Low pH Dimerization Behavior of Active-Site Derivatives of Bovine α-Chymotrypsin†

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ABSTRACT: The qualitative similarity but quantitative difference between the dimerization of bovine α-chymotrypsin A and diisopropylphosphoryl chymotrypsin evident from a study of the effect of pH and temperature (Horbett and Teller, manuscript in preparation) led to this study of the dimerization behavior of a series of inhibited derivatives of chymotrypsin. These were found to fall into three classes with respect to dimerization. Class I derivatives dimerize like chymotrypsin, and include acetyl, propionyl, butyryl, valeryl, isobutyryl, trimethylacetyl, benzoyl, and diethylphosphoryl chymotrypsin. Class II derivatives show reduced dimerization, and include furoyl, hydrocinnamoyl, diisopropylphosphoryl, and nitrated chymotrypsin. Class III derivatives dimerize very little, and include phenylmethanesulfonyl, toluenesulfonyl, *p*-iodophenylsulfonyl, *p*-nitrobenzoyl, cinnamoyl, diphenylcarbamyl, tosylamidophenylethylmethyl, and carboxypeptidase A treated diisopropylphosphoryl chymotrypsin. The competitive inhibitor *N*-acetyl-L-tryptophan strongly inhibits dimerization of chymotrypsin. A study of difference spectra and optical rotatory dispersion for representatives of each class suggests that there is no correlation between these phenomena and the dimerization behavior of the derivatives. These data and many earlier studies are discussed with the purpose of elucidating the specific chemical mechanism of the dimerization reaction. The chemical mechanism proposed here is based on the partly hydrophobic, partly ionic interaction between the carboxyl terminal Tyr-146 and the active-site His-57. This interaction is proposed to closely resemble an enzyme–product complex.

The elucidation of the chemical forces responsible for the low pH dimerization of bovine α-chymotrypsin has been the subject of extensive experimentation in our laboratory for several years. An understanding of the nature of these interactions is important for several reasons. First, chymotrypsin is a proteolytic enzyme whose biological functioning must involve formation of macromolecular complexes with other proteins. The quaternary interactions displayed in the dimerization of chymotrypsin may be very similar to those interactions involved in the proteolysis of other molecules since the formation of chymotrypsin itself involves limited autolysis, a process which must involve formation of a macromolecular aggregate between chymotrypsin molecules (Miller *et al.*, 1971). Second, understanding of the dimerization of chymotrypsin should provide insight into the *specific* chemical nature of quaternary interactions. Such interactions are widespread in biochemical systems and have been recognized as basic to the functioning of these systems. Finally, it is desirable to learn how much information is required to specify the mechanism of a self-association reaction.

The low pH self-association of chymotrypsin offers unique opportunities to attempt to define the specific chemical nature of a protein–protein interaction. The amino acid sequence (Hartley, 1964) and three-dimensional structures are now known (Sigler *et al.*, 1968). Several related forms of the molecule are available including chymotrypsinogen, δ-, γ-, and β-chymotrypsins, and the more recently isolated κ-chymotrypsin (Miller *et al.*, 1971). Most importantly, many synthetic active-site chemical derivatives of chymotrypsin have been prepared and studied.

The earliest studies on the association properties of chymotrypsin derivatives involved the use of relatively nonspecific or poorly characterized modifications. McLaren and Finkelstein (1950) observed greatly decreased sedimentation coefficients and activity upon 235-mμ irradiation of chymotrypsin. Similar observations were made by Smith and Brown (1952) on a periodate-oxidized derivative of chymotrypsin and by Jandorf *et al.* (1955) and by Egan *et al.* (1957) on a photooxidized derivative of chymotrypsin. One histidine and several tryptophan residues were shown to be absent from the latter derivative by Jandorf *et al.* (1955), but subsequently Koshland *et al.* (1962) also determined that a methionine residue is transformed into the sulfoxide form in this derivative.

The sedimentation velocity technique used in all previous studies of chymotrypsin derivative association is relatively insensitive to small changes in dimerization and is not a reliable means for determining equilibrium constants or stoichiometry. Thus, the actual association behavior of iPr_2P-chymotrypsin[1] at low pH has not previously been clearly defined. Smith and Brown (1952) measured the sedimentation behavior of iPr_2P-chymotrypsin between pH 4 and 9.6 and found it similar to that for chymotrypsin, with a single maximum sedimentation coefficient at pH 4.0. Sedimentation measurements on iPr_2P-chymotrypsin by Jandorf *et al.* (1955) indi-

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[1] Abbreviations used are: TPCK, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone; PMSF-, tosyl-, and pipsyl-chymotrypsin result from chymotrypsin reaction with phenylmethanesulfonyl, toluenesulfonyl, or *p*-iodophenylsulfonyl fluorides, respectively. iPr_2P and Et_2P are diisopropylphosphoryl and diethylphosphoryl, respectively. iPr_2FP and Et_4PP are the inhibitors diisopropylfluorophosphate and tetraethylpyrophosphate. K_2 is the equilibrium dimerization constant in units of liter mole^{-1}.

cated that the degree of dimerization was significantly lower for iPr_2P-chymotrypsin than for chymotrypsin and, furthermore, another maximum in the sedimentation coefficient *vs.* pH profile was observed at pH 7.9. Gladner and Neurath (1954) did not detect any difference in the sedimentation behavior of iPr_2P-chymotrypsin and chymotrypsin.

The question of the relation of the active site of chymotrypsin to its aggregation site has been the subject of interest and controversy for almost as long as the aggregation has been known to occur. iPr_2P-chymotrypsin, which totally inhibits chymotrypsin activity, does not prevent dimerization, as noted above. An early observation by Schwert and Kaufmann (1951, footnote 1) that the competitive inhibitor β-phenyl propionate does not greatly affect the aggregation of chymotrypsin has been confirmed by Sarfare *et al.* (1966) and in our laboratory. Carboxypeptidase A treatment eliminates dimerization but not activity (Gladner and Neurath, 1954; this work). All of these experiments are incompatible with the hypothesis that the dimerization and active sites of chymotrypsin are the same. There is, however, almost as much evidence in favor of this hypothesis. Thus, photooxidation destroys both activity and dimerizability (Jandorf *et al.*, 1955). Egan *et al.* (1957) reported that the rates of reaction of iPr_2FP and of a specific ester substrate with chymotrypsin showed a smaller increase with increasing chymotrypsin concentration than would be expected if all the added chymotrypsin were capable of reacting. Similar kinetic experiments with similar results have been reported by Booman and Niemann (1957), Martin and Niemann (1958), Kezdy and Bender (1965), and Inagami and Sturtevant (1965). All of these kinetic experiments were performed under high enough chymotrypsin concentration to assure the presence of considerable dimer. As pointed out by Martin and Niemann (1958), however, experiments of this type do not allow one to decide whether the binding of substrate, or formation of the acyl-enzyme intermediate, or the catalytic breakdown of the acyl intermediate, or all three are impaired or prevented in the dimer form of chymotrypsin.

