A carboxypeptidase Y pulse method to study the accessibility of the C-terminal end during the refolding of ribonuclease A

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Carboxypeptidase Y pulses, applied after various times of refolding, were employed to probe the accessibility of the C-terminus of RNAase A during the refolding process. The increase in resistance against proteolytic cleavage was measured by determination of the amount of liberated C-terminal amino acids and by activity assays. The results indicate that the C-terminus of RNAase becomes inaccessible early in the course of refolding, if folding is carried out at low temperatures under conditions that effectively stabilize the native state. At higher temperatures (25 °C) or under conditions of marginal stability, intermediates are not populated and protection against proteolytic cleavage is not detectable before the formation of the native state. The method described may be used to monitor the accessibility of the C-terminus of various proteins during refolding. However, intermediates on the folding pathway can only be observed if the native state is stable against carboxypeptidase attack.

INTRODUCTION

Bovine pancreatic RNAase A is a monomeric enzyme that can be refolded quantitatively from its unfolded form. Several unfolded forms coexist in denatured RNAase that differ by the conformation of their proline peptide bonds [1–6]. Under conditions that effectively stabilize the native state, 80 % of the unfolded RNAase molecules refold on the slow U_s -to-N pathway [5]. This major slow refolding reaction starts from an unfolded state that contains at least one incorrect proline isomer.

Two folding intermediates, I_1 and I_N , have been detected on the major U_s -to-N pathway. The first step U_s to I_1 leads to the protection of both the amide protons within the β -sheet of native RNAase [7–10] and the site Phe-120–Asp-121 against cleavage by pepsin [11]. Then a change in tyrosine absorbance monitors the formation of the native-like intermediate I_N , which is already active and stable against cleavage by trypsin. However, I_N still contains an incorrect Pro-93 isomer [5,12–14] and it is less stable than native RNAase [15,16]. Isomerization of Pro-93 occurs in the final I_N -to-N step and can be detected by tyrosine fluorescence [5,17]. Thus the minimal kinetic mechanism for the folding of U_s is:

Under conditions of marginal stability, the intermediates I_1 and I_N are not populated and refolding is very slow.

Here we probe the accessibility of the C-terminal end during refolding with the use of carboxypeptidase Y. Refolding is allowed to proceed for different times in the absence of the proteinase. Then a proteinase pulse is applied to determine the increase in resistance against proteolytic cleavage during the refolding process. The method is analogous to the proteinase pulse experiments

carried out previously with pepsin [11] and trypsin [18], but in contrast with those it can be easily transferred to other proteins. Possible intermediates, however, can only be detected if the C-terminus is buried in the native protein. In accordance with the pulse experiments carried out with pepsin as a proteolytic probe, the present results indicate that the C-terminal end of RNAase is involved in the structure of the early intermediate I_1 .

MATERIALS AND METHODS

Materials

RNAase A (type XII-A), *p*-hydroxymercuribenzoate, 2',3'-cyclic CMP and carboxypeptidase Y from yeast were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). *o*-Phthalaldehyde, triethylamine, Brij 35, acetic acid and acetonitrile (h.p.l.c. grade) were supplied by Fluka (Buchs, Switzerland). Urea was from Roth (Karlsruhe, Germany). All other reagents were analytical-grade substances from Merck (Darmstadt, Germany). Quartz-double-distilled water was used throughout.

Absorbance and fluorescence measurements of RNAase A refolding

A Cary 118C spectrophotometer with a thermostatically controlled cell holder was employed to monitor the absorbance change at 287 nm as a function of time during RNAase A refolding. To unfold RNAase A the freeze-dried protein was dissolved in 10 M-urea in 0.1 M-sodium acetate buffer, pH 6.3, and incubated for 1 h at 35 °C. At zero time, the unfolded RNAase A was diluted in 0.1 M-sodium acetate buffer, pH 6.3, to different final urea concentrations. Then the solution was thoroughly

Abbreviations used: RNAase A, bovine pancreatic ribonuclease A (EC 3.1.27.5) with disulphide bonds intact; U_s and U_f , slow-folding and fast-folding species of unfolded RNAase A; I_1 and I_N , folding intermediates of RNAase A.

