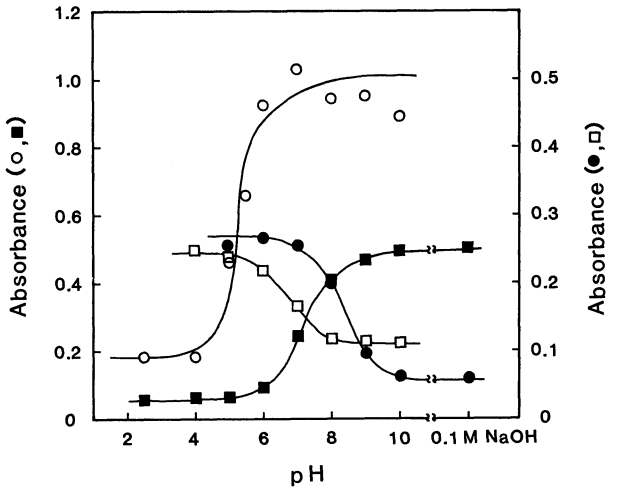

Fig. 5. Spectrophotometric titration curves for the 1-BNK(□)- and 2-BNK(○)-modified ChT derivatives and for the model sulfonium salts, 1-NSS (■) and 2-NSS (●). Wavelength monitored (nm): ○, 325; ●, 360; □, 350; ■, 270.

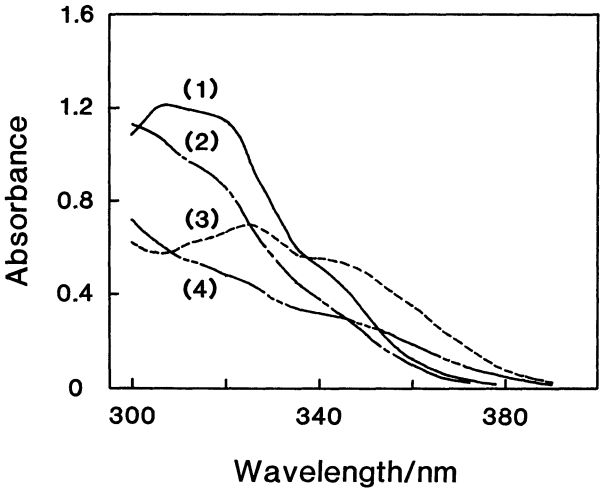

Fig. 6. Absorption spectra of 2-NSY (5.0×10^{-5} M) in ethanol (1), methanol (2), dimethyl sulfoxide (3), and dioxane (4) at 25±1°C.

red shift of the ylide absorption band.

As shown in Figs. 4A and 4B, we also observed the spectral behavior of 1-BNK-ChT, which corresponded well with that of the model compounds 1-NSS and 1-NSY. From an analysis of the titration curves collected in Fig. 5, we determined the pK_a values of the model sulfonium salts and the reporter groups incorporated into the enzyme active site: pK_a=7.5 (1-NSS), 6.6 (1-BNK-ChT), 8.4 (2-NSS), and 5.2 (2-BNK-ChT) at 25±1°C. Interestingly, the enzyme active site provides an environment which promotes proton dissociation of the 2-BNK-derived chromophore to a much larger extent compared with that of the 1-BNK-derived chromophore. Our pK_a of 2-NSS is consistent with the literature value (8.2)[8] within experimental error.

On the other hand, solvent effects on the absorption spectra of four model compounds revealed that the spectrum of 2-NSY undergoes pronounced solvent effects in the wavelength region 300—400 nm (Fig. 6), whereas those of 2-NSS, 1-NSY, and 1-NSS are not so large. Thus, the solvent effects may be useful to explore the microscopic environment surrounding a 2-BNK-derived reporter group which adopts the ylide form. A close comparison of both intensity (molar extinction coefficients at 320 nm=22000 M^{-1} cm^{-1} for 2-BNK-ChT at pH 8.0 and 23000 M^{-1} cm^{-1} for 2-NSY in EtOH) and the shape of the first absorption bands shown in Figs. 3A (curve 1) and 6 (curve 1) suggests the existence of ethanol-like circumstances in the vicinity of the 2-isomer pendant introduced into the substrate binding site of ChT.

