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THE EFFECTS OF CHEMICAL MODIFICATION ON THE REFOLDING TRANSITION OF α-CHYMOTRYPSIN

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The role of several active site residues of α-chymotrypsin in the prototypical refolding transition between active and inactive forms of this enzyme is examined using chemical modification. Oxidation of Met-192 to the sulfoxide results in a derivative which remains entirely in an active state from pH 6 to 9. The derivative becomes inactive only at high pH with $pK_a = 10.3$, $\Delta H^0 = 9.5$ kcal and $\Delta S^0 = -15$ eu., indicating the sulfoxide group supplies about 2.1 kcal of active state stabilization relative to the unoxidized methionine side chain. The refolding transition of N-methyl-His-57-α-chymotrypsin, in which a nitrogen of the "charge relay" histidine is methylated, displays one ionization process with an apparent pK_a of 9.45. The absence of an additional ionization process with a pK_a near 7 provides evidence that one of the ionizations in the six state mechanism which describes this transition in α-chymotrypsin is linked to the charge relay system. We also demonstrate, using α-chymotrypsin, Met-192-sulfoxide-α-chymotrypsin and N-methyl-His-57-α-chymotrypsin, that the 230 nm circular dichroism band is a quantitative probe of the active-inactive equilibrium, although the chromophore or chromophores responsible for this and another very large negative band at 202 nm have not been identified. Circular dichroism was used to observe the active-inactive equilibrium in methane sulfonyl-α-chymotrypsin and phenylmethane sulfonyl-α-chymotrypsin. The enhanced stability of the active state of these derivatives relative to α-chymotrypsin can be rationalized in terms of steric effects in the substrate side chain binding site.

1. Introduction

It is known that α-chymotrypsin can exist in two major conformations at neutral and alkaline pHs, only one of which is active [1–5]. The active species contains an internal ion-pair between the N-terminal α-amino group of Ile-16 and the carboxylate of Asp-194 [6]. The impotent species is formed by rupture of this salt bridge with conformational rearrangements, making the Ile-16 α-amino group at least partially exposed to solvent. We have examined this transition in α-chymotrypsin under a wide variety of conditions [7,8] and now add chemical modification to our list of variables. Previous work on the chemical modification of α-chymotrypsin is extensive [9–15]. We have selected a few simple, well defined active-site modifications and have determined the equilibrium between the active and inactive isomers of these derivatives as a function of pH and, in a particularly interesting case, temperature.

Crystallographic results [16], heat-capacity results [8,17] and the instability of inactive conformation dimers [7] suggest that the conformational transition between the active and inactive isomers involves changes throughout the molecule rather than only near the Ile-16-Asp-194 ion-pair and the substrate binding site. Therefore, we would expect that chemical modification at any site on the protein could affect the active-inactive equilibrium. In support of this view, it has been shown that oxidation of the Met-192 side chain increases the stability of the active conformation by greater than 1.5 kcal [14]. In this work, the proflavin assay employed previously [4,7] was used to observe the equilibrium between the two conformations

in α-chymotrypsin derivatives that bound proflavin. Unfortunately, many derivatives with active-site modifications do not bind proflavin. Optical rotation and circular dichroism spectra show changes upon activation of chymotrypsinogen to α-chymotrypsin and during the active to inactive transition [1,18,19]. We have correlated the circular dichroism band at 230 nm with the proflavin assay results for α-chymotrypsin and have used circular dichroism to investigate the conformational transition in those derivatives which do not bind proflavin.

2. Materials and methods

Salt-free three times crystallized α-chymotrypsin was obtained from either Worthington Biochemical Co. (Type CDI, Let numbers 3AF, 34S895, 35H793, 35A970, 35A912 and 35P692) or Miles Laboratories, Inc. (Lot number 1695C). Salt-free six times crystallized chymotrypsinogen A was purchased from Worthington Biochemical Co. (Type CGC, Lot number 35E636). These proteins were purified, stored and assayed as described previously [7]. Active site cinnamoyl imidazole titrations were between 88 and 100%. The circular dichroism results were slightly dependent on the purity of the enzyme, so these experiments on α-chymotrypsin and its derivatives were carried out using purified protein from a single lot number (3AF). Phenylmethane sulfonyl-α-chymotrypsin and methane sulfonyl-α-chymotrypsin were prepared as described previously [9] and were shown to contain less than 2% active enzyme by activity assays with N-acetyl-L-tryptophan ethyl ester [20]. Met-192-sulfoxide-α-chymotrypsin was prepared according to published methods [14].