Neet and Brydon (1970) reported sedimentation velocity studies of the association of several derivatives of chymotrypsin. These workers found that at pH 4.4, monoacetyl chymotrypsin associates while PMSF-, tosyl-, cinnamoyl-, and TPCK-chymotrypsin do so to a very limited or nonexistent degree, in agreement with the sedimentation equilibrium studies reported in this paper. However, at pH 5.4 and 6.2, PMSF-, tosyl-, and cinnamoyl-chymotrypsin were found to associate almost as well as chymotrypsin. Neet and Brydon have interpreted their results in terms of a shift in the pK of a group important in the association process while we have explained the decreased association of these derivatives at pH 4.4 quite differently (see Discussion). Shifts in pK do seem to occur during the dimerization reaction, as predicted by Aune and Timasheff (1971). Aune and Timasheff (1971) have postulated a pK shift mechanism to explain the pH dependence of the dimerization reaction. We have measured changes in proton binding during dilution of chymotrypsin at various pH values which are in close accord with the predictions of Aune and Timasheff's mechanism. These developments provide additional indirect support of Neet and Brydon's conclusions. However, the issue remains clouded since the difference in free energy of association between chymotrypsin and iPr_2P-chymotrypsin is apparently independent of pH (Horbett and Teller, manuscript in preparation). In addition, Neet and Brydon's data do not make clear whether the trimerization reaction known to occur with chymotrypsin at pH 6.2 caused the apparent increased "dimerization" of the PMSF-, tosyl-, cinnamoyl-, and TPCK-chymotrypsin derivatives at this pH.

We report the dimerization constants of a large number of specific active-site derivatives of chymotrypsin at pH 4.4 in 0.2 M KCl–0.01 M acetate. Many derivatives dimerize as well as chymotrypsin itself and some show reduced dimerization, while many dimerize very little if at all. Optical rotatory dispersion parameters and difference spectra for a selected number of these derivatives are also presented. Changes in these structural parameters do not correlate with changes in dimerization behavior of a derivative. The variation in dimerization behavior among the derivatives is interpreted to be due to specific, identifiable chemical properties of each derivative. Correlation among these properties, and with much preexisting data, is done at length in order to define a chemical mechanism for the dimerization reaction.

Materials

Enzymes. Several lots of α-chymotrypsin from each of two suppliers were used. One type was the three times recrystallized, salt-free from ethanol material with control number 6397 from the Nutritional Biochemical Co. The other type was the CDI grade from Worthington Biochemical Corporation and was a three times recrystallized, dialyzed, salt-free preparation. Carboxypeptidase A, CDA grade, was also from Worthington. Pipsyl- and tosyl-chymotrypsin were gifts of P. B. Sigler of D. M. Blow's laboratory.

Assay Substrates. *N*-Acetyl-L-tyrosine ethyl ester and *N*-benzoyl-L-tyrosine ethyl ester were Calbiochem A grade materials. Some *N-trans*-cinnamoylimidazole was a gift of Dr. Hans Neurath but the majority was purchased from Sigma Chemical Co.

Reagents Used to Make Chymotrypsin Derivatives. *p*-Nitrophenyl acetate was a gift of Dr. Hans Neurath. Phenylmethanesulfonyl fluoride (twice sublimed) was a gift of Dr. Philip E. Wilcox. *p*-Nitrophenyl trimethylacetate and tetranithromethane were Aldrich Chemical Co. products. iPr_2FP was purchased from Merck and Co. Et_4PP was supplied by the American Potash and Chemical Co. (Los Angeles, Calif.) as a 40% solution. Acetic, propionic, butyric, and valeric acid anhydrides, benzoyl chloride, and diphenylcarbamyl chloride were purchased from Eastman Organic Chemicals (Distillation Products Industries). Isobutyric anhydride was a J. T. Baker Chemical Co. product. L-1-Tosylamido-2-phenylchloromethyl ketone was purchased from Cyclo Chemicals. Anhydrous ethyl ether used for the alkyl acyl derivative preparations was a Baker and Adamson Co. product. [1-^{14}C]-Valeric anhydride was obtained from New England Nuclear Co. Furoylimidazole and hydrocinnamoylimidazole were synthesized according to the procedure of Caplow and Jenks (1962).

Miscellaneous Materials. 2-Furoyl chloride and hydrocinnamoyl chloride were obtained from Eastman Organic Chemicals (Distillation Products Industries). Imidazole was Sigma crystalline grade III material. *p*-Nitrobenzoyl chloride was a J. T. Baker Chemical Co. product. Sephadex was obtained from Pharmacia Fine Chemicals AB. Tris(hydroxymethyl)aminomethane was purchased as "Trizma HCl" from the Sigma Chemical Co. Acetic acid and potassium chloride used for preparation of the standard pH 4.4 buffer (0.2 M-KCl–0.01 M potassium acetate, pH 4.40) were both Mallinckrodt Analytical Reagent grade. *N*-Acetyl-L-tryptophan was obtained from Mann Research Laboratories. All other compounds were of reagent grade or the finest commercially

available grade. All materials were used without further purification.

Methods

Protein Concentration. The optical density at 282 mμ in a 0.5-cm cell was taken to be equal to the protein concentration of the cuvet's contents in milligrams/milliliter, which corresponds to a value of 20.0 for $E_{282}^{1\%,\,1cm}$ (Laskowski, 1955).

Extinction Coefficients. Determination of $E_{282}^{1\%,\,1cm}$ values for certain derivatives was done using the B-S differential refractometer (Phoenix Precision Instrument Co.). The refractometer was calibrated with known solutions of NaCl and the refractive increments given by Stacey (1956). The calibration was checked each time the instrument was used by running a chymotrypsin control [for which $E_{282}^{1\%,\,1cm}$ = 20.0 was assumed (Laskowski, 1955)]. We used the refractive index increments of 0.185 (at 546 mμ) and 0.194 (at 436 mμ) ml/g reported by Wilcox *et al.* (1957) for chymotrypsinogen and chymotrypsin. Absorbance measurements were made using the Zeiss PMQ II spectrophotometer.

pH 8.0 Rate Assays. A volume of 2.5 ml of substrate solution containing 0.01 M *N*-acetyl-L-tyrosine ethyl ester, 0.01 M Tris, 0.2 M KCl, and 0.01 M Ca^{2+}, pH 8.0, was put into the reaction vessel of the Radiometer pH-Stat apparatus and maintained at 25.0°. To this was added enough chymotrypsin or chymotrypsin derivative to give a reproducible rate of base uptake. Chymotrypsin controls were always run on the day of measurement and activities of the tested material expressed as the ratio of the rate of base uptake per milligram of tested material to the rate of base uptake per milligram of chymotrypsin multiplied by 100 to give per cent activities.

pH 6.2 Rate Assays. These assays were done exactly as for the pH 8.0 assays except 0.01 M acetate, pH 6.2, was used instead of the 0.01 M Tris and the base uptake was recorded at pH 6.2.

Modified Spectrophotometric Assay of Chymotrypsin. This assay of chymotrypsin employs *N*-benzoyl-L-tyrosine ethyl ester as substrate and is essentially the same as reported by Hummel (1959) except that the 25.6% (w/w) methanol has been omitted from the buffer solution. This change greatly improves the sensitivity and linearity of this assay.

Active-Site Titrations. Method A of Schonbaum *et al.* (1961) using *N-trans*-cinnamoylimidazole was followed exactly, using a Gilford or Cary 16 spectrophotometer. Chymotrypsin controls were always run and activities expressed as the per cent of the active sites per mole observed for the chymotrypsin control.

