vide the 5.4.5 ring system **6**. The former step presumably is preferred over the alternative loss of bromide and rearrangement to give a second isocyanate function; the latter step is commonly observed in the formation of alkylacylureas as by-products in the Hofmann rearrangement.[33] Finally, debromination of **6** on warming in aqueous acid to give the rearrangement product **7** is a step with long-established precedent.[34,35]

This scheme satisfactorily accounts for the lack of oxygen-18 incorporation into the final product during the above reaction sequence. Although no closely analogous process is known, a similar ring cleavage leading to the formation of an isocyanate has been proposed to account for the rearrangement of certain cytosine derivatives.[36]

It is clear from the proposed mechanism that the formation of *cis,cis,cis*-3-carbonamido-1,7-dimethyl-3,5,9-triazatricyclo[5.3.0.0^{2,6}]deca-4,8,10-trione (**7**) is possible only as a result of *cis–syn* stereochemistry of thymine dimer.

Acknowledgments. This research was supported in part by the award of an SRC Research Studentship (to R. J. D.). $D_2{}^{18}O$ was a generous gift from Professor F. Sondheimer. Dr. D. Kerridge gave valuable assistance with cultures of *E. coli*.

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The Environment of a Reporter Group at the Active Site of Chymotrypsin[1]

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Abstract: The reaction of 2-bromoacetamido-4-nitrophenol with chymotrypsin produces a reporter-labeled protein in which the phenolic group is covalently attached to the methionine residue, Met_{192}, three residues away from the active serine. The modified enzyme is still active but the reporter group apparently is bound at or near the binding site of the enzyme and decreases the velocity of acetyltyrosine ethyl ester hydrolysis. The pH dependence of the absorption spectra indicates that a positively charged group of pK = 7 is spatially close to the hydroxyl group of the phenol and that the positively charged group perturbs the pK of the phenol strongly. Other groups of pK's greater than 9 affect the spectra appreciably but less strongly. Binding of substrate or reaction with phenylmethanesulfonyl fluoride eliminates the pK = 7 effect apparently by displacing the reporter group from its nearness to the pK = 7 group. The pK = 7 group is tentatively identified as the histidine at the active site. The results illustrate the value of such environmentally sensitive groups for exploring the microscopic environment of parts of the active site.

The advent of crystallography and the improved methods for protein modification have made the study of the mechanism of enzyme action both more rewarding and more precise. As these studies unfold, it is imperative that the nature of the active site be probed with increasingly sophisticated techniques to discover those environmental influences which make enzymes so effective. Although many physical properties of the protein can be followed as a function of substrate binding, *e.g.*, the over-all hydrodynamic properties, the absorption spectra, optical rotatory dispersion, etc.,[2] these methods suffer from the disadvantage that they represent the integrated changes of a number of different groups. Thus, the change in one group toward a more hydrophobic environment might be nullified by the change in another group toward a more hydrophilic environment. By placing a sensitive "reporter group" at one specific position in a protein molecule the changes in its local environment can be examined. Thus, it was shown that the attachment of an environmentally sensitive nitrophenol moiety near the active site of chymotrypsin produced absorption spectra of the reporter group that were sensitive to substrate binding and that varied with the individual substrates absorbed.[3] In the present paper the environment of this reporter group is probed further both in relation to its immediate neighbors at the active site and in relation to the influence of bound substrate molecules.

(1) The authors wish to acknowledge the generous support of the National Science Foundation and the National Institutes of Health during the course of this work. Abbreviations: Rp, reporter group; RpBr, 2-bromoacetamido-4-nitrophenol; RpH, 2-acetamido-4-nitrophenol; ChTr-Met_{192}Rp, chymotrypsin with above reporter group attached to Met_{192} through a sulfonium linkage; ChTr-Met_{192}Rp-Ser_{195}-PMS, chymotrypsin with reporter group on Met_{192} and phenylmethanesulfonyl group on Ser_{195}; ATEE, acetyltyrosine ethyl ester; DIP, diisopropylphosphoryl; PMSF, phenylmethanesulfonyl fluoride. An account of this work was presented at the 152nd National Meeting of the American Chemical Society, Miami Beach, Fla., April 1966.

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Experimental Section

Synthesis of 2-Bromoacetamido-4-nitrophenol (RpBr). The procedure of Newbery and Phillips[4] for treatment with acetyl halides was followed in general. Crystals of the product, which formed on the dropwise addition of 9.3 ml (0.11 mole) of bromoacetyl

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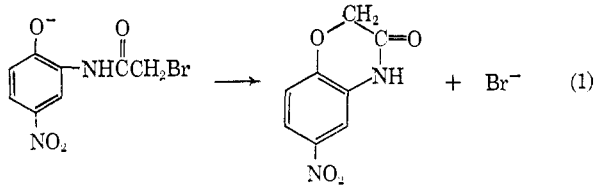
bromide to 10 g (0.07 mole) of 2-amino-4-nitrophenol with 80 ml of acetone, were filtered and recrystallized from 95% ethanol, mp 212–213° (sealed tube). *Anal.* Calcd for $C_8N_2O_4BrH_7$: C, 34.93; N, 10.19; Br, 29.05; H, 2.57. Found: C, 35.31; N, 10.01; Br, 29.91; H, 2.62. The infrared spectrum of the product in a KBr pellet has a single sharp absorption band at 3360 cm^{-1} in the normal hydrogen stretching region and a broad band in the 3100–3000-cm^{-1} region. These and other features of the infrared spectra compared with known spectra[5-9] showed that the infrared spectrum is consistent with the structure 2-bromoacetamido-4-nitrophenol and inconsistent with the isomeric compound in which the bromoacetyl group is on the phenolic oxygen.

