Inactivation during denaturation of ribonuclease A by guanidinium chloride is accompanied by unfolding at the active site

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Inactivation of pancreatic RNAase A occurs in guanidinium chloride (GdmCl) at low concentrations before the unfolding of the molecule as a whole can be detected [Liu and Tsou (1987) *Biochim. Biophys. Acta* **916**, 455–464]. We have now shown that the rate of digestion of the RNAase molecule by either trypsin or proteinase K increases significantly at low concentrations of GdmCl where the enzyme is largely inactivated, but fluorescence

and absorption measurements reveal no conformational changes. N-Terminal sequence analysis of the peptide fragments generated shows that proteolysis occurs primarily at or near the active site. The decrease in activity of RNAase at low concentrations of GdmCl is therefore due to partial unfolding of the molecule, particularly at the active site and not to an inhibition by the denaturant.

INTRODUCTION

In a series of studies from this laboratory (Yao et al., 1984; Tsou, 1986, 1993; Liu and Tsou, 1987) on the comparison of activity and conformational changes of enzymes during unfolding by chemical denaturants, we have shown that inactivation generally precedes unfolding of the molecules as a whole, leading to the suggestion that enzyme active sites are situated in limited and flexible regions and are therefore more easily perturbed than the rest of the molecules. However, it has been suggested by some authors that inhibition by guanidinium chloride (GdmCl) rather than partial unfolding at the active site of the enzyme molecules could be responsible for the activity decrease observed in dilute GdmCl solutions (Creighton, 1990; Kelly et al., 1993). Recent results from this laboratory on D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Lin et al., 1990) and adenylate kinase (Zhang et al., 1993) show that, during thermal denaturation of these enzymes, inactivation also precedes significant conformation change and is most probably due to partial unfolding because of the absence of an inhibitor. Probes introduced at the active sites of GAPDH (Xie and Tsou, 1987) and creatine kinase (Zhou et al., 1993) have actually demonstrated conformational changes at the active sites of these enzymes at low concentrations of GdmCl before gross conformation changes of the molecules as a whole can be observed.

Although in a previous paper (Liu and Tsou, 1987), evidence has been presented to show that the decrease in activity of RNAase is very likely due to partial unfolding rather than inhibition by GdmCl, the latter cannot be completely ruled out, especially as GdmCl inhibition of RNAase activity was recorded in the literature (Anfinsen et al., 1955). It is now shown that the susceptibility of RNAase to digestion by trypsin or proteinase K is markedly increased in dilute GdmCl and sequence analysis of the peptide fragments liberated suggests conformation change at the active site concurrently with inactivation during GdmCl denaturation.

MATERIALS AND METHODS

Materials

Bovine pancreatic RNAase (type III-A), trypsin [type XIII, tosylphenylalanylchloromethane ('TPCK')-treated], proteinase K from *Tritirachium album*, 2':3'-cyclic CMP, o-phthalaldehyde (OPTA), fluorescamine {4-phenylspiro[furan-2(3H),1'-(3'H)-isobenzofuran]-3,3'-dione}, dithiothreitol (DTT) and SDS were Sigma products. N^ε-benzoyl-L-arginine ethyl ester (BAEE) and N^ε-benzoyl-L-tyrosine ethyl ester (BTEE) were from Dongfeng Biochemicals, Shanghai, China. GdmCl was obtained from ICN Biochemicals. Bio-Gel P-2 was from Bio-Rad.

Concentrations of RNAase (ϵ_{284}^1 , 9700 M⁻¹·cm⁻¹), trypsin ($A_{280,1\text{cm}}^{1\%}$ 14.4) and proteinase K ($A_{280,1\text{cm}}^{1\%}$ 14.2) were determined by measuring the absorbance.

