Kinetic and Thermodynamic Thermal Stabilities of Ribonuclease A and Ribonuclease B[†]

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ABSTRACT: The thermal stabilities of ribonuclease A (RNase A) and ribonuclease B (RNase B), which possess identical protein structures but differ by the presence of a carbohydrate chain attached to Asn34 in RNase B, were studied by proteolysis and UV spectroscopy at pH 8.0. Proteolysis was quantified by sodium dodecyl sulfate−polyacrylamide gel electrophoresis and densitometry. Increasing protease concentrations led to a hyperbolic increase of the rate constants of proteolysis. With thermolysin, which attacks the unfolded molecules only, the thermal unfolding constants were determined by extrapolating the rate constants of proteolysis to infinite concentration of protease. With trypsin, the unfolding constants of RNase A could be confirmed. Subtilisin attacked even the native RNases, where RNase B was more stable toward proteolytic degradation. Kinetic stabilities ($\Delta G^{\ddagger}$) calculated from the unfolding constants for temperatures between 52.5 and 65 °C revealed a higher kinetic stability of RNase B, which results from enthalpic effects only, whereas entropic effects counteract stabilization. $\Delta\Delta G^{\ddagger}$ at the transition temperature of RNase A (60.4 °C) was 2.2 ± 0.3 kJ mol^{-1}. Thermodynamic stabilities (ΔG) were estimated from the thermal transition curves at 287 nm for the temperature range from 55 to 70 °C. For 17.5−25 °C, ΔG values were determined from transition curves of unfolding induced by guanidine hydrochloride and extrapolation of the free energy values to those in the absence of denaturant. At all temperatures, RNase B proved to be more stable than RNase A with essentially the same enthalpy and entropy of unfolding. $\Delta\Delta G$ was 2.5 ± 0.2 kJ mol^{-1} at 60.4 °C and 2.3 kJ mol^{-1} at 25 °C.

Despite great efforts, the molecular mechanisms of protein stabilization developed by nature in biological systems or arising from artificial modifications of the protein molecule such as carrier binding, chemical modifications of the amino acid residues, or amino acid exchanges by site-directed mutagenesis are not yet sufficiently understood. Glycosylation belongs to those types of naturally occurring modifications that are mostly connected with increased protein stability (Lis & Sharon, 1993; Wang et al., 1996). Even if stabilization is probably not the primary function of glycosylation in biological systems, glycosylated and nonglycosylated proteins with otherwise identical structure are excellent models for studying stabilization mechanisms. Ribonuclease A (RNase A)[1] and ribonuclease B (RNase B), both of which occur in bovine pancreas, are such a pair of proteins differing only by an oligosaccharide chain N-linked to Asn34 (Plummer et al., 1968). The carbohydrate chain in RNase B molecules ($GlcNAc_2Man_{5-9}$) is not uniform but differs in the number of mannose units, resulting in an increase of the molecular mass from 13 683 Da (RNase A) to 14 899−15 547 Da (RNase B of the Man_{5-9}-type). X-ray diffraction (Williams et al., 1987) as well as circular dichroism (CD), optical rotatory dispersion (ORD), and ultraviolet (UV) spectroscopy (Puett, 1973), ^{13}C nuclear magnetic resonance (NMR) (Berman et al., 1981), and ^1H NMR spectroscopy (Joao et al., 1992) revealed the same conformation for the protein component of RNase A and RNase B. However, some proof was obtained for stability differences between the two RNase species. From equilibrium spectral measurements in the presence of guanidine hydrochloride (GdnHCl) or acid, the free energies of unfolding in the absence of denaturant (ΔG) were calculated. ΔG of RNase B′, a preparation from pancreatic juice containing an average of 1 mol of glucosamine and 2−3 mol of mannose per mole of protein, was determined to be several hundreds of calories higher than ΔG of RNase A, indicating a small stabilizing effect due to the carbohydrate moiety (Puett, 1973). In the studies on unfolding and refolding in GdnHCl, differences were observed in the unfolding kinetics and transition curves but not in the refolding kinetics (Grafl et al., 1987). Joao et al. (1992) and Joao and Dwek (1993) determined the amide proton H/D-exchange rates of selected amino acid residues close to and remote from the glycosylation site for individual forms of RNase B. A comparison with RNase A showed a higher global stability of RNase B, being in agreement with an increase of the thermodynamic stability by 5 kJ mol^{-1} as determined by CD analysis of thermal denaturation at pH 3.35. The decrease of structural fluctuations due to glycosylation was also confirmed for RNase B forms with homogeneous carbohydrate moieties isolated by capillary electrophoresis and characterized by 1D and 2D ^1H NMR spectroscopy, catalytic activity, and susceptibility toward proteolysis by Pronase (Rudd et al., 1994).