The product 2-bromoacetamido-4-nitrophenol (RpBr) rapidly cyclizes in neutral or basic solutions to give a colorless benzoxazine derivative, 3,4-dihydro-6-nitro-1,4-benzoxazol-3-one.[10] The half-life of the cyclization at pH 7 and room temperature is about 18 min as determined by the decrease in absorption at 410 mμ. At



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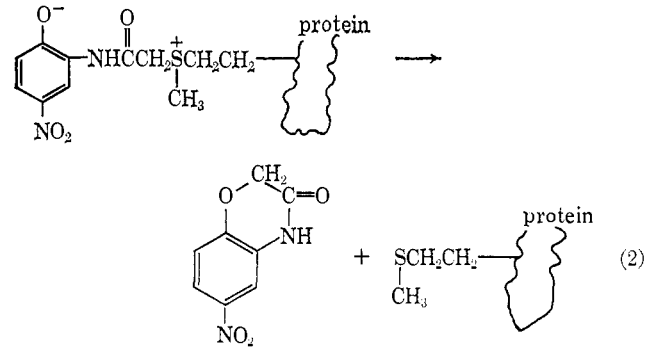
pH 3 the hydroxyl group of the RpBr is not ionized and cyclization does not occur to any appreciable extent.

2-Acetamido-4-nitrophenol (RpH). This compound was prepared according to the method of Hewitt and King.[11] Crystals with a melting point of 277° (sealed tube) and an infrared absorption in ethanol of 3410 cm^{-1} (sharp) and 3000–2850 cm^{-1} (broad) were obtained. The differences in the absorption frequencies for RpBr and RpH are explained by the electron-withdrawing power of the bromine in RpBr. As would be expected from the structure, the 2-acetamido derivative is stable in neutral and basic solutions.

Preparation of a Chymotrypsin Labeled with 2-Acetamido-4-nitrophenol (ChTr-Met$_{192}$Rp). The reagent 2-bromoacetamido-4-nitrophenol was dissolved in methanol and added slowly to a solution of 1–2 mg of bovine α-chymotrypsin in 0.001 *N* HCl at 20° until a 50 m*M* solution in 20% methanol was obtained. This solution and a control with the acetamido derivative in place of the bromoacetamido compound were incubated in the dark for 12–21 days at pH 3, 20°. The activities of the two solutions were followed by the efficiency assay using acetyltyrosine ethyl ester.[12] The activity of the bromoacetamido-treated protein tended to level off in 15 days and remained about 45% active relative to a control in the absence of the reagent. The enzymatic solutions were then filtered with paper and exhaustively dialyzed at 9° against 0.001 *N* HCl. The protein was further purified on Sephadex G-25 (coarse grain), lyophilized, and stored at −18°.

When the enzyme, purified in this manner, was dissolved in buffered solutions at neutral pH values, its absorption at 410 mμ gradually decreased with time. A simultaneous increase in the activity of the enzyme indicated that the reporter group was being liberated from the enzyme. The fading is probably caused by intramolecular cyclization of the phenolic moiety on the protein with the thioether of the methionine residue acting as the leaving group (*cf.* eq 2). The product of such a cyclization would be the colorless benzoxazine derivative that is observed during the intramolecular cyclization of the RpBr compound. Fortunately the half-life of the cyclization from the protein at pH 6.5 and 22° is about 15 hr. Therefore, difference spectra could be obtained with negligible perturbation from cyclization. To minimize cyclization in the preparation of solutions large batches of enzyme were frozen at pH 6.5 and thawed immediately before use. A typical experiment



(2)

involved freezing in small aliquots a solution of 200 mg of protein dissolved in 100 ml of ice-cold 0.05 *M* phosphate buffer, pH 6.5, which had been previously centrifuged to remove undissolved protein. Spectra were run immediately to minimize errors and in many cases checked to ensure that no appreciable drift in time had occurred. Reproducible difference spectra which showed no effect of the freezing and thawing procedure were obtained in this way.

Preparation of DIP-Chymotrypsin. A 40 m*M* solution of bovine α-chymotrypsin in 0.05 *M* phosphate buffer, pH 6.5, was treated with a 2.7-fold excess of diisopropyl fluorophosphate for 45 min.[13] The modified protein had no measurable activity. It was exhaustively dialyzed against 0.001 *N* HCl, lyophilized, and stored at −18°.

Assays for Chymotrypsin. The all-or-none assay[14] for α-chymotrypsin activity was performed by treating the enzyme with C^{14}-labeled phenylmethylsulfonyl fluoride[15] (3.4 μmoles in 0.1 ml of dioxane) in 0.05 *M* phosphate buffer, pH 6.5, and 25°. The protein was separated from excess C^{14}-labeled PMSF on a Sephadex G-25 column in 0.001 *N* HCl. The 6–8 mg of protein that appeared were lyophilized, weighed, and plated in 2-mg portions on planchettes. Corrections for C^{14} self-absorption were performed.

