CD PROPERTIES OF THE FAST- AND SLOW-FOLDING FORMS OF UNFOLDED RIBONUCLEASE A

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1. Introduction

Under unfolding conditions several small proteins do not exist as a single species, but are composed of an equilibrium mixture of fast-refolding (U_F) and slow-refolding (U_S) molecules [1–4]. To explain the molecular mechanism of this observation, the proline model of protein folding was proposed, which suggests that the slow isomerization about peptide bonds preceding proline is responsible for the formation of slow-folding species after unfolding [5]. For RNase A it could be shown that the kinetics of the $U_F \rightleftharpoons U_S$ equilibration reaction after the fast protein unfolding step $N \rightarrow U_F$ shows all properties expected for the *cis* $\rightleftharpoons$ *trans* isomerization of prolyl residues as known from studies on model compounds with X–Pro bonds [6].

An important feature of the proline isomerization model is that U_F is not a partially folded intermediate, but that both U_F and U_S are completely unfolded molecules, which only differ in the configuration of X–Pro peptide bonds. This part of the model is supported by experiments, which show that:

(i) the $U_F \rightleftharpoons U_S$ equilibrium is not shifted to U_S by high temperatures or by increasing concentrations of denaturants such as 6 M GuHCl or 8.5 M urea [1,7];

(ii) >97% of the change in tyrosine absorbance upon unfolding occurs in the $N \rightarrow U_F$ step; U_F and U_S both show absorbance properties of the unfolded protein [8,9].

Abbreviations: RNase A, bovine pancreatic ribonuclease A (EC 3.1.27.5) with disulfide bonds intact; N. native RNase A; U_S, U_F, slow- and fast-folding species of unfolded RNase A, respectively; GuHCl, guanidine hydrochloride; CD, circular dichroism; τ, time constant of a chemical reaction (reciprocal of the apparent rate constant)

However, in [9] tyrosine fluorescence of U_S was ~20% higher than the emission of U_F. This difference was tentatively ascribed to strictly local changes in the environment of one tyrosine residue, coupled to proline isomerization in the unfolded polypeptide chain. However, it could not be excluded definitely that the fluorescence difference between U_F and U_S was caused by the presence of residual ordered structures in U_F in contradiction to the assumption of the proline model and to [1,5,7]. Measurements of the circular dichroism in the amide region provide the most useful spectroscopic approach to detect such residual ordered structure. In this study the far-UV CD spectra of U_F and U_S are determined together with the CD spectrum of the native protein. A comparison of these 3 spectra shows that within a few percent, U_F and U_S are identical in their state of unfolding, as far as secondary structures are concerned.

2. Materials and methods

Ribonuclease A (type XII A, lot no. 49C-8047) was purchased from Sigma (St Louis MO); GuHCl (ultrapure) from Schwarz-Mann (Orangeburg NY); sodium cacodylate from Serva (Heidelberg).

RNase A concentrations were determined spectrophotometrically using a molar absorbance at 278 nm of 9800 $M^{-1} \cdot cm^{-1}$ [10]. Measurements of ellipticity were carried out with a JASCO J500A spectropolarimeter equipped with a model DP 500N data processor. All spectra and CD kinetics were measured in 1 mm cells at 10°C. The spectrum of U_F was recorded at a fast rate (50 nm/min), starting 15 s after the $N \rightarrow U_F$ unfolding step. The spectrum of the equilibrium mixture of 20% U_F and 80% U_S was recorded 15 min after unfolding using the same sample. Kinetic unfolding

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experiments were started by a 20-fold dilution of native RNase A (in 0.1 M Na-cacodylate (pH 6.0)) into a solution of 6.3 M GuHCl in 0.1 M HCl, (pH 1) or 6.3 M GuHCl in 0.1 M Na-cacodylate (pH 6), pre-equilibrated in the CD cell. Changes in ellipticity were recorded at 225 nm with a band width of 2 nm.

3. Results and discussion

At low pH and high concentrations of GuHCl the unfolding reaction of RNase A ($N \rightarrow U_F$) is very fast [9,11] compared to the subsequent isomerization reaction ($U_F \rightleftharpoons U_S$). At pH 1, 6 M GuHCl, 10°C the $N \rightarrow U_F$ step is complete within the time required for manual mixing, whereas the $U_F \rightleftharpoons U_S$ reaction shows $\tau = 200$ s [6]. These unfolding conditions were used to transiently populate U_F and to monitor the CD spectrum of this species. The resulting spectrum, recorded 15 s after unfolding from 240–210 nm with a high scan speed of 50 nm/min is shown in fig.1b. The spectrum was complete 45 s after initiating unfolding. At this time >80% of the unfolded molecules were still in the U_F state. The $U_F \rightleftharpoons U_S$ equilibration reaction is over after 15 min and the CD spectrum of the resulting equilibrium mixture of 20% U_F and 80% U_S is recorded (fig.1c). For a comparison, the molar ellipticity of native RNase A was also measured at 10°C and included into fig.1a. Inspection of the spectra in fig.1 shows that almost the entire change in ellipticity occurs during the fast $N \rightarrow U_F$ unfolding reaction, which produces the fast-folding species U_F. The CD spectrum of U_F is already almost identical to the spectrum of the 20% U_F/80% U_S mixture, present in unfolding conditions at equilibrium. There is a small deviation in the range of 220–230 nm, which is maximal at ~225 nm. At this wavelength the difference between spectra b and c is only ~3% of the total change in ellipticity upon unfolding (fig.1a,c). This effect could be caused by slight differences in secondary structure but there are other possible sources for CD changes as well, such as changes in the orientation of aromatic side-chains [12].

