The Role of α-Chymotrypsin Active Site in the Proton Dissociation of Aromatic Sulfonium-Salt Pendants Attached to the Methionine-192 Residue

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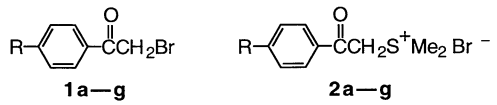
Hammett and ^{13}C NMR analyses of proton-dissociation processes for sulfonium-salt pendants on α-chymotrypsin which had been modified by 2-bromoacetophenone and its derivatives (as well as for their corresponding model sulfonium salts) show that a hydrophobic environment around the pendants destabilizes the sulfonium-salt form so as to substantially promote its proton-dissociation reaction. This produces a sulfonium ylide with a keto structure.

The existence of methionine-192 residue (Met-192) at the α-chymotrypsin (ChT) active site has been manifested by an analysis of its three-dimentional structure.[1] The alkylating agents that modify Met-192 inactivated ChT to a different extent, suggesting that the reporter groups which are attached to this amino-acid residue occupy a part of the substrate-binding region in the active site.[2-5] Researches that indicated that the substrate-binding area near Met-192 provides a favorable environment in regards to sulfonium ylides rather than for sulfonium salts[4,6,7] motivated us to explore the factors that control the proton dissociation of the sulfonium salts that are bound to Met-192 and to clarify the structure of the sulfonium ylide that was formed in the ChT active site by using 2-bromoacetophenone and its derivatives (**1a—g**).



1a—g **2a—g**

a: R= OMe b: R= Me c: R= H d: R= Cl
e: R= Br f: R= CN g: R= NO_2

The reaction of ChT (5.0×10^{-5} M, 1 M= 1 mol dm^{-3}; Sigma, Type I-S) with **1c** (2.5×10^{-4} M), (which selectively alkylates the Met-192 of this enzyme, thus forming sulfonium salt,[3]) in 0.1 M phosphate buffer [ionic strength, I= 0.25 (KCl); pH= 7.0] at room temperature resulted in a more than 90% inactivation of ChT within a few hours. The removal of excess **1c** by centrifugation and sufficient dialysis against buffers with different pH values (I= 0.25; pH= 2.5—10.0) followed by the purification of a dialysed enzyme solution by membrane filtration, gave pH-dependent UV absorption spectra as is shown in Figure 1. A comparison with the pH dependence of the UV spectrum of the model sulfonium salt **2c** establishes that the observed spectral changes of ChT that was modified by **1c** are due to the interconversion between the sulfonium salt and the sulfonium ylide forms in the active site. Similar UV spectral behavior was observed for ChT that was modified by the **1c** derivatives except for **1g**. The **1g**-derived pendant showed UV spectral changes (which are virtually independent of pH) in a range of 2.5 to 10.0. Its absorption spectrum can be regarded as the superposition of the spectra of native ChT and the sulfonium ylide even at a pH of 2.5. Thus, the introduction of a nitro substituent promotes the proton dissociation of the sulfonium-salt pendant to a much greater extent than that expected from the substituent constant σ for this group.

Table 1. Proton dissociation constants (pKa) of the model sulfonium salts (**2**) and sulfonium-salt pendant on modified ChT (**2**-ChT) at 24±2 °C

Substituent (σ)	pKa		ΔpKa[a]
	2	**2**–ChT	
OMe (–0.27)	9.1	5.2	3.9
Me (–0.17)	8.9	5.0	3.9
H (0)	8.6	4.8	3.8
Cl (0.23)	8.1	4.2	3.9
Br (0.27)	8.0	4.3	3.7
CN (0.66)	6.9	3.7	3.2
NO_2 (0.78)	6.7	<2.5	

[a] ΔpKa= pKa (**2**) – pKa (**2**–ChT).

Analysis of the pH titration curves for **1**-modified ChT and **2** allowed us to determine the pKa values of the model sulfonium salts and the reporter groups that had been incorporated into Met-192 (Table 1). Evidently, the ChT active site offers an environment that accelerates the proton-dissociation reaction of any sulfonium salt pendant that we examined (ΔpKa>3). The proton dissociation of the sulfonium salts that were attached to Met-192 possessed a smaller reaction constant (ρ= 1.6±0.1), as compared with that (ρ= 2.3±0.1) for the dissociation of **2a—g** [ρ values were evaluated from the slopes of the pKa(R=H) – pKa(R)

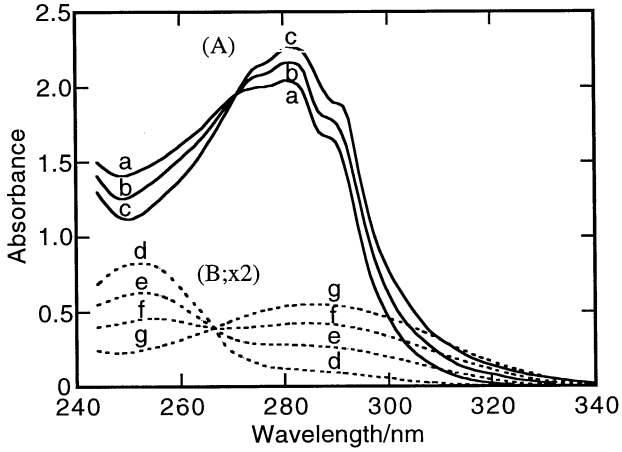
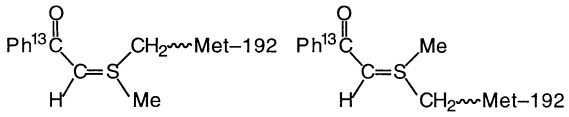