N-methyl-His-57-α-chymotrypsin was prepared by two methods. The derivative was prepared in large amounts by exhaustive reaction with methyl p-nitrobenzene sulfonate as described by Nakagawa and Bender [12]. The enzyme was also modified and purified on a turkey ovomucoid affinity column as described by Ryan and Feeney [15] to eliminate the products of undesirable side reactions. No differences between the two preparations of the derivative were noted in proflavin dissociation constants, circular dichroism and the active to inactive equilibrium.

Stopped-flow determinations of the fraction of protein in the active conformation were performed using the proflavin fraction-active assay procedure described previously [4,7]. Results are reported as the fraction of total protein in an active conformation (E_A/E_T). Proflavin dissociation constants were determined at 25°C for N-methyl-His-57-α-chymotrypsin and as a function of temperature for Met-192-sulfoxide-α-chymotrypsin with spectrophotometric techniques [7]. Non-specific binding of proflavin to the proteins was corrected for by replacing α-chymotrypsin with either chymotrypsinogen or phenyl methane sulfonyl-α-chymotrypsin in a typical stopped-flow experiment.

Circular dichroism spectra from 350 nm to 200 nm were recorded on a Cary Model 6001 spectropolarimeter, which was calibrated with the 290 nm band of d-camphor sulfonic acid. Enzyme concentrations were about 1.0×10^{-5} M. A 0.5 cm pathlength cell was used between 350 nm and 230 nm and a 0.1 cm pathlength cell was used between 230 nm and 200 nm. Molar ellipticities were calculated using a mean residue molecular weight of 105.5 gm residue^{-1} and are reported in units of deg cm^2 decimole^{-1}.

3. Results

3.1. Active-inactive equilibrium for derivatives which bind proflavin

3.1.1. Proflavin dissociation constants

Proflavin dissociation constants for Met-192-sulfoxide-α-chymotrypsin are reported as a function of temperature in fig. 1. Calculated enthalpy and entropy changes are 9.8 ± 1.2 kcal and 11.9 ± 4.2 e.u. respectively. The proflavin dissociation constant for N-methyl-His-57-α-chymotrypsin was determined to be 4.26×10^{-5} M (0.055 M phosphate, 0.1 M KCl, 25°C, pH 7). Non-specific binding of proflavin to the chymotrypsin derivatives was assumed to be identical to that observed for chymotrypsinogen and phenyl methane sulfonyl-α-chymotrypsin [7].

3.1.2. Met-192-sulfoxide-α-chymotrypsin

The fraction of enzyme in the active conformation was determined for Met-192-sulfoxide-α-chymotrypsin as a function of pH and temperature using the stopped-flow proflavin assay procedure (fig. 2). Between approximately pH 6 and 8.5, no transients due to the

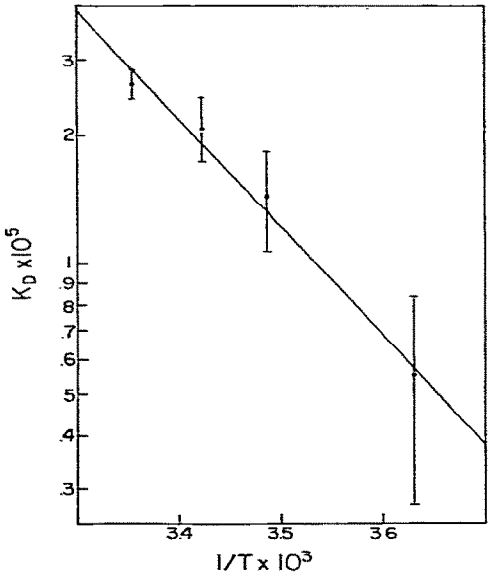


Fig. 1. Temperature dependence of proflavin-Met-192-sulfoxide-α-chymotrypsin dissociation constant. Conditions: Met-192-sulfoxide-α-chymotrypsin concentration ca. 2.0×10^{-5} M, proflavin concentration varied between 1.0×10^{-5} M and 1.0×10^{-4} M, 0.055 M phosphate, 0.1 M KCl. Reported errors are two standard deviations. The line is a weighted least squares fit to a straight line.

