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Kinetics of tryptic hydrolysis of the arginine–valine bond in folded and unfolded ribonuclease T_1

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We have used ribonuclease T_1 and its chemically modified derivatives as substrates, and trypsin as proteinase, to investigate the kinetics of proteolysis of a specific peptide bond in the folded and unfolded conformations of a protein. Steady-state kinetic studies showed that $K_m = 0.27$ mM and $K_{cat.} = 2.45\,s^{-1}$ for the tryptic hydrolysis of the $Arg_{(77)}$–$Val_{(78)}$ peptide bond in unfolded ribonuclease T_1. This K_m is somewhat lower than, and the $k_{cat.}$ value similar to, values found for the tryptic hydrolysis of comparable bonds in small peptides. Our data for the initial velocity of hydrolysis of the $Arg_{(77)}$–$Val_{(78)}$ bond in a solution of the folded protein indicate that the bond is at least 1700 times less rapidly hydrolysed in the folded than in the unfolded conformation of ribonuclease T_1, and do not exclude the possibility that the bond is completely resistant to hydrolysis in the folded protein.

Peptide-bond cleavage plays an essential role in many important biological processes, including the post-translational processing steps of protein synthesis, the generation of polypeptide hormones, assembly of intracellular organelles, blood clotting, intracellular protein degradation, tumour invasion and alimentary digestion. It is therefore desirable that we understand the kinetics of peptide-bond cleavage by proteolytic enzymes. Most of our knowledge of the action of proteinases is based on kineties studies of the hydrolysis of small peptides, so that much is known of the influence of covalent structure on the kinetics of proteolysis, but there is little information on the influence of conformation, since most small peptides do not form stable folded conformations in aqueous solution (Blundell & Wood, 1982).

It is well established that most proteins are rendered much more susceptible to proteolysis by denaturation. The significance of this has been much debated since the seminal work of Linderstrøm-Lang's laboratory in the late 1940s (Linderstrøm-Lang, 1949), but few firm conclusions have been reached, partly because quantitative information on the effects of substrate conformation on the rate of action of proteinases has been hard to come by. For example, no one has yet reported quantitative measurements of the rate of hydrolysis of specific peptide bonds in the folded and unfolded conformations of a protein. This is not surprising, because such measurements can be made only with a substrate protein that can be either folded or unfolded under conditions in which the proteolytic enzyme of interest is active, and which preferably contains only a single peptide bond that is cleaved by the enzyme. Ribonuclease T_1 and trypsin provide such a system. Oobatake *et al.* (1979) have shown that ribonuclease T_1 unfolds completely when the two disulphide bonds are broken. In addition, ribonuclease T_1 contains only two bonds susceptible to cleavage by trypsin, $Lys_{(41)}$–$Tyr_{(42)}$ and $Arg_{(77)}$–$Val_{(78)}$, and it has been shown that both the lysine and the arginine side chains can be individually modified (Takahashi & Inoue, 1977; Takahashi, 1976), so that it is possible to prepare derivatives of ribonuclease T_1 that contain only a single peptide bond that can be cleaved by trypsin.

In the present paper we describe our study of the steady-state kinetics of the tryptic hydrolysis of peptide bonds in the folded and unfolded conformations of derivatives of ribonuclease T_1 and a comparable model peptide, Ac-Arg-Val-OMe.

Abbreviations used: the abbreviations used for amino acid derivatives and *N*-terminal groups are based on the standard conventions [*Biochem. J.* (1972) **126**, 773–780]. Ribonuclease T_1 derivatives: Ac, *N*-acetyl; Cm, *S*-carboxymethyl; Suc, *N*-2-carboxypropionyl.

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Materials and methods

Preparation of ribonuclease T_1 and its derivatives

Ribonuclease T_1 was purified from Sanzyme powder, purchased from Calbiochem, by a procedure based on that of Fields *et al.* (1971). The batchwise DEAE-cellulose step for removal of pigment was eliminated; instead, to remove the yellow–brown colour that remains after the final chromatography on DEAE-cellulose, the protein was dialysed, freeze-dried and run on a Sephadex G-50 column (1.6cm × 96cm) in 50mM-NH_4HCO_3.