The efficiency assay was performed using acetyltyrosine ethyl ester following the procedure of Schwert and Takenaka.[12] The decrease in absorption at 237 mμ of 3 ml of 0.001 *M* ATEE was followed after the addition of 5–100 μl of enzyme.

Preparation of ChTr-Met$_{192}$Rp-Ser$_{195}$PMS. Preparation of PMS-labeled chymotrypsin containing the reporter group was performed on a large scale using the method of the all-or-none assay as described above. A frozen solution of this material (*c* 0.5 mg/ml) was prepared for the spectrophotometric titrations as discussed for the standard preparation of reporter-labeled enzyme.

Determination of the Number of Reporter Residues per Molecule of Chymotrypsin. Samples of reporter-labeled chymotrypsin, RpBr and RpH, were hydrolyzed in 6 *N* HCl for 24 hr. The hydrolysis products of RpBr and RpH were verified to be 2-amino-4-nitrophenol by their spectra in half-normal NaOH ($\epsilon_{455\ m\mu}$ 1.35 × 10^4). The moles of reporter group per mole of enzyme was calculated from the absorbance of a basic solution of hydrolyzed reporter-labeled chymotrypsin at 445 mμ. A correction was made for the absorption of hydrolyzed native chymotrypsin (ϵ_{445} 0.3 × 10^4). The concentration of the enzyme was determined by amino acid analysis of a fraction of the hydrolysis products using the automatic amino acid analyzer.[16] To ensure that all the reporter groups assayed by this procedure were initially covalently bonded a sample of the modified protein was dissolved in 20% methanol, precipitated with trichloroacetic acid, washed with ether, and dried before hydrolysis in 6 *N* HCl. The same number of reporter residues per molecule of chymotrypsin was found in this preparation as in the standard preparations used for the difference spectra.

Spectral Measurements. Spectra were measured with a Cary 14 recording spectrophotometer equipped with a 0 to 0.1 optical density slide wire, a scan feed of 25 A/sec, a chart speed of 5 in./min. Temperature was maintained at 22° using a circulating water thermostat outside the cell compartments. Corrections were made for air *vs.* air absorption. For the spectrophotometric titrations an

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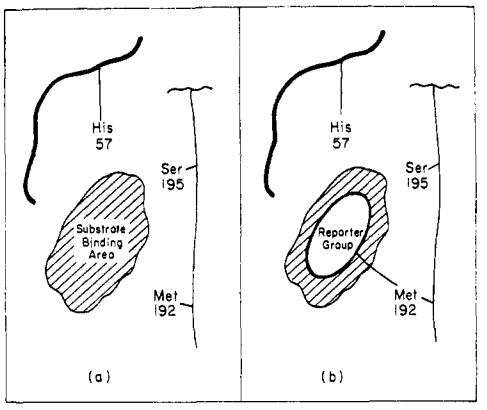

Figure 1. Schematic illustration of active site with and without reporter group. Reporter group attached to methionine-192 which is not essential for enzyme action is absorbed to substrate binding area and thus acts as competitive inhibitor of substrate.

unfrozen stock solution containing the reporter-labeled enzyme was diluted to one-third with 0.05 *M* phosphate buffer and pipetted into the quartz cuvette. Aliquots (10 μl) of the appropriate concentration of acid or base were added; the solution was mixed thoroughly and a spectral measurement made with water as a reference solution. The spectrum was completed less than 5 min after the addition of acid or base.

Results

Characterization of Reporter-Labeled Protein. The reporter-labeled enzyme was treated with C^{14}-labeled phenylmethanesulfonyl fluoride in a typical all-or-none procedure. It was found that the phenylmethanesulfonyl fluoride reacted stoichiometrically whereas no counts were incorporated into DFP-treated enzyme (Table I). In previous studies it has been shown that Met_{192} of the Hartley sequence[17] can be oxidized[18-20] or alkylated[21-23] without complete loss of enzyme activity. Studies of Knowles[19] and in our laboratory[18,20] show that oxidation of this methionine produces only mild decrease in reactivity, but alkylation causes larger changes. Lawson and Schramm[21,22] and Kézdy, Feder, and Bender[23] investigated the properties of alkylated chymotrypsin and found a significant increase in both K_M and k_{cat}. The finding that this reporter-labeled enzyme is still active is, therefore, consistent with previous findings of the nonessentiality of this methionine residue for catalytic activity.

Table I. Reaction of Chymotrypsin Preparations with PMSF[a]

Protein	Counts of C^{14} covalently bonded/mg of protein
Native chymotrypsin	3920
ChTr-Met_{192}Rp	3710
DIP-ChTr	24

[a] Conditions: 8×10^{-5} *M* protein, 5×10^{-4} *M* PMSF, pH 6.5, 25°, 1.5 hr.