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mixed before the measurement. The final RNAase A concentrations were the same as for the pulse experiments.

In addition to absorbance measurements, the fluorescence kinetics were monitored in the presence of 0.25 M-urea by using a Perkin MPF 44A fluorescence spectrophotometer with a thermostatically controlled cell holder. The instrument was run in the ratio mode with an excitation wavelength of 268 nm and an emission wavelength of 305 nm.

Carboxypeptidase Y pulse experiments

The freeze-dried carboxypeptidase was dissolved in water 1 day before use. In order to restore full activity, the solution had to be kept overnight at -20°C [19].

Unfolding and initiation of refolding was carried out as described above. After various times of refolding carboxypeptidase Y was added and proteolysis was allowed to proceed for 2 min. Carboxypeptidase Y action was stopped by addition of *p*-hydroxymercuribenzoate dissolved in 0.5 M- Na_2HPO_4 , pH 10.5, to a final concentration of 5 mM in the refolding assay. The enzyme is stoichiometrically inhibited by *p*-hydroxymercuribenzoate [19] and no further cleavage by carboxypeptidase Y was observed after this treatment. The reaction mixture was heated at 100°C for 10 min in order to precipitate the proteins. Then amino acid analysis was performed as described below. Competition experiments between refolding and cleavage by carboxypeptidase Y (experiments where the proteinase is already present at the beginning of refolding) were carried out in order to determine the time of cleavage and the amount of proteinase that is necessary to cleave all RNAase molecules at the beginning of refolding. Under marginally stable conditions the presence of 2 mg of carboxypeptidase Y/ml during the first 2 min of renaturation is sufficient to cleave all RNAase molecules. The concentration of RNAase in the refolding assay system can be varied between 3.75 mg/ml and 15 mg/ml without changing the results. A higher RNAase concentration is, however, recommended to avoid interference of residual urea, which disturbs the exact determination of small amounts of amino acids. In order to shorten the time of proteolysis pulse, a higher carboxypeptidase Y concentration can be used. But this may lead to more autolytic products, which can interfere with the correct determination of cleaved RNAase molecules.

Under all refolding conditions cleavage by carboxypeptidase Y is much slower than the fast U_F -to-N refolding reaction [20], by which 20% active RNAase A is generated. An RNAase concentration of 3.75 mg/ml and a carboxypeptidase Y concentration of 2 mg/ml at 25°C and of 2.5 mg/ml at 10.5°C was chosen to study the competition between cleavage and the slow folding reaction U_S to N. Under conditions that effectively stabilize the native state, only one-quarter of all slowly refolding molecules escape from cleavage of the last amino acid when carboxypeptidase Y is present already at the beginning of refolding.

Control experiments under identical final conditions starting with native RNAase A confirm that folded RNAase A is not attacked by carboxypeptidase Y.

The fraction of uncleaved RNAase A can also be determined by measuring the enzymic activity after complete refolding (30 min) using assays with 2',3'-cyclic CMP. In these experiments the activity of RNAase A is assayed without inactivating carboxypeptidase Y.

Amino acid analysis

Amino acid analysis was performed by using pre-column reaction with *o*-phthalaldehyde and h.p.l.c. The h.p.l.c. apparatus included the following Spectra Physics

Fig. 1. (a) Elution profile of *o*-phthalaldehyde derivatives of an amino acid standard mixture and (b) linearity of response for the *o*-phthalaldehyde derivatives of alanine ($\blacktriangle$), aspartic acid (∇), serine ($\circ$) and valine ($\bullet$)

Chromatographic conditions: solvent A, 18.75 mM-triethylamine, pH 7.5; solvent B, acetonitrile (h.p.l.c. grade); linear gradient from 0% to 38% (v/v) solvent B over 38 min, then from 38% to 70% (v/v) solvent B over 10 min; flow rate, 2.0 ml/min; column, Serva Si 100 Polyol RP 18 ($5\ \mu\text{m}$ particle size) ($4.6\ \text{mm} \times 250\ \text{mm}$).