Dimerization Behavior of Hydrocinnamoyl and Cinnamoyl Chymotrypsin. Portions of a 10-mg/ml solution of chymotrypsin in the standard pH 4.4 buffer were inhibited at room temperature by addition of a tenfold molar excess of solid hydrocinnamoyl- or cinnamoylimidazole. After 11 min, the solutions were cooled on ice. The two derivatives and the chymotrypsin control solutions were then subjected to sedimentation velocity analysis at 60,000 rpm simultaneously, using a four-hole titanium rotor and wedge window cells. The rotor, cells, and centrifuge chamber were all precooled to 5°, or less. Schlieren peak height positions were measured at 10× magnification on a Nikon microcomparator. Dimerization constants were calculated from the sedimentation coefficients by a procedure similar to that used by Smith and Schachman (1971).

Stock solutions maintained at 5° were assayed with active-site titrations at the end of the sedimentation velocity experiment. The activities were 0 and 30% for the cinnamoyl and hydrocinnamoyl chymotrypsin preparations, respectively.

Preparation and Characterization of Chymotrypsin Derivatives. In all cases where not stated otherwise, the final steps in preparation of a derivative were dialysis *vs.* 0.001 N HCl in a 5° cold room followed by lyophilization.

MONOACETYL CHYMOTRYPSIN. Monoacetyl chymotrypsin was prepared exactly according to the procedure of Balls and Wood (1956) and was *not* dialyzed *vs.* 0.001 N HCl before lyophilization. Active-site titration of two separate batches gave activities of 7.0 and 3.0%, and both were completely reactivated by 20-min exposure to pH 8. It was found expedient to perform sedimentation equilibrium experiments on this and several other acyl derivatives at 5.0° to avoid reactivation to the extent of 20% which occurs during the course of a typical 8-hr sedimentation equilibrium experiment at 20°. Less than 5% reactivation occurs at 5° under the pH conditions (4.4) used in these experiments.

CARBOXYPEPTIDASE-TREATED iPr_2P-CHYMOTRYPSIN. This derivative was prepared according to the procedures of Gladner and Neurath (1954). Amino acid analysis of acid-hydrolyzed samples of the iPr_2P-chymotrypsin control and the carboxypeptidase-treated iPr_2P-chymotrypsin was done according to the procedure of Spackman *et al.* (1958) using the Beckman-Spinco amino acid analyzer. This showed the absence of 1.01 tyrosines (out of a total of four in the molecule; Hartley, 1964) from the carboxypeptidase treated iPr_2P-chymotrypsin.

DIISOPROPYLFLUOROPHOSPHORYLATED AND DIETHYLPHOSPHORYLATED CHYMOTRYPSIN. Recrystallized iPr_2P- and Et_2P-chymotrypsin were prepared with iPr_2FP and Et_4PP and thrice recrystallized according to the procedure of Balls and Jensen as given by Laskowski (1955). The residual activity (modified spectrophotometric rate assay) was less than 1%.

PROPIONYL, BUTYRYL, ISOBUTYRYL, AND VALERYL CHYMOTRYPSINS. These derivatives were prepared according to the procedure of Dixon and Neurath (1957) who used a low molar excess of the corresponding acid anhydride. Residual activities of all these derivatives (measured by active-site titration) were 15% or less.

CINNAMOYL AND FUROYL CHYMOTRYPSINS. These derivatives were prepared by a procedure which was essentially a scaled-up active-site titration with *N-trans*-cinnamoylimidazole or furoylimidazole (Schonbaum *et al.*, 1961). Residual activities were 4.1 and less than 10%, respectively, using active-site titration. A refractometrically determined extinction coefficient of $E_{282}^{1\%,\,1cm}$ = 28.0 was used for cinnamoyl chymotrypsin, while the extinction coefficient for furoyl chymotrypsin was indistinguishable from chymotrypsin.

TOSYLAMIDOPHENYLETHYL CHLOROMETHYL KETONE CHYMOTRYPSIN (TPCK-CHYMOTRYPSIN). TPCK-chymotrypsin was prepared in a manner essentially the same as that of Schoellman and Shaw (1963). The reaction was carried out at pH 6.5 in 0.1 M phosphate buffer for 5 hr with a 17.9-fold molar excess of TPCK. The residual (pH 8.0 ATEE rate assay) activity was 1.6%.

DIPHENYLCARBAMYL CHYMOTRYPSIN. This derivative, first reported by Erlanger and Cohen (1963), was prepared as follows. Twenty-five milliliters of a 20-mg/ml solution of chymotrypsin in H_2O was brought to pH 7.0 with 0.1 N NaOH and to this was immediately added 1.25 ml of a 0.024 M solution of the inhibitor, diphenylcarbamyl chloride, in methanol. The reaction was done at 25° and the pH was kept constant and base uptake recorded using the Radiometer pH-Stat apparatus. After 35 min, 0.35 ml of a 0.24 M inhibitor solution

TABLE I: Effect of Various Treatments on Dimerization Constants of Chymotrypsin Samples.[a]

Material	Treatment	$K_2 \times 10^{-3}$	$\pm K_2 \times 10^{-3}$
NBC-6397	None	14.0	2.00
NBC-6397	Recrystallized	11.8	1.34
NBC-6397	Expose to pH 6, room temp, for 0.5 hr	12.4	3.26
CDI-8VS	None	16.9	0.95
CDI-8VS	Expose to pH 5.6, room temp, for 14 hr	20.2	2.13
CDI-8VS	Expose to pH 5.6, 5°, for 14 hr	18.0	2.88
CDI-8VS	Expose to pH 8, room temp, for 0.5 hr	21.2	2.49

[a] All experiments were performed at 20° in 0.2 M KCl–0.01 M acetate, pH 4.4.

was added, but no further base uptake occurred. The final molar excess of inhibitor was approximately 6. After 2 hr, the pH was brought to pH 4.0 and the protein precipitated by bringing the solution to 80% saturation in $(NH_4)_2SO_4$. Attempts to crystallize this material according to the procedure used for iPr_2P-chymotrypsin (Laskowski, 1955) failed, and the protein was then dialyzed and lyophilized as usual. The residual (pH 8.0 rate assay) activity was 0.6%.

PHENYLMETHANESULFONYL CHYMOTRYPSIN (PMSF-CHYMOTRYPSIN). PMSF-chymotrypsin, first described by Gold and Fahrney (1964), was prepared in a manner quite similar to that used for the preparation of diphenylcarbamyl chymotrypsin described above, except that the final molar excess of inhibitor, phenylmethanesulfonyl fluoride, was 30. One preparation was fractionated on G-75 Sephadex in 0.01 M acetate–0.2 M KCl, pH 4.4, prior to the sedimentation equilibrium run. The residual activity (pH 8.0 rate assay) of all preparations was less than 0.2%.

BENZOYL CHYMOTRYPSIN. This derivative was made using benzoyl chloride according to the procedure of Dixon and Neurath (1957). The remaining activity was 9.6% (active-site titration). The extinction coefficient was determined refractometrically and found to be the same as that of chymotrypsin within experimental error.

TRIMETHYLACETYL CHYMOTRYPSIN. This derivative was prepared using the inhibitor *p*-nitrophenyl trimethylacetate as described by McDonald and Balls (1957) except that the excess inhibitor and *p*-nitrophenol were removed by exhaustive dialysis *vs.* 0.001 N HCl in the cold and the protein was then lyophilized. The residual activity (active-site titration) was 13.7%.

