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Molecular Conformation of Chymotrypsinogen and Chymotrypsin by Low-Angle X-Ray Diffraction*

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ABSTRACT: Low-angle diffraction measurements are reported for chymotrypsinogen A and four chymotrypsins in 0.1 M NaCl at pH 7.0. Chymotrypsinogen is found to have a radius of gyration, R, of 18.1 Å and an axial ratio 2.0. The equivalent scattering ellipsoid has semiaxes 33.0 and 16.5 A, and a surface-to-volume ratio of 0.155 A^{-1}. The latter quantity agrees with that measured for the molecule in solution, 0.160 $Å^{-1}$, indicating that chymotrypsinogen has a smooth surface free of major protuberances. The principal product of slow activation, α-chymotrypsin, has very nearly the same size and shape (R = 18.0 Å and axial ratio 2.0), while the rapid activation end product, δ-chymotrypsin, is slightly larger (R = 19.0 Å) and somewhat more symmetrical (axial ratio 1.8). These results indicate that the activation of chymotrypsinogen is not accompanied by a gross change of the molecular conformation in solution. Two minor products of the slow activation process, β- and γ-chymotrypsin, are aggregated to some extent in 0.1 M NaCl. Chymotrypsinogen and chymotrypsin also have nearly the same molecular conformations in the crystalline state, but these are significantly smaller and more symmetrical than those deduced from our solution studies.

Proteolytic enzymes are secreted in the form of an inactive precursor, or zymogen. Zymogen activation, which creates the active enzyme, involves splitting of the polypeptide chain at specific sites. Neurath, in an excellent review of proteolytic enzymes (Neurath, 1964), has pointed out the importance of obtaining information concerning the conformational changes which accompany zymogen activation if one is to understand how the specificity site is created in this process.

One such zymogen is chymotrypsinogen, an inactive pancreatic enzyme first isolated by Kunitz and Northrop (1933). These workers also discovered that chymotrypsinogen is converted into the active pancreatic enzyme, chymotrypsin, by minute amounts of trypsin. One obtains from cattle pancreatic juice approximately equal quantities of two chymotrypsinogens designated A and B. At pH 8 the former is cationic, while the latter is anionic. Structural studies have been largely confined to chymotrypsinogen A, and this form is implied in all further references to chymotrypsinogen.

Both trypsin and chymotrypsin are capable of hydrolyzing the peptide bonds of chymotrypsinogen, so that different conditions of hydrolysis lead to different active forms. Slow activation of chymotrypsinogen in the

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presence of low trypsin concentrations leads ultimately to α-chymotrypsin. It is postulated that the Thr 147-Asp 148 dipeptide is first split out by chymotrypsin, yielding neochymotrypsin. This undergoes two further peptide cleavages (one due to trypsin and the other to chymotrypsin), resulting in the loss of the Ser 14-Arg 15 dipeptide and the production of α-chymotrypsin. In the presence of high trypsin concentrations (fast activation), the Arg 15–Ile 16 bond is first split by trypsin, yielding π-chymotrypsin. Further action of chymotrypsin removes the Ser 14-Arg 15 dipeptide, giving δ-chymotrypsin. Autolysis of δ-chymotrypsin, liberating the Thr 147-Asn 148 dipeptide, provides a second route for the production of α-chymotrypsin (Desnuelle, 1960). Two additional active forms, designated β- and γ-chymotrypsin, have been isolated (Kunitz, 1938). These are formed in small amounts during slow activation, and have the same terminal residues, specificity, and activity, but their mode of formation is as yet unclear (Desnuelle, 1960). It has been shown (Corey *et al.*, 1965) that α- and γ-chymotrypsins are interconvertible by change in pH, which implies that these may differ only in molecular conformation. In summary, the primary structures of the active and inactive forms of this enzyme differ by one (δ-chymotrypsin) or two (α-, β-, and γ-chymotrypsins) dipeptides.