Cm-ribonuclease T_1 was prepared by reducing the protein (12mg/ml) for 3h at 20°C with 60mM-dithiothreitol in a solution containing 6M-guanidinium chloride, 2mM-EDTA and 0.1M-Tris/HCl buffer, pH8.2, and then adding an equal volume of a solution containing 0.5M-potassium iodoacetate and 0.25M-Tris/HCl buffer, pH7.5. After 5min, the solution was desalted by use of a column of Sephadex G-25 in 0.1M-sodium phosphate, pH8.0.

Portions of the Cm-ribonuclease T_1 were succinylated (2-carboxypropionylated) or acetylated by adding a total of 2mg of succinic anhydride/mg of protein during a period of 2h, or 2mg of acetic anhydride/mg of protein during a period of 1h, respectively. In each case, the pH was maintained near 7.0 by addition of 1M-NaOH, at 20°C. The acetylated protein was left to stand overnight at pH10.5 to promote deacetylation of tyrosine residues. Both proteins were desalted by use of a column of Sephadex G-25 in 0.1M-sodium phosphate buffer, pH8.0. The derivatives ran as single bands in electrophoresis in 15% polyacrylamide slab gels with the discontinuous buffer system of Davis (1964), and their relative mobilities were consistent with the expected differences in net charge. The fluorescence yields in the reaction with fluorescamine (see Table 2) showed the extent of blocking of the amino groups in Cm,Suc-ribonuclease T_1 and Cm,Ac-ribonuclease T_1 to be about 96% and 94% respectively. One batch of Cm-ribonuclease T_1 was acetylated by this procedure, but with only 1mg of acetic anhydride/mg of protein; the extent of acetylation was then 86%. This sample of Cm,Ac-ribonuclease T_1 was used for the steady-state kinetic experiments to estimate K_m and $V_{max.}$. Native ribonuclease T_1 was succinylated (to give Suc-ribonuclease T_1) by use of succinic anhydride as above, and gave a product in which the amino groups were over 98% blocked.

The concentrations of solutions of ribonuclease T_1 and its chemically modified derivatives were determined from A_{278} measurements of solutions that had been passed through 0.45μm-pore-size Millipore filters, on the basis that $\varepsilon = 2.1 \times 10^4 M^{-1} \cdot cm^{-1}$ (Takashaski *et al.*, 1970).

Other materials

Pig trypsin (Sigma type IC) was acetylated as described by Labouesse & Gervais (1967) to decrease its reaction in the fluorescamine assay and stabilize it against autolysis. The active-site molarity of the stock solution was determined by titration with *p*-nitrophenyl *p'*-guanidinobenzoate as described by Chase & Shaw (1967). The result served to calibrate a rate assay with Bz-DL-Arg-$NPhNO_2$ as substrate (Kassell, 1970), which was then used for routine monitoring of the concentration of the active enzyme.

Ac-Arg-Val-OMe was kindly supplied by Dr A. P. Lobo and Dr. W. B. Lawson (Division of Laboratories and Research, New York State Department of Health, Albany, NY, U.S.A.). The concentration of Ac-Arg-Val-OMe was determined by following the complete tryptic hydrolysis of a sample as described below, and then quantifying the Val-OMe released by use of the fluorescamine procedure and standard curves prepared with Val-OMe. All of the amino acids and peptides in Table 2 were purchased from Sigma Chemical Co.

Fluorimetric determination of amino groups

To each sample (2.0ml), 0.5ml of a fresh 0.15mg/ml solution of fluorescamine (Sigma) in acetone was added with vigorous mixing (De Bernado *et al.*, 1974). The fluorescence intensity (390nm excitation, 475nm emission) was measured after 10–20min, in a Perkin–Elmer model LS-3 spectrofluorimeter. The temperature of the thermostatically controlled cell holder was maintained at 30°C for all measurements.

Measurement of rate of peptide-bond hydrolysis

The rates of tryptic hydrolysis of Ac-Arg-Val-OMe, Cm-ribonuclease T_1, Cm,Suc-ribonuclease T_1 and Cm,Ac-ribonuclease T_1 were measured by determining the increase in concentration of α-amino groups as a function of time. The reaction was in 0.1M-sodium phosphate buffer, pH8.0, at 30°C. The reaction was initiated by adding acetylated trypsin (final concentration 0.1–0.3μM) to a solution (typically 0.5 or 1.0ml) of the substrate equilibrated at 30°C. Samples (20–200μl) were withdrawn at 1–3min intervals, and immediately mixed with a solution of soya-bean trypsin inhibitor (Sigma type 1-S) in 0.1M-sodium phosphate buffer, pH8.0, so that there was a 1.5–2-fold weight excess of trypsin inhibitor over trypsin. To the mixture, now 2.0ml, was added fluorescamine solution (0.5ml) to complete the assay as above.

