The Reaction of 4-Nitrocatechol Cyclic Sulfate with α-Chymotrypsin

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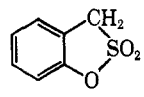
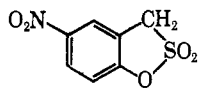
Abstract: 4-Nitrocatechol cyclic sulfate (VII) has been found to react rapidly with α-chymotrypsin to give a sulfonated enzyme (VIII). Measurements with a stopped-flow spectrophotometer of the kinetics of the formation of VIII show that the function k_2/K_s has a bell-shaped pH dependence. Ionizing groups on the enzyme with pK values of 7.09 and 8.76 appear to be responsible for this pH behavior. The results thus obtained are very similar to those found previously for the sulfonylation of chymotrypsin by 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone (II) to give a sulfonyl enzyme (IV). However, in contrast to IV which desulfonylates over a wide pH range at a conveniently measurable rate, the rate of decomposition of VIII is negligible. The nitrophenol chromophore in VIII has been titrated spectrophotometrically, and the results have been compared to those found for IV.

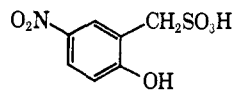
Recent investigations have shown that certain five-membered cyclic sulfonates (I and II) are good substrates for α-chymotrypsin[3,4] and carbonic anhydrase.[5] In the case of the chymotrypsin-catalyzed



I

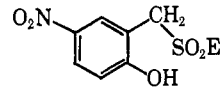


II



III

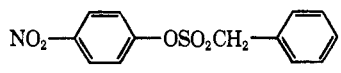
hydrolysis of II to its product acid III, a rapid sulfonylation of the active site of the enzyme by II to give the sulfonyl enzyme IV occurred, followed by a relatively slow decomposition of IV to the product III while



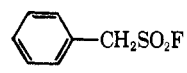
IV

regenerating active enzyme.[3,4] The rate of sulfonylation was conveniently measured with a stopped-flow spectrophotometer by following the appearance of the absorption maximum of the phenolate form of IV at 3900 Å. The rate of desulfonylation was measured in a conventional spectrophotometer by following the appearance of the phenol III or its phenolate ion.

The properties of the cyclic sulfonates I and II are in remarkable contrast to those of the acyclic compounds V and VI. *p*-Nitrophenyl-α-toluenesulfonate (V) does not appear to react at all with α-chymotrypsin.[3] In



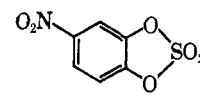
V



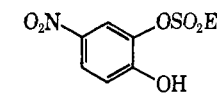
VI

the case of VI a fully inhibited species, α-toluenesulfonyl-α-chymotrypsin is formed and desulfonylation does not occur under normal conditions.[6-8] It has been concluded, therefore,[3,4] that the phenolic hydroxyl group in IV is necessary for desulfonylation to occur. Both the sulfonylation and desulfonylation reactions have bell-shaped rate *vs.* pH dependencies.

The present work involves the extension of the work on 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone (II) to the isoelectronic compound nitrocatechol cyclic sulfate (VII). It has been found that this compound



VII



VIII

reacts rapidly with α-chymotrypsin to give an apparently inactive sulfonated enzyme VIII. In contrast to the sulfonyl enzyme IV obtained from the nitrosultone II, decomposition of the ester VIII does not appear to occur under normal conditions. The kinetics of the formation of VIII have been measured with a stopped-flow apparatus in the range of pH 4.6–9.4. A bell-shaped pH profile has been found for this reaction, analogous to that of the sulfonylation of the enzyme with II.[4]

As in the case of IV, the nitrophenol chromophore present in VIII has been titrated spectrophotometrically. An examination of the spectral data shows that they do not conform to the shape of a theoretical sigmoid curve. A possible interpretation for these results similar to that proposed for the data observed in the spectrophotometric titration of the nitrophenol chromophore of IV is that the ionization of the phenolic proton in the sulfonated enzyme, VIII, is affected by the ionization of another nearby group occurring at a similar pH. By analogy to the situation in IV it seems probable that this group might be the imidazole ring of a histidine residue.