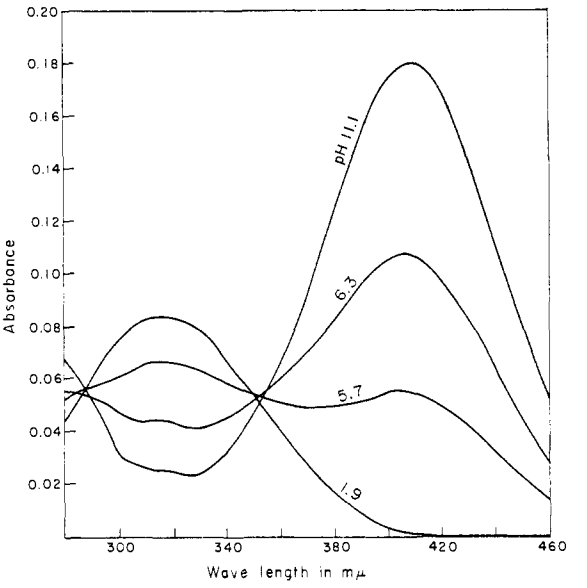

Figure 2. Experimentally determined absorption spectra of the reporter group, RpH. Condition: 0.05 *M* ion phosphate ions.

Using the acetyltyrosine ethyl ester efficiency test, however, the enzyme activity dropped appreciably as might be expected. Since the enzyme was only partially labeled because of the slow reactivity of the methionine residue with the RpBr, it is difficult to put a precise limit on the enzyme activity. However, it appears that the reporter-labeled enzyme, ChTr-Met_{192}Rp, is at least ten times less reactive toward ATEE than the native enzyme. Because of the bulkier side chain compared to iodoacetamide and the (N-2-carboxyisopropyl)carbamylmethyl groups, this greater drop might be expected. In fact the decreased activity strongly suggests that the aromatic ring of the reporter compound is attracted to the active site in such a manner as to hinder binding of the ATEE without damaging the catalytic power of the enzyme (*cf.* Figure 1).

Determination of reporter groups incorporated by the spectrophotometric procedures described in the Experimental Section and determination of the methionines modified by performic acid oxidation[24] gave mutually consistent results of 0.6 Rp residue per chymotrypsin molecule. From this correlation and the finding that no other amino acids were affected, it can be concluded that only methionine is modified by this procedure. Cyanogen bromide[25] cleavage showed that Met_{192} was modified and Met_{180} was not. This is also consistent with the oxidations and alkylations reported previously.[18-23]

Spectrophotometric Measurements on Reporter-Labeled Chymotrypsin. In Figure 2 are shown the absorption spectra of the 2-acetamido-4-nitrophenol at various pH's. A gradual change from the un-ionized form with an absorption maximum at 318 mμ to the ionized form with an absorption maximum at 410 mμ was observed. When the data at 315 and 410 mμ, respectively, are plotted to indicate the fraction of ionized and un-ionized forms, Figure 3 is obtained in which the points represent experimentally determined values and the solid lines are calculated for a single

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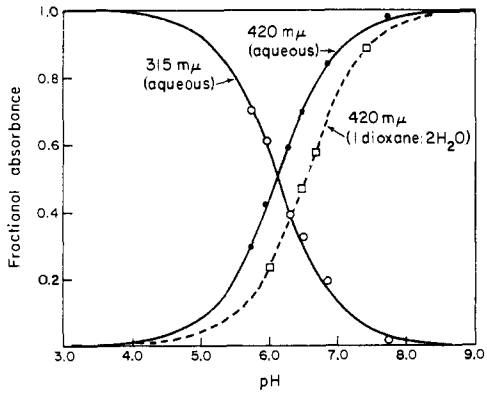

Figure 3. Relative concentrations of the un-ionized and ionized phenolic forms of the reporter group in the 2-acetamido-4-nitrophenol (RpH). The solid lines are calculated for the ionization of a weak acid with a pK of 6.12 for the aqueous solution (solid lines) and a pK of 6.52 for the dioxane–water mixture (dashed line). The experimental points were measured at 315 mμ (○), 410 mμ (●), 370 mμ (△), and 450 mμ (×) for the aqueous solution and 410 mμ (□) for the dioxane–water solution. Points at pH 2 not shown are 1.0 for 3.5 mμ and 0.0 for 410 mμ. All aqueous solutions are 0.05 M in phosphate ions. RpH is 13 μM.

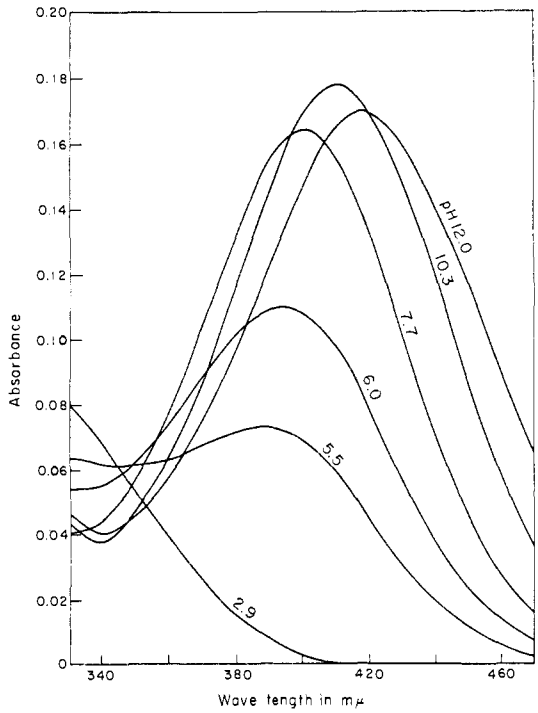

Figure 4. Experimentally determined absorption spectra of the reporter group, Rp, in the environment of chymotrypsin. A shift in the wavelength maximum with pH which is not due to the absorption of the 315-mμ peak can be seen. Conditions: 23 μM ChTr-Met$_{192}$Rp, 0.05 M in phosphate ions.