NITRATED CHYMOTRYPSIN. Two preparations were made under slightly different conditions but both were performed by exposing 10^{-3} M chymotrypsin to an approximately 50-fold molar excess of tetranitromethane at pH 8.0, room temperature. In one reaction, chymotrypsin in H_2O was brought to and maintained at pH 8 with 0.1 N NaOH; tetranitromethane in 95% ethanol was added, and after 20 min, enough 0.1 N HCl was added to bring the pH to 3. In the second reaction, chymotrypsin was dissolved in 0.1 M Tris-HCl–0.1 M $CaCl_2$–5% ethanol; tetranitromethane in 95% ethanol was added, and after 60 min an equal volume of 0.2 M acetate, pH 2.8, was added. Products of both reactions were dialyzed exhaustively against 10^{-3} N HCl at 5° and nitrotyrosine content determined in this solvent using the 381-mμ (pH isospestic) molar extinction for nitroacetyltyrosine given as 2200 by Sokolovsky *et al.* (1966). Protein concentrations were determined refractometrically. The 20- and 60-min reaction products were estimated to contain 0.7 and 1.4 mol of nitrotyrosine/mol of chymotrypsin, respectively.

Optical Rotatory Dispersion Measurements. A Cary 60 recording spectropolarimeter was used with a 10-cm pathlength cylindrical cell filled with approximately 1 mg/ml of protein. Optical rotation was recorded in the range 600–300 mμ. The base lines were carefully determined using a buffer-filled cell, as any error in base-line position was found to strongly affect the resulting parameters. Readings at 5-mμ intervals were taken from the chart and calculations were made with a computer program. An average of 60 data points was processed for each scan and the Drude equation parameters were obtained by least-squares calculation.

Difference Spectra. Spectra were recorded with the 0.1 o.d. full scale slide wire on a Cary 15 spectrophotometer. Measurements were made at room temperature in standard 1-cm quartz cuvets. A 2-mg/ml enzyme solution (3.0 ml) in 0.033 M Tris-HCl–0.033 M $CaCl_2$, pH 6.9, was carefully pipetted into each of two cuvets and a base-line scan was done. Then 0.001 ml of inhibitor (iPr_2FP or Et_4PP, each 0.33 M) was added to the sample cuvet and 0.001 ml of anhydrous isopropyl alcohol (the dilutant used to make inhibitor solutions) was added to the reference cuvet. Thus, the final solution was 4×10^{-5} in chymotrypsin and 1.1×10^{-4} M in inhibitor. Mixing was done by capping with parafilm and inverting the cuvet at least five times. Five minutes after putting the cuvets back into the spectrophotometer, at least two scans separated by about 10 min were done. The spectra were read off directly using a small piece of the same chart paper as a measuring device. Readings were taken every 5 mμ and in addition peak heights and wavelengths were measured. The data were plotted and a smooth curve was drawn through the points.

Ultracentrifugal Methods. The procedure used for sedimentation equilibrium experiments was the same as described by Miller *et al.* (1971) except for runs on unstable acyl-enzymes (*e.g.*, monoacetyl chymotrypsin). For these, the lyophilized derivative was dissolved in ice-cold buffer and adjusted to the correct concentration. The rotor and chamber were precooled to 5° or below and the solutions were kept on ice during cell filling. Equilibrium dimerization constants (K_2) were calculated from the observed molecular weight distributions using the methods described by Hoagland and Teller (1969).

Results

Controls for Effect of Reaction Conditions on Dimerization of a Derivative. Table I shows the results of exposing chymotrypsin to some of the conditions used in making derivatives.[2,3] The first and second lines show that recrystallization conditions used in some preparations of iPr_2P- and PMSF-chymotrypsin have no great effect on K_2. The exposure of the

[2] To facilitate presentation of the data, the chemical structure of each of the derivatives is indicated in Figure 1.

[3] The data in Tables I–IV are arranged so that the K_2 for each material can be compared to K_2 for the proper control material. In each case, the comparison is to the *first* chymotrypsin sample encountered upon reading upward in the table, starting from the material under consideration.

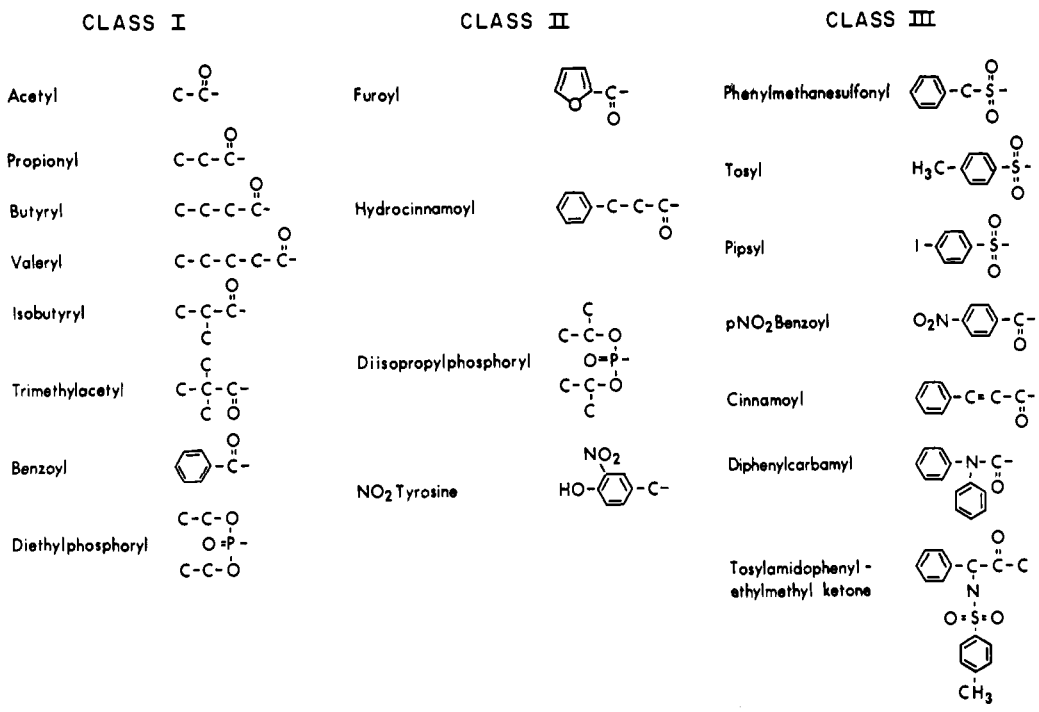

FIGURE 1: Structure of derivatives of chymotrypsin.

material to the conditions used to prepare cinnamoyl, furoyl, hydrocinnamoyl, and *p*-nitrobenzoyl chymotrypsin (pH 6 for 0.5 hr at room temperature) is also without great effect, as the third line shows. The last three sets of conditions provide a control for the pH and temperature of the reaction conditions used in preparation of the derivatives TPCK- (pH 5.6, room temperature), monoacetyl, propionyl, butyryl, valeryl, isobutyryl, trimethylacetyl, and benzoyl chymotrypsin (pH 5.6, 5°), and iPr_2P-, Et_2P-, diphenylcarbamyl, and PMSF-chymotrypsin (pH 8, room temperature). Compared to the untreated material, there was no great change in K_2 accompanying these conditions. The main point of these results is that conditions of pH and temperature very similar to or the same as those commonly employed in derivative preparations have no large effects on K_2. Other less easily controlled factors may have affected the results with other derivatives, however. For example, rapid acidification to pH 3.0 to end a reaction and lyophilization are inherently much less reproducible than might be adequate. Another problem is the fact that the derivative may behave differently toward certain treatments than the native enzyme. For example, the control for iPr_2P-chymotrypsin involves exposing chymotrypsin to pH 8 where it is able to undergo autolysis, which the iPr_2FP-inhibited enzyme cannot do. These examples illustrate that exact reproduction of conditions is not always possible, or if so, does not necessarily result in a completely valid control.