The Kinetics of Formation of the Sulfonated Enzyme

The sulfonated enzyme VIII has two absorption maxima: one, due to the nitrophenol chromophore

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(2) To whom inquiries concerning this paper should be addressed.
(3) J. H. Heidema and E. T. Kaiser, *J. Am. Chem. Soc.*, **89**, 460 (1967).
(4) J. H. Heidema and E. T. Kaiser, *ibid.*, **90**, 1860 (1968).
(5) K. W. Lo and E. T. Kaiser, *Chem. Commun.*, 834 (1966).
(6) A. M. Gold and D. E. Fahrney, *Biochemistry*, **3**, 783 (1964).
(7) A. M. Gold, *ibid.*, **4**, 897 (1965).
(8) D. E. Fahrney, Ph.D. Thesis, Columbia University, 1963.

has a λ_{max} of 3200 Å, and the other due to the nitrophenolate anion, has a λ_{max} of 3850 Å. The kinetics of the reaction were conveniently followed at 3900 Å in a stopped-flow spectrophotometer. Nitrocatechol cyclic sulfate is rapidly hydrolyzed by hydroxide ions and also by various buffer anions (*e.g.*, phosphate, acetate). The determination of the rate of formation of the sulfonated enzyme using nitrocatechol cyclic sulfate in excess was therefore found to be impractical, hence the kinetic parameters were measured exclusively with the enzyme in excess.[4] Under the conditions and in the pH range that these measurements were made, the error caused by the hydroxide ion and buffer ion catalyzed hydrolyses of VII was found to be negligible.

The reaction of nitrocatechol cyclic sulfate with chymotrypsin is believed to follow a reaction sequence of the following type

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} EP \qquad (1)$$

where E represents the enzyme, S is the cyclic sulfate, ES is a noncovalent complex of the two reactants, and EP is a covalent species, the sulfonated enzyme. With a sufficiently large excess of enzyme a pseudo-first-order reaction with a rate constant, k, should be observed, where

$$k = \frac{k_2[E_0]}{(k_{-1} + k_2)/k_1 + [E_0]} = \frac{k_2[E_0]}{K_s + [E_0]} \qquad (2)$$

assuming $k_{-1} \gg k_2$ and $K_s = k_{-1}/k_1$. Measurement of the pseudo-first-order rate constants over a range of enzyme concentrations permits the evaluation of k_2 and K_s separately by using the double reciprocal plot method of Lineweaver and Burk. In principle, therefore, the pH dependence of k_2 and K_s could normally be determined. However, plots of $1/k$ against $1/[E_0]$ gave intercepts on the $1/k$ axis which were zero within experimental error for the range of enzyme concentration studied. Hence, to a good approximation, expression 2 may be simplified to

$$k = (k_2/K_s)[E_0] \qquad (3)$$

It was therefore possible to obtain a pH profile for k_2/K_s and attempts to evaluate the two parameters separately were abandoned, as in the work on the nitrosultone.[4]

The pH dependence of the acylation step of chymotrypsin is usually found to be dependent on two ionizable groups[9] on the enzyme, an acid and a base. Hence, the pH dependence of k_2/K_s will give a direct estimate of the prototropic equilibria of chymotrypsin which directly influence the reaction rate. The pH dependence of k_2/K_s is usually found to be a bell-shaped curve which may be represented by the equation[9]

$$\frac{k_2}{K_s} = \frac{k_2/K_s(\text{lim})}{1 + ([H]/K_1) + (K_2/[H])} \qquad (4)$$

The experimental results of this investigation are plotted in Figure 1, which shows that the points lie on a well-defined, bell-shaped curve. A least-square