Digestion of RNAase by trypsin and proteinase K in GdmCl

Unless otherwise specified, digestion of RNAase A with trypsin was carried out in 0.05 M Tris/HCl (pH 7.8)/0.01 M CaCl₂ at 35 °C. The reaction system contained 0.1 mM RNAase and 0.01 mM trypsin with a molar ratio of 10:1 at the GdmCl concentrations indicated. A high molar ratio of trypsin to RNAase had to be used because of the decreased activity of trypsin in GdmCl. Digestion with proteinase K was carried out, unless otherwise specified, in 0.01 M Tris/HCl buffer, pH 8.0, with a molar ratio of RNAase to proteinase K of 40:1 at GdmCl concentrations as indicated and the mixture was thermostatically controlled in an ice bath. To stop the proteolytic reaction, 0.03 M phenylmethanesulphonyl fluoride (PMSF) in methanol as an inhibitor of proteinase K was used with the molar ratio of 1200:1. In both cases the disulphide bonds in the digestion products were reduced by adding DTT with a molar ratio of RNAase of 5:1 before chromatographic separations.

Abbreviations used: BAEE, N^ε-Benzoyl-L-arginine ethyl ester; BTEE, N^ε-benzoyl-L-tyrosine ethyl ester; DTT, 1,4-dithiothreitol; GAPDH, D-glyceraldehyde-3-phosphate dehydrogenase; GdmCl, guanidinium chloride; OPTA, o-phthalaldehyde; PMSF, phenylmethanesulphonyl fluoride; TFA, trifluoroacetic acid.

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H.p.l.c. and f.p.l.c. systems

Analysis of the trypsin digestion products of RNAase was carried out with a Beckman ODS reverse-phase column on a Beckman Gold h.p.l.c. system and eluted with a linear gradient of 0.1 % trifluoroacetic acid (TFA) in water and acetonitrile at a flow rate of 1 ml/min and the effluent was monitored at 214 nm. Separation of peptide fragments from the digestion products was performed on a Pharmacia f.p.l.c. system using a phenyl-Superose HR 5/5 column with a linear gradient of decreasing ionic strength in 0.1 M potassium phosphate buffer, pH 7.0, from 1.7 M $(\text{NH}_4)_2\text{SO}_4$ to the same buffer alone, with the detector set at 214 nm. Each peak was collected, pooled from several separations when necessary, dialysed against distilled water in dialysis tubing (molecular-mass cut-off < 3500) and freeze-dried before being subjected to N-terminal sequence analysis.

The intact RNAase remaining in the proteinase K digestion mixture was determined by the f.p.l.c. profile with a PepRPC column. Separation of the peptide fragments was performed on a Beckman Gold System using a Waters ODS column. In both cases elution conditions were the same as in analysis of trypsin digestion products.

Fluorescence labelling of the N-terminal residues

The OPTA labelling of the trypsin digestion products was carried out in 0.05 M borate buffer (pH 7.8)/0.01 M CaCl_2 (Cronin and Hare, 1977). To 300 μl aliquots of the digestion mixture, 30 μl of 0.6 M OPTA in methanol and 2.6 μl of 2-mercaptoethanol were added with stirring and then diluted with 2.7 ml of buffer. Fluorescence emission at 452 nm was measured with a Hitachi-MPF spectrofluorimeter with excitation at 358 nm. Fluorescamine labelling (Weigele et al., 1972) was achieved with fluorescamine dissolved in anhydrous acetonitrile at a concentration of 0.06 M, and 60 μl of the solution was added to 3 ml of the 100-fold-diluted digestion mixture with vigorously stirring. The fluorescence emission was measured at 488 nm with an excitation wavelength of 395 nm.

The labelling of the proteinase K digestion products was done in a similar way, the concentration of RNAase were 0.01 and 0.001 mM in OPTA and fluorescamine modification respectively.

Electrophoresis

Electrophoresis was performed on a Pharmacia Phast System using PhastGel 8-25. The digestion mixtures, containing various concentrations of GdmCl, were passed through a desalting column packed with Bio-Gel p-2 gel (fine) to remove GdmCl and then freeze-dried. Samples were dissolved in 0.05 M Tris/HCl (pH 7.8)/2.5 % SDS/1 % DTT for electrophoresis, and staining was with Coomassie Blue.

Determination of N-terminal sequence

N-terminal sequences of the fragments were determined with a Milli Gen-6600 Microprotein Sequencer through Edman auto-degradation.