The molecular weight of chymotrypsinogen is 25,767 (Hartley, 1964a). The molecule in solution has been represented as a prolate ellipsoid having an axial ratio of 4 from sedimentation studies (Schwert, 1951); however, viscosity and diffusion measurements (Smith *et al.*, 1951) gave a frictional ratio of only 1.12, which indicates a more symmetrical molecule. Unit cell dimensions and space group assignments have been given for three crystalline modifications of chymotrypsinogen (Bluhm and Kendrew, 1958). Type D crystals selected for further study belonged to space group $P2_12_12_1$ and had unit cell dimensions $a = 42.6$ Å, $b = 54.6$ Å, and $c = 91.9$ Å, corresponding to a molecular weight of 25,000 + 800. Examination of the low-order reflections from a type D crystal (Kraut, 1958) indicated that the over-all molecular shape could be well approximated by a sphere of radius 21 Å. Further refinement of data from a type F crystal (Kraut *et al.*, 1964) to 4-Å resolution revealed that the molecule in the crystalline state is slightly ellipsoidal, having semiaxes 25, 20, and 20 Å.

Studies concerning changes in the secondary and tertiary structures of molecules in solution during activation have led to conflicting conclusions. An early observation of a decrease in levorotation during fast activation was interpreted as indicating a more constrained arrangement of the peptide chains in chymotrypsin (Rupley *et al.*, 1955). More recent investigations of optical rotatory dispersion have indicated no change (Jirgensons, 1959), or a twofold increase in helical content (Imahori *et al.*, 1960). Study of the Cotton effect near 225 mμ has indicated no change in the amount of helical character during either slow or fast activation (Raval and Schellman, 1965); however, a circular dichroism study of the same band has been interpreted as indicating that 10–15 residues enter the helical region during activation (Fasman *et al.*, 1966). The latter authors also conclude from changes in the Cotton effect at 222 mμ, the appearance of a Cotton effect at 290 mμ, and differences in the circular dichroism curves in the 260–300-mμ range, that there is a significant change in the environment of the aromatic chromophores and disulfide bonds during activation.

Very recently the crystal structure of tosyl α-chymotrypsin at 2-Å resolution has been reported (Matthews *et al.*, 1967). Through comparison with the known sequence determined chemically (Hartley, 1964b; Hartley and Kauffman, 1966; Keil *et al.*, 1963; Meloun *et al.*, 1966), it was possible to follow the chains over most of their course. Only eight residues at the C terminus are in an α helix. The remainder of the molecule appears to be stabilized by hydrogen bonds, since there is a tendency for folding so that adjacent portions of the chain run parallel at a separation of 4.5–5 Å for appreciable distances. Matthews *et al.* conclude that there is no significant conformational change during activation, since the four terminal groups created in this process are in positions compatible with cleavage of a zymogen having essentially the same structure.

Despite the availability of this detailed structural knowledge concerning the molecular conformation in the crystal, it would also be desirable to have information about the shape of the molecule in solution. Sedimentation studies of α- and γ-chymotrypsins (Schwert, 1949) indicated a very symmetrical particle shape. Some years ago a low-angle diffraction study of an undesignated chymotrypsin was performed (Kratky and Sekora, 1943). A single measurement upon a 9% solution in acidified water yielded a radius of gyration of 17.8 Å, which they interpreted as indicating a spherical particle of radius 23 Å. In view of the lack of really comparable conformational data for the various components of the chymotrypsinogen–chymotrypsin system in dilute solution, we have undertaken a low-angle X-ray investigation of chymotrypsinogen, and of α-, β-, γ-, and δ-chymotrypsins in 0.1 M NaCl.

Experimental Section

Materials and Apparatus. Chymotrypsinogen A and the four chymotrypsins were obtained from Nutritional Biochemical Corp. in three-times-recrystallized, salt-free form. Chymotrypsin undergoes a reversible dimerization (Steiner, 1953), but α-chymotrypsin is reported to be nearly entirely in the monomeric form if the ionic strength is 0.1 or higher (Tinoco, 1957). The present measurements were therefore performed using 0.1 M NaCl solution at pH 7.0 (without added buffer) as the solvent.