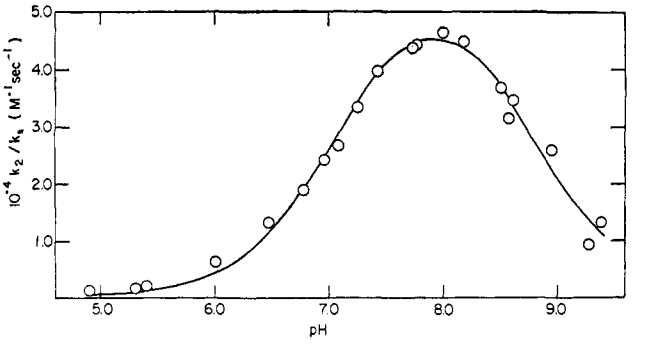


Figure 1. pH profile for the sulfonation of α-chymotrypsin by 4-nitrocatechol cyclic sulfate at 25.0°. The curve is a theoretical one for $pK_1 = 7.090$, $pK_2 = 8.758$, and $k_2/K_s(\text{lim}) = 6.105 \times 10^4\ M^{-1}\ sec^{-1}$ (a computer-calculated, least-squares fit to the experimental points).

fit[10] of eq 4 to these results yielded the following best values for the ionization equilibria.

$$pK_1 = 7.090$$

$$pK_2 = 8.758$$

$$k_2/K_s(\text{lim}) = 6.105 \times 10^4\ M^{-1}\ sec^{-1}$$

The solid curve in Figure 1 is a theoretical one utilizing the above values.[11]

Inhibition Study

If nitrocatechol cyclic sulfate reacts at the normal site of chymotrypsin (hydroxyl group of serine-195), it would be expected that a known specific substrate which binds at this site would inhibit the rate of formation of the sulfonated enzyme. A brief study using the known specific substrate N-acetyl-L-tryptophanamide was made at pH 8.0 in Tris–HCl buffer, using the method outlined by Heidema and Kaiser.[4] If competitive inhibition is assumed, and k and k' are the pseudo-first-order rate constants in the presence and absence of the inhibitor, we have (under conditions where $[E] \ll K_m$)

$$K_I = \frac{[I]}{(k/k') - 1} \qquad (5)$$

Here K_I is the inhibition constant, and [I] is the inhibitor concentration. The results observed at pH 8 are outlined as follows (concn of inhibitor, M (k, sec^{-1}): 0 (3.39) and 6.1×10^{-3} (1.40). From eq 5, a value of K_I of $4.3 \times 10^{-3}\ M$ was obtained. This value agrees quite well with those obtained for N-acetyl-L-

(9) M. L. Bender, G. E. Clement, F. J. Kezdy, and H. d'A Heck, *J. Am. Chem. Soc.*, **86**, 3680 (1964).

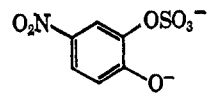
(10) A computer program written by Dr. P. L. Hall was used. See P. L. Hall, Ph.D. Thesis, University of Chicago, 1967.

(11) The possibility that the step indicated by k_2 might be reversible to a significant extent (*i.e.*, that a k_{-2} step should be considered) was briefly examined by Mr. John H. Heidema of this laboratory by an experimental approach suggested by Professor F. J. Kezdy. If the k_2 step were reversible this would mean that recyclization of the sulfate in the EP complex (VIII) would be taking place releasing the free enzyme. Aliquots of a concentrated solution of chymotrypsin at pH 7.1 which had been modified with VII were diluted and after standing for various lengths of time they were assayed at this pH with a high concentration of the specific substrate, N-acetyl-L-tryptophan methyl ester. Even after 20 hr of standing no more than 10% of the original activity of the unmodified chymotrypsin was observed. Similar results were found at pH 5.5. We conclude that reversal of the k_2 step is negligible on the time scale of the stopped-flow kinetic measurements described in this paper.

tryptophanamide by other workers,[12] and indicates that nitrocatechol cyclic sulfate binds at the normal active site of chymotrypsin.