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[1] Abbreviations: CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid disodium salt; GdnHCl, guanidine hydrochloride; NMR, nuclear magnetic resonance; ORD, optical rotatory dispersion; RNase, ribonuclease; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; UV, ultraviolet.

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Our studies on the thermal denaturation of RNase A by limited proteolysis (Arnold et al., 1996) have shown that the structural region near Asn34, where the carbohydrate moiety is attached to the protein molecule in RNase B, becomes particularly exposed during thermal unfolding. The primary cleavage sites were identified to be Asn34−Leu35 and Thr45−Phe46 for thermolysin, and Lys31−Ser32 and Arg33−Asn34 for trypsin. Therefore, the stabilizing effect in RNase B might be caused by the fixation of this labile surface region consisting of the loop structure between the α-helix Asn24−Asn34 and the β-sheet Lys41−His48. Hence, we were interested in comparing the thermal stabilities of RNase A and RNase B.

Protein folding and unfolding processes have been intensively studied under different aspects for many years. The aim of most studies was to elucidate the folding code determining the specific three-dimensional structure of proteins (Creighton, 1990; Jaenicke, 1991). Thereby, also the fundamental mechanisms of protein stability and stabilization are expected to become better understood. These studies mostly concentrate on the determination of thermodynamic stabilities (ΔG) of model proteins and their kinetic pathway of refolding from the denatured state. Unfolding rates and kinetic stabilities ($\Delta G^{\ddagger}$) are of minor interest in this consideration. A different approach is practiced from the view of applied research, since reversible denaturation is often succeeded by one or more irreversible consecutive reactions (Gupta, 1991). In these cases, the unfolding rate and the corresponding kinetic stability are preferably used as criteria of stability. Accordingly, also procedures of protein stabilization are evaluated by different criteria regarding either thermodynamic or kinetic stabilities. If feasible, both ΔG and $\Delta G^{\ddagger}$ should be determined as an approach to distinguish between several stabilization mechanisms such as stabilization of the native state, destabilization of the denatured state, or destabilization of the transition state of denaturation.

In the case of reversibly denaturing proteins, thermodynamic stability is usually determined from transition curves obtained from spectroscopic measurements if the denaturation can be described as a two-state transition between the native (N) and the denatured (D) state:

$$\mathrm{N} \underset{k_f}{\overset{k_U}{\rightleftharpoons}} \mathrm{D} \qquad (1)$$

with the equilibrium constant $K_D = [D]/[N] = k_U/k_f$. The determination of the rate constant of the unfolding reaction (k_U) and hence the calculation of $\Delta G^{\ddagger}$ by the same methods, however, may be connected with some difficulties. k_U cannot be measured directly but must be calculated from the observed unfolding rate constant k_{obs} ($k_{obs} = k_U + k_f$) and K_D according to

$$k_U = k_{obs} K_D/(1 + K_D) \qquad (2)$$

in the simplest case. If the denatured state consists of more than one conformational species being in equilibrium with rate constants influencing the overall unfolding rate constant, as it is known for prolyl cis-trans isomerizations in RNase A, the reaction scheme is even more complicated, and the calculation of k_U requires a complex mathematical system (Kiefhaber et al., 1992). The determination of k_U, however, becomes very simple if unfolding can be coupled with a consecutive irreversible reaction such as H−D exchange as practiced by Kiefhaber and Baldwin (1995). Even proteolysis can be used as the irreversible reaction (Imoto et al., 1986) under consideration of the following prerequisites: (i) the native protein molecule must not be susceptible toward proteolysis by the protease used; (ii) the protease must degrade the unfolded protein molecule regardless of isomerization equilibria; (iii) the specificity of the protease must not limit the digestibility of the protein; and (iv) the protease must be stable enough to possess constant activity over the period of degradation. Equation 1 is then extended to

$$\mathrm{N} \underset{k_f}{\overset{k_U}{\rightleftharpoons}} \mathrm{D} \underset{k_b}{\overset{k_a[P]}{\rightleftharpoons}} \mathrm{D{-}P} \xrightarrow{k_c} \mathrm{P} + \text{peptides} \qquad (3)$$

where P is the protease, D−P is the enzyme−substrate complex in proteolysis, and k_a, k_b, and k_c are the rate constants of the proteolytic reaction. Under conditions where unfolding and not proteolysis is rate limiting, the decrease of N yields first-order reaction kinetics with the rate constant k_U.

In the present study, the unfolding rate constants of RNase A and RNase B at different temperatures have been determined by proteolysis with thermolysin and subsequent quantification of the remaining intact RNase by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and densitometry. In addition, the susceptibility of RNase A and RNase B toward proteolysis by subtilisin, which is able to attack both native RNase species, has been measured. The calculated parameters of the kinetic stabilities have been compared with the thermodynamic stability parameters obtained from UV spectroscopic equilibrium measurements.