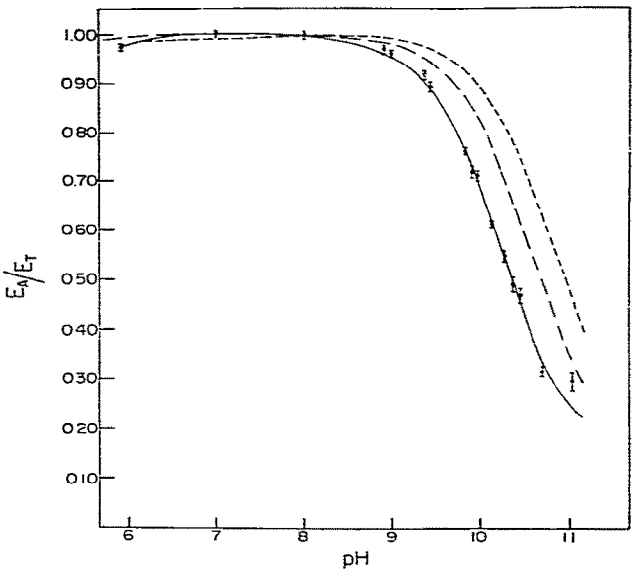
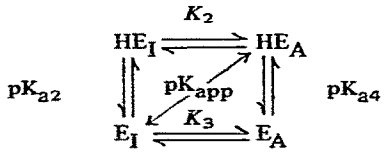


Fig. 2. pH and temperature dependence of the fraction of Met-192-sulfoxide-α-chymotrypsin in the active conformation at ionic strength 0.2 determined by the proflavin binding assay. Stopped-flow conditions: Initial Met-192-sulfoxide-α-chymotrypsin solution, approximately 4.0×10^{-5} M Met-192-sulfoxide-α-chymotrypsin, 0.005 M phosphate from pH 6.0 to 8.0 and pH 10.5 to 11.0, 0.005 M borate from pH 8.0 to pH 10.5, ionic strength 0.2 by addition of KCl, pH as shown on figure; initial proflavin solution, approximately 1.8×10^{-4} M proflavin, 0.1 M phosphate, pH 7.0. (——) 25°C, (—·—·—) 13°C, (— — —) 1°C. The lines are weighted least squares fits to scheme I. Fitted constants are reported in table 1.

slow inactive to active isomerization were observed upon mixing the enzyme with proflavin, thus we conclude that more than 98% of the protein is in the active conformation. These data confirm previous reports on this derivative [14]. Data above pH 9 could be described by scheme I; however, we note that HE_I is not observed to exist in measurable concentrations. Equilibrium constants obtained as described previously [7], are reported in table 1.



(scheme I)

There are large uncertainties in the constants, especially at low temperatures, because the complete titration curves cannot be obtained. The van't Hoff plot of pK_{app} is linear, yielding an enthalpy change of 9.5 ± 2.3 kcal and an entropy change of −15.3 ± 5.0 e.u.

3.1.3. *N-methyl-His-57-α-chymotrypsin*

The pH dependence of the fraction of protein in the active conformation, determined by the stopped-flow proflavin assay procedure, for N-methyl-His-57-α-chymotrypsin is shown in fig. 3. No differences were noted for the two methods of protein preparation. The data show a single ionization and can be fit by scheme I. Calculated equilibrium constants are reported in table 1. The pH profile of α-chymotrypsin shows an ionization near pH 7. However, based on the fitting criterion that chi squares values less than one indicate a good fit, the data for N-methyl-His-57-α-chymotrypsin show no evidence for this process.

3.2. *Circular dichroism*

The circular dichroism spectrum of α-chymotrypsin

Table 1
Summary of equilibrium constants used to fit the met-192-sulfoxide-α-chymotrypsin and N-methyl-His-57-α-chymotrypsin data

α-chymotrypsin derivative	Temperature,	K_2	K_3	pK_{a2}	$pK_{apparent}$
Met-192-sulfoxide [a]	25.0	–	0.13 ± 0.02	–	10.3 ± 0.1
	13.0	–	0.11 ± 0.10	–	10.6 ± 0.2
	1.0	–	0.10 ± 0.13	–	10.9 ± 0.2
N-methyl-His-57 [b]	25.0	21.7 ± 3.0	0.146 ± 0.020	8.12 ± 0.06	9.45 ± 0.10