The diffraction data were obtained using a 1964 series medium-resolution Kratky camera with copper radiation and a proportional counter. The scattered intensities were placed on an absolute scale by comparison with a Lupolene standard of known scattering power (Kratky *et al.*, 1966). Slit desmearing was performed using a FORTRAN program (Brumberger and Kent, 1964) modified for an IBM 7072 computer.

Chymotrypsinogen A was studied at three concentrations ranging from 1.832 to 5.525 g per 100 ml. The diffraction results are shown in Figure 1, where the logarithm

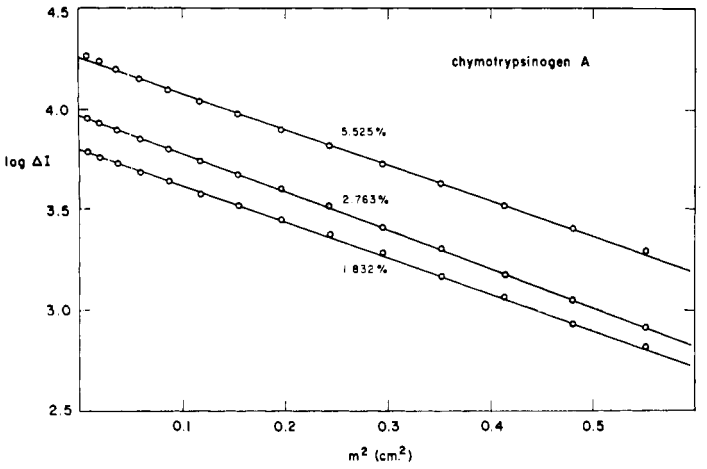

FIGURE 1: Guinier plot of the data for three concentrations of chymotrypsinogen A in 0.1 M NaCl at pH 7.0.

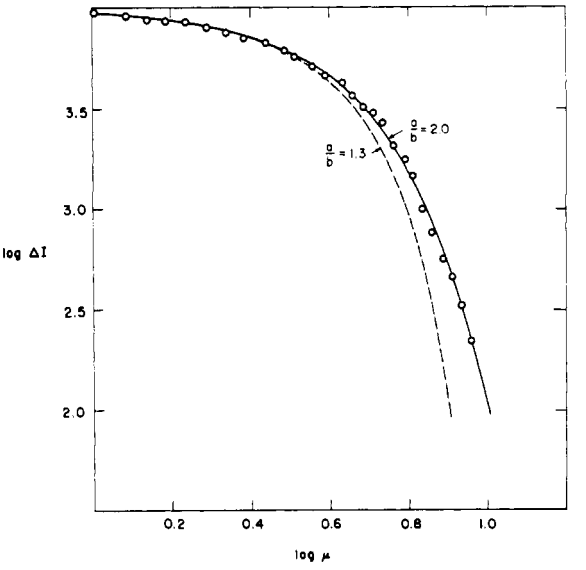

FIGURE 2: Comparison of the data for a 1.832% solution of chymotrypsinogen A (circles) with the theoretical scattering curve for an ellipsoid having axial ratios 2:1 (full curve) and 1.3:1 (dashed curve).

of the excess intensity, ΔI, is plotted against m. The latter parameter is related to the scattering angle, 2θ, in the following way

$$m/a = \tan 2\theta \cong 2\theta \qquad (1)$$

where a, the distance from the sample to the detector, is 21.0 cm. The Guinier plot is seen to be linear down to the smallest angles investigated, indicating the absence of aggregation. Further, the linear behavior extends outward over an extensive angular range, a behavior typical of particles having a small, but finite, asymmetry.