MATERIALS AND METHODS

RNase A from Serva and RNase B from Sigma were purified using an FPLC MONO S (Pharmacia) column resulting in single bands in the SDS−PAGE. The proteases thermolysin and subtilisin Carlsberg from Sigma and trypsin (treated with N^{α}-tosyl-L-phenylalanine chloromethyl ketone) from Serva were used without further purification.

Acrylamide, *N*,*N*′-methylenebis(acrylamide), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, and ammonium persulfate were purchased from Pharmacia, tris(hydroxymethyl)aminomethane (Tris), *N*-[tris(hydroxymethyl)methyl]glycine, and Coomassie Brillant Blue G250 from Serva, calcium chloride and phenylmethanesulfonyl fluoride from Merck, ultrapure GdnHCl from Schwarz-Mann Biotech, and 1,4-dithioerythritol, ethylenediaminetetraacetic acid disodium salt (EDTA), and SDS from Sigma. All other reagents were the purest ones commercially available.

Thermal Denaturation and Proteolysis. In a typical experiment, 80 μL of 50 mM Tris-HCl buffer, pH 8.0, was preincubated in a RM 6 thermostat (LAUDA, accuracy ±0.1 °C). At zero time, 10 μL of protease solution (0.00156−1.5 mg/mL) of trypsin or subtilisin in 50 mM Tris-HCl buffer, pH 8.0, or thermolysin in 50 mM Tris-HCl buffer, 10 mM $CaCl_2$, pH 8.0, and 10 μL of RNase (2.5 mg/mL) was added. After distinct time intervals, samples of 15 μL were removed and mixed with 5 μL of 50 mM EDTA in the case of thermolysin or 5 μl of 50 mM phenylmethanesulfonyl fluoride (dissolved in 2-propanol) in the case of trypsin and subtilisin.

Electrophoresis and Densitometric Evaluation. Electrophoresis was carried out on a Midget electrophoresis unit (Hoefer) according to Schägger and von Jagow (1987) but using 10, 14, and 18% acrylamide for the sampling, spacer, and separating gels. Reducing conditions were applied in order to obtain linear peptide fragments. The SDS−PAGE gels were stained with Coomassie Brillant Blue G250 for at least 2 h followed by destaining with methanol solutions (20−40% methanol). The gels were scanned at 595 nm using a CD 60 densitometer (Desaga).

Determination of the Unfolding Rate Constants. From the decrease of the peak areas of intact RNases in the scanned SDS−PAGE gels as a function of time of proteolysis, the rate constants of proteolysis ($k_{proteolysis}$) were calculated for proteolysis by trypsin and thermolysin according to

$$A_t = A_0 \exp(-k_{proteolysis} t) \quad (4)$$

and $k_{proteolysis}$ and $k'_{proteolysis}$ for proteolysis by subtilisin according to

$$A_t = A_0[a \exp(-k_{proteolysis} t) + (1 - a) \exp(-k'_{proteolysis} t)] \quad (5)$$

where A_t is the peak area at time t, A_0 is the peak area at $t = 0$, and a is the fraction of the fast reaction phase. The constants of proteolysis were determined for several protease concentrations, $[P]_0$, and temperatures. From the respective pairs of $k_{proteolysis}$ and $[P]_0$ of thermolysin and trypsin, k_U values were estimated according to

$$k_{proteolysis} = \frac{k_U [P]_0}{B + [P]_0} \quad (6)$$

in adaption to the approach by Imoto et al. (1986). This equation is derived from eq 3 for steady-state conditions [d[D]/dt = 0 and d[D−P]/dt = 0], where

$$B = \frac{(k_b + k_c)k_f}{k_a k_c} \quad (7)$$

and

$$\lim k_{proteolysis} = k_U \text{ (for } [P]_0 \rightarrow \infty) \quad (8)$$

Calculation of the Energetic Parameters of Kinetic Stability. $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$, and $\Delta G^{\ddagger}$ were calculated according to Eyring's equation:

$$\ln (k_U/T) = \ln (K/h) + \Delta S^{\ddagger}/R - \Delta H^{\ddagger}/RT \quad (9)$$

where K, h, and R are Boltzmann's, Planck's, and the gas constant, respectively, and the Gibbs' equation

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger} \quad (10)$$

Determination of Thermal Transition Curves. The absorbance of RNase (85 μM) in 50 mM Tris-HCl buffer, pH 8.0, was measured at 287 nm after equilibration at different temperatures using a U-2000 spectrophotometer (Hitachi) and stirred water-jacketed quartz cuvettes (1 cm) connected to a WK14 thermostat (Colora), accuracy ±0.1 °C. Temperature was controlled in the cuvette.

Determination of the Transition Curves in GdnHCl. The procedure was the same as above, but the temperature was kept constant and the GdnHCl concentration controlled by the refractive index (Pace et al., 1989) was varied.