Activity measurements of the enzymes

Ribonuclease activity of the digestion mixture was determined by the method of Crook et al. (1960) by monitoring changes in A_{284} with 0.1 mg/ml CTP as the substrate in 0.05 M Tris/HCl, pH 7.8, for trypsin and 0.01 M Tris/HCl, pH 8.0, for proteinase K digestion respectively. Activities of trypsin at pH 7.8, 35 °C, and proteinase K at pH 8.0, 30 °C, were measured with BAEE and

BTEE as substrates respectively by the pH-stat method (Walsh and Wilcox, 1970).

RESULTS

Decrease of native RNAase during trypsin digestion in GdmCl

RNAase loses about 50 % of its activity without any detectable change in either the intrinsic fluorescence or u.v. absorbance, and very little activity remains in 2 M GdmCl, where the molecule appears to be still largely folded (Liu and Tsou, 1987). During the course of tryptic hydrolysis at different concentrations of GdmCl, the amounts of intact RNAase molecule remaining as detected by the h.p.l.c. profile of the reaction mixtures are shown in Figure 1(a). Native RNAase is fairly resistant to trypsin digestion in the absence of GdmCl, with only a slight decrease after 10 h, and GdmCl increases the rate of digestion strikingly. The increase in the digestion rate is not due to an activation effect of GdmCl on trypsin, as control experiments show that the activity of trypsin towards BAEE decreases significantly in GdmCl and is only about 35 % as active in 2 M GdmCl as in its absence (Figure 2). The difference between the digestion rates of native and partially denatured RNAase can therefore only be explained by changes in the RNAase molecule, most probably an opening up of its folded structure. A comparison with the results

Figure 1 Trypsin digestion of RNAase in GdmCl

The digestion mixtures containing 0.1 mM RNAase, 0.01 mM trypsin in 0.05 M Tris/HCl/0.01 M CaCl_2 , pH 7.8 and different concentrations of GdmCl as indicated were incubated at 35 °C. Aliquots were taken at different time intervals. (a) Decrease of RNAase by h.p.l.c. analysis with the effluent monitored at 214 nm after the addition of DTT. (b) Activity change. Aliquots were diluted 100-fold for assay of RNAase activity.

Figure 2 Trypsin activity in different concentrations of GdmCl

Trypsin was incubated with different concentrations of GdmCl for 5 h in 0.01 M Tris/HCl, pH 7.8. Assays were made on a pH-stat thermostatically controlled at 35 °C by titration with 0.1 M NaOH during the hydrolysis of 0.01 mM BAEE.

of Liu and Tsou (1987) shows that in increasing concentrations of GdmCl, the increase in the digestion rate by trypsin parallels the inactivation of RNAase and precedes significant conformation changes of the molecule as a whole.

Activity decrease during trypsin digestion in GdmCl

The time courses of activity decrease during trypsin digestion of RNAase in different concentrations of GdmCl are given in Figure 1(b). The inactivation of RNAase by GdmCl has been shown to be completely reversible (Liu and Tsou, 1987), and, as the reaction mixture was diluted 100-fold for activity measurements, the effect of any remaining GdmCl can be neglected. Control experiments show that RNAase activity with cyclic CTP as substrate is stable by incubation with trypsin for 9 h in the absence of GdmCl. In general the result of activity measurement is in agreement with the intact RNAase molecule remaining as shown above. However, by closer comparison of Figures 1(a) and 1(b), it can be seen that, in 0.5 or 1 M GdmCl, the activity decrease is somewhat slower than the disappearance of intact RNAase. It is possible that some of the partly digested enzyme can have some residual activity. In this connection it is known that the fragments of RNAase produced by subtilisin digestion can form an enzymically active complex (Kalman et al., 1955).

Liberation of peptide fragments during trypsin hydrolysis

The time course of the liberation of digestion products during tryptic proteolysis of RNAase in the presence of GdmCl has been examined by the generation of free amino groups and by the electrophoresis pattern of the reaction mixture. Both OPTA and fluorescamine have been used to detect free N-terminal amino groups liberated. Figure 3 shows the increase of fluorescence emission of OPTA-modified fragments produced by trypsin digestion of RNAase in different concentrations of GdmCl. Similar results (not shown) were obtained by reaction with fluorescamine. In both cases very few free amino groups are liberated in the absence of GdmCl, and the rates of amino-group liberation increase significantly with the increase of GdmCl concentration. The above results are in general agreement with the decrease both in activity and in intact RNAase molecules at different GdmCl concentrations.