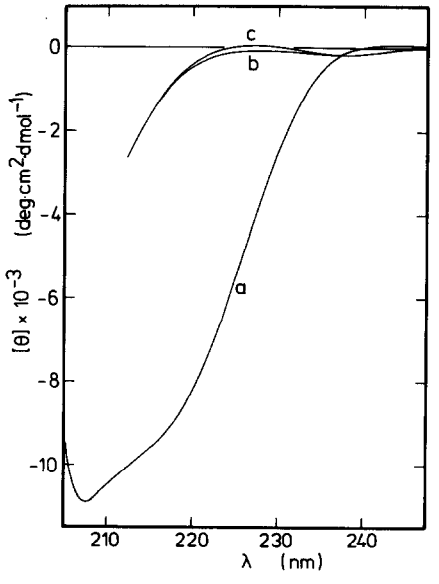

Fig.1. Molar ellipticity of native and unfolded forms of RNase A: (a) Native RNase A in 0.1 m Na-cacodylate, pH 6, 10°C; (b) ellipticity of fast-folding species U_F. Recording was started 15 s after unfolding at pH 1, 6 M GuHCl, 10°C; (c) ellipticity of the equilibrium mixture of 20% U_F and 80% U_S, recorded 15 min after unfolding at pH 1, 6 M GuHCl, 10°C. RNase A was 32 μM.

In a second set of experiments the time dependence of the small changes in ellipticity occurring in parallel to the $U_F \rightleftharpoons U_S$ reaction was monitored at 225 nm. Three kinetic measurements were performed:

(i) To get the total change in ellipticity, unfolding was carried out at pH 6, 10°C and 5 M GuHCl. Under these conditions (at neutral pH) the $N \rightarrow U_F$ reaction is slow ($\tau = 200$ s) [9] and yields the entire amplitude of unfolding (fig.2a);

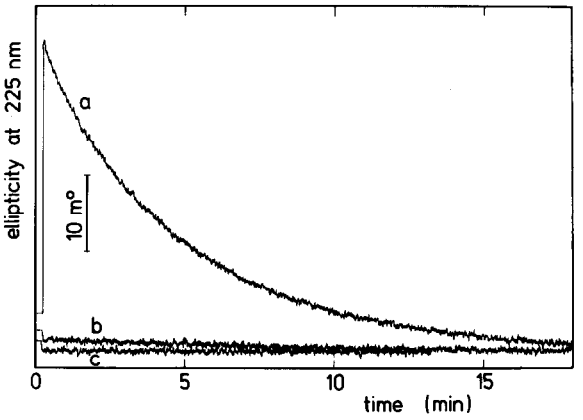

Fig.2. Kinetic changes of ellipticity at 225 nm upon unfolding of RNase A. Unfolding conditions: (a) 5 M GuHCl, 0.1 M Na cacodylate, pH 6, 10°C; (b) 6 M GuHCl, 0.1 M HCl, pH 1, 10°C. Final concentration of RNase A: 85 μM. Unfolding was initiated by a 20-fold dilution of native RNase A (in 0.1 M Na cacodylate, pH 6) into the final unfolding solution. (c) Mixing control experiment: RNase A, unfolded at equilibrium by 6 M GuHCl, pH 1 was diluted 20-fold with 6 M GuHCl, pH 1, at 10°C.

(ii) Unfolding at acid pH (pH 1, 6 M GuHCl, 10°C), where the $N \rightarrow U_F$ step is extremely fast, results in an immediate jump in ellipticity (which accounts for almost the entire CD change) and subsequent slow kinetics with a minor amplitude equivalent to the transition from b–c in fig.1. These kinetics are shown in fig.2b.

(iii) To exclude mixing or temperature artifacts, unfolded RNase A (in 6 M GuHCl) was diluted into 6 M GuHCl, pH 1 under identical experimental conditions as were employed in expt (ii). Fig.2c shows that after the deadtime of ~20 s the CD signal remains constant.

The kinetic results in fig.2 demonstrate that unfolding of RNase A at pH 1 and 6 M GuHCl results in an instant loss in ellipticity during the fast $N \rightarrow U_F$ unfolding reaction followed by a slow reaction which lies in the time range of the $U_F \rightleftharpoons U_S$ reaction with an amplitude of ~3% of the total change. There are no slow changes of ellipticity visible, when unfolding is monitored at 210 nm or at 235 nm, as expected from the spectra in fig.1.

4. Conclusions

(1) The fast-refolding (U_F) and the slow-refolding (U_S) forms, which coexist in unfolded RNase A differ by ~3% in ellipticity in the wavelength region of amide absorption. Hence both species are unfolded to approximately the same extent as assumed by the proline model of generation of slow-folding molecules [5,6] and as expected from the kinetics of RNase A folding [1,7,11].

(2) The difference in ellipticity between U_F and U_S is significant, but it cannot be interpreted in molecular terms, as the effect is very small.

(3) The results corroborate the explanation of the difference in tyrosine fluorescence between U_F and U_S given previously. The effect was discussed to be caused by strictly local changes around one or more tyrosine residues caused by isomerization of neighbouring proline peptide bonds in the unfolded chain [9].

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