Exactly the same procedure was used for ribonuclease T_1 and Suc-ribonuclease T_1, except that samples were taken over a period of 25h because the tryptic hydrolysis was very slow. The results

obtained were corrected for the slow generation of amino groups due to the autolysis of trypsin by use of parallel control incubations.

Results

Unfolding of ribonuclease T_1

Oobatake *et al.* (1979) have shown that reduced ribonuclease T_1 and reduced carboxymethylated ribonuclease T_1 appear to be as completely unfolded in water as they are in 8M-urea. We have used intrinsic-fluorescence measurements to investigate the conformation of ribonuclease T_1 and the chemically modified derivatives. Previously it was found that, when ribonuclease T_1 is unfolded by urea or guanidinium chloride, the wavelength maximum of fluorescence emission increases by about 20nm and the fluorescence intensity at 320nm decreases more than 6-fold (C. N. Pace, unpublished work). The data in Table 1 indicate that Cm-ribonuclease T_1, Cm,Ac-ribonuclease T_1 and Cm,Suc-ribonuclease T_1 were completely unfolded in 0.1M-Tris/HCl buffer, as the fluorescence properties were similar to those observed in 6.4M-guanidinium chloride. In contrast, the fluorescence properties of Suc-ribonuclease T_1 resembled those of the native protein in 0.1M-Tris/HCl buffer, indicating that succinylation of the *N*-terminal α-amino group and the ε-amino group of Lys-41 did not cause unfolding of the polypeptide chain.

Table 1. *Intrinsic fluorescence of ribonuclease T_1 and its derivatives*

Measurements of intrinsic fluorescence were made at pH8.0 at 30°C in a Perkin–Elmer LS-3 spectrofluorimeter, with 1 μM-protein, in the solvents indicated. The *F* values were for excitation at 278nm and emission at 320nm.

Solvent ...	0.1M-Tris/HCl		6.4M-Guanidinium chloride	
Protein	$\lambda_{max.}$	*F*	$\lambda_{max.}$	*F*
Ribonuclease T_1	333	227	355	36
Suc-ribonuclease T_1	333	204	356	23
Cm-ribonuclease T_1	354	20	356	30
Cm,Suc-ribonuclease T_1	355	20	355	28
Cm,Ac-ribonuclease T_1	354	21	354	24

Table 2. *Fluorescence intensity of the fluorescamine reaction for several compounds*

Assays were as described in the Materials and methods section. For each compound, the sample volume (2.5ml) contained 0.5–50nmol, and the *F* values (arbitrary units) were adjusted to 1nmol on the basis of the linear fluorescence–concentration response observed.

Compound	*F*
Valine	4.3
Valine amide	22.6
Valine methyl ester	26.9
Val-Val	17.3
Val-Gly-Gly	24.5
Ribonuclease T_1	27.0
Suc-ribonuclease T_1	0.5
Cm-ribonuclease T_1	55.4
Cm,Suc-ribonuclease T_1	2.5
Cm,Ac-ribonuclease T_1	3.5

Calibration of the fluorescamine assay

In the calibration of the fluorescamine reaction with which we followed peptide-bond hydrolysis, it was necessary to take account of the large differences in response of the assay to primary amines of generally similar structure (Table 1). It can be seen that valine derivatives in which the carboxy group is blocked yielded much more fluorescence than did valine itself. For studies of the hydrolysis of Ac-Arg-Val-OMe, we used Val-OMe as reference compound. For studies of the hydrolysis of the ribonuclease T_1 derivatives in which the ε-amino group of Lys-41 is blocked, the standard curve should ideally have been based on the peptide containing residues 78–104 of ribonuclease T_1, and the situation was still more complicated for ribonuclease T_1 and Cm-ribonuclease T_1 in which there are two bonds that can be cleaved and three possible products with free α-amino groups. However, our finding of relatively small differences among the results for Val-NH_2, Val-OMe and Val-Gly-Gly, taken together with the results obtained by Furihata *et al.* (1978) with a variety of other peptides, suggested that a standard curve based on Val-Gly-Gly would serve as a reasonable approximation for the proper peptides. Consequently, all of the results of kinetic studies of the hydrolysis of ribonuclease T_1 and its derivatives were quantified on the basis of standard curves with Val-Gly-Gly.