The radius of gyration, R, is evaluated from the initial slope of the Guinier plot, $(\tan \alpha)_0$, using the relation

$$R = [(3 \ln 10)^{1/2}/2\pi]\lambda a(\tan \alpha)_0^{1/2} \qquad (2)$$

The results are collected in Table I. Column two gives the radius of gyration, $\tilde{R}$, evaluated using slit smeared intensities, while the corresponding values after desmearing, designated R, are listed in column three. We observe that desmearing has little effect upon the radius of gyration, as anticipated from the long linear region in the Guinier plot. Further, the values of the radius of gyration are nearly independent of concentration, so interparticle interference effects are small for this system.

We next consider the shape of the chymotrypsinogen molecule in solution. A comparison of the scattering behavior of the 1.832% solution with the theoretical scattering curve of an ellipsoid of revolution having axial ratio 2:1 appears in Figure 2. Here the parameter μ is $r_e h$, where $h = (4\pi/\lambda) \sin \theta$, λ being the wavelength, and r_e is given in terms of the semiaxes, a and b, by $r_e^2 = (a^2 + b^2)/2$. The agreement between the experimental and the theoretical scattering curves is quite satisfactory. Further confirmation of this assignment is provided by the "characteristic function" (Porod, 1951a,b), $\gamma_0(r)$, defined as

$$\gamma_0(r) = (1/2\pi^2\rho^2 V)\int_0^\infty h^2F^2(h)[(\sin hr)/hr]dh \qquad (3)$$

where ρ is the excess electron density of the particle having volume, V, and $F(h)$ is the structure factor. The indicated transform of the scattering curve was performed using a computer program (Brumberger and Kent, 1964). The function $\gamma_0(r)$ obtained from the experimental scattering curve agreed well with that computed using the theoretical scattering curve of a 2:1 ellipsoid of revolution. If $\gamma_0(r)$ is expressed as a polynomial in r

$$\gamma_0(r) = 1 - (S/4V)r - \ldots \qquad (4)$$

we see that the surface to volume ratio of the scattering particle may be evaluated from the initial slope of $\gamma_0(r)$ *vs.* r. Values so obtained from the transforms of the experimental scattering curves are listed in column four of Table I. Extrapolation to infinite dilution yields $S/V = 0.160$ Å^{-1}. If the radius of gyration is taken as 18.1 Å, the equivalent scattering ellipsoid has semiaxes 33.0 and 16.5 Å, and the surface-to-volume ratio for this

TABLE I: Size and Shape Parameters for Chymotrypsinogen A.

10^2c (g/ml)	$\tilde{R}$ (Å)	R (Å)	S/V (Å^{-1})	$(I_0/c)^a$
1.832	18.0	18.0	0.157	3840
2.743	18.2	18.2	0.156	3830
5.525	18.0	18.2	0.152	3670

[a] I_0 in counts per second and c in grams per milliliter. For the calibrated Lupolene standard at 150 Å, $\tilde{I}_L$ = 1864 cps.

equivalent scattering particle is 0.155 Å^{-1}. The near agreement of this value with that calculated from the scattering curve suggests that the surface of the chymotrypsinogen molecule must be reasonably smooth and free of protuberances. The outer part of the scattering curve did not exhibit a Porod region, in which the desmeared intensity varies as m^{-4}, so that the invariant (and hence the volume of the molecule) could not be evaluated.

Chymotrypsins. Figure 3 shows the Guinier plot for five concentrations of α-chymotrypsin in 0.1 M NaCl. The absence of aggregation is indicated by the fact that a linear behavior is still exhibited at the smallest angles investigated. Further, the lowest concentration studied, 0.822 g/100 ml, was a fraction from a Sephadex G-75 column. The variation of ultraviolet absorption with fraction number indicated the presence of only one species, and the curve for this solution falls in the correct order with those of the other concentrations (which were not chromatographed).