Calculation of the Energetic Parameters of Thermodynamic Stability. The fraction of native protein (f_N) was calculated according to

$$f_N = \frac{A - A_D}{A_N - A_D} \quad (11)$$

where A is the absorption of the sample. A_N and A_D are the absorption of the native and the denatured protein extrapolated to the corresponding temperature or GdnHCl concentration from the linear pre- and posttransitional segments of the transition curves. From f_N, the unfolding equilibrium constant (K_D) was calculated according to

$$K_D = \frac{1 - f_N}{f_N} \quad (12)$$

From the van't Hoff equation:

$$d(\ln K_D)/d(1/T) = -\Delta H/R \quad (13)$$

The transition temperature (T_m) and the enthalpy at T_m (ΔH_m) were calculated by setting $\ln K_D = 0$. The entropy at T_m (ΔS_m) was calculated from the Gibbs equation ($\Delta G = 0$) according to

$$\Delta S_m = \Delta H_m/T_m \quad (14)$$

From

$$\Delta G = -RT \ln K_D \quad (15)$$

the free energies for different temperatures (ΔG) between 55.0 and 70.0 °C were calculated. ΔG data between 17.5 and 25.0 °C were obtained from the determination of ΔG_{GdnHCl} as a function of the concentration of GdnHCl with

$$\Delta G_{GdnHCl} = -RT \ln K_{D(GdnHCl)} \quad (16)$$

and linear extrapolation to the free energies in the absence of denaturant according to Santoro and Bolen (1988):

$$\Delta G_{GdnHCl} = \Delta G - m[GdnHCl] \quad (17)$$

where m is a constant. With this enlarged set of ΔG values, the heat capacities of thermal unfolding (ΔC_p) and $\Delta G_{25\ °C}$ were fitted according to the Gibbs-Helmholtz equation:

$$\Delta G = \Delta H_m(1 - T/T_m) - \Delta C_p[(T_m - T) + T \ln (T/T_m)] \quad (18)$$

The temperature of maximum stability (T_{max}) was calculated from

$$T_{max} = T_m \exp[-\Delta H_m/\Delta C_p T_m) \quad (19)$$

Data Processing. The experiments for the determination of k_U values by thermolysin and the thermal transition curves were repeated at least 3 times. Errors are given as standard deviations. The determination of k_U by trypsin and the evaluation of the proteolysis by subtilisin resulted from single

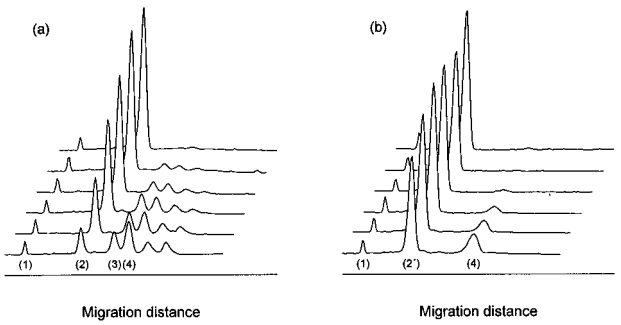

FIGURE 1: Densitometric chromatograms obtained from Coomassie-stained SDS−PAGE gels after proteolysis of RNase A (a) and RNase B (b) by thermolysin at 62.5 °C. The RNase:thermolysin ratio was 40:1 by weight. Time of incubation was 0, 1.0, 1.5, 2.0, 2.5, and 3.0 min (from back to front). The peak numbers represent thermolysin (1), RNase A (2), RNase B (2′), fragment 35−124 (3), and fragment 46−124 (4).

run experiments. The rate constants of proteolysis were determined from at least six data points.

All calculations were performed using the program SigmaPlot (Jandel Scientific).

RESULTS

Thermal Unfolding and Proteolysis by Thermolysin and Trypsin. From several proteases tested, thermolysin proved to be most appropriate for the determination of the unfolding constants of RNase A and RNase B. As shown by Arnold et al. (1996) for RNase A, and demonstrated for RNase B in this study, only the unfolded enzyme molecules are degraded by this protease. Furthermore, thermolysin is thermoresistent enough to withstand temperatures where both RNase species are denatured. Thermal unfolding of RNase A and RNase B was performed in the presence of thermolysin at different concentrations of the protease and followed by quantification of the intact RNase by SDS−PAGE and densitometry as demonstrated in Figure 1. From its decrease, which follows first-order kinetics until less than 10% of the original RNase amount, rate constants of proteolysis ($k_{proteolysis}$) were determined. As derived by Imoto et al. (1986), the dependence of $k_{proteolysis}$ on the protease concentration follows a Michaelis−Menten type function, which yields the unfolding constant (k_U) for infinite protease concentrations. In Figure 2a,b the constants of proteolysis of RNase A and RNase B by thermolysin are compared for different temperatures in the range of 52.5−65.0 °C. The lines show the curves fitted according to eq 6. A distinct difference of the k_U values between RNase A and RNase B was found which decreases with increasing temperature. Figure 3 shows the Eyring plot of the data from which the activation enthalpies of unfolding ($\Delta H^{\ddagger}$) and the activation entropies of unfolding ($\Delta S^{\ddagger}$) were determined (Table 1).