Circular Dichroism Spectra of ChT Derivatives. Because an enzyme active site possesses a chiral environment, the induced circular dichroism (ICD) of a reporter group is expected to be observed on incorporating it into the active site. Blout and his co-workers have found that the NPB-derived ylide pendant in the ChT active site exhibits a strong positive ICD band in the wavelength range of 300 to 420 nm.[8] Thus, the finding that all of

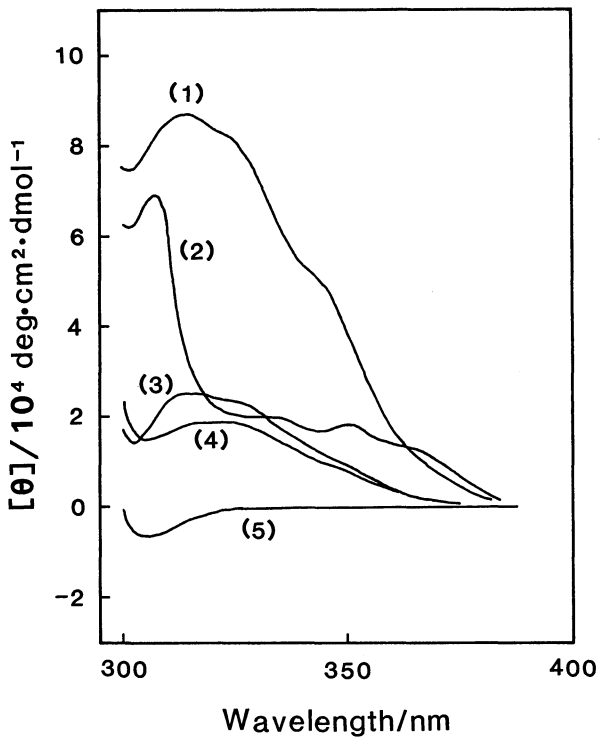

Fig. 7. Circular dichroism spectra of native ChT (5), 2-BNK–ChT (1, 2), and 1-BNK–ChT (3, 4) at pH 2.5 (2, 3, 5) and pH 10.0 (1, 4, 5) at 25±1°C. Concentration (M): (1), 4.4×10^{-5}; (2), 4.2×10^{-5}; (3), 5.5×10^{-5}; (4), 4.9×10^{-5}; (5), 5.0×10^{-5}.

the ChT derivatives give definitely positive ICD bands at wavelengths longer than 300 nm (Fig. 7) allowed us to conclude that our probe molecules were present in the enzyme active site. Denaturation of these modified enzymes by 6 M guanidine hydrochloride at pH 2.5 and 10.0 resulted in the complete disappearance of the long-wavelength ICD bands. In addition, removal of this denaturant by dialysis against 0.1 M buffer, pH 2.5 or 10.0, made it possible to restore the corresponding long-wavelength ICD bands. These observations constitute further evidence for our conclusion.

When 2-BNK–ChT was denatured by 6 M guanidine hydrochloride at pH 7.0, a dramatic UV spectral change caused by the conversion of the sulfonium ylide into the sulfonium salt was observed as shown in Fig. 8. By dialysis of the denatured ChT derivative against 0.1 M phosphate buffer, pH 7.0, we could reproduce the absorption spectrum obtained before denaturation. This supports the idea that the active-site region surrounding the 2-BNK-derived reporter group has the ability to greatly stabilize the ylide structure. As evident from a comparison of the pK_a (6.6) of the 1-BNK-derived reporter group on ChT with that (7.5) of the model compound, 1-NSS, there is no pH where the pronounced spectral change of 1-BNK–ChT is detected on denaturation.