[a] Least squares fit of the data in fig. 2 to scheme I. [b] Least squares fit of the data in fig. 3 to scheme I.

contains two strong negative bands, one centered at 230 nm and the other at 202 nm. As pH is increased from neutral pH, where the active conformation is predominant, to high pH, where the inactive conformation is predominant, the band at 230 nm become less negative while the band at 202 nm becomes more negative. These observations are consistent with previous results [1,2,14]. Instrumental difficulties prevent quantitative measurements with the necessary precision at 202 nm, but the molar ellipticity at 231 nm (θ_{231}) has been determined as a function of pH for α-chymotrypsin (fig. 4). When these data are fit to a one-proton ionization curve with variable high- and low-pH endpoints, the apparent pK_a obtained is within error equal to that obtained by the proflavin assay method (pK_{app} = 8.81 for circular dichroism data. Table 2;

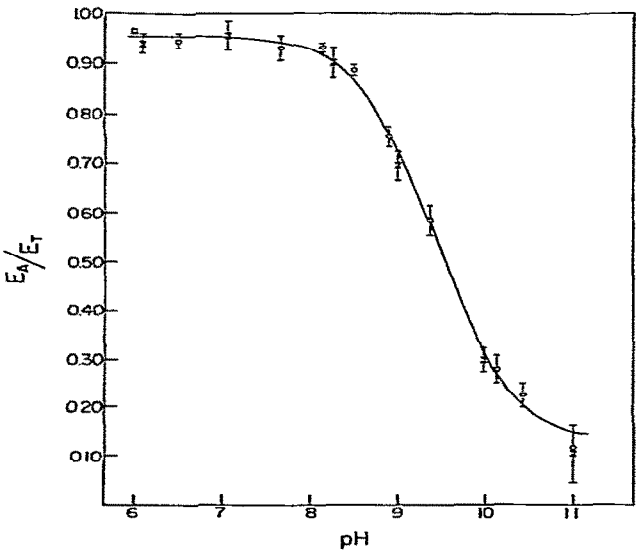


Fig. 3. pH dependence of the fraction of N-methyl-His-57-α-chymotrypsin in the active conformation at 25°C and ionic strength 0.2. Stopped flow conditions: Initial N-methyl-His-57-α-chymotrypsin solution, approximately 4.0×10^{-5} M protein, 0.005 M phosphate from pH 6.0 to 8.0 and from pH 10.5 to 11.0, 0.005 M borate from pH 8.0 to 10.5, ionic strength 0.2 by addition of KCl, pH as shown on figure; initial proflavin solution, approximately 1.8×10^{-4} M proflavin, 0.1 M phosphate, pH 7.0. ⨀ protein prepared as described by Nagakawa and Bender [12]. ⨍ protein prepeared as described by Ryan and Feeney [15]. The line is a weighted least squares fit to scheme I. Fitted constants are given in table 1.

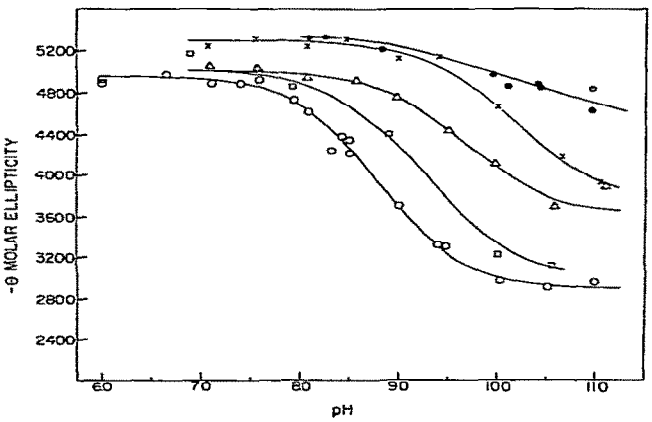


Fig. 4. pH dependence of θ_{231} for chymotrypsin and derivatives. Conditions: approximately 1.0×10^{-5} M protein, 25°C, 0.05 M phosphate from pH 6.0 to 8.0 and pH 10.5 to 11.0, 0.05 M borate from pH 3.0 to 10.5, ionic strength 0.2 by addition of KCl. (○) α-chymotrypsin, (□) N-methyl-His-57-α-chymotrypsin, (△) Methanyl sulfonyl-α-chymotrypsin, (X) Met-192-sulfoxide-α-chymotrypsin, (●) phenyl-methane sulfonyl-α-chymotrypsin. All lines, except for the phenyl-methane sulfonyl-α-chymotrypsin line which was hand drawn, represent a least squares fit to a single ionization using the pK_a's and endpoint values reported in table 2.