Titration of the Nitrophenol Chromophore in the Sulfonated Enzyme VIII

We have titrated the nitrophenol chromophore in VIII spectrophotometrically with the intention of comparing our results to those already reported for IV.[13] Solutions of the sulfonated enzyme used in the



IX

titration experiments were prepared by adding a known concentration of VII to an excess of enzyme. With excess enzyme present the danger that spontaneous hydrolysis of the substrate might occur was diminished because of the rapidity with which 4-nitrocatechol cyclic sulfate (VII) reacts with chymotrypsin relative to the rate of spontaneous hydrolysis of VII. A known amount of the solution of the sulfonated enzyme was added to buffers of varying pH, and a spectrum from 3200 to 4200 Å was taken at each pH. The pH dependence of the spectral data recorded at either 3200 or 3850 Å does not conform to the shape of a theoretical sigmoid curve. In contrast, the data of a similar pH *vs.* absorbance profile for the monosulfate IX do fit a theoretical sigmoid for a pK_a of 6.38. These results for VIII are similar to those found earlier for the sulfonyl enzyme IV,[4] and we have concluded that the ionization of the phenolic proton in the sulfonated enzyme VIII is influenced by the ionization of a nearby group on the enzyme, possibly the imidazole ring of a histidine residue. Hence, the scheme in eq 6 below which is identical with that proposed to account for the spectral titration of the sulfonyl enzyme IV[4] is suggested also to account for our results with VIII. In this scheme HONH is the sulfonated enzyme protonated at both the phenolic group and the imidazole

$$\begin{array}{ccc} \text{HONH} & \overset{K_1}{\rightleftharpoons} & \text{ONH} \\ \Big\uparrow\!\!\downarrow K_2 & & \Big\uparrow\!\!\downarrow K_3 \\ \text{HON} & \underset{K_4}{\rightleftharpoons} & \text{ON} \end{array} \qquad (6)$$

ring of the histidine, ON is the sulfonated enzyme with both groups deprotonated, and ONH and HON are species singly protonated at the imidazole ring and the phenolic group, respectively. The colored species absorbing at 385 mμ would be ONH and ON. If the assumption that the two latter species have the same extinction coefficients at 385 mμ is made, then eq 7 may be derived.[4] In this equation $M = K_1 + K_2$,

$$\frac{\Delta A}{\Delta A_\infty} = \frac{N/[\text{H}] + K_1/M}{1 + [\text{H}]/M + N/[\text{H}]} \qquad (7)$$

$N = K_4/[1 + (K_1/K_2)]$, ΔA represents the absorbance at the hydrogen ion concentration, [H], minus the absorbance at low pH, and A_∞ represents ΔA at high pH. Equation 7 was solved by using the values of $\Delta A/\Delta A_\infty$ and [H] at three points on the experimental titration curve and subsequently solving three equations in three unknowns. As in the case of the sulfonyl enzyme IV, if pK values derived in this way, assuming the scheme given in eq 6, are used to predict a titration curve for the sulfonated enzyme VIII, a good fit to the experimental data is obtained. There are, of course, many other schemes which could be postulated to account for the spectral titration data, but at present there seems to be no reason to devise another, perhaps more complex, scheme to explain the results found for VIII.[14] The pK values found for IV and VIII are compared in Table I.

Table I

	Sulfonyl enzyme IV	Sulfonated enzyme VIII
pK_1	6.69	5.07
pK_2	6.73	5.31
pK_3	7.77	5.88
pK_4	7.72	5.64

The results given in Table I indicate that the pK values for the ionizations observed in the case of VIII are considerably lower than those for IV. This is not too surprising since as mentioned already the pK_a for the ionization of the phenolic group in the monosulfate IX is 6.38 which is far lower than the pK_a of 7.10 found for the nitrophenol moiety in the sulfonic acid III.[4] Apparently then, the oxygen atoms adjacent to the aromatic ring and attached to sulfur in both the species VIII and IX cause substantial increases in the acidity of the nitrophenol groups as compared to the corresponding groups in the species III and IV where a methylene group is adjacent to the ring.