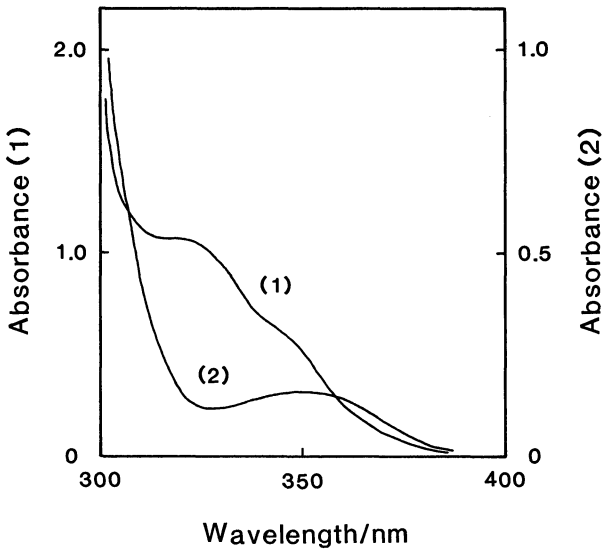

Fig. 8. Absorption spectra of 2-BNK–ChT in the absence (1) and presence (2) of guanidine hydrochloride (6 M) in 0.1 M phosphate buffer, pH 7.0 at 25±1°C. Concentration (M): (1), 5.6×10^{-5}; (2), 3.3×10^{-5}.

Discussion

Our alkylating agents, 1-BNK and 2-BNK, reacted with ChT following second-order kinetics to inactivate the enzyme to an appreciable extent. This bimolecular modification reaction was in accord with the attachment of one reagent molecule to a single amino acid residue on the enzyme. Evidence for the 1 : 1 stoichiometry came from the observation that the absorption spectrum of 2-BNK–ChT at pH 2.5 corresponded well to the sum of the spectra of 2-NSS and native ChT measured under the same conditions. This observation also indicated that 2-BNK reacts with a methionine residue to form the sulfonium salt. A ChT molecule has two methionine residues:[1] One is methionine-192, which is exposed and is present near the substrate binding site, and the other is methionine-180, which is buried and is unreactive toward alkylations as well as oxidations.[4,6,7,9] Thus, the observation that the competitive inhibitor, indole, suppresses the inactivation of the enzyme by BNK reagents, and the fact that many other alkylating agents,[5,6,8] whose structures are similar to that of BNK, modify methionine-192 selectively, allow us to conclude that only methionine-192 is alkylated by 1-BNK and 2-BNK. Furthermore, the presence of BNK-derived pendants, being attached to this methionine residue, at the active site was verified by the appearance of dramatic changes in their ICD and UV spectra on denaturation of the ChT derivatives and also by the observation of similar changes in the ICD and UV spectra of the NPB-derived ylide pendant on denaturation.[8]

Taking into account that alkylating agents containing an aromatic ring have some ability to complex with ChT,[2,5] the modification reaction is suggested to proceed

by way of a Michaelis–Menten-type complex. If so, we may explain the relatively large difference in reactivity between 1-BNK and 2-BNK in terms of a conformational difference in the BNK chromophore in the BNK·ChT complex. A 2-BNK molecule is likely to adopt a more favorable conformation for reacting with methionine-192 than does a 1-BNK molecule. However, we could not obtain supporting evidence for this idea because of the much larger dissociation constants of BNK·ChT complexes than the initial concentrations of BNK examined.