Kinetics of hydrolysis of the substrates

The fluorescence intensity (*F*) in fluorescamine reactions as described above was measured for samples withdrawn periodically from the incubation mixtures. The fluorescence intensity at zero time was obtained by extrapolation. The increase in concentration of α-amino groups as a function of time of incubation could then be assessed by reference to the standard curve. Fig. 1 shows typical results obtained for Ac-Arg-Val-OMe and Cm,Ac-ribonuclease T_1, at the highest and lowest substrate concentrations used in Fig. 2. The acetylated trypsin made only a small contribution to the blank fluorescence, but the trypsin inhibitor made a substantially greater contribution.

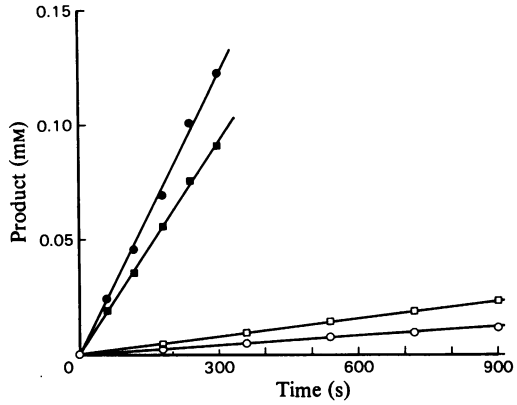

Fig. 1. *Product concentration as a function of the time of tryptic hydrolysis of Ac-Arg-Val-OMe and Cm,Ac-ribonuclease T_1.*

Hydrolysis was in 0.1M-sodium phosphate buffer, pH8.0, at 30°C, and product concentrations were determined as described in the text. Key: □, 0.154mM-Ac-Arg-Val-OMe; ■, 11.2mM-Ac-Arg-Val-OMe; ○, 0.0142mM-Cm,Ac-ribonuclease T_1; ●, 0.575mM-Cm,Ac-ribonuclease T_1.

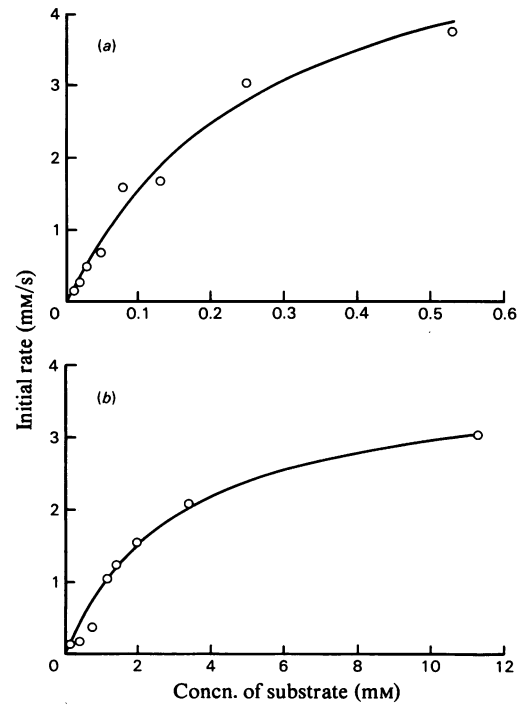

Fig. 2. *Initial velocity as a function of substrate concentration for the hydrolysis of Cm,Ac-ribonuclease T_1 (a) or Ac-Arg-Val-OMe (b) by trypsin*

Hydrolysis was in 0.1M-sodium phosphate buffer, pH8.0, at 30°C. The concentration of acetylated trypsin was 0.24μM for (*a*) and 0.12μM for (*b*). The continuous lines are calculated from the kinetic constants given in Table 3.