Trypsin proved also to be appropriate to determine unfolding constants of RNase A, yielding the same constants as thermolysin within experimental errors (Figure 3), whereas it failed with RNase B. In this case, no experimentally reasonable conditions could be found where unfolding and not proteolysis was rate-limiting.

Proteolysis of Native RNase A and RNase B by Subtilisin. Subtilisin is known to cleave RNase A between Ala20 and Ser21 even under native conditions (Richards & Vithayathil, 1959), which is due to the high flexibility of this region in the native state. For characterizing conformational differences between native RNase A and RNase B, the proteolysis of the two RNase species at different concentrations of subtilisin at 25 °C was followed. In all cases, the decrease of the intact RNase concentration as a function of time followed biphasic kinetics which could be fitted to an equation with two exponential terms (eq 5). The calculated constants of proteolysis ($k_{proteolysis}$ and $k'_{proteolysis}$) were distinctly higher for RNase A than for RNase B at all concentrations of subtilisin (Figure 4).

Thermal Unfolding Equilibria. The conformational stabilities of RNase A and RNase B were compared by UV spectroscopy. The transition curves (Figure 5) show a small but unambiguous difference with an increase of T_m by 1.5 °C for RNase B (Table 2). The van't Hoff plots of the corresponding equilibria constants (K_D) are shown in Figure 6. The enthalpies of thermal denaturation at the transition temperatures (ΔH_m) and the corresponding entropies (ΔS_m) are given in Table 2.

In order to compare stabilities of RNase A and RNase B at 25 °C, the Gibbs−Helmholtz function (eq 18) was used to fit ΔG as a function of temperature. Since ΔG values obtained from thermal denaturation were restricted to a limited temperature range (55.0−70.0 °C), in adaptation to the approach by Pace and Laurents (1989), ΔG values for lower temperatures (17.5−25.0 °C) were completed from measurements of GdnHCl-induced denaturation using eqs 16 and 17. Figure 7 shows the ΔG values experimentally determined as a function of temperature and the corresponding fitted curves. The thermodynamic stabilities at 25 °C ($\Delta G_{25\ °C}$) calculated from the fitted functions (Table 2) show that RNase B is more stable than RNase A even under native conditions. The heat capacities (ΔC_p) and the temperatures of maximum stability (T_{max}) were estimated as well (Table 2).

DISCUSSION

As demonstrated in this paper, proteolysis is a useful method for the determination of unfolding constants. In comparison with measurements of unfolding kinetics by spectroscopic methods, it possesses significant advantages. First, it allows the determination of k_U independent of the rate and mechanism of refolding or isomerization equilibria of the unfolded protein. Second, the proteolytic method enables the experimental determination of unfolding constants at much lower temperature. The differences in spectroscopic signals such as UV absorbance, fluorescence emission, or CD signals are too small for exact kinetic measurements of RNase unfolding at temperatures where f_N is higher than 0.85. The proteolytic degradation by thermolysin, however, could be well followed down to 52.5 °C, where f_N values are 0.990 and 0.995 for RNase A and RNase B, respectively.

To our knowledge, the method of proteolysis for the determination of unfolding rate constants has been applied only by the group of Imoto for the denaturation of lysozyme, β-lactoglobulin, and RNase A at 40 and 50 °C (Imoto et al., 1986) and of chemically modified and nonmodified lysozyme at 40 °C (Yamada et al., 1993). These authors used Pronase, thermolysin, and chymotrypsin as proteases, and the protein degradation was quantified by HPLC. In our studies, the proteolytic method was combined with SDS−PAGE for the determination of thermal unfolding constants of RNase A