Other complications arise from the variation in K_2 among various lots of chymotrypsin (Miller *et al.*, 1971) and the fact that derivatization may change the chemical homogeneity of the preparation. For all these reasons, in comparing K_2 values for a derivative to the control material, some latitude must be allowed, and it is largely for this reason that classes of derivatives rather than individual K_2 values are considered the proper viewpoint for the results presented below.

Dimerization of Derivatives of Chymotrypsin. The derivatives are grouped into three classes with respect to dimerization (see Figure 1): class I derivatives dimerize like chymotrypsin, class II derivatives display reduced dimerization, like iPr_2P-chymotrypsin, and class III derivatives dimerize very little or not at all. In order to make the difference in K_2 between these classes more clear, Figure 2 shows the observed M_w *vs.* concentration plots for a representative of each class.

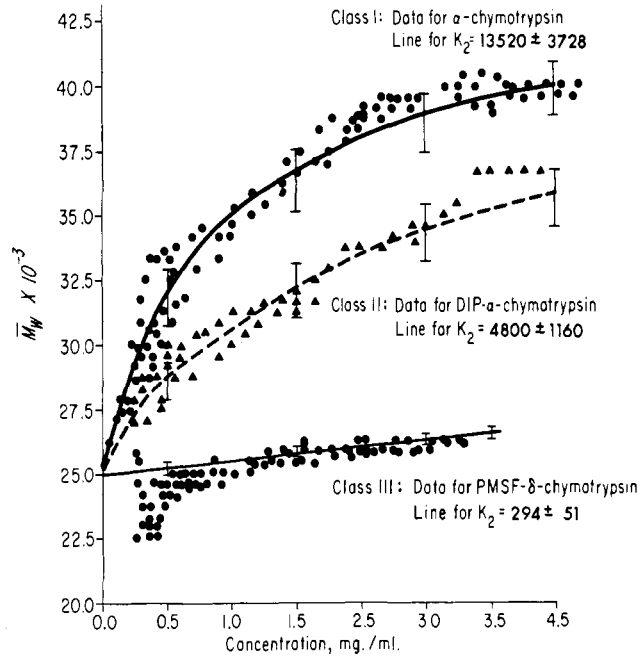

FIGURE 2: Molecular weight distributions for examples of the three classes of chymotrypsin dimerization. The points plotted are observed molecular weights while the lines were calculated using the equilibrium constants shown. Sedimentation equilibrium experiments were performed at 20°, 32,000 rpm, with a 0.3-cm column height and initial concentration of 0.75 mg/ml. The solvent was 0.01 M acetate–0.2 M KCl, pH 4.4.

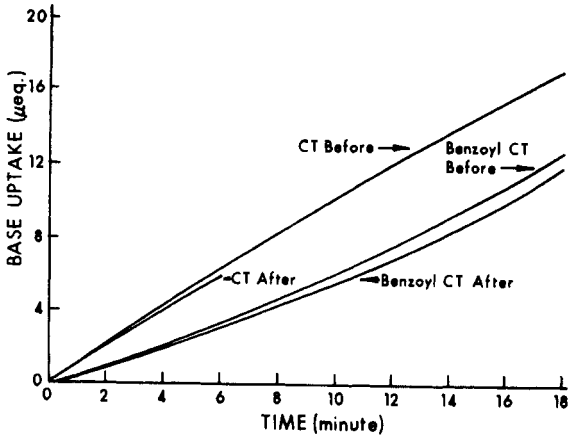

FIGURE 3: Stability of benzoyl chymotrypsin under sedimentation equilibrium conditions.

The differences in these distributions are clear, and are reflected in the K_2 values on Figure 2 and in the tables of data now to be discussed.

CLASS I DERIVATIVES. Table II shows the dimerization constants for the class I derivatives of chymotrypsin, all of which associate to approximately the same degree as chymotrypsin itself. The K_2 values for some of the derivatives listed in Table II are seen to be considerably larger than for chymotrypsin itself, but the general fact that all these derivatives still do associate at least as well as chymotrypsin itself is felt to be the most significant observation. The only derivative which exhibits a K_2 outside the range of values observed for different chymotrypsin lots (see Miller *et al.*, 1971) is isobutyryl chymotrypsin. The reactivation of this material by brief exposure to pH 8 resulted in a material with a K_2 essentially identical with the starting material, as seen from the last 5° entry in Table II. This fact disallows nonspecific, irreversible changes, such as denaturation-induced aggregation, as explanations for the high K_2 for isobutyryl chymotrypsin. The preparation was repeated but the K_2 observed was again much higher than for chymotrypsin and almost identical with the value for isobutyryl chymotrypsin in Table II. No evidence for trimerization or higher degrees of association was apparent in the data for isobutyryl chymotrypsin.

Since many of the derivatives listed in Table II will deacylate and form native enzyme, suitable controls for this possible problem were performed. A typical experiment is one performed on benzoyl chymotrypsin, represented in Figure 3. The stock solution used for the sedimentation equilibrium experiment was assayed at times corresponding to "before" and "after" the equilibrium experiment using a pH 6.2 rate assay in which the reactivation reaction occurs more slowly and thus is readily measurable. As seen in Figure 3, the before and after assays are nearly identical, especially in the initial region, where increased activity would be most apparent. The only difference is that the after assay shows slightly lowered activity, but since this also occurred in a chymotrypsin control, it is quite likely due to some small change in measurement sensitivity, such as an unnoticed shift in the temperature at which the activity was measured.

It is known that the acetyl group in monoacetyl-chymotrypsin is covalently linked to Ser-195 (Oosterbaan and von Adrichem, 1958). The asterisked monoacetyl chymotrypsin*

TABLE II: Dimerization Constants for Class I Derivatives of Chymotrypsin.[a]

Material	Temp (°C)	$K_2 \times 10^{-3}$	$\pm K_2 \times 10^{-3}$
Chymotrypsin	5	11.1	2.74
Monoacetyl chymotrypsin	5	21.2	10.6
Monoacetyl chymotrypsin*	5	23.0	6.48
Propionyl chymotrypsin	5	13.2	1.00
Butyryl chymotrypsin	5	12.1	3.36
Valeryl chymotrypsin	5	16.2	5.82
Isobutyryl chymotrypsin	5	63.2	9.60
Trimethylacetyl chymotrypsin	5	25.9	2.25
Benzoyl chymotrypsin	5	9.4	0.90
Reactivated isobutyryl chymotrypsin	5	13.8	2.55
Chymotrypsin	20	17.2	0.26
Et_2P-chymotrypsin	20	18.6	0.70

[a] All experiments were performed in 0.2 M KCl–0.01 M acetate, pH 4.4. The asterisked monoacetyl chymotrypsin was prepared with acetic anhydride.

in Table II was prepared with acetic anhydride, while monoacetyl chymotrypsin is prepared with *p*-nitrophenyl acetate. The similar lack of effect on dimerization of the acetylation when done these two ways together with the very similar rates of reactivation observed for the two preparations (unpublished experiments by T. Horbett) provide evidence that the acetyl group in asterisked monoacetyl chymotrypsin* is also on Ser-195. This was important to interpretation of the dimerization results for the other alkyl acyl derivatives of chymotrypsin made with acidic anhydrides (propionyl, butyryl, isobutyryl, and valeryl chymotrypsin) since it therefore seems unlikely that these groups are located elsewhere than Ser-195. The stoichiometry of valeric anhydride inhibition also supports this view, since 1.00 ± 0.03 mol of [1-^{14}C]valeric anhydride groups were present per mole of cinnamoylimidazole titratable sites lost.