For both Ac-Arg-Val-OMe and Cm,Ac-ribonuclease T_1, initial velocities were measured as a function of substrate concentration, and the results are shown in Fig. 2. Even at the lowest substrate concentration (0.15mM), and the longest incubation time (15min), the extent of hydrolysis of the Ac-Arg-Val-OMe reached only 12.5%, and in most cases it was less than 4%, so that it was unnecessary to correct the data for substrate depletion. However, with Cm, Ac-ribonuclease T_1, substrate concentrations down to 0.014mM were used, and it was then necessary to correct the results obtained for substrate depletion by the method described previously (Pace, 1980).

The steady-state kinetic parameters K_m and $V_{max.}$ were derived from the data of Fig. 2 by the procedure of Wilkinson (1961). The results are given in Table 3. Note that our K_m and $V_{max.}$ values for Ac-Arg-Val-OMe differ substantially from those reported by Lobo *et al.* (1976). In part, this probably reflects the differences in solvent and enzyme used. We could not use Tris buffer because it interferes with the fluorescamine assay, and we used acetylated porcine trypsin because it is more stable than the bovine enzyme (Travis & Liener, 1965; Labouesse & Gervais, 1967).

Because of limited solubility of Cm,Ac-ribonuclease T_1 (0.5–0.6mM), the highest substrate concentration in Fig. 2 is only about twice the K_m, so that the estimates of K_m and $V_{max.}$ are less reliable than they would have been if results could have been obtained at higher substrate concentrations. As noted above, most of the data in Fig. 2(*a*) were obtained with a sample of Cm,Ac-ribonuclease T_1 in which the amino groups were only 86% acetylated. We were concerned that hydrolysis of the Lys–Tyr bond might be making an appreciable contribution to the kinetics, but two observations suggest that this was not the case. First, the rate of hydrolysis of the Arg–Val and Lys–Tyr bonds are comparable (see Table 4 below), and, secondly, velocities measured on the Cm,Ac-ribonuclease T_1, which was 94% acetylated, did not differ significantly from those measured on the 86%-acetylated protein.

Table 4 gives values for the initial velocities of hydrolysis for ribonuclease T_1 and all of the chemically modified derivatives at a substrate concentration of 0.025mM. Because of the large differences in the rates of hydrolysis, values for the unfolded substrates Cm-, Cm,Ac- and Cm,Suc-ribonuclease T_1 were obtained over a 15min period, whereas results for the folded substrates ribonuclease T_1 and Suc-ribonuclease T_1 were obtained over a 25h period. It is possible that there was some loss of activity of the trypsin during the longer period, in which case the estimates of rate for the folded substrates may be low.

Table 3. *Steady-state kinetic constants for the tryptic hydrolysis of the Arg–Val peptide bond in Cm,Ac-ribonuclease T_1 and several peptides*

		K_m (mM)	$k_{cat.}$ (s^{-1})	$k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)
I	Cm,Ac-ribonuclease T_1*	0.27±0.05	2.45±0.26	9070
II	Ac-Arg-Val-OMe*	2.97±0.17	3.18±0.08	1070
III	Ac-Arg-Val-OMe†	6.9	2.2	320
IV	Ac-Gly-Arg-Val-OMe†	3.6	20	5600
V	Ac-Pro-Arg-Val-OMe†	1.5	200	135000
VI	Gly-Pro-Arg-Val-OEt†	0.68	135	200000
VII	Ac-Gly-Pro-Arg-Val-OEt†	4.7	290	62000
VIII	Ac-Val-Arg-Gly-Pro-Arg†	0.37	4.2	11
IX	Val-Arg-Gly-Pro-Ala-NH_2‡	37.6	0.94	25
X	Gly-Val-Arg-Gly-Pro-Ala-NH_2‡	3.5	2.3	670
XI	Gly-Gly-Arg-Gly-Pro-Ala-NH_2‡	11.7	4.2	360
XII	Gly-Gly-Gly-Arg-Gly-Pro-Ala-NH_2‡	2.31	1.6	680
XIII	Gly-Val-Arg-Gly-Pro-Arg-Leu‡	2.0	1.24	612
XIV	Gly-Val-Arg-Gly-Gly-Arg-Leu‡	4.9	8.64	1775
XV	Gly-Val-Arg-Gly-Pro-Gly-Leu‡	0.93	1.83	2750

* The data of Fig. 3 were analysed by the method of Wilkinson (1961) to give K_m, $V_{max.}$ and the standard errors for each.