The results of electrophoresis of the trypsin digestion products are shown in Figure 4(a). In agreement with the results presented above, RNAase digested by trypsin for 5 h at 35 °C shows only

Figure 3 Liberation of free amino groups during trypsin digestion in GdmCl

Conditions for trypsin digestion were as for Figure 1, except that 0.05 M borate and 0.01 M CaCl₂, pH 7.8, were used with GdmCl concentrations as indicated. Aliquots were taken at time intervals for reaction with OPTA (for details, see the text). Fluorescence emission was measured at 452 nm with excitation at 358 nm.

a faint band of hydrolysis product, but, in the presence of 0.5 or 1 M GdmCl, at least three fragments can be clearly seen, together with the decrease of unhydrolysed RNAase in the digestion mixture.

Decrease of native RNAase during proteinase K digestion in GdmCl

Similar results were obtained in proteinase K-catalysed proteolysis of RNAase. It is known that proteinase K is more resistant to GdmCl denaturation than trypsin and about 60 % of the original activity remains with BTEE as substrate, even in 5 M GdmCl (results not shown). RNAase is hydrolysed by proteinase

Figure 4 SDS/PAGE of RNAase digested by trypsin or proteinase K in different concentrations of GdmCl

(a) Trypsin digestion. Conditions were as for Figure 1. Before electrophoresis, the digestion mixtures were gel-filtered on Bio-Gel P-2 gel columns to remove GdmCl (for details, see the text). Lane a, RNAase alone; lane b, RNAase with trypsin; lane c, as lane b, but with 0.5 M GdmCl; lane d, as lane b, with 1 M GdmCl. (b) Proteinase K digestion. The reaction mixture contained 0.074 mM RNAase and 0.0018 mM proteinase K in 0.01 M Tris/HCl, pH 8.0, with different GdmCl concentrations as indicated and was kept in an ice bath for 1 h. PMSF was then added to stop the reaction and, after removal of GdmCl as described in the text, samples were taken for electrophoresis. Lane a, RNAase alone; lane b, RNAase with proteinase K; lane c, as lane b, but with 0.5 M GdmCl; lane d, as lane b, but with 1 M GdmCl; lane e, as lane b, but with 2 M GdmCl. In both cases, the sample was run on a PhastGel 8-25 and stained with Coomassie Blue.

Figure 5 Proteolysis of RNAase by proteinase K in GdmCl

Conditions for proteinase K digestion were as for Figure 4(b), with GdmCl concentrations as indicated. Aliquots were taken at different time intervals. (a) Intact RNAase remaining was detected by f.p.l.c. with the addition of DTT by monitoring the effluent at 214 nm. (b) Activity decrease was measured after dilution of aliquots taken at different time intervals.

Figure 6 Liberation of free amino groups during proteinase K digestion in GdmCl

Conditions for digestion were as for Figure 5(b), except that 0.05 mM borate buffer, pH 8.0, was used with GdmCl concentrations as indicated. Aliquots were taken at time intervals and diluted for reaction with fluorescamine (for details, see the text). Fluorescence emission was recorded at 488 nm with excitation at 395 nm.

K, even in the absence of GdmCl, and the hydrolysis rates increase with GdmCl concentration (Figure 5a). In 0.5 M GdmCl the hydrolysis rate of RNAase is already about twice as rapid as in the absence of GdmCl, indicating the loosening up of the structure of RNAase, even though no noticeable change can be observed by intrinsic fluorescence and u.v. absorbance measurements up to 2 M GdmCl. In 5 M GdmCl, where the unfolding of RNAase is nearly complete (Liu and Tsou, 1987), the digestion rate increases about 10-fold. A Guggenheim plot shows that the digestion of RNAase by proteinase K in 1 M GdmCl is a first-order reaction (results not shown).

In contrast with trypsin, during digestion by proteinase K the

activity of RNAase decreases significantly even in the absence of GdmCl. However, the rate of inactivation increases markedly with increase in GdmCl concentration (Figure 5b). Even though f.p.l.c. was employed for peptide separation, it has not been possible to make an examination at short digestion periods, as can be easily done for activity assay, in general the decrease in activity again parallels RNAase hydrolysis. However, a closer comparison of Figure 5(a) with Figure 5(b) shows somewhat higher activity than can be accounted for by intact RNAase remaining, especially at longer times of digestion, and this could be for the same reason as discussed above for trypsin digestion.