From amino acid and spectroscopic analyses of ChT alkylated with NPB, Blout and his co-workers found that the reporter group, being bound to methionine-192 in the form of a sulfonium salt, has a marked tendency to dissociate into the corresponding sulfonium ylide.[8] They proposed that the presence of an amino acid side chain(s) with a positive charge(s) near methionine-192 should be responsible for promoting the formation of the sulfonium ylide at the enzyme active site. The pH dependence of the absorption spectra of 2-BNK–ChT and 2-NSS demonstrates that the active-site local environment surrounding the 2-BNK-derived reporter group affects the sulfonium salt so as to reduce its pK_a by 3.2 units relative to the model 2-NSS. In contrast, the pK_a of the 1-BNK-derived sulfonium-salt pendant on the enzyme decreases by only 0.9 units relative to the model 1-NSS. These findings suggest that the configuration of these isomeric chromophores in the active site must be different from each other. Since the absorption spectrum of the 2-BNK-derived pendant at pH 8.0 (sulfonium-ylide form) is very similar to that of 2-NSY in ethanol, the 2-isomer pendant is proposed to be in an ethanol-like environment at the active site. It is well-known that there are three serine side chains (serine-189, -190, and -195) near the substrate binding site of ChT.[3] On the other hand, spectroscopic studies on dimethylsulfonium phenacylide indicated the ylide adopted the enolate structure.[10] The IR and ^{1}H NMR spectra of 2-NSY agree with this structure. Thus, if one of the three serine hydroxyl groups hydrogen bonds with the ylide carbonyl oxygen with a negative charge in a relatively rigid configuration (Fig. 9), this hydrogen-bond formation should stabilize the sulfonium ylide and cause a substantial decrease in the pK_a of the 2-NSS pendant as observed. In contrast, the absorption spectrum of the 1-BNK-derived pendant at pH 10.0 (sulfonium-ylide form) has a distinct shoulder around 320 nm, which is absent from the spectrum of 1-NSY in protic solvents. It resembles the spectra obtained in aprotic solvents such as dimethyl sulfoxide. This observation implies that the 1-NSY pendant on the enzyme occupies a site where the conversion of the sulfonium salt into the sulfonium ylide is facilitated by the hydrogen bonding mentioned above to only a small or a negligible extent. As a result, we notice the small difference in the pK_a (ΔpK_a) between 1-NSS and 1-BNK–ChT, compared with ΔpK_a between 2-NSS and 2-BNK–ChT.

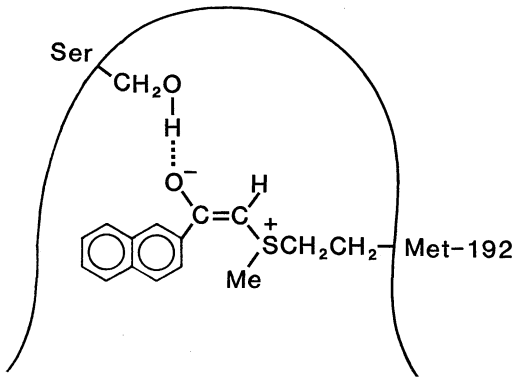

Fig. 9. Schematic illustration of the substrate binding area of ChT with a 2-NSY pendant.

Table 1. pH Dependence of the Remaining Activity (RA) of Modified ChT at 25±1°C

pH	RA/%	
	1-BNK–ChT	2-BNK–ChT
2.5	—[a]	5
4.0	5	5
5.0	7	7
5.5	—[a]	7
6.0	7	5
7.0	24	6
8.0	31	5
9.0	30	4
10.0	30	8

a) Not determined.

Interestingly, 2-BNK–ChT has a remaining activity of 6±2% which is independent of pH in the range of 2.5 (sulfonium-salt form, curve 4 in Fig. 3) to 10.0 (sulfonium-ylide form, curve 1 in Fig. 3), whereas the activity of 1-BNK–ChT is sensitive to pH changes as shown in Table 1. Alterations in the pH of a 1-BNK–ChT solution from 10.0 (sulfonium-ylide form, curve 3 in Fig. 4) to 4.0 (sulfonium-salt form, curve 1 in Fig. 4) and from 4.0 to 10.0 by dialysis gave the same remaining activities as those initially obtained at pH 4.0 and 10.0 within experimental error, respectively, thus establishing the reversibility of the observed pH dependence of the activity. An abrupt change in the activity of 1-BNK–ChT was observed between pH 6.0 and 7.0. The occurrence of this change around the pK_a (6.6) of the 1-NSS pendant on the enzyme suggests that the sulfonium-salt reporter group mainly occupies the substrate binding site, but on its conversion to the sulfonium-ylide form, the reporter group is brought far from the binding site to result in some recovery of enzyme activity. This movement of the 1-NSY moiety in the active site might be due to repulsive interactions between the ylide adopting the enolate structure and neighboring amino acid residues. The insensitivity of 2-BNK–ChT activity to pH changes is consistent with our conviction that the 2-BNK-derived ylide pendant is subject to stabilization through a

hydrogen-bonding interaction between this pendant and a serine residue present near the substrate binding site.