ionization with a pK of 6.12. To indicate the effect of the medium a similar titration curve was performed in a mixture of one volume of dioxane to two volumes of water (Figure 3). Thus, decreasing the dielectric constant of the medium shifts the pK of the absorbing group to that of a weaker acid (pK of 6.52). It was also found that this more hydrophobic medium shifts the absorbance maximum to longer wavelengths.

The measured absorbances of the reporter group attached to chymotrypsin are shown in Figure 4. Some general characteristics to be expected from the

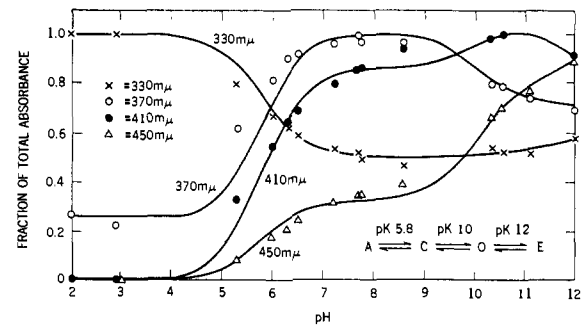

Figure 5. Spectrophotometric titration of ChTr-Met$_{192}$Rp and calculated curves assuming one pK value below pH 9. The solid lines were calculated assuming that there were three dissociating groups with pK values of 5.8, 10, and 12. The experimental points are shown for 330 mμ (×), 370 mμ (○), 410 mμ (●), and 450 mμ (△). Conditions and experimental points identical with Figure 4.

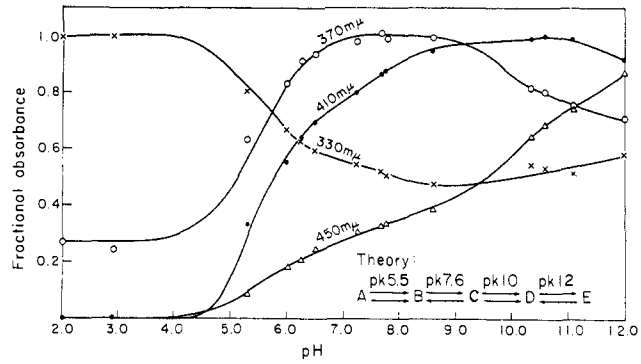

Figure 6. Spectrophotometric titration and calculated titration curves for the reporter group, Rp, in the environment of chymotrypsin assuming two pK values below pH 9. The solid lines are calculated from the estimated absorptivity values in Figure 7 and the equilibria for the ionization of four groups with pK values of 5.5, 7.6, 10, and 12. The dashed line is the best fit at 410 mμ assuming pK values of 5.5, 8.0, 10, and 12 and the appropriate absorptivity values. The experimental points shown for 330 mμ (×), 370 mμ (○), 410 mμ (●), and 450 mμ (△) are from the spectra in Figure 5 and other measurements of identical protein solutions.

behavior of RpH in solution are obtained *i.e.*, a decrease of 340 mμ absorption as the pH is increased and a concomitant increased contribution of the absorption at 410 mμ. However, it is immediately apparent (a) that the spectra are far more complex than spectra of the reporter group free in solution, (b) that no isosbestic point exists, and (c) that both the spectral changes and the pK values are complicated functions.

It is apparent that the observed spectra will be influenced by all the molecular species existing in solution at a given pH and the absorption spectra of the species. At a new pH the relative proportions of the species should change in a predictable manner based on the pK's of the ionizing groups. By appropriate simultaneous equations and assumptions of number of ionizing species and their pK's, all the species and their spectra could be deduced. In practice it was found easier to obtain the initial acid spectrum at low pH's (1.9 and 2.89) and to use this spectrum in simultaneous equations to obtain the spectrum of the next ionizing species, and proceed similarly to the high pH solutions. Assuming a single ionizing group below pK = 8.5, the fit of theory to experiment was poor. A typical relation of "theory," with experimental data to give the "best fit" for such a case is shown in Figure 5. How-