† Data from Lobo *et al.* (1976). Experimental conditions: 0.1M-Tris/HCl, 5mM-$CaCl_2$, 80mM-NaCl, pH8.2, 30°C, with bovine trypsin.

‡ Data from Liem & Scheraga (1974). Experimental conditions: 50mM-Tris/HCl, 150mM-KCl, pH8.0, 25°C, with bovine trypsin.

Table 4. *Comparison of initial velocities of tryptic hydrolysis of folded and unfolded derivatives of ribonuclease T_1*

The initial velocities were measured at a substrate concentration of 25 μM and a trypsin concentration of 0.24 μM in sodium phosphate buffer, pH8.0, at 30°C.

Substrate	Initial velocity (pM/s)
Cm-ribonuclease T_1	47000
Cm,Suc-ribonuclease T_1	28000
Cm,Ac-ribonuclease T_1	27000
Ribonuclease T_1	54
Suc-ribonuclease T_1	30

Discussion

The results in Table 1 indicate that Cm-ribonuclease T_1 is completely unfolded in water, as reported by Oobatake *et al.* (1979). The results also show that the protein remains unfolded when the two amino groups are either succinylated or acetylated. In contrast, Suc-ribonuclease T_1 retains the native globular conformation of ribonuclease T_1 (Heinemann & Saenger, 1982). Indeed, analysis of Suc-ribonuclease T_1 by urea-gradient gel electrophoresis (Creighton, 1979) has shown that the midpoints of the urea denaturation curves and hence the conformational stabilities of Suc-ribonuclease T_1 and ribonuclease T_1 are similar (C. N. Pace & T. E. Creighton, unpublished work). Since the ε-amino group of Lys-41 was at least 94% blocked in Suc-, Cm,Ac- and Cm,Suc-ribonuclease T_1, the results of the tryptic hydrolysis of Cm,Ac- and Cm,Suc-ribonuclease T_1 reflect the kinetics of tryptic hydrolysis of the single peptide bond $Arg_{(77)}$–$Val_{(78)}$ when the protein is completely unfolded, whereas the data for Suc-ribonuclease T_1 should reflect cleavage of the same bond when the protein is folded in its globular conformation (see

also below). Cm-ribonuclease T_1 (unfolded) and ribonuclease T_1 (folded) represent a similar system, but with both the $Lys_{(41)}$–$Tyr_{(42)}$ and $Arg_{(77)}$–$Val_{(78)}$ bonds available for tryptic hydrolysis.

We wished to compare the kinetic parameters of tryptic hydrolysis of a peptide bond in an unfolded protein with those for small peptides. Kinetics of tryptic hydrolysis of a variety of peptides containing from two to seven residues have been reported (Liem & Scheraga, 1974; Lobo *et al.*, 1976; Pozsgay *et al.*, 1981), but exceptionally low K_m values tend to be found for substrates such as tripeptide *p*-nitroanilides (Pozsgay *et al.*, 1981) and high $k_{cat.}$ values for some of the peptides with only one amino acid residue on the *C*-terminal side of the bond cleaved. The results reported indicate that trypsin has at least six subsites, three on either side of the catalytic site, which can interact with the amino acid residues of the substrate and affect the steady-state kinetic constants. The interactions between trypsin and its protein inhibitors revealed by crystallography suggest that the active site may be even larger (Huber & Bode, 1978; Sweet *et al.*, 1974).

Table 3 includes some results from the work of Lobo *et al.* (1976) and Liem & Scheraga (1974), in which tryptic cleavage occurred at an Arg–Val bond, as it does in Cm,Ac-ribonuclease T_1, or at an Arg–Gly bond. The K_m observed with Cm,Ac-ribonuclease T_1 was lower than those of any of the small peptides containing the Arg–Val bond (II–VII) or with Arg–Gly bonds (VIII–XV). Thus it can be concluded that trypsin binds Cm,Ac-ribonuclease T_1 better than it does most peptides, but it is not clear whether this is due mainly to a more favourable amino acid sequence or the possibility of interactions with a greater number of residues.