The Guinier plot of α-chymotrypsin resembles that of the zymogen in having an extensive linear region, hence the comments made for the latter apply here as well. Values deduced for the radius of gyration of α-chymotrypsin appear in Table II. These are only slightly affected by slit desmearing, and interparticle interference effects are quite small. The equivalent scattering particle for α-chymotrypsin, as deduced by the two procedures described above, is an ellipsoid of revolution having axial ratio 2.0, and the S/V ratio evaluated from the plot of the "characteristic function" is 0.157 Å^{-1}. These values are nearly identical with those obtained for chymotrypsinogen.

Two concentrations, approximately 2 and 4%, were investigated for each of the β-, γ-, and δ-chymotrypsins. The derived parameters are collected in Table II. For δ-chymotrypsin the radius of gyration is 19.0 Å, the S/V ratio is 0.146 Å^{-1}, and the equivalent scattering ellipsoid has axial ratio 1.8. The Guinier plots of the other two chymotrypsins exhibited some curvature in the inner portions. For γ-chymotrypsin the desmeared radius of gyration is 18.3 Å, S/V is 0.153 Å^{-1}, and the axial ratio of the equivalent ellipsoid is 1.9. The Guinier plot of the desmeared intensities for β-chymotrypsin exhibited so much curvature in the inner portion that an accurate evaluation of the radius of gyration was not possible.

TABLE II: Size and Shape Parameters for the Chymotrypsins.

Type	10^2c (g/ml)	$\tilde{R}$ (Å)	R (Å)	$(I_0/c)^a$
α	0.822	17.8	18.0	3430
	2.113	18.0	18.1	3480
	3.170	18.2	18.3	3440
	4.227	18.0	18.1	3400
	6.340	17.9	18.2	3380
β	1.410	17.2		3490
	4.230	17.4		3220
δ	2.095	18.6	19.1	4660
	4.190	18.8	19.2	4640
γ	2.143	17.9	18.4	3290
	4.285	18.1	18.5	3050

[a] I_0 in counts per second and c in grams per milliliters. $\tilde{I}_L$ = 1864 cps at 150 Å.

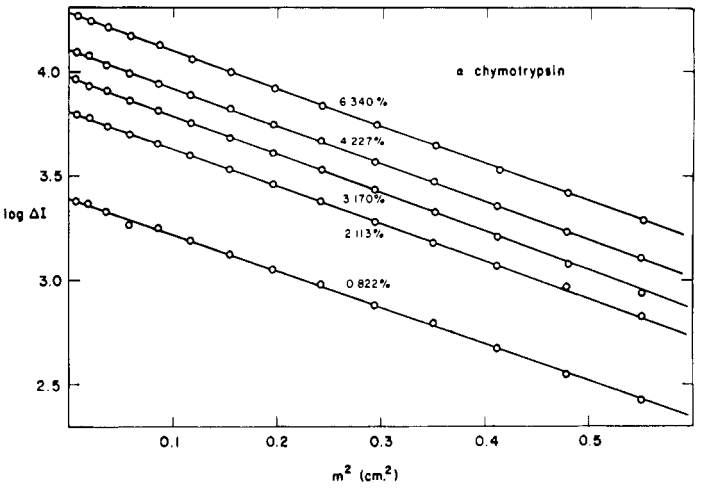

FIGURE 3: Guinier plot of the data for five concentrations of α-chymotrypsin in 0.1 M NaCl at pH 7.0.

Molecular Weights. The molecular weight may be evaluated from the scattering curve if the intensities are measured on an absolute scale. The intensity, I_0, at zero angle, obtained by extrapolating the Guinier plot, is related to the molecular weight in the following way

$$M = \frac{21(a/d)}{(z_2 - \rho_1\bar{v}_2)^2}\,\frac{(I_0/c)}{\tilde{I}_L}\,\frac{0.0123}{A_S} \qquad (5)$$