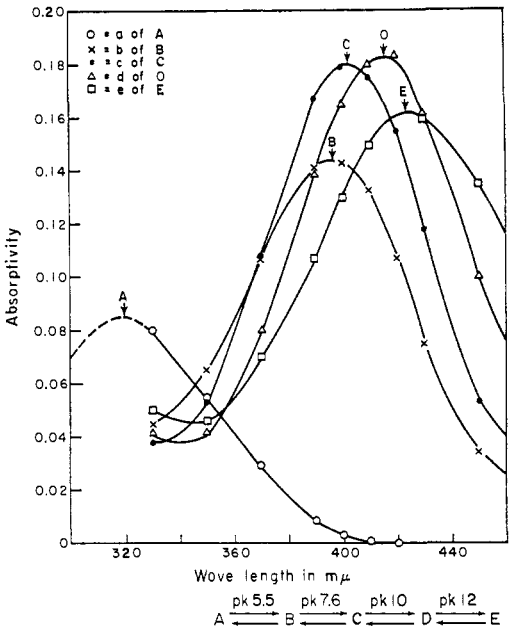

Figure 7. Estimated absorption spectra of the five postulated forms of the chymotrypsin–reporter complex. The estimated absorptivity values delineate the spectra of the five forms, A (○), B (×), C (●), D (△), and E (□), of the enzyme–reporter complex resulting from the assumption that there are four ionizing groups with pK values of 5.5, 7.6, 10, and 12 which influence the absorption of Rp in the enzyme environment.

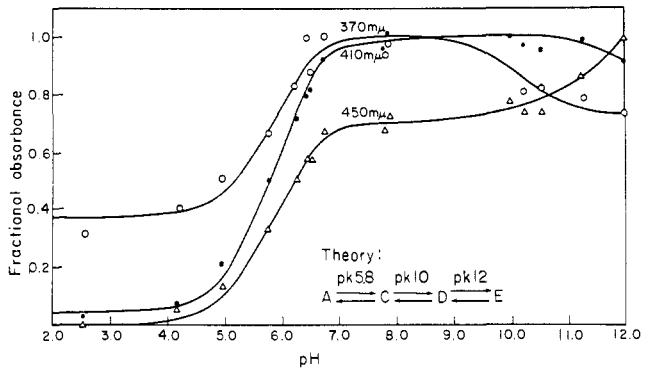

Figure 8. Spectrophotometric titration of a chymotrypsin–reporter-labeled enzyme treated with phenylmethanesulfonyl fluoride (ChTr-Met$_{192}$Rp-Ser$_{195}$PMS). The solid lines are calculated from the estimated absorptivity values in Figure 9 and the equilibria for the ionization of only three groups with pK values of 5.8, 10, and 12. The data below pH 9 is fit with a single pK. The experimental points are shown for 370 mμ (○), 410 mμ (●), and 450 mμ (△). Conditions: about 20 μM ChTr-Met$_{192}$Rp-Ser$_{195}$PMS, and 0.05 M in phosphate ions.

ever, with two pK's below 8.5, the agreement between experimental data and assumed molecular species could be excellent, as shown in Figure 6. These "theoretical" curves are calculated with the assumed pK's of 5.5, 7.6, 10, and 12 and the appropriately calculated spectra for the hypothetical molecular species shown in Figure 7. Although it cannot be said that this fit proves these identical ionization constants, it does give strong presumptive evidence for the pK's of 5.5 and 7.6. Attempts to fit the data with curves based on pK's as much as two-tenths of a pK unit different from these values gave data which was at appreciable variance from the experimental facts. As might be expected from the observed spectral changes in Figure 3, the change in basic solution is small and therefore more leeway in

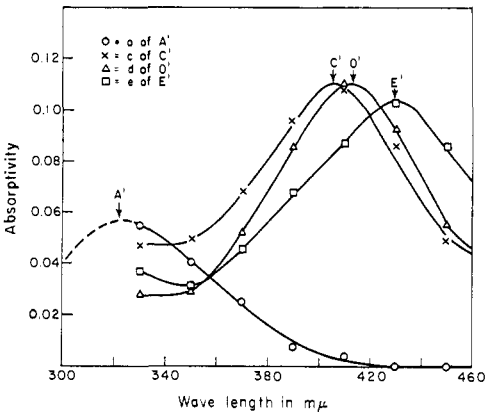

Figure 9. Estimated absorption spectra of the reporter group in the postulated forms of the ChTr-Met$_{192}$Rp-Ser$_{195}$PMS. In the presence of the covalently bonded "substrate," phenylmethylsulfonyl, only four forms are needed to fit the data and only the forms **A′** and **B′** occur below pH 9. The assumed pK values are 5.8, 10, and 12.

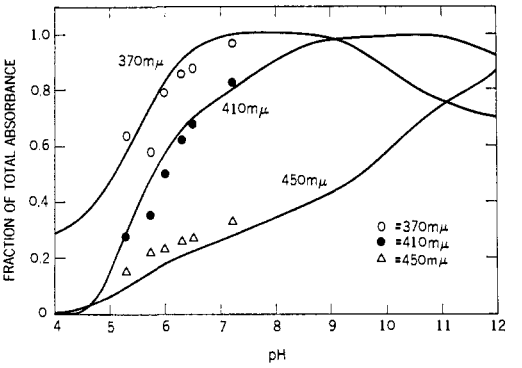

Figure 10. pH titration of ChTr-Met$_{192}$Rp in the presence of benzoylphenylalanine. The experimental points are shown for 370 mμ (○), 410 mμ (●), and 450 mμ (△). The points are normalized to the maximal absorption in the absence of substrate. The solid lines are those calculated for ChTr-Met$_{192}$Rp (of Figure 5) in the absence of substrate. Conditions: 23 μM enzyme, 0.01 M benzoyl-L-phenylalanine, 0.05 M phosphate ions.

the choice of these particular pK's is available. The fit of the experimental data therefore gives strong support for groups of pK = 5.5 and 7.6 and indicates generally that groups with pK's in the alkaline region cause further perturbations of this absorption spectrum. Whether two or more groups in that region are involved cannot be said from the data and therefore two groups of pK = 10 and 12 were chosen as first approximations. Of course, the postulation of many additional groups could also fit the spectrum even more precisely, but this added complexity is not justified.