Values of $k_{cat.}$ for the eight comparable peptides (VIII–XV) range from 0·94 to 8·6 s^{-1}, with an average of 3.1 s^{-1}, which is similar to our value of 3.2 s^{-1} for Cm,Ac-ribonuclease T_1. The values of $k_{cat.}/K_m$ for the same eight peptides average 2300 $M^{-1} \cdot s^{-1}$. $k_{cat.}/K_m$ for Cm,Ac-ribonuclease T_1 (9070 $M^{-1} \cdot s^{-1}$) was substantially higher than the values for most of these peptides because the K_m value was lower. We conclude that in general the kinetics of hydrolysis of a peptide bond in the randomly coiled polypeptide chain are comparable with those observed with peptides that are much smaller.

Several conclusions can be drawn from the results in Table 4. The initial velocities for Cm,Ac-ribonuclease T_1 and Cm,Suc-ribonuclease T_1 did not differ significantly, showing that the group used to block the α- and ε-amino groups of the substrate did not modify the interaction with trypsin. This was not surprising, since these groups are 36 and 77 residues respectively from the bond being cleaved. The greater initial velocity observed with Cm-ribonuclease T_1 was expected, since the molecule contains two bonds sensitive to trypsin. The results suggest that the rates of tryptic cleavage of the Arg–Val and Lys–Tyr bonds in unfolded ribonuclease T_1 are comparable in magnitude. For the folded proteins the situation was similar: a greater rate of hydrolysis was observed with ribonuclease T_1, in which two bonds can be cleaved, than with Suc-ribonuclease T_1, in which only the Arg–Val bond can be cleaved.

The most significant results in Table 4 are the actual rates of hydrolysis for the three unfolded proteins Cm-, Cm,Ac- and Cm,Suc-ribonuclease T_1 relative to those for the two folded proteins ribonuclease T_1 and Suc-ribonuclease T_1. The Arg–Val bond was hydrolysed 930 times faster in the unfolded Cm,Suc-ribonuclease T_1 than it was in Suc-ribonuclease T_1. In ribonuclease T_1 and Cm-ribonuclease T_1, where two bonds can be cleaved, the rate of hydrolysis was 870 times greater in the unfolded protein than in the folded protein. Thus, although the side chains of Lys-41 and Arg-77 are known to be on the surface of the ribonuclease T_1 molecule (Heinemann & Saenger, 1982), it is clear that the folding of the native protein severely limits the hydrolysis at these residues by trypsin.

The most natural explanation for the resistance of native ribonuclease T_1 (and most other native proteins) to tryptic hydrolysis is that the lack of conformational freedom of the folded polypeptide prevents the potentially susceptible peptide bonds from being bound in the active site of the enzyme. Indeed, inspection of the crystallographic structure of ribonuclease T_1 (Heinemann & Saenger, 1982) strongly suggests that the Val-78 side chain is not well placed to fit the specificity subsite S_1' of a proteinase. This raises the possibility that the tryptic hydrolysis of the protein occurs exclusively through the unfolded form. Small concentrations of unfolded protein always exist in equilibrium with the native conformation of a globular protein in solution (Pace, 1975; Privalov, 1979), and, on the basis of the steady-state kinetic parameters for hydrolysis of the Arg–Val bond in unfolded ribonuclease T_1 (Table 3), we calculate that an unfolded protein concentration of 14 nM would completely account for the observed rate of hydrolysis (3.0×10^{-11} M/s) in the solution of the folded protein. This concentration of unfolded ribonuclease T_1 would be predicted to be present in the 25 μM solution used if the native conformation is 18.8 kJ/mol (4.5 kcal/mol) more stable than the unfolded conformation. Analysis of the urea denaturation curve of ribonuclease T_1 by standard procedures (Ahmad & Bigelow, 1982) indicates that the globular conformation is, in fact, 21 kJ/mol (5 kcal/mol) more stable than the unfolded con-

formation (C. N. Pace, unpublished work). This would lead to a 6.1 nM concentration of the unfolded protein, which would in turn account for about half (1.3×10^{-11} M/s) of the observed rate of hydrolysis. The remainder may truly represent hydrolysis of the native conformation of the protein at approx. 1700-fold lower rate (at non-saturating concentrations) than the unfolded protein, but the results do not convincingly exclude the possibility that there is indeed no hydrolysis of the native conformation.

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