CLASS II DERIVATIVES. Table III lists the K_2 values for the intermediate dimerization derivatives of chymotrypsin. The K_2 for hydrocinnamoyl chymotrypsin of Table III can only be properly compared with the K_2 for the chymotrypsin listed directly above it since both values were determined using a sedimentation velocity experiment. It is to be noted here that cinnamoyl chymotrypsin was also examined in that same sedimentation velocity experiment and was found to associate very much less than either chymotrypsin or hydrocinnamoyl chymotrypsin, in agreement with separate sedimentation equilibrium experiments on cinnamoyl chymotrypsin (see Table IV).

CLASS III DERIVATIVES. Table IV lists the K_2 values observed for class III derivatives of chymotrypsin, all of which show essentially no dimerization. The K_2 for the materials of Table IV average 9.3 ± 4.6% of the K_2 for the corresponding control material. The individual K_2 values are seen to be subject to considerable error; often the standard error of K_2 is as large or larger than the K_2 value itself. This magnitude of error is expected with such slightly associated materials, since the dimer present is only a small percentage of the total mass

TABLE III: Dimerization Constants for Class II Derivatives of Chymotrypsin.[a]

Material	Temp (°C)	$K_2 \times 10^{-3}$	$\pm K_2 \times 10^{-3}$
Chymotrypsin	5	11.1	2.74
Furoyl chymotrypsin	5	3.8	0.71
Chymotrypsin[b]	7.6	16.5	2.0
Hydrocinnamoyl chymotrypsin[b]	7.6	4.1	0.71
Chymotrypsin[c]	20	17.2	0.26
Chymotrypsin[d]	20	14.0	2.00
iPr_2P-chymotrypsin[c]	20	3.9	0.65
iPr_2P-chymotrypsin	20	3.8	0.51
0.7 nitrated chymotrypsin[d,e]	20	5.1	1.24
1.4 nitrated chymotrypsin[c,e]	20	6.6	1.96

[a] All experiments were performed in 0.2 M KCl–0.01 M acetate, pH 4.4. [b] Sedimentation velocity experiment estimate. [c] CDI-8VS-chymotrypsin. [d] NBC-6397-chymotrypsin. [e] Numbers preceding these derivatives are moles of nitrotyrosine per mole of enzyme.

TABLE IV: Dimerization Constants for Class III Derivatives of Chymotrypsin.[a]

Material	Temp (°C)	$K_2 \times 10^{-3}$	$\pm K_2 \times 10^{-3}$
Chymotrypsin	5	11.1	2.74
Carboxypeptidase treated iPr_2P-chymotrypsin	5	0.51	0.84
TPCK-chymotrypsin	5	1.08	0.20
PMSF-chymotrypsin	5	1.84	0.80
Diphenylcarbamyl chymotrypsin	5	0.90	0.39
p-Nitrobenzoyl chymotrypsin	5	1.32	0.56
Cinnamoyl chymotrypsin	5	1.90	0.88
Chymotrypsin[b]	7.6	16.5	2.0
Cinnamoyl chymotrypsin[b]	7.6	0.90	0.26
Chymotrypsin	20	17.2	0.26
PMSF-chymotrypsin (Sephadexed)	20	0.86	0.65
Tosyl chymotrypsin	20	1.39	1.14
Pipsyl chymotrypsin	20	0.72	0.56

[a] All experiments were performed in 0.2 M KCl–0.01 M acetate, pH 4.4. [b] Sedimentation velocity experiment estimate.

so that any source of difficulty usually encountered will be magnified in this case. For example, base-line shifts (Horbett and Teller, 1972) and the presence of even small amounts of "light" or "heavy" contaminants in the preparation will often overshadow the basic homogeneity or lack of dimerization. The results for the two PMSF-chymotrypsin preparations in Table IV indicate the difficulties, for the lack of association of this derivative is much more clearly demonstrable from the K_2 for the second, Sephadexed preparation (line 11) than it is for the first preparation (line 4). The weight-average molecular weight *vs.* concentration plots for the first material (line 4) showed the molecular weight was virtually constant at 27,000 g/mol from 0.5 to 14 fringes. Thus, despite the fact that no reversible association was indicated by the uniform plot, the use of a monomer molecular weight of 25,000 resulted in a substantial K_2 value. Any number of systematic errors may have caused the "high" molecular weight level of the one PMSF-chymotrypsin (Teller *et al.*, 1969; Horbett and Teller, 1972).

Optical Rotatory Dispersion of Chymotrypsin and Some Chymotrypsin Derivatives. The optical rotatory dispersion of several of the chymotrypsin derivatives has been investigated in order to determine any relation between conformational changes and changes in dimerization behavior. The results were obtained with the same solvent system, pH, and temperature used in measuring dimerization behavior, so that these data should be directly applicable to interpretation of the dimerization results. It is seen from Table V that chymotrypsin, isobutyryl chymotrypsin, and PMSF-chymotrypsin all have very similar λ_c values, while cinnamoyl and diphenylcarbamyl chymotrypsin have significantly lower λ_c values. PMSF-chymotrypsin does not dimerize, nor do cinnamoyl and diphenylcarbamyl chymotrypsin, yet the λ_c values for PMSF-chymotrypsin are not different from chymotrypsin, while those of diphenylcarbamyl and cinnamoyl chymotrypsin are. Others have detected small changes in the optical rotatory dispersion (ORD) parameters of iPr_2P- and monoacetyl chymotrypsin compared to chymotrypsin (Wootton and Hess, 1962; Havsteen and Hess, 1963). Monoacetyl chymotrypsin associates normally while iPr_2P-chymotrypsin shows much reduced dimerization, as noted above. It is clear from all these data that changes in the dimerization behavior of these derivatives are not directly correlated with changes in the ORD parameters.