When a similar experiment is performed on the phenylmethanesulfonyl-treated reporter-labeled chymotrypsin, the data of Figure 8 are obtained. The data can be fit assuming four forms of the enzyme and three ionization constants of 5.8, 10, and 12. The absorption of the individual hypothetical species corresponding to these pK's is shown in Figure 9. It is notable that the raw data in this case are considerably simpler than in the case of the ChTr-Met$_{192}$-Rp. Below pH 8.5 the data fit easily with a single pK and the ionization constant is close to that of the free reporter group.

An effect similar to that of treatment with the PMSF is obtained by adding substrate to the labeled enzyme (Figure 10). Addition of 0.01 M benzoylphenylala-

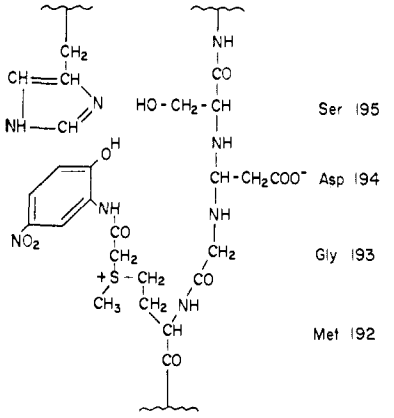

Figure 11. Schematic illustration of reporter group at active site with phenolic hydroxyl near 1 *N* of histidine residue. Distances are schematic but can correspond to an actual space-filling model of the site folded appropriately.

nine causes the ionization of the phenolic hydroxyl to be shifted to a higher p*K* value, indicating again a tendency of substrate to separate the phenolic group from the influence of a positively charged group of p*K* = 7. Although these spectral shifts are not quite as elegant as in the case of the PMS-induced shifts of the reporter group (because of the difficulty of obtaining sufficient solubility of the substrate), the qualitative effects are very similar.

Discussion

The observed spectral changes of the reporter group at the active site of chymotrypsin reveals (a) that the attachment of the nitrophenol reporter group to the methionine of chymotrypsin causes a spectral shift in a direction expected for a change to a more polar environment than H_2O, (b) that the p*K* of the reporter group is shifted to that of a stronger acid, (c) that the titration of a group at p*K* = 7.6 affects the spectra of the ionized phenolic species quite strongly, (d) that further changes at alkaline pH indicate that groups of p*K* $\gtrsim$ 9 affect spectra slightly, and (e) that substrate or covalently attached substrate analogs eliminate the interaction of the positively charged group of p*K* = 7.6 with the reporter group and shift the p*K* of the reporter phenolic group to more basic values.

These facts can be most simply explained by the scheme shown in Figure 11. The reporter hydroxyl group must be near a positively charged group whose normal p*K* is 7. The most obvious candidate for such a group is the histidine at the active site which is known to give a p*K* of 6.7 in titration studies[26] and activity–pH profiles.[27,28] In strong acid this group would be positively charged and its juxtaposition to the phenolic hydroxyl would increase the acidity of that group so that a p*K* of 5.5 would be understandable instead of the 6.1 of the free reporter group. As the solution is made more basic, the phenolic group is converted to its ionized form, clearly evident from the spectral shifts, thus placing a negatively charged O^- immediately adjacent to the imidazolium group. As a result the p*K* of that group is shifted to more basic values, *i.e.*, to a p*K* of 7.6 instead of 6.7. Since the initial titration causes the spectral shift from molecular species A, the un-ionized phenolic form, to molecular species B, the ionized phenolic form, all further changes in spectrum involve perturbations on this ionized form (Figure 7). The titration of the imidazolium group causes the most significant shift, from molecular species B to molecular species C. This is also consistent with a shift to a longer wavelength as the environment becomes more nonpolar. At more alkaline pH's further changes in the spectrum are observed (species D and E), but these changes are smaller in magnitude. It seems probable that the interaction of the phenolic hydroxyl of the reporter group and the imidazolium of histidine is direct because of the magnitude of the p*K* and spectral shifts. The further spectral changes at pH's greater than 10 could be a direct effect of more distant groups or more probably indirect conformational effects. In this regard it would have been interesting to observe an effect on the reporter group of an amino acid with p*K* = 8.5 in view of the interesting conformational studies of Himoe, Parks, and Hess.[28] A small effect may be present but, as mentioned above, the spectra in the alkaline region are not sufficiently sensitive to establish or eliminate such an effect.

The identification of the imidazolium group of histidine is, of course, not definitive since any group of p*K* = 7 would suffice to explain the results reported here. However, the group would have to be positively charged in the acid form to explain the p*K* shift of the phenolic group and it would have to be accessible to titration. The α-amino group of isoleucine which has been identified with chymotrypsin activity by Hess and co-workers[28] satisfies the positively charged criterion but its p*K* is shifted to higher values than is normal for an α-amino group and it appears to be buried at neutrality.