Experimental Section

Materials. α-Chymotrypsin, three times recrystallized and lyophilized, was obtained from two sources. The kinetic studies and some of the pK titration measurements were done with material obtained from Worthington Biochemical Corp. (lot no. CDI6JF and CDI6JC) which was used without further purification. Other pK titration measurements were carried out with several preparations purchased from Sigma Chemical Co. Centrifugation of the enzyme solutions was not found to be necessary. The activity of each batch of enzyme was determined by spectrophotometric titration with N-*trans*-cinnamoyl imidazole.

N-Acetyl-L-tryptophanamide, a product of Mann Research Laboratories (mp 192–194°), was used without further purification.

(12) (a) Published values vary over the range 5.0–7.5 × 10^{-3} *M*. *Cf.* R. J. Foster and C. Niemann, *J. Am. Chem. Soc.*, 77, 1886 (1955) and ref 12b and 12c. (b) M. L. Bender, M. J. Gibian, and D. J. Whelan, *Proc. Natl. Acad. Sci. U. S.*, **56**, 833 (1966). (c) F. J. Kezdy, J. Feder, and M. L. Bender, *J. Am. Chem. Soc.*, **89**, 1009 (1967).

(13) A solution of the sulfonated enzyme VIII underwent no appreciable spectral change near its absorption maximum of 3850 Å over a period of more than 12 hr. The nitrophenolate group in the monosulfate IX has its absorption maximum at a different wavelength, 4050 Å. Thus, the spectral stability of the solution of VIII indicates that further hydrolysis of the sulfonated enzyme to give IX is insignificant in the vicinity of the neutral pH range for the time periods involved in the spectrophotometric titration experiments reported here. However, when a solution of the sulfonated enzyme VIII in 0.2 *N* NaOH was allowed to stand overnight desulfonation occurred, and the spectrum of the product formed was identical with that of the monosulfate IX in a solution of the same alkalinity.

(14) In a recent paper by M. B. Hille and D. E. Koshland (*J. Am. Chem. Soc.*, **89**, 5945 (1967)) the spectrophotometric titration of a nitrophenol chromophore covalently bound to a methionine residue in the vicinity of the chymotrypsin active site was reported. This species was produced by the reaction of the enzyme with 2-bromoacetamido-4-nitrophenol.

N-*trans*-Cinnamoyl imidazole (mp 132.5–133.4°), prepared according to the method of Schonbaum, *et al.*,[15] was a gift from Mr. J. H. Heidema. 4-Nitrocatechol cyclic sulfate, recrystallized from ethanol (mp 68°) was a gift from Dr. K. W. Lo as was potassium 2-hydroxy-5-nitrophenylsulfate. All inorganic compounds used in preparing buffer solutions were of analytical reagent grade. 2-Amino-2-hydroxymethyl-1,3-propanediol (Tris) was purchased from both Mann Research Laboratory and Matheson Coleman and Bell and recrystallized once from a mixture of ethyl acetate and ethanol. 2-Amino-2-methyl-1,3-propanediol (Ammediol) was purchased from Matheson Coleman and Bell and recrystallized from a mixture of ethyl acetate and ethanol. Sodium barbital, obtained from the Fisher Scientific Co., was recrystallized once from aqueous ethanol. Succinic acid was recrystallized once from water (mp 188°). Acetic acid was obtained as analytical reagent grade and used without further purification. Acetonitrile (J. T. Baker Chemical Co.) was dried over phosphorus pentoxide and fractionally distilled (bp 80.0°). All water used in this work was obtained by passing distilled water through a mixed-bed, ion-exchange column (Continental Demineralization Service). Standard acids and bases were purchased from the Fisher Scientific Co.