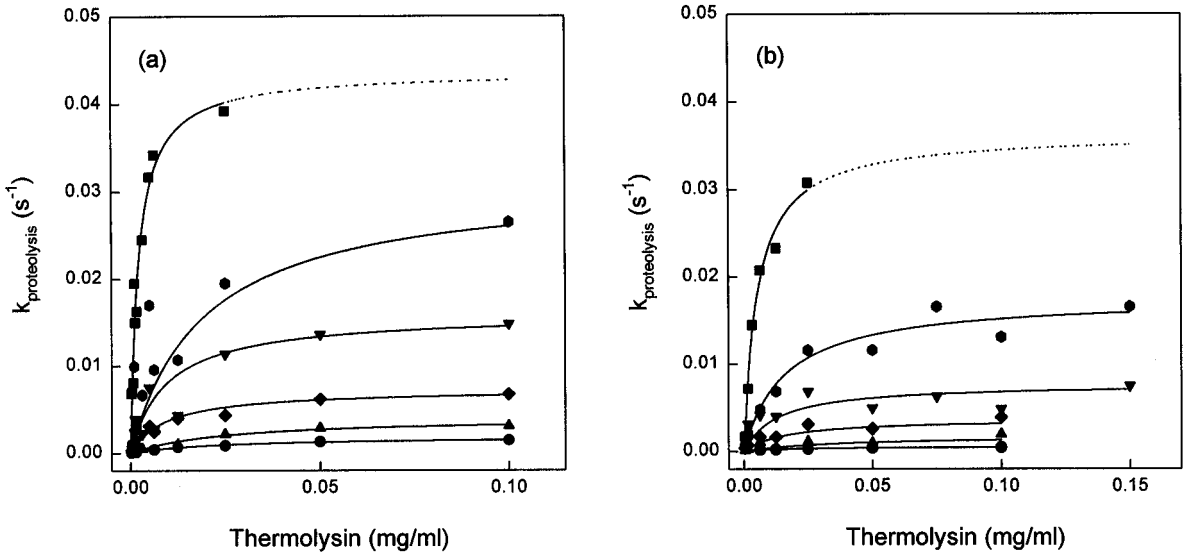

FIGURE 2: Rate constants of proteolysis by thermolysin for RNase A (a) and RNase B (b) at different temperatures. Temperatures were 52.5 (●), 55.0 (▲), 57.5 (◆), 60.0 (▼), 62.5 (⬢), and 65.0 °C (■). The dotted lines represent extrapolations according to eq 6. The unfolding constants k_U (s^{-1}) of RNase A and RNase B were determined to be 1.78×10^{-3} and 5.16×10^{-4} (52.5 °C), 3.96×10^{-3} and 1.75×10^{-3} (55.0 °C), 7.20×10^{-3} and 3.67×10^{-3} (57.5 °C), 1.59×10^{-2} and 7.65×10^{-3} (60.0 °C), 3.10×10^{-2} and 1.76×10^{-2} (62.5 °C), and 4.36×10^{-2} and 3.27×10^{-2} (65.0 °C).

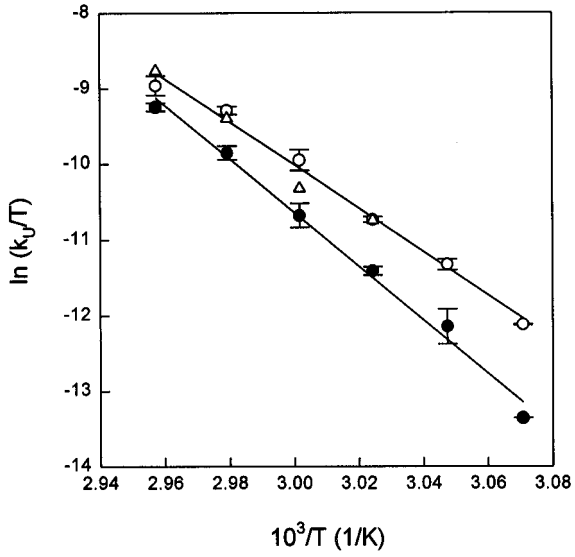

FIGURE 3: Eyring plot for the unfolding constants of RNase A determined by thermolysin (open circles) and trypsin (open triangles) and RNase B by thermolysin (filled circles).

and RNase B in the temperature range below and above the transition temperatures (52.5−65.0 °C). Thermolysin possessing at least 32 potential cleavage sites in both RNase species proved to be unspecific and stable enough to be suitable for the determination of unfolding constants for RNase A and RNase B (Figures 1−3). In the case of RNase A, thermal unfolding constants could be determined also with trypsin (Figure 3). The accordance of the unfolding constants determined with thermolysin and trypsin confirms the reliability of the method. The unfolding constants determined by Imoto et al. (1986) for RNase A at 50.0 °C and pH 8.0 [$(10.1 \pm 0.4) \times 10^{-4}$ s^{-1} by thermolysin and $(9.8 \pm 0.6) \times 10^{-4}$ s^{-1} by pronase] correspond to that calculated from our data [$(9.19 \pm 0.54) \times 10^{-4}$ s^{-1}]. The reason for the failing of trypsin in the experiments with RNase B is presumably found in the steric hindrance of proteolysis by the carbohydrate chain which is attached to the protein just at that position (Asn34) where the primary cleavage sites for trypsin (Lys31−Ser32 and Arg33−Asn34; Arnold et al., 1996) are located.

Interestingly, despite the small differences of kinetic stabilities between RNase A and RNase B ($\Delta\Delta G^{\ddagger}$ decreases from 3.0 ± 0.6 kJ mol^{-1} at 52.5 °C to 1.7 ± 0.5 kJ mol^{-1} at 65.0 °C), the energetic parameters $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ differ remarkably (Table 1). They show that the increase of the kinetic stability caused by glycosylation is due to enthalpic effects only, whereas entropic effects counteract stabilization but are overcompensated by the increase of enthalpy.