Liberation of peptide fragments during proteinase K hydrolysis

The digestion of RNAase by proteinase K, unlike that by trypsin, is noticeable, even in the absence of GdmCl, since faint signs of digestion products are discernible by electrophoresis (Figure 4b). This is also the case for the liberation of free amino groups, as shown by reaction with OPTA (results not shown) and fluorescamine (Figure 6). Both the production of digestion products and liberation of free amino groups increase markedly with increasing GdmCl concentrations.

Separation and identification of the digestion products

By trypsin digestion of RNAase in 1 M GdmCl for 5 h at 35 °C, seven peaks can be identified by hydrophobic chromatography, as shown in Figure 7(a). Peak II was identified as intact RNAase, control experiments showed that peaks IV and V were derived from trypsin. Peaks I, III, VI and VII have been subjected to N-terminal sequence analysis and the results for I, III and VI are summarized in Table 1. The sequences are unique, so that one is able to identify peak I as the N-terminal peptide and the identification of cleavage points at Arg¹⁰-Gln¹¹ and Arg³³-Asn³⁴. The sequencing of peak VII has not been possible because the amount available was too small and because of the presence of impurities therein.

Figure 7(b) shows the h.p.l.c. profile for the peptide fragments from digestion with proteinase K in 1 M GdmCl. The first peak

Figure 7 Separation of digestion products of RNAase

(a) F.p.l.c. separation of trypsin digestion products of RNAase in 1 M GdmCl. For conditions of f.p.l.c., see the text. (b) H.p.l.c. profile of proteinase K digestion products of RNAase in 1 M GdmCl. Conditions for digestion were as for Figure 5(b), except that the period of digestion was 2 h. Results of sequence analysis of the fragments are given in Table 1.

Table 1 N-terminal sequences of peptides released from partial proteolytic digestion of RNAase

(a) From Trypsin digestion (see Figure 7a)

Peak	Sequence
I	Lys ¹ -Glu-Thr-Ala-Ala-Ala.....
III	Gln ¹¹ -His-Met-Asp-Ser-Ser-Thr-Ser-Ala-Ala.....
VI	Asn ³⁴ -Leu-Thr-Lys.....

(b) From proteinase K digestion (see Figure 7b)

Peak	Sequence
II	Ser ²¹ -Ser-Ser-Asn-Tyr-Cys-Asn.....
III	Val ⁴³ -Asn-Thr-Phe-Val.....
IV	Ser ²¹ -Ser-Ser-Asn-Tyr-Cys.....
V	Lys ⁷ -Phe-Glu-Arg.....

appears to be a mixture, and sequencing has not been possible. The results of sequence analysis of peaks II, III, IV and V are given in Table 1. Peaks VI and VII are RNAase and proteinase K respectively. Peaks II and VI have the same N-terminal sequence and are probably derived from the same sequence, but of different lengths. Again, the sequences are unique and allow the cleavage points to be identified as Ala⁶-Lys⁷, Ala²⁰-Ser²¹ and Pro⁴²-Val⁴³. The points of cleavage of the RNAase molecule at low GdmCl concentrations by trypsin and proteinase K are indicated in Figure 8, together with the known essential residues at the active site of this enzyme.

Figure 8 Cleavage positions in the RNAase molecule by proteolysis with trypsin or proteinase K in dilute GdmCl

The peptide bonds hydrolysed by trypsin digestion are indicated by '→' and those by proteinase K by '⇨'. The essential residues at the active site, His¹², Lys⁴¹, Thr⁴⁵, and His¹¹⁹ are marked with '●'.