Preparation of Solutions for Kinetic Studies. Buffer solutions were made up by dissolving weighed quantities of the appropriate salts in distilled, deionized water. The ionic strengths of all the buffer solutions were adjusted to 0.4 by adding the calculated quantity of sodium chloride. The pH of each buffer was checked on a Radiometer pH meter, type PHM 4c, calibrated against an appropriate standard buffer solution (Fisher Certified Standard Buffer solutions).

Enzyme solutions were prepared by accurately weighing the required amount of enzyme in a volumetric flask (5 or 10 ml) and dissolving this in a few milliliters of the appropriate buffer. When the enzyme had completely dissolved, buffer solution was added until the flask was filled up to the mark. The stoppered flask was carefully inverted once to ensure complete homogeneity in the solution without inducing the formation of bubbles. Solutions were stored in a refrigerator at 4° until required for use.

For the nitrocatechol cyclic sulfate, a stock solution (*ca.* 1×10^{-2} *M*) was prepared by dissolving an accurately weighed quantity of the solid in acetonitrile. This solution was found to be stable for several weeks if stored in a sealed amber container. To make a substrate solution, a 20-μl aliquot of the stock solution was diluted to 50 ml with distilled, deionized water and used immediately. All substrate solutions were made with an acetonitrile concentration of 0.4% v/v.

The Determination of the Activity of α-Chymotrypsin. The activity of each batch of chymotrypsin used in the kinetic measurements was determined by a spectrophotometric method using N-*trans*-cinnamoyl imidazole as described by Schonbaum, *et al.*[15] The titration was carried out on a Cary 15 recording spectrophotometer at a wavelength of 3350 Å. Duplicate titrations were carried out in each case and the estimated accuracy of the procedure was ±2%. Assuming a molecular weight of 2.48×10^4 for the pure enzyme, the purities of the enzyme preparations used in this work were estimated to be in the range 80–87%.

The Stopped-Flow Apparatus. The rate studies on the formation of the sulfonated enzyme were carried out on a Durrum-Gibson stopped-flow spectrophotometer (Durrum Instrument Corporation, Palo Alto, Calif.). The flow system of this apparatus was constructed of Kel-F with glass syringes and ceramic plungers. The syringes, mixing chamber, and optical cell were thermostated at 25.00 ± 0.02° with water circulated from a thermostat apparatus. The temperature was checked periodically with an NBS standard thermometer. At least 10-min thermostating time was allowed after each charge of reactants was introduced into the drive syringes. Light of the appropriate wavelength was supplied from a tungsten lamp and monochromator. The path length of the optical cell in the apparatus was 20 mm. The capacity of each drive syringe was 2.8 ml, thus allowing the flow system to be completely filled with reactants and cleared for flushing purposes. With 10 ml of each reactant solution the drive syringes could then be filled twice more for kinetic runs. Each "shot" required a total volume of 250 mμ of each reactant to ensure that all the reacted material was completely swept out of the system; 200 μl was found to be insufficient for this purpose. Traces of each kinetic run were retained on the oscilloscope screen by using the "storage mode." Ideal reproducibility was obtained if the traces from two consecutive runs were superimposed on each other; a photograph was then taken with a Polaroid camera. When reproducibility was not ideal, photographs of at least two runs were taken. An "infinity" trace was recorded in every case. The reacted solution from all the kinetic runs was collected in a glass vial and the pH measured immediately to ±0.02 pH units on the Radiometer pH meter.