(San José, CA, U.S.A.) equipment: an SP 8700 solvent delivery system, an SP 8750 organizer and an SP 4270 integrator. A Gilson Spectra-Glo fluorimeter (Gilson Medical Electronics, Middleton, WI, U.S.A.) with a 20 μ l flow cell and filters for the excitation at 360 nm and the emission at 455 nm was used to detect the fluorescent *o*-phthalaldehyde-amino acid adducts. A Serva h.p.l.c. precision column (4.6 mm $\times$ 250 mm) with Servachrom packing Si 100 Polyol RP 18 (5 μ m particle size) was applied for the separation of the amino acid adducts. The *o*-phthalaldehyde reagent was prepared as described by Larsen & West [21]. Formation of *o*-phthalaldehyde derivatives was carried out in the following manner. A 40 μ l portion of the Millipore-filtered *o*-phthalaldehyde reagent was placed in a micro test tube. A 20 μ l sample of the probe was added and then mixed for 20 s. After 1 min 10 μ l of the reaction mixture was injected into the h.p.l.c. apparatus. All sample injections were performed with a Rheodyne injection valve (model 7120) equipped with a 10 μ l loop. Immediately after injection of the reaction mixture the gradient was started. The aqueous portion of the mobile phase consists of filtered 18.75 mM-triethylamine, pH 7.5. The organic component of the mobile phase was acetonitrile. All solvents, both aqueous and organic, were degassed with He before use. A linear gradient was applied starting at injection (time zero) from 0% to 38% (v/v) acetonitrile over 38 min and then from 38% to 70% (v/v) acetonitrile over 10 min. Immediately after termination of the gradient, the column was washed with 100% acetonitrile for 5 min. All chromatographic runs were made at room temperature at a solvent flow rate of 2 ml/min.

Different crystalline amino acids were dissolved in water. Their *o*-phthalaldehyde derivatives were formed and then analysed in order to determine the relative retention time of each. A 2.5 μ M standard amino acid solution (AA-S-18 from Sigma, Munich, Germany) was diluted 1:5 with water and then used to determine a complete profile of all amino acids (Fig. 1a).

The C-terminus of RNAase A is characterized by the

sequence Asp-121-Ala-122-Ser-123-Val-124. As shown in Fig. 1(b), all C-terminal amino acids can be detected quantitatively. To maintain the reproducibility, the conditions for derivative formation and h.p.l.c. elution were always exactly the same.

RESULTS AND DISCUSSION

Even under marginally stable conditions (1.5–5 M-urea in the assay medium) native RNAase A is resistant against carboxypeptidase Y attack. The extent of cleavage by carboxypeptidase during refolding of RNAase was measured either by the amount of released C-terminal valine after the proteinase pulse by h.p.l.c. or by the amount of unmodified RNAase by measuring the activity after refolding has gone to completion (after 30 min). The coincidence of the results obtained by the two methods indicates that, although it is still partially active [22], des-Val-124-RNAase is sensitive to carboxypeptidase Y and is further degraded during the time of incubation (30 min). Indeed, amino acid analysis of these samples shows an almost complete degradation.

We have studied the accessibility of the C-terminal end during RNAase A refolding under different refolding conditions where the native protein is either only marginally stable, such as in the presence of high urea concentrations, or very stable, at 25 °C and 12.5 °C.