Table 2
Summary of fitting parameters for pH dependence of θ_{231} [a]

α-chymotrypsin derivative	pK_{app}	θ_{231} (neutral pH) [b),d)]	θ_{231} (high pH) [c),d)]
Parent	8.81 ± 0.05	−4964	−2891
Met-192-sulfoxide	10.17 ± 0.10	−5294	−3750
N-methyl-His-57	9.26 ± 0.2	−5008	−3009
Methane sulfonyl	9.67 ± 0.10	−5009	−3625
Zymogen	pH independent	−2320	

a) Least squares fit of the data in fig. 4. b) Neutral pH theoretical endpoint. c) High pH theoretical endpoint.
d) Reported in ° cm^2 $decimole^{-1}$. Errors are ±50.

pK_{app} = 8.78 for proflavin assay [7]). The errors in these circular dichroism experiments are such that the small perturbation due to the pK_a near 7 found previously [7] cannot be detected. It is reasonable to conclude that the pH dependency of the molar ellipticity at 231 nm is caused by the pH dependency of the active to inactive transition.

The circular dichroism spectrum of Met-192-sulfoxide-α-chymotrypsin at pH 7 is similar to that of α-chymotrypsin at pH 7. The pH dependence of θ_{231} is similar to the pH dependence of E_A/E_T, measured with the proflavin assay (fig. 4). When fitted to a single ionization process with variable high and low pH endpoints, the apparent pK_a's for the two methods agree to within error (pK_{app} = 10.3 ± 0.1 for proflavin assay; pK_{app} = 10.2 ± 0.1 for θ_{231} results).

The circular dichroism spectrum of chymotrypsinogen is pH independent and resembles the circular dichroism spectrum of α-chymotrypsin at pH 11; however, θ_{231} is only approximately equal to that observed at high pH for α-chymotrypsin, indicating possible conformational differences between chymotrypsinogen and inactive α-chymotrypsin.

Circular dichroism spectra and the θ_{231} pH dependence of N-methyl-His-57-α-chymotrypsin were examined (fig. 4) and again the circular dichroism effects and the proflavin assay results are correlated (tables 1 and 2). The apparent pK_a affecting θ_{231} is within error equal to the apparent pK_a controlling the active-inactive equilibrium of α-chymotrypsin although in this case the errors are quite large.

Phenylmethane sulfonyl-α-chymotrypsin and

Table 3
Comparison of results obtained by direct E_A/E_T assay and by circular dichroism

α-chymotrypsin derivative	pH	θ_{231} (observed) [c)]	E_A/E_T (calculated) [d)]	E_A/E_T (observed) [e)]
Met-192-sulfoxide	neutral [a)]	−5294	1.02	> 0.98
	high [b)]	−3750	0.40	0.12
N-methyl-His-57	neutral [a)]	−5008	0.90	0.96
	high [b)]	−3009	0.10	0.13
Methane sulfonyl	neutral [a)]	−5009	0.90	–
	high [b)]	−3625	0.35	–
Phenyl methane sulfonyl	pH < 9	−5300	1.02	–

a) Neutral pH titration curve endpoint. b) High pH titration curve endpoint.
c) Obtained from table 2 in ° cm^2 $decimole^{-1}$.
d) Calculated assuming θ_{231} = −5256 ° cm^2 $decimole^{-1}$ for the active isomer and θ_{231} = −2761 ° cm^2 $decimole^{-1}$ for the inactive isomer (see text).
e) Obtained from the data reported in table 1.

methane sulfonyl-α-chymotrypsin do not bind proflavin, so our only observable for the conformational transition is circular dichroism. The pH dependence of θ_{231} is shown in fig. 4. The active conformation is stabilized in both derivatives, the phenyl group of phenylmethane sulfonyl-α-chymotrypsin having a much larger effect than the smaller methyl group of methane sulfonyl-α-chymotrypsin. The apparent pK_a and the high pH and low pH endpoints describing the pH dependence observed for methane sulfonyl-α-chymotrypsin are given in table 2.