Experimental

Instrumentation. UV absorption and ICD spectra were recorded on a Shimadzu UV-210A spectrophotometer and a JASCO J-600 spectrodichrometer, respectively. A cell with a 10 mm pathlength was used. The purification of α-chymotrypsin (ChT) and its derivatives was performed on a Hitachi CR-20B3 centrifuge at room temperature. IR and ^{1}H NMR spectra were taken on a Hitachi 270-30 infrared spectrometer and a JEOL FX-200 spectrometer, respectively.

Materials and Solvents. Bromomethyl 2-naphthyl ketone (2-BNK) and bromomethyl 1-naphthyl ketone (1-BNK) were prepared according to the method of Cowper and Davidson[11] and were purified by repeated recrystallization from methanol (2-BNK) and by column chromatography over silica gel (70—230 mesh, Merck) using benzene–hexane (1 : 1 v/v) as eluent (1-BNK).

2-BNK, mp 84—85°C; IR (KBr) 1690 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ=4.55 (2H, s) and 7.50—8.45 (7H, m).

1-BNK, oily liquid; IR (neat) 1700 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ=4.50 (2H, s) and 7.38—8.64 (7H, m). The ^{1}H NMR spectrum showed that 1-BNK was contaminated with about 2 mol% of the starting methyl 1-naphthyl ketone.

1-BNK and 2-BNK were converted into their sulfonium salts, dimethyl(1-naphthoylmethyl)sulfonium bromide (1-NSS) and dimethyl(2-naphthoylmethyl)sulfonium bromide (2-NSS), by the method of Blout et al.[8] Recrystallization of the crude products from methanol (2-NSS) or from acetone-methanol (1-NSS) gave analytically pure samples.

1-NSS, mp 119—120.5°C (lit,[12] mp 123—124°C); IR (KBr) 3440 (broad) and 1660 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ=3.14 (6H, s), 5.86 (2H, s), and 7.64—8.94 (7H, m).

2-NSS, mp 122.5—124.5°C (lit, mp 113—115°C,[8] 128—129°C[12]); IR (KBr) 3520, 3460 (broad), and 1680 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ=3.15 (6H, s), 5.87 (2H, s), and 7.65—8.86 (7H, m).

Dimethyl(1-naphthoylmethyl)sulfonium ylide (1-NSY) and dimethyl(2-naphthoylmethyl)sulfonium ylide (2-NSY) were synthesized via treatment of the corresponding sulfonium salts with aqueous sodium hydroxide.[13] Analytical grade 1-NSY and 2-NSY were obtained by recrystallization of the crude products from ethyl acetate (1-NSY) or from ethyl acetate–hexane (2-NSY).

1-NSY, mp 171—172°C; IR (KBr) 1520 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ=2.92 (6H, s), 3.96 (1H, s), and 7.34—8.50 (7H, m). Found: C, 73.06; H, 6.21%. Calcd for $C_{14}H_{14}OS$: C, 73.01; H, 6.13%.

2-NSY, mp 136—137°C; IR (KBr) 3260 (broad), 1520, and 1500 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ=2.88 (6H, s), 4.42 (1H, somewhat broad), and 7.44—8.32 (7H, m). Found: C, 72.05; H, 6.16%. Calcd for $C_{14}H_{14}OS\cdot(1/6)H_2O$: C, 72.07; H, 6.19%.

Three-times-crystallized ChT and *N*-acetyl-L-tyrosine ethyl ester (ATEE) were purchased from Sigma and used as received. All other chemicals employed were obtained from commercial sources and were of the highest grade available.