iPr_2FP- and Et_4PP-Induced Difference Spectrum on Chymotrypsin, Purified Chymotrypsin, and Nitrated Chymotrypsin. The results are shown in Figure 4. Figure 4A shows iPr_2FP inhibition causes the difference spectrum observed by Wootton and Hess (1962). Figure 4B shows that this still occurs with peptide Sepharose-purified chymotrypsin (see Miller *et al.*, 1971), seemingly eliminating the possibility that the difference spectrum might have arisen from the displacement of impurities from the active site of chymotrypsin. Such a possibility existed because it is known that the iPr_2FP reaction allows one to prepare a purer product with respect to NH_2-terminal groups (Jansen *et al.*, 1950; Rovery *et al.*, 1953). Figure 4C shows that even nitrated chymotrypsin (0.7 mol of nitrotyrosine/mol of chymotrypsin) exhibits this difference spectrum. Since it is likely that this preparation had at least Tyr-146 nitrated, and it is known that nitration dramatically increases the ultraviolet extinction of tyrosine (from 1360 to 4000 at 275 mμ, pH 8.0; Sokolovsky *et al.*, 1966), it seems likely that the ultraviolet difference in absorption spectrum cannot be due to Tyr-146. The results of Figure 4D further confirm this, for it is seen that Et_4PP gives a difference spectrum very similar to that observed for iPr_2FP, although apparently of smaller magnitude. Formation of monoacetyl chymotrypsin also gives a smaller difference spectrum than iPr_2P-chymotrypsin as shown by Wootton and Hess (1962). As shown above Et_2P-chymotrypsin associates normally, unlike iPr_2P-chymotryspin. These results agree with the ORD results presented above, which suggest that the small conformational changes seemingly accompanying the formation of iPr_2P-chymotrypsin and other derivatives cannot explain the changes in dimerization in these derivatives. These

TABLE V: Drude Equation Optical Rotatory Dispersion Parameters for Chymotrypsin Derivatives in 0.01 M Acetate–0.2 M KCl, pH 4.4.[a]

Material	Temp (°C)	λ_c	$\pm\lambda$	$-K \times 10^{-7}$	$\pm K \times 10^{-7}$
Chymotrypsin	25	236.3	2.3	1.64	0.06
Chymotrypsin[b]	25	235.5	1.6	1.66	0.04
iPr_2P-chymotrypsin	25	237.1	0.4	1.62	0.004
iPr_2P-chymotrypsin[b]	25	238.4	0.5	1.56	0.002
Chymotrypsin	5	234.9	1.3	1.64	0.24
Isobutyryl chymotrypsin	5	234.0	0.7	1.52	0.15
PMSF-chymotrypsin	5	236.4		1.70	
Cinnamoyl chymotrypsin	5	229.6	1.3	1.75	0.13
Diphenylcarbamyl chymotrypsin	5	231.0	1.3	1.53	0.08

[a] The λ_c and K data presented are averages of least-squares slope and intercepts, while the $\pm\lambda_c$ and $\pm K$ data represent the standard deviations of these averages. [b] These materials were from Worthington Biochemicals, Lot CDI-6LD, whereas all other materials were from Nutritional Biochemicals, Control No. 6397.

results also help rule out the perturbation of Tyr-146 as a significant source of the iPr_2FP-induced difference spectrum.

Discussion

The experiments reported in this paper and previous or simultaneous results of others provide a great deal of evidence which strongly suggest the following conclusions. The chemical mechanism of the low pH dimerization has as its principal feature the partly hydrophobic, partly ionic interaction between the phenyl ring and carboxylate ion of Tyr-146 in each monomer with the histidine ring and imino ion of His-57 of the other monomer. The side-chain carboxyl group of Asp-102 may play an indirect role in the mechanism by its tendency to form an intramolecular bridge with His-57, which would prevent maximum interaction of the carboxyl function

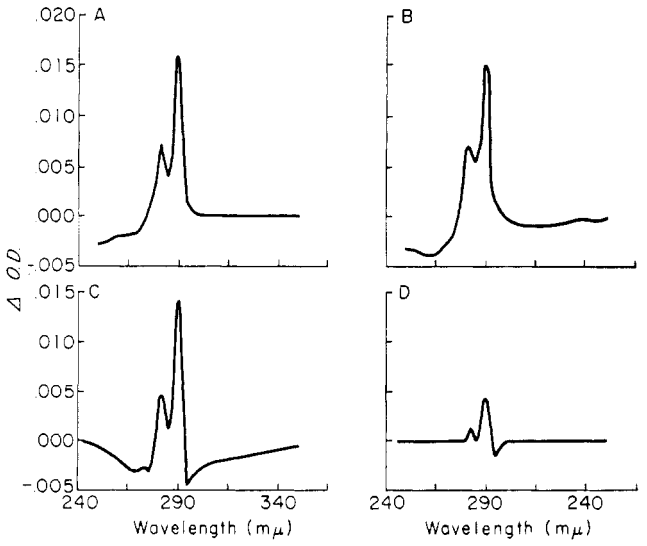

FIGURE 4: iPr_2FP- and Et_4PP-induced difference spectra. In each case shown, 4×10^{-5} M protein solutions in 0.033 M Tris-HCl–0.033 M $CaCl_2$, pH 6.9, at room temperature were treated with 1.1×10^{-4} M inhibitor solutions and the spectra were then recorded. (a) Chymotrypsin + iPr_2FP; (b) peptide Sepharose purified chymotrypsin + iPr_2FP; (c) nitrated chymotrypsin + iPr_2FP; and (d) chymotrypsin + Et_4PP.

of Tyr-146 with the imino group of His-57. The low pH dimer of chymotrypsin is proposed to be an enzyme–product complex in which each monomer acts simultaneously as enzyme and product by both receiving the interaction of Tyr-146 of the other monomer with its histidine and donating its tyrosine to the histidine of the other monomer.

The evidence for the involvement of Tyr-146 in the dimerization seems quite convincing. Carboxypeptidase removal of this residue from iPr_2P-chymotrypsin abolishes dimerization (Gladner and Neurath, 1954; also confirmed in this work). Chymotrypsinogen and δ-chymotrypsin lacking C-terminal Tyr-146 do not dimerize, while γ-chymotrypsin has a C-terminal tyrosine and does associate more than chymotrypsinogen, although not as well as chymotrypsin (Miller *et al.*, 1971). The lower association of γ-chymotrypsin is perhaps explainable by a slightly altered orientation of Tyr-146 in γ-chymotrypsin as first proposed by Wright *et al.* (1968). Nitration of Tyr-146 markedly decreases dimerization. The lack of free solution dimerization of chymotrypsinogen and δ-chymotrypsin has its analog in the absence of crystalline dimer formation in these forms in contrast to the presence of such a dimer in crystalline chymotrypsin (Wright *et al.*, 1968; Sigler *et al.*, 1968). Tyr-146 becomes carboxyl terminal by a chymotrypsin-catalyzed hydrolysis of a peptide bond (Cunningham, 1965), showing there is a tendency for this residue to enter the active-site region of another chymotrypsin molecule and also demonstrating that this residue is indeed accurately described as a product of chymotrypsin catalysis. This reaction also occurs at low pH (Miller *et al.*, 1971). Tyrosine analogs such as *N*-acetyldibromo-L-tyrosine bind to chymotrypsin at low pH and are competitive inhibitors of activity of chymotrypsin showing that the Tyr-146 could bind in the proposed product-like fashion at low pH (Doherty and Vaslow, 1952). Finally, Tyr-146 is known to be one of the residues involved in the crystalline dimer interactions of chymotrypsin (Sigler *et al.*, 1968).

The evidence for the involvement of His-57 in the association reaction is less extensive. Ultraviolet radiation (McLaren and Finkelstein, 1950), periodate oxidation (Smith and Brown, 1952), and Methylene Blue photooxidation (Egan *et al.*, 1957) all caused simultaneous decreases in activity and dimerization. TPCK reacts with His-57 to form a protein which does not self-associate. This result is interpretable in terms of a steric blocking effect because of the bulky nature of the TPCK

group. Nevertheless, the lack of self-association shows that His-57 is in a position where the carboxyl function of a bound substrate or product (or Tyr-146) could interact with it. Lastly, it is thought to interact with Tyr-146 in the crystal dimer (Sigler *et al.*, 1968).