In contrast to the experiments with thermolysin and trypsin, which served for characterizing unfolding kinetics of RNase A and RNase B, studies with subtilisin aimed at the comparison of the native states of the two RNase species. Proteolysis did not follow first-order kinetics which suggests that the rate of proteolysis not only is determined by the first cleavage in the intact molecule (Ala20−Ser21) but also is influenced by additional factors, e.g., the presence of the cleavage products. Under all conditions, however, distinct differences in the proteolysis rates were found for RNase A and RNase B (Figure 4), suggesting that there is a higher flexibility of the sensitive region around residue 20 in RNase A. A decrease of the accessibility for the protease by steric hindrance due to the carbohydrate chain attached to Asn34 can be excluded, since in this case differences in the proteolytic rate constants between RNase A and RNase B should decrease with increasing protease concentration. In our experiments, however, both $k_{proteolysis}$ and $k'_{proteolysis}$ values diverge with increasing concentrations of subtilisin. These findings are in good agreement with the results obtained by Joao et al. (1992) and by Rudd et al. (1994), who concluded from differences in the NH−ND exchange rates of RNase A and RNase B as well as in the proteolysis by Pronase that glycosylation decreased dynamic fluctuation throughout the molecule.

Thermodynamic stabilities determined from UV spectroscopic equilibrium studies of unfolding (Table 2) reveal small differences (2.5 ± 0.2 kJ mol^{-1}) between RNase A and RNase B at the T_m of RNase A. A similar difference (2.3

Table 1: Energetic Parameters of the Kinetic Stabilities of RNase A and RNase B

	$\Delta H^{\ddagger}$ (kJ mol^{-1})	$\Delta S^{\ddagger}$ (J mol^{-1} K^{-1})	$\Delta G^{\ddagger}$ at T_m[a] (kJ mol^{-1})	$\Delta\Delta G^{\ddagger}$ at $T_{m(\text{RNase A})}$ (kJ mol^{-1})
RNase A (by trypsin)[b]	253	477	94	
RNase A (by thermolysin)	247 ± 9	460 ± 27	93.6 ± 0.2	
RNase B (by thermolysin)	284 ± 12	565 ± 35	94.9 ± 0.5	2.2 ± 0.3

[a] T_m values, see Table 2. [b] Determined from single run experiments.

Table 2: Parameters of Thermodynamic Stabilities of RNase A and RNase B

	T_m (°C)	ΔH_m (kJ mol^{-1})	ΔS_m (kJ mol^{-1} K^{-1})	$\Delta G_{60.4\ °C}$ (kJ mol^{-1})	$\Delta G_{25\ °C}$ (kJ mol^{-1})	T_{max} (°C)	ΔC_p (kJ mol^{-1} K^{-1})
RNase A	60.4 ± 0.1	574 ± 4	1.72 ± 0.01	0	42.6	4.6 ± 0.6	9.40 ± 0.06
RNase B	61.9 ± 0.1	576 ± 8	1.72 ± 0.02	2.5 ± 0.2	44.9	2.3 ± 1.4	8.78 ± 0.11

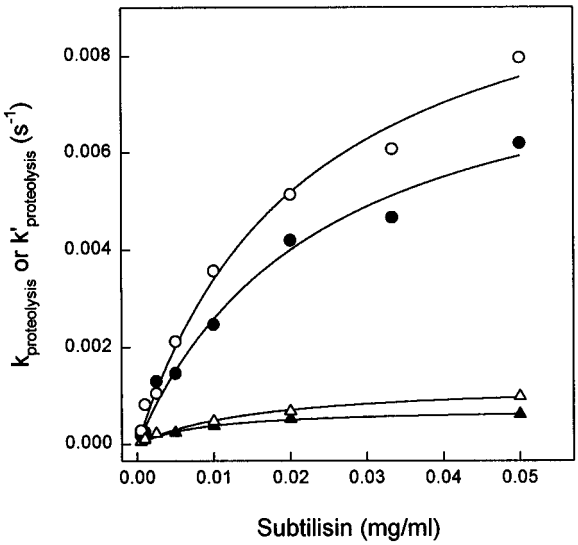

FIGURE 4: Proteolysis of RNase A (open symbols) and RNase B (filled symbols) by subtilisin at 25 °C. The constants of proteolysis $k_{proteolysis}$ (circles) and $k'_{proteolysis}$ (triangles) were determined according to eq 5.