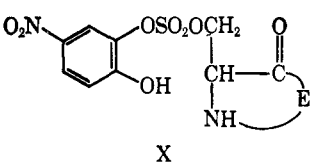
The Treatment of the Kinetic Results. Readings from the oscilloscope photographs were set out in tabular form. The vertical scale of all the traces were linear with respect to light transmittance and these readings were converted to optical density units (OD) using the known transmittance of the infinity reading. Plots of $(OD_{inf} - OD)/OD_{inf}$ against time were made on logarithmic graph paper. The slope of the linear plots gave values of $k/2.303$.

pK_a Determinations. In a typical spectrophotometric titration of the nitrophenol group in VIII, 250 μl of a 1×10^{-2} *M* solution of 4-nitrocatechol cyclic sulfate (VII) in acetonitrile was added to 5 ml of a 6×10^{-4} *M* chymotrypsin solution (0.05 *M* Tris–sulfate buffer, $\mu = 0.2$). Then 200 μl of the resulting solution was added to 2 ml of buffer at the pH desired, and at each pH a spectrum was taken from 3200 to 4200 Å on a Cary 15 spectrophotometer. The same pipets were used in the spectral determinations over the whole pH range. A similar procedure was used to determine the pK_a of the nitrophenol chromophore in the monosulfate IX. For the spectral measurements below pH 5, 0.05 *M* acetate buffers were employed. In the range pH 5.0 to 8.7 phosphate buffers were used and above pH 8.7 measurements were made in Ammediol buffers.

Conclusions

The pH dependence of the formation of a sulfonated α-chymotrypsin from the reaction of the enzyme with 4-nitrocatechol cyclic sulfate has been determined. The results reveal a bell-shaped pH profile for the ratio k_2/K_s with pK values of 7.09 and 8.76 indicating that the reaction is dependent on two ionizing groups on the enzyme. Similar results for the sulfonylation[4] and acylation[9] of chymotrypsin have been found. The group with a pK near 7.0 can be ascribed to an imidazole ring of a histidine residue[9] which is catalytically active only in its unprotonated form. The ionization with a pK which generally appears to fall between 8.6 and 9.0 has recently been attributed to a group affecting binding, possibly an N-terminal isoleucine group in the enzyme.[12b,16,17]

The stopped-flow kinetic studies together with the observations on the inhibition of the sulfonation reaction by N-acetyl-L-tryptophanamide suggest that 4-nitrocatechol cyclic sulfate reacts at the normal active site serine hydroxyl of chymotrypsin. In other words it appears that the structure of the sulfonated enzyme VIII can be written as X.[18]



X

(15) G. R. Schonbaum, B. Zerner, and M. L. Bender, *J. Biol. Chem.*, **236**, 2930 (1961).

(16) A. Himoe, P. C. Parks, and G. P. Hess, *J. Biol. Chem.*, **242**, 919 (1967).

(17) M. L. Bender and F. C. Wedler, *J. Am. Chem. Soc.*, **89**, 3052 (1967).

(18) The active site in chymotrypsin is stoichiometrically sulfonated by 4-nitrocatechol cyclic sulfate. All of the reagents which behave in a similar manner and lead to complete inhibition of chymotryptic activity have been shown to be attached either to serine-195 or histidine-57. Evidence obtained in many other investigations indicates that all acyl-, phosphoryl-, and sulfonylchymotrypsins previously investigated are modified at serine-195. Two recent pertinent references are: T. C. Bruice and S. J. Benkovic, "Bioorganic Mechanisms," W. A. Benjamin, Inc., New York, N. Y., 1966, pp 228–242; M. L. Bender and F. J. Kezdy, *Ann. Rev. Biochem.*, **34**, 49 (1965).

Previous results[4] indicate that the phenol group in the sulfonyl enzyme IV participates in the desulfonylation of this species since α-toluenesulfonyl-α-chymotrypsin does not desulfonate at an appreciable rate in the neutral pH range. We had anticipated therefore that the sulfonated enzyme VIII might also undergo desulfonation at a significant rate with participation of the *ortho* phenolic group in this reaction. However, as indicated before, we have found that VIII does not desulfonate appreciably to give the monosulfate IX near the neutral pH range over a time period of more than 12 hr. At present we do not know the reasons for the difference in the stability of the modified enzymes IV and VIII. Also, we mention again here that while our spectrophotometric titration results show that the spectral behavior of the nitrophenol chromophore in VIII can be understood in terms of reaction scheme 6, as is the case with IV, the pK values obtained for the various ionizations shown in the scheme are quite different for the two modified enzymes.