As the θ_{231} and proflavin fraction-active assay data appear to show similar pK_a's, a more quantitative comparison is in order. Knowledge of θ_{231} and the fraction of protein in an active conformation, E_A/E_T, at two pHs allows calculation of θ_{231} for the active and inactive conformations. We have used the high and neutral pH titration-curve endpoints for α-chymotrypsin to minimize errors in our calculation ($\theta_{231} = -4964\ ^\circ\ cm^2\ decimole^{-1}$, $E_A/E_T = 0.883$ at neutral pH; $\theta_{231} = -2891\ ^\circ\ cm^2$ decimole, $E_A/E_T = 0.052$ at high pH) and have found $\theta_{231} = -5256\ ^\circ\ cm^2$ decimole^{-1} for the active conformer of the native enzyme and $\theta_{231} = -2761\ ^\circ\ cm^2$ decimole^{-1} for the inactive conformer. With the additional assumption that θ_{231} values for the active and inactive species do not depend on chemical modification, these values can be used to compute E_A/E_T from the circular dichroism data for the four derivatives of α-chymotrypsin studied. These calculations, with comparison to direct E_A/E_T measurements where possible, are shown in table 3. Agreement between the two methods is within error in most cases, indicating our assumptions are at least approximately valid. The only exception is for the inactive form of Met-192-sulfoxide-α-chymotrypsin where the discrepancy indicates a probable difference in the θ_{231} values for the inactive forms of α-chymotrypsin and Met-192-sulfoxide-α-chymotrypsin. For derivatives on which direct proflavin fraction active assays are impossible, we must rely on our tentative assumption that θ_{231} does not depend on chemical modification.

4. Discussion

4.1. Circular dichroism

Biltonen et al. [18] have demonstrated the presence of large optical rotation effects correlated with modifications of α-chymotrypsin. These are observed in circular dichroism spectra as a negative band at 230 nm and a positive band at 202 nm, superimposed on a larger fixed negative band. The changes in these two bands with chemical modification and pH are approximately but not exactly linearly correlated. Nevertheless, it is possible that they are derived from the same chromophore or chromophores. The bands were later discussed by Lumry and Biltonen [21] who emphasized the fact that the optical rotation effects, if due to one or two protein chromophores, are among the largest ever observed. Subsequent consideration of the bands by others [22] has emphasized a basis in β-sheet structural change, but nearly 30% of all protein residues would have to shift from a β-sheet to a random structure to explain the large optical rotation effects seen on activation of chymotrypsinogen to α-chymotrypsin. Nothing of this sort is seen by crystallography [23,24]. Even if there are remarkably coincidental readjustments in the extensive but highly disordered β-sheet, Madison [25] has shown that disordered β-sheet structure in proteins can produce effects of varying sign, peak position and rotational strength, in contrast with the much simpler behavior of homopolypeptides. Parker and Kim [26] have noted that pH changes and many types of covalent modifications and non-covalent bonding produce aromatic difference spectra with a unique pattern for each modification. It is probable that the circular dichroism effects are another manifestation of changes in a very few chromophores. The magnitude of the effects and their possible origin in a perturbation of the circular dichroism chromophores by the fields of the two saltbridge ions are of considerable interest.

The "captive" situation in proteins of the chymotrypsin type is attractive for fundamental development of field perturbations in circular dichroism theory. From the enzymologists point of view the circular dichroism bands may provide a means to implicate and explain the role of the ion-pair in function. Several chromophores which might cause these effects are: 1) Try-141, which is near the carboxylate of Asp-194

in the zymogen [23,27]; 2) His-57 [28,29]; and 3) the Cys-58-Cys-42 disulfide bond which is in close contact with Ser-195 in the zymogen [24].

Whatever the source of the circular dichroism changes, the pH dependence of θ_{231} has been shown to be coupled to the conformational transition between the active and inactive forms of α-chymotrypsin [1,2,14]. We have confirmed this quantitatively for α-chymotrypsin and have extended the result to include Met-192-sulfoxide-α-chymotrypsin and N-methyl-His-57-α-chymotrypsin.