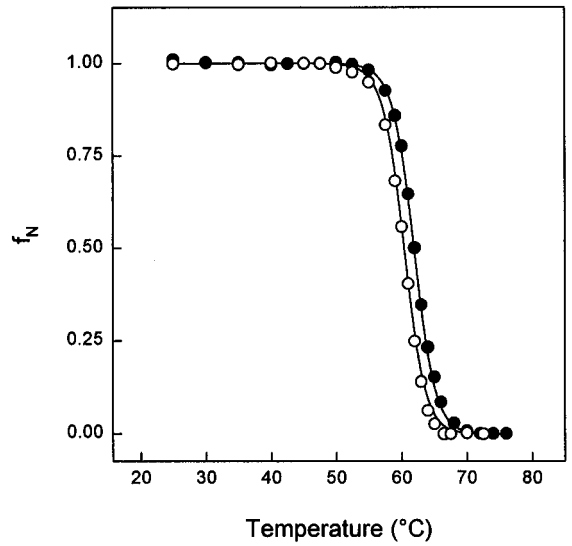

FIGURE 5: Thermal transition of RNase A (open circles) and RNase B (filled circles) by UV spectroscopy. The solid lines represent the f_N values calculated from the fitted ΔG values (Figure 7) according to eqs 15 and 12.

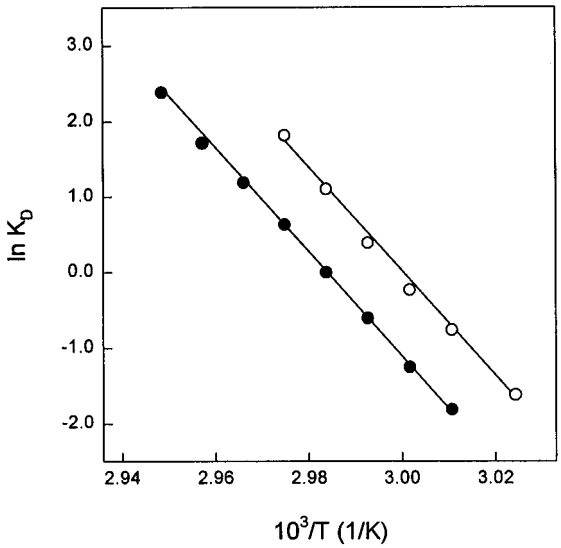

FIGURE 6: van't Hoff plot for thermal unfolding of RNase A (open circles) and RNase B (filled circles).

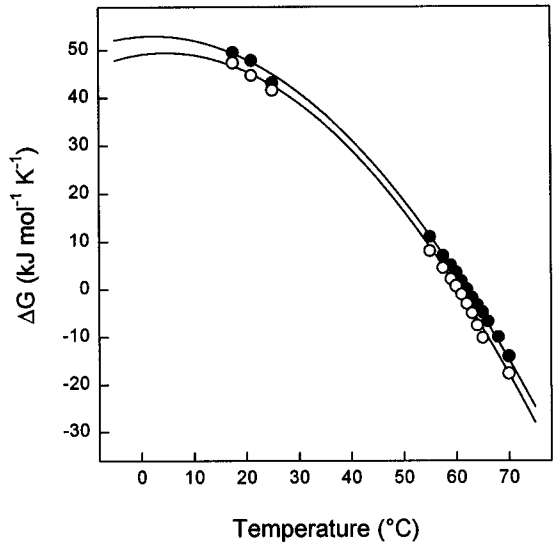

FIGURE 7: Thermodynamic stabilities (ΔG) of RNase A (open circles) and RNase B (filled circles) as a function of temperature.

kJ mol^{-1}) was estimated for 25 °C. These values are in accordance with results of measurements of GdnHCl-induced denaturation (Puett, 1973) and CD measurements of thermal unfolding (Joao & Dwek, 1993). An exact comparison of our values with those published in the literature, however, is not possible because the studies were performed at different pH values. In contrast to $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$, ΔH_m and ΔS_m characterizing energetic differences of the native and denatured states are very similar for RNase A and RNase B. As illustrated also in Figure 7, differences of thermodynamic stabilities are hardly changed by temperature, whereas kinetic stabilities converge at higher temperatures. With some precaution, it can be concluded from the

temperature dependence of ΔG that the temperature of maximum stability (T_{max}) for RNase B is somewhat lower than for RNase A (Table 2).

One of the most intriguing aspects in the comparison of RNase A and RNase B is the question whether thermodynamic stabilization by glycosylation is caused by a decrease of the free energy of the native state (stabilization of the native state) or by an increase of the free energy of the denatured state (destabilization of the denatured state) or both. Even if a final decision of this question cannot be given, the variant that only destabilization of the denatured state is the reason for higher stability can be excluded. In this case, $\Delta\Delta G^{\ddagger}$ should be zero, being not the case. A stabilization of the native state can be concluded from the results of proteolysis with subtilisin, while the distinct differences in the activation parameters $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ suggest that also the transition state of unfolding is changed due to glycosylation.

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