Further studies on the modification of enzymes and other proteins with highly reactive cyclic esters are currently under way in our laboratory.[19]

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(19) The nitrosultone II has recently been discovered to be an excellent reagent for probing the action of the sulfhydryl enzyme, papain (M. Iwatsuru, unpublished results).

Ultraviolet Circular Dichroism of Cupric and Nickel Ion Complexes of Amino Acids and Peptides[1]

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Abstract: Nickel ion complexes of L-amino acids exhibit negative circular dichroism near 210 mμ in agreement with the prediction of the octant rule for an n → π* carbonyl group transition. This transition is evidently obscured by a charge transfer transition in cupric complexes. Optically active charge transfer transitions also occur at 230–280 mμ in the peptide complexes of both transition metal ions. Cupric complexes of di- and tripeptides exhibit circular dichroism from 300 to 315 mμ where no discernible shoulders or maxima appear in the absorption spectra. It is suggested that this low-absorption intensity optically active transition might be due to an n → π* transition moved to longer wavelengths in the cupric chelates of peptides with ionized amide hydrogens. The CD of aromatic transitions is enhanced by incorporation of the amino acid into a dipeptide, and a lesser augmentation in CD intensity occurs on chelation by cupric ion.

Amino acids and peptides bearing aliphatic side chains exhibit absorption maxima only at less than 200 mμ, but a weaker n → π* transition gives rise to measurable optical activity at longer wavelengths. Aromatic side chains introduce further absorption at less than 300 mμ. It is part of the purpose of this paper to describe and analyze the changes wrought in the circular dichroism (CD) through these absorption bands by cupric and nickel ion chelation of these ligands. Urry and Eyring have already shown us how to apply the octant rule to the n → π* carbonyl group transition in metal ion complexes of amino acids.[2] In addition, new ultraviolet absorption bands are introduced by cupric and nickel ion chelation.

Upon addition of base to solutions containing peptides and cupric or nickel ions, amide hydrogen ionization is promoted even in neutral solutions.[3] All the peptide complexes referred to in this paper have undergone peptide hydrogen ionizations. Since earlier conclusions from solution studies regarding the geometry of metal ion complexes of amino acids and peptides have been confirmed by X-ray diffraction,[4] interpretation of CD results of these complexes may be predicated on well understood and often relatively rigid structures. CD involving d–d transitions on the metal ions in the visible region of the spectrum have already been reported[5] for the cupric[6] and planar nickel[7] ion complexes of the amino acids and peptides presented in this paper. Since the transitions reported here involve primarily the ligands, the ultraviolet CD results for the free ligands and chelates with both transition metal ions are discussed together.

(1) This paper is abstracted from the Ph.D. dissertations of Drs. J. M. Tsangaris and J. W. Chang (both 1967), and the research was supported by a grant from the National Science Foundation.

(2) D. W. Urry and H. Eyring, *J. Am. Chem. Soc.*, **86**, 4574 (1964).

(3) R. B. Martin, M. Chamberlin, and J. T. Edsall, *ibid.*, **82**, 495 (1960).

(4) H. C. Freeman, *Advan. Protein Chem.*, **22**, 257 (1967).

(5) R. B. Martin, J. M. Tsangaris, and J. W. Chang, *J. Am. Chem. Soc.*, **90**, 821 (1968).

(6) J. M. Tsangaris and R. B. Martin, submitted for publication.

(7) J. W. Chang and R. B. Martin, submitted for publication.