Here d is the sample thickness in centimeters, $\tilde{I}_L$ is the intensity scattered by the Lupolene standard at 150 Å, A_S is the fractional transmission of the sample, and $z_2 - \rho_1\bar{v}_2$ is the electron density difference in moles per gram. It preferable to determine the latter quantity by varying the electronic density of the solvent until the excess scattering vanishes (Malmon, 1957). We have, however, approximated this parameter by computing the electronic density of the solute, z_2, as 0.530 mole/g from the known composition, taking for the electronic density ρ_1 of the solvent 0.555 mole/ml, and for the partial specific volume of the solute $\bar{v}_2$ = 0.736 cc/g (Schwert and Kauffman, 1951). With A_S = 0.68 and d = 0.10 cm, the following values are obtained for the molecular weights: chymotrypsinogen A, 25,000; α-chymotrypsin, 22,000; β-chymotrypsin, (23,000); γ-chymotrypsin, 22,000; and δ-chymotrypsin 30,000. These molecular weight values carry an uncertainty of ±20% due to the uncertainty in the electronic density difference; however, they do furnish further evidence that we are dealing with solutions of the monomeric species.

TABLE III: Comparison of the Size and Shape Parameters for Chymotrypsinogen and the Chymotrypsins.

Substance	R (Å)	S/V (Å^{-1})	Axial Ratio
Chymotrypsinogen	18.1	0.160	2.0:1
α-Chymotrypsin	18.0	0.157	2.0:1
β-Chymotrypsin		0.155	2.0:1
δ-Chymotrypsin	19.0	0.146	1.8:1
γ-Chymotrypsin	18.3	0.153	1.9:1

Discussion

We have collected in Table III the parameters characterizing the molecular dimensions in solution of chymotrypsinogen and the four chymotrypsins. Examination of these results leads to the conclusion that there can be no major change in molecular conformation during the activation of chymotrypsinogen. The parameters obtained for chymotrypsinogen and α-chymotrypsin, the principal slow activation end product, are virtually identical. The rapid activation end product, δ-chymotrypsin, is slightly larger and more symmetrical than the zymogen. Whereas the foregoing are almost entirely in the monomeric form in 0.1 M NaCl, β- and γ-chymotrypsins are partially aggregated under the same conditions.

It is interesting to compare the results of dilute solution and crystallographic studies. Recent crystallographic studies at 5-Å resolution (Kraut *et al.*, 1967) led to the conclusion that, although there may be minor local rearrangements on activation, the gross conformations of chymotrypsinogen and π-, δ-, and γ-chromotrypsin molecules in the crystalline state are very similar. Our solution studies lead to the same conclusion. Chymotrypsinogen in the type F crystal has been represented as an ellipsoid having semiaxes 25 Å × 20 Å × 20 Å, while the over-all shape of tosyl α-chymotrypsin is approximated by an ellipsoid 22.5 Å × 19 Å × 17.5 Å. These data indicate an axial ratio of 1.3 ± 0.1. The theoretical scattering curve for an ellipsoid having an axial ratio of 1.3:1, represented by the dashed curve in Figure 2, is seen to differ significantly from the observed scattering behavior for chymotrypsinogen. If one further assumes the mass to be more or less uniformly distributed within the molecule, a radius of gyration of 16 ± 1 Å is computed. These values differ significantly from those measured in solution (semiaxes 32 Å × 16 Å × 16 Å, axial ratio 2.0 ± 0.1, and radius of gyration 18.0 ± 0.1 Å). Thus the zymogen and active enzyme have similar shapes in the crystalline state, and also in dilute solution, but both molecules are more asymmetrical in solution than in the crystal. This implies that during dissolution some portion of the molecule opens to give a more asymmetrical shape. It appears that this conformational change is not associated with creation of the active site, since both the zymogen and active enzyme change in the same manner on going into solution.

Single crystal studies are unique in furnishing detailed information concerning the three-dimensional structure in the crystalline state. On the other hand, it is the conformation of the molecule in aqueous solution which is of biochemical interest. The present study provides a warning that the biochemist should not base his interpretation of the biological activity of a molecule in solution solely upon information gleaned from the crystal structure. A molecule in the crystal is surely surrounded by quite different force fields than one in solution. The rigidity of the structure will determine the magnitude and type of conformational change accompanying dissolution. One might anticipate a smaller change for molecules possessing a large helix content, but the available data are too limited to permit an adequate test of this hypothesis. For this reason a knowledge of the molecular conformation in solution, as provided by low-angle diffraction, is a necessary complement to the more detailed information gained from single crystal studies.