Kinetics of Inactivation. The inactivation of ChT by our BNK reagents was studied by adding a freshly-prepared solution (100 μl) of 1-BNK or 2-BNK in acetonitrile to the enzyme (2 ml) dissolved in 0.1 M phosphate buffer, pH 7.0 (I=0.25, KCl) at 25±1°C. The initial concentrations of 1-BNK, 2-BNK, and ChT were 2.2—8.9×10^{-4}, 7.4—95×10^{-5}, and 7.4×10^{-6} M, respectively. At the same time, a control enzyme solution containing 4.8 vol% acetonitrile was prepared under the same conditions except for the exclusion of the BNK reagents. The enzyme concentration was estimated using a molar extinction coefficient (ε) of 52000 M^{-1} cm^{-1} at 280 nm.[14] At suitable time intervals, a 100 μl aliquot of the reaction mixture was taken and added to a solution (3 ml) of ATEE (6.5×10^{-4} M) to record the decrease in absorbance at 237 nm of this substrate at 25±1°C. A control solution was treated in the same way to determine the remaining percent activity. Pseudo-first-order rate constants for the inactivation reactions of ChT (k_{obsd}) were evaluated by the use of Eq. 1 and determined at least in triplicate for each concentration of 1-BNK or 2-BNK.

$$-\ln[(a_t - a_\infty)/(a_0 - a_\infty)] = k_{obsd}t \quad (1)$$

$$k_{obsd} = k_i[BNK]_0$$

where a_t is the percent activity at time t, a_∞ is the final percent activity, a_0 is the percent activity at t=0, k_i is the second-order rate constant of inactivation, and $[BNK]_0$ is the initial concentration of 1-BNK or 2-BNK.

Preparation of ChT Derivatives. ChT modified by BNK was prepared by allowing a solution of ChT (5.0—7.0×10^{-5} M) and BNK (1.0—1.5×10^{-3} M) in 4.8 vol% acetonitrile–0.1 M buffer of a given pH (2.5—10.0) and ionic strength (I=0.25, KCl) to stand with stirring at room temperature. The buffers used were glycine (pH 2.5), acetate (pH 4.0—5.5), phosphate (pH 6.0—7.0), and borate (pH 8.0—10.0). The progress was monitored by following the rate of loss of the enzyme activity toward ATEE. After the reaction was completed (a change in the remaining activity was no longer detected), the precipitate was removed by centrifugation for 30 min at 18000 rpm, room temperature. The supernatant was then dialyzed at least three times against 0.1 M buffer with a given pH (2.5—10.0) and I (0.25) to remove excess alkylating agent. Modified enzyme was stored in a refrigerator after the activity was measured. When a slight turbidity reappeared during dialysis or storage, the dialyzed enzyme solution was centrifuged again just before the spectroscopic measurement of alkylated ChT at 25±1°C. A control enzyme solution containing no BNK was prepared according to exactly the same procedure as that described above.

Another procedure to prepare control and sample enzyme solutions was also used. After ChT (5.0—7.0×10^{-5} M) was allowed to react with excess BNK (1.0—1.5×10^{-3} M) in 4.8 vol% acetonitrile–0.1 M phosphate buffer, pH 7.0, I=0.25, until there was no further change in the remaining activity, the supernatant obtained by centrifugation of the reaction mixture was dialyzed several times against 0.1 M buffer (I=0.25) with pH ranging from 2.5 to 10.0.

These purification procedures of the enzyme derivatives were found to affect the remaining activities to only a very small extent. Because a small but perceivable dilution of the enzyme solutions to a different extent was observed during dialysis, the absorption spectra obtained at each pH were corrected for this dilution by using the native enzyme which was treated in exactly the same way except for the omission of the alkylating agents. Contrary to the model sulfonium ylides, the absorption spectra of the model sulfonium salts are not subject to pronounced solvent effects, so that molar extinction coefficients of 1-NSS (ε at 330 nm=7100 M^{-1} cm^{-1}) and 2-NSS (ε at 350 nm=5000 M^{-1}

cm^{-1}) can be used to estimate concentrations of the alkylated enzymes at low pH where reporter groups exist predominantly in the form of the sulfonium salts. The concentrations of the ChT derivatives thus determined were in reasonable agreement with those evaluated from the extent of dilution observed during dialysis.

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