The observations that the reporter group inhibits the ATEE assay but not the all-or-none assay, and that the free substrate, benzoylphenylalanine, or the "bound substrate," PMS, nullify the effect of the p*K* = 7 group, are mutually consistent and agree with the universally accepted conclusions that histidine[26,27,29] and serine[30–33] are at or near the active site. Thus, the reporter group in this preparation acts as a competitive inhibitor which happens to be covalently bound to the enzyme by a group which is not essential for activity. Its presence inhibits binding of substrate but not catalysis; its displacement by substrate removes it from the juxtaposition with the histidine residue. (This kind of covalently bound competitive inhibition will exist in all cases of "affinity labeling" and therefore the loss of activity with affinity labeling cannot *per se* lead to identification of an essential catalytic group.)

It is interesting that the environmental probe used here reveals an environment more polar than water whereas it has been widely stated that the active site of chymotrypsin is hydrophobic[34] and the use of a similar reporter compound by Kallos and Avatis[35] has re-

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vealed that the nitrobenzenesulfonyl group shows a spectral shift in the direction of a more hydrophobic environment. There is nothing inconsistent with these results and the more polar environment of the OH group reported here. The explanation lies in the heterogeneity of the active site and the particular effectiveness of reporter groups in probing micro regions. The specificity pattern of chymotrypsin certainly indicates that hydrophobic bonding must play a key role in the attraction of substrates, *e.g.*, the increased activity of phenylalanine and tyrosine derivatives in preference to derivatives of alanine and glycine. Thus the specificity site undoubtedly contains a hydrophobic region and this hydrophobic region probably accounts for the spectral shifts observed by Kallos and Avatis.[35] The same hydrophobic bonding probably occurs in our compound which contains a nitrobenzene portion. This hydrophobic bonding of the aromatic portion of the phenol, however, must place the OH of the phenolic group in a microscopic environment more polar than water, *e.g.*, next to the positive charge of histidine. The combination of hydrophobic and hydrophilic regions is a key to active site behavior, the subtleties of their juxtaposition undoubtedly playing a key role in enzyme action. Reporter groups tailored to explore different facets of the active site will therefore be particularly valuable in exploring these subtleties. The recent demonstration that a change in the relative orientation of bromacetamido and phenolic groups causes changes in the difference spectra is consistent with this interpretation.[36] Different techniques may also provide special new insights. The nuclear magnetic techniques of Cohn,[37] McConnell,[38] Baldeschwieler,[39] and their coworkers look particularly attractive in this regard. The use of fluorescent probes exploited so well by Weber,[40] Stryer,[41] and Edelman[42] also provide new information about the binding of small molecules in specific locations.

The elegant X-ray crystallographic studies of Phillips, *et al.*,[43] and Kendrew, *et al.*,[44] describe specific substrate–enzyme and small molecule–protein interaction in ways that would never be possible by more indirect physical probes, but the existence of conformational changes in solution and the need to probe the relationship of a wide variety of ligand–protein interactions mean that X-ray crystallography alone may not be able to probe all the interactions at the enzyme surface. Thus, a demand is created for complementary tools of which the reporter group may represent a powerful example.[45]

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(45) NOTE ADDED IN PROOF. The structure of chymotrypsin has recently been reported by B. W. Mathews, P. B. Sigler, R. Henderson, and D. M. Blow (*Nature*, **214**, 652 (1967)). There is no conflict between the conclusions of this article and that structure where the facts are relevant. On the other hand, the deductions about binding and the p*K* perturbations cannot be established or rejected by mere obser vation of the structure, and, therefore, must await further work.

Communications to the Editor

Metastable Ion Characteristics. II.[1] Variation of Metastable Ion Abundances in Mass Spectra with Vibrational Degrees of Freedom[2]

Sir:

Recent communications have described the use of the mass, relative abundance, and kinetic energy of formation of "metastable ions" resulting from the decomposition of a particular ion in order to characterize the structure and energy state of that ion.[1,3] The abundances of the different ions resulting from the metastable decompositions of ions of a particular structure were constant relative to each other, but there was indication that the abundances of these ions relative to that of the precursor ion decreased with molecular weight.[1] Careful abundance measurements for 17 different metastable ion transitions now indicate that the ratio of the log abundance of a particular "metastable ion" relative to the abundance of ions of the same elemental composition as the precursor is *inversely proportional to the number of vibrational degrees of freedom in the original molecule*, as shown in Figures 1 and 2.[4] This relationship is a distinctive characteristic of the structure of the precursor ion and can be measured for many systems for which the other "metastable ion characteristics[1,3]

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(2) The generous financial support of the National Institutes of Health (GM 12755) is gratefully acknowledged. The Hitachi RMU-6D mass spectrometer used in this work was purchased through National Science Foundation Grant GP 4335.
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(4) Relative peak heights measured from spectra recorded on a Hitachi RMU-6D mass spectrometer with an electron energy of 75 v. Data for 2-nonadecanone were measured on a CEC 21-110 with defocusing of the normal ions by the electrostatic analyzer;[5] relative abundances obtained by this technique for the other 2-alkanones were essentially identical with data from the Hitachi instrument.
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