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Acetimidation of Bovine Pancreatic Ribonuclease A*

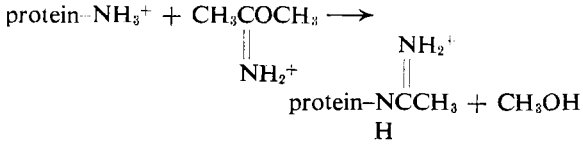
John H. Reynolds

ABSTRACT: All of the amino groups of bovine pancreatic ribonuclease A have been substituted with methyl acetimidate to give an enzymatically inactive protein which resembles ribonuclease A in its physical and chemical properties. The facts that completely acetimidated ribonuclease is not hydrolyzed by trypsin and that its optical rotatory dispersion spectrum, ultraviolet spectrum (ϵ 9900), and sedimentation coefficient ($s^0_{20,w}$ = 2.3 S) are almost identical with that of ribonuclease A indicate that no large structural changes have occurred upon acetimidation. Full ribonuclease activity can be regained by deacetimidation with concentrated aqueous ammonia. The product of this reaction is not chromatographically identical with that of ribonuclease A on SE-Sephadex but the amino acid content is identical with that of the original protein. When the disulfide bonds in completely acetimidated ribonuclease are reduced, reoxidized, and the acetimido groups removed, enzymatic activity is regained. Performic acid oxidized acetimidated ribonuclease is susceptible to trypsin-catalyzed hydrolysis only at arginyl peptide bonds. The five resulting peptides have been identified by paper chromatography, cellulose acetate electrophoresis, and ion-exchange chromatography.

Thus acetimidation can provide a method for selectively blocking the action of trypsin at lysyl bonds while allowing the protein to retain much of its original structure.

Several reports in the recent literature have dealt with the reaction of imido esters with proteins. Insulin (Hunter and Ludwig, 1962) and bovine serum albumin (Wofsy and Singer, 1963; Habeeb, 1966) have been modified with methyl acetimidate and the products were studied in some detail. A review has recently appeared (Ludwig and Hunter, 1967). Hartman and Wold (1967) have reacted RNase with a bifunctional imido ester to produce an intramolecular cross-linked active enzyme. Because our work required a selective reversible modification of lysine amino groups in proteins which would block tryptic digestions we chose to study the reaction of methyl acetimidate with bovine pancreatic RNase A.

We have found that all of the amino groups of RNase react with methyl acetimidate according to the scheme below to produce an inactive protein whose physical and chemical properties are very similar to native RNase. Complete enzymatic activity can be regenerated upon removal of the acetimido groups. Trypsin activity is confined to the arginyl peptide bonds in performic acid oxidized acetimido RNase (AIROX).[1] The details of the chemistry of completely acetimidated ribonuclease (AIR) and its preparation and regeneration to active RNase are reported here.



Experimental Section

Materials. Bovine pancreatic RNase was purchased from Worthington Biochemical Corp. as a three-times-recrystallized crude mixture and also as pure RNase A. The crude material is heterogeneous on SE-Sephadex and contains about 50% impurities and RNase B and 50% RNase A. Methyl acetimidate hydrochloride was prepared from hydrogen chloride, acetonitrile, and methanol according to published procedures (Hunter

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[1] Abbreviations used: RNase, bovine pancreatic ribonuclease; AIR, completely acetimidated RNase; RIBOX, performic acid oxidized RNase; AIROX, performic acid oxidized AIR; TPCK, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone; TNBS, trinitrobenzenesulfonic acid. For other abbreviations, see *Biochemistry 5*, 1445 (1966).