Figure 1. UV absorption spectra of **1c**-modified ChT (A) and **2c** (B) (5.0×10^{-5} M; I= 0.25) at room temperature as a function of pH: (a) pH 2.5; (b) pH 5.0; (c) pH 8.0; (d) 0.01 M HCl; (e) pH 8.0; (f) pH 9.0; (g) 0.1 M NaOH.

vs. σ plots]. Application of the Hammond postulate[8] to the above shows that the transition state for the reaction in the enzyme active site (as compared to that for the model sulfonium salt) is more similar to the starting sulfonium salt. Thus, Hammett analysis reveals that there are two reasons for the observed large differences in p*K*a: One is that the substrate-binding region around Met-192 considerably destabilizes the sulfonium-salt form relative to the sulfonium ylide and the other is the significant stabilization of the ylide structure in this binding area. It was found that the p*K*a of a cationic acid decreases by about 1.6 units owing to the destabilization of the charged species when the acid is incorporated into the hydrophobic environment of a nonionic micelle.[9] Thus, the hydrophobic nature of the substrate-binding region of ChT should destabilize the sulfonium salt, thereby bringing about a substantial decrease in its p*K*a.[1,10] In addition, we predicted that the nitro-substituted sulfonium-salt pendant with double charges would undergo additional destabilization. Our observation agrees with this prediction, thereby supporting the former reason. There may be a measurable contribution of the stabilization of the ylide structure by the surrounding amino-acid residue(s) to the decrease in the p*K*a of a given sulfonium-salt reporter group on the derivatized enzyme.

I **II**

If the substrate-binding pocket on ChT is able to strongly suppress the generation of a charged species, the sulfonium ylide that is formed in this pocket will be able to adopt keto structure I but not enolate II. In order to determine which structure is preferable at the ChT active site, **1c**-*carbonyl*-^{13}C (99+ atom%, 1.5×10^{-3} M) was allowed to react with ChT (3.0×10^{-4} M) in 0.1 M phosphate buffer (pH= 7.5) at room temperature. Centrifugation and subsequent dialysis against a HCl aqueous solution (0.01 M, *I*= 0.25) or a phosphate buffer (0.1 M, pH 7.5, *I*= 0.25) (and, finally, the membrane filtration of a dialysed enzyme solution) enabled us to record the ^{13}C NMR spectrum of the sulfonium salt or the sulfonium ylide pendant that was bound to Met-192.[11] For the sake of comparison, ^{13}C NMR spectra of ^{13}C-labeled **2c** were measured in HCl (0.01 M, *I*= 0.25) and NaOH (0.1 M, *I*= 0.25) aqueous solutions under the same conditions. The carbonyl carbon signal of the model sulfonium salt was observed at 192.9 ppm, and upon conversion of the salt into the sulfonium ylide, this carbon signal was subject to a relatively large upfield shift ($\Delta\delta$= 13.0). This is in agreement with the result that aromatic ylides are best represented by enolate structure II.[12] On the other hand, the sulfonium-salt pendant that was attached to Met-192 exhibited its carbonyl carbon signal at 198.3 ppm. Significantly, the ^{13}C resonance of the sulfonium salt (which was accommodated into the ChT active site) underwent a downfield shift, giving two signals at 200.3 and 202.4 ppm when the pendant was converted into the ylide. Since no modified enzyme showed indications of denaturation during the NMR measurements, the observed downfield shift of the ylide carbonyl carbon signal presents strong evidence in support of keto structure I in the active site. Taking into account the fact that aromatic sulfonium ylides exist as two interconvertible geometrical isomers,[12] isomers III (*Z*) and IV (*E*) are a likely candidate for the observed ^{13}C signals. The three-dimentional structure of the ChT active site around Met-192 suggests that both of the isomer pendants can be generated without undergoing any steric restraint of the surrounding amino-acid residues.[13]



III **IV**

The proton dissociation reaction of the aromatic sulfonium salts that was described in this paper can be used to estimate the extent of hydrophobicity about these salts after they are introduced into a specific enzyme.

References and Notes

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11 ^{13}C NMR spectra of the ^{13}C-labeled **2c** (1—2×10^4 scans) and ChT modified by ^{13}C-labeled **1c** (3—4×10^4 scans) were measured in buffered aqueous solutions containing 10 vol% deuterated water and 0.1 vol% 1,4-dioxane (internal standard). The chemical shifts of the observed carbon signals were converted to the tetramethylsilane standard.
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