The proposed involvement of the carboxyl side chain of Asp-102 in the dimerization reaction is suggested by its spatial proximity to His-57 in the structure of tosyl chymotrypsin (Blow *et al.*, 1969). A salt bridge between these residues is believed to explain the pH dependence of binding of anionic inhibitors to chymotrypsin (Johnson and Knowles, 1966; Blow *et al.*, 1969). We suggest this same salt bridge affects the pH dependence of the dimerization reaction, but in a different manner. If the pK of the aspartic acid carboxyl side chain were about 5, then, as the pH is decreased from 6 to 4, the salt bridge with His-57 would be disrupted, resulting in a net positive charge partially buried in the enzyme's structure. This unfavorable situation could be overcome by interaction of Tyr-146 carboxylate of another molecule with this histidine, thus leading to the observed enhancement of dimerization between pH 6 and 4. Involvement of a group with a pK of 5 was postulated by Aune and Timasheff (1971) to explain the pH dependence of chymotrypsin dimerization. We have measured changes in proton binding during dilution of chymotrypsin solutions which are in accord with Aune and Timasheff's predictions (Horbett and Teller, manuscript in preparation).

The evidence for the specific manner of interaction proposed for the tyrosine and histidine residues is indirect but fairly extensive. Reaction at the active-site region of groups which might be expected to occupy the enzymatic binding site usually eliminate dimerization, as evidenced by cinnamoyl, PMSF-, tosyl, pipsyl, *p*-nitrobenzoyl, diphenylcarbamyl, and TPCK-chymotrypsin. Acylation of Ser-195 by groups not possessing phenyl rings does not decrease dimerization as evidenced by the class I derivatives monoacetyl, propionyl, butyryl, valeryl, isobutyryl, and trimethylacetyl chymotrypsin. Groups which can freely rotate around the acyl linkage, and thus perhaps assume a position of minimal interference with the binding site, have less of an effect on dimerization, as evidenced by the derivatives benzoyl, furoyl, and hydrocinnamoyl chymotrypsin. The variety of effects of covalently linked groups at the active site shows both the involvement of this region in the dimerization reaction and the very localized, specific, sterically sensitive nature of the dimerization reaction, properties very similar to those usually encountered with the substrate binding site. The lack of effect of the diethylphosphoryl group in Et_2P-chymotrypsin in comparison to the large effect of the diisopropylphosphoryl group in iPr_2P-chymotrypsin clearly suggests the smallness of the region involved and the sensitive steric requirements for the dimerization reaction.

The occurrence of crystalline dimers of PMSF-, tosyl-, and pipsyl chymotrypsin of very nearly the same sort as proposed to occur in solution, despite the fact that these derivatives show very little free solution dimerization, is a puzzling fact. The explanation we favor is that the orientation of the inhibitor moieties is different in the solution and crystalline enzyme in accord with Hein and Niemann's proposal (Cunningham, 1965) of multipoint interaction of substrates and inhibitors with the active site of chymotrypsin.

The proposal that the tyrosine–histidine dimer interaction resembles or duplicates the normal enzyme–product complex is supported by several lines of evidence. The low pH binding of *N*-acetyldibromo-L-tyrosine to chymotrypsin provides a model of the proposed interaction. Since two tyrosine–histidine interactions are involved in the proposed mechanism of dimer formation, one may regard the dimerization process as the sum of two tyrosine–chymotrypsin equilibria: 2Tyr-146 + 2 chymotrypsin = 2[Tyr-146·chymotrypsin]. The free energy of this process is −6.1 kcal/mol with peptide Sepharose purified chymotrypsin (Miller *et al.*, 1971). Vaslow and Doherty (1953) reported a free energy of approximately −3 kcal for binding of the model compound to chymotrypsin at pH 4, so that one would predict a free energy of −6 kcal for the dimer formation at this pH. Furthermore, the enthalpy of binding of the model compound at pH 4 is approximately 0, while the enthalpy of chymotrypsin dimerization at this pH and temperature is also quite small (about −0.5 kcal) (Aune and Timasheff, 1971). It is therefore clear that the proposed mechanism predicts a free energy of dimerization which is in accord with available data for such a mechanism.

The proposed similarity of the dimerization of chymotrypsin to product binding by chymotrypsin is also supported by the finding that a model product, *N*-acetyl-L-tryptophan, strongly inhibits the pH 4.4 dimerization of chymotrypsin. The K_2 observed (in 0.01 M inhibitor added to the standard buffer, the only concentration so far studied) was 429 ± 39 l./mol in comparison to 17,200 ± 256 for the control material. The dimerization was restored when the inhibitor was removed from the protein solution by dialysis. This finding is also in accord with the observation by Steitz *et al.* (1969) that diffusion of *N*-formyl-L-tryptophan into chymotrypsin crystals (at pH 5.6) results in a mode of binding of this inhibitor at the active site which is accompanied by a displacement of Tyr-146 of the other monomer by approximately 2 Å.

The mechanism of dimerization proposed here has sufficient evidence to serve as a model for further work. The mechanism of dimerization has been proposed to involve pK shifts (Aune and Timasheff, 1971) and we verified this model (Horbett and Teller, manuscript in preparation). Thus, it is of considerable interest to extend the study of these derivatives to other pH and temperature values in order to verify the conclusion that the dimer formation resembles an enzyme–product complex.

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Uricase Reaction Intermediate. Mechanism of Borate and Hydroxide Ion Catalysis†

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ABSTRACT: It has been previously shown that an intermediate is produced during the uricase reaction which absorbs strongly in the ultraviolet (uv) region where uricase is typically assayed (293 nm). A thorough knowledge of the kinetics of this intermediate is mandatory for detailed spectrophotometric study of the uricase reaction. The decay rate of this compound has been shown by others to be enhanced by borate and hydroxide ions. The present studies show that this decay rate is not influenced by changes in oxygen or uricase concentration. Kinetic studies with both borate and hydroxide ion indicate that they act catalytically by independent, parallel processes. Both catalysts show saturation behavior over the concentration range studied but only if the temperature is above 23°. Activation parameters for the separate processes are reported. An empirical rate equation has been obtained and a partial mechanism proposed for each catalyst. It is further shown that the previously reported substrate inhibition with urate was due to the accumulation of intermediate and is not an inherent property of the enzymic catalysis.

Transitory intermediates have been reported to occur during the uricase, urate:oxygen oxidoreductase (EC 1.7.3.3), catalyzed oxidation of urate (Praetorius, 1948; Mahler *et al.*, 1956). One of these intermediates absorbs strongly in the region 270–330 nm and thus may interfere with the commonly used spectrophotometric assay at 293 nm (Priest and Pitts, 1972). Structure I has been proposed for this intermediate, 1-carboxy-2,4,5,8-tetraazabicyclo[3.3.0]octa-4-ene-3,7-dione (Mahler *et al.*, 1956). Borate and hydroxide ions enhance the decay of this intermediate to stable products (Praetorius, 1948). Detailed kinetic studies of the uricase mechanism of action necessitate a thorough prior understanding of the

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