Fig. 2 shows the increase in resistance against carboxypeptidase cleavage during refolding of RNAase A in the presence of 1.5 M-, 3 M- and 5 M-urea compared with the kinetics of the absorbance change during refolding under the same marginally stable conditions. Under these conditions refolding is very slow, and there is no evidence for any folding intermediates [5,11,17]. The kinetics of the protection against carboxypeptidase Y cleavage correspond to the formation of the native state as measured by absorbance. Protection against proteolytic cleavage does not occur before the rate-limiting step of refolding of U to N.

At 25 °C under strongly native conditions, no

Fig. 2. (a) Increase in resistance towards carboxypeptidase Y cleavage during refolding of RNAase A in the presence of urea and (b) elution profiles of released valine after various times of refolding in the presence of 3 M-urea

(a) Increase in resistance towards carboxypeptidase Y cleavage during refolding of RNAase A in the presence of 5 M- (●), 3 M- (▲) and 1.5 M-urea (■). Absorbance-detected folding kinetics under the same conditions (○, △ and □) are included for comparison. The kinetic progress curves are matched so that initial and final values coincide. (b) Elution profiles of released valine after various times of refolding in the presence of 3 M-urea. The profile obtained after 10 min refolding can be attributed to the autolytic digestion of carboxypeptidase Y.

Fig. 3. Increase in resistance towards carboxypeptidase Y during the slow refolding pathway of RNAase A in the presence of 0.25 M-urea at 25 °C

Refolding was initiated at zero time by a 40-fold dilution of unfolded RNAase A. Carboxypeptidase Y was added after the indicated time intervals, and the competition between unfolding and cleavage was allowed to proceed for 2 min. The residual RNAase activity (○) and the determined amount of valine (●) is shown as a function of the time when the proteinase was added. Absorbance-detected (▼) and fluorescence-detected (▽) folding kinetics are included for comparison. The kinetic progress curves are matched so that initial and final values coincide.

difference is perceptible between the absorbance-detected formation of I_N , the fluorescence-detected formation of the native state and the regain of stability against carboxypeptidase cleavage, as shown in Fig. 3. At this temperature intermediates are not populated and all kinetics coincide.

Fig. 4. Increase in resistance towards carboxypeptidase Y cleavage during the slow refolding reaction of RNAase A in the presence of 0.25 M-urea at 10.5 °C

All details are as described in Fig. 3 legend.

Under the same strongly native conditions but at 10.5 °C, the sensitivity towards cleavage of the last amino acid residue Val-124 is lost during refolding more rapidly than the absorbance change related to the formation of the native-like intermediate I_N (Fig. 4). Under comparable conditions the time course of the increase in resistance against carboxypeptidase cleavage is very similar to the time course of the loss of the Phe-120–Asp-121 peptic cleavage site [11] and to the time course of the decrease of incorporation of tritium label into refolding RNAase [19]. This suggests that the formation and stabilization of a structure around the C-terminus are associated with the formation of hydrogen-bonded structures during the refolding process. Recently, a study of the hydrogen-exchange reaction between the protein amide groups and solvent has shown that essentially the β -sheet of native RNAase A, which includes the C-terminus, is formed in an early step of refolding [10].

In accordance with these results, the carboxypeptidase Y pulse experiments prove the importance of the C-terminal region of RNAase A for the pathway of folding as well as for the stability of the native protein.

CONCLUSION

This paper presents a new method for studying the accessibility of the C-terminus of a polypeptide chain during refolding involving proteolytic cleavage by carboxypeptidase Y, a proteinase that shows a broad specificity and a high stability in urea [19]. The released amino acids can be determined quickly and quantitatively after pre-column reaction with *o*-phthalaldehyde by using a reversed-phase h.p.l.c. column and detection by fluorescence. Several precautions and limitations of the method must be considered. First, the native protein must be stable towards the proteinase. Moreover, it is important to start the refolding of the protein in the absence of the proteinase in order to avoid interference of proteinase binding with the folding process itself. The amount of protein that is proteinase-resistant can be determined by proteinase pulse after various times of refolding. This method was tested by using RNAase A as a model, but it can be easily transferred to other proteins.

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