For derivatives which do not bind proflavin, we have used circular dichroism to observe the conformational transition. It is possible to measure the apparent pK_a for these derivatives, but we cannot determine the actual fraction of protein in the active conformation at any pH because the magnitude of θ_{231} in either the active or inactive state may change relative to these values for α-chymotrypsin. In fact, we have shown that for Met-192-sulfoxide-α-chymotrypsin, where both circular dichroism and direct proflavin fraction active measurements can be obtained, the circular dichroism of the inactive conformation shows changes relative to the circular dichroism of the inactive conformation of α-chymotrypsin (table 3).

4.2. The effects of chemical modification

4.2.1. Met-192-sulfoxide-α-chymotrypsin

Oxidation of Met-192 has previously been shown to stabilize the active conformation of α-chymotrypsin [14]. We have confirmed these results. The apparent pK_a for the active to inactive transition is shifted from 8.78 to 10.3, implying the sulfoxide supplies about 2.1 kcal of stabilization relative to the unoxidized methionine side chain. Note that this stabilization could occur in either of the two steps which affect the apparent pK_a (see scheme I). Other similar proteases, such as trypsin or elastase, have a polar residue in this position [30] and remain in an active conformation until near pH 10.0 [31–33]. This suggests that a non-polar residue at this position in the polypeptide chain destabilizes the active form. The Met-192 side chain is observed to move from a buried position to a surface position during activation of chymotrypsinogen to α-chymotrypsin [23], while the Met-192 side chain of α-chymotrypsin rotates 180° about the C_α–C_β bond to a new surface position as the ion-pair breaks at pH 8.3 in the crystal [16]. Electron-spin-resonance experiments also show that titration of the ion-pair causes increases in the rotational freedom of Met-192 [34,35]. The various data present an unclear and sometimes conflicting view of what happens to Met-192 during the active to inactive transition. However, it is apparent that the environment of this group does change during the process. The destabilization of the active isomer by a non-polar residue at position 192 could be due to a hydrophobic interaction, such as placing the unoxidized Met-192 on the surface of the protein in contact with water when it is energetically more favorable for this side chain to be buried as in the zymogen. A specific effect, such as hydrogen bonding between the oxidized side chain of residue 192 and another group, could also cause the 2.1 kcal of stabilization observed with a polar residue. We cannot distinguish between these possibilities.

It is possible that the mechanism of the isomerization between active and inactive forms of Met-192-sulfoxide-α-chymotrypsin is not identical to that observed for α-chymotrypsin; e.g., the apparent pK_a may not represent Ile-16 or the two conformations may differ from their counterparts in α-chymotrypsin. The enthalpy and entropy changes of the apparent pK_a differ considerably from those observed in α-chymotrypsin. They are similar to the enthalpy and entropy changes on ionization of an α-amino group [36]. The apparent pK_a results from two coupled subprocesses so care must be taken in interpretation. We also note that the circular dichroism spectra of the inactive forms of Met-192-sulfoxide-α-chymotrypsin and α-chymotrypsin differ, suggesting these conformations are different.

4.2.2. N-methyl-His-57-α-chymotrypsin

The conformational transitions in N-methyl-His-57-α-chymotrypsin and α-chymotrypsin appear similar to each other; however, the active conformation is slightly more stable in N-methyl-His-57-α-chymotrypsin and only a single pK_a process, that of Ile-16, is needed to describe the pH dependence in this derivative. The lack of a pK_a near pH 7 provides evidence that His-57 (or possibly Asp-102) is the group responsible for this ionization. The stabilization of the active conformation can be explained by steric effects at the active site, especially in the inactive conformation where close contacts are thought to exist [24], or by electronic ef-

fects, perhaps because the "charge relay" system cannot be formed.

4.2.3. Phenylmethane sulfonyl-α-chymotrypsin; methane sulfonyl-α-chymotrypsin

The phenyl group of phenylmethane sulfonyl-α-chymotrypsin presumably occupies the neck of the substrate side chain site, thus preventing its collapse in the inactive species. The effect is one of stabilizing the active conformation; e.g., even at pH 11 phenylmethane sulfonyl-α-chymotrypsin remains predominantly in the active conformation. The methyl group of methane sulfonyl-α-chymotrypsin is not large enough to reach the binding pocket [21] and probably as a result provides a much smaller amount of stabilization.

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