DISCUSSION

Conformational changes at the active site during inactivation

The proposition that the conformation of active sites is held together by relatively weak forces and is consequently more flexible than the enzyme molecule as a whole is based on the observations that, during denaturation of a number of enzymes by GdmCl or urea, inactivation occurs before noticeable conformational changes of the molecule as a whole (Tsou, 1986, 1993). However, this has been challenged (Creighton, 1990; Kelly et al., 1993) by the suggestion that the decrease in activity could be explained by GdmCl inhibition, as reported for RNAase by Anfinsen et al. (1955). However, for GAPDH and creatine kinase, conformational changes at the active sites in dilute GdmCl have been demonstrated (Xie and Tsou, 1987; Zhou et al., 1993). Moreover, inactivation precedes noticeable conformational changes during thermal denaturation of GAPDH and adenylate kinase (Lin et al., 1990; Zhang et al., 1993) which cannot be ascribed to inhibition, as no inhibitor has been added.

Susceptibility to proteolysis has been shown to be a sensitive method of detecting subtle conformational changes in protein molecules (Wilson, 1991; Arnone et al., 1992; Mast et al., 1992). Native proteins are generally resistant to proteolysis, and their susceptibility increases conspicuously even during a slight unfolding of the folded structure (Betton et al., 1989). It is now shown that, concurrent with inactivation of RNAase in dilute GdmCl, conformational change in the RNAase molecule does

occur, as indicated by increased susceptibility to digestion by both trypsin and proteinase K. Binding of GdmCl as an inhibitor would have resulted, if anything, in protection against proteolysis, particularly at the active site. Fragment separation and N-terminal sequence determination reveal the hydrolysis of the bonds Ala⁶–Lys⁷, Arg¹⁰–Gln¹¹, Ala²⁰–Ser²¹, Arg³³–Asn³⁴ and Pro⁴²–Val⁴³. The location of these sites are indicated in Figure 8, and it can be seen that, with the exception of Ala²⁰–Ser²¹, all the bonds cleaved are situated close to the active-site region, especially the Arg¹⁰–Gln¹¹ and the Pro⁴²–Val⁴³ bonds next to His¹², Lys⁴¹ and Thr⁴⁵ known to be essential for RNAase activity (Richards and Wyckoff, 1971; Seshadri et al., 1993). Partial unfolding in dilute GdmCl apparently opens up the active-site cleft, resulting in the separation of His¹², Lys⁴¹ and Thr⁴⁵, thus leading to inactivation. Residues Ala²⁰–Ser²¹ are situated in a surface loop at the back of the molecule and are probably therefore prone to hydrolysis. The above results clearly show that, although gross unfolding of the RNAase molecule as a whole cannot be detected by conventional methods, a subtle change in conformation has indeed occurred at its active site during inactivation in dilute GdmCl.

Relation of the inactivated to the 'molten-globule' state

The inactivated state of RNAase in low concentrations of GdmCl before gross conformational changes occur resemble in a way the molten-globule state which was first proposed for the partially denatured state of α -lactalbumin (Dolgikh et al., 1981). The consensus on the concept of molten-globule state has been defined as high contents of secondary structure, considerable molecular compactness, non-specific tertiary structure and significant flexibility (Kuwajima, 1989; Uversky et al., 1992; Haynie and Freire, 1993). The molecule of RNAase inactivated in dilute GdmCl is partly loosened up, especially in the active-site region and thus open to attack by proteolytic hydrolysis without noticeable changes in its secondary structure. For the inactivation of creatine kinase in dilute GdmCl, increased flexibility has been revealed by a fluorescent probe introduced at the active site (Zhou et al., 1993) and general compactness of the molecule as a whole by viscosity measurements (Liu et al., 1989). All the above are consistent with the concept of the molten-globular state. It would be interesting to examine whether the above holds for the other enzymes which have been shown to lose their activities without noticeable conformational changes of the molecules as a whole during denaturation by chemical denaturants or physical factors.

Sequential conformation change during unfolding of RNAase

It has been suggested that the GdmCl denaturation of RNAase followed the two-state model (Creighton, 1990). Subtle conformation change at the active site has now been shown to accompany inactivation of this enzyme before gross conformation change can be detected by conventional methods. The increase in rates of proteinase K digestion with the increase of

GdmCl concentration also suggests the gradual opening up of the enzyme molecule, and similar results have been obtained with trypsin digestion. In this connection, n.m.r. data have suggested that some changes do occur at GdmCl concentrations lower than required for overall unfolding of the molecule (Benz and Roberts, 1975), and a sequential model for the folding of denatured RNAase has been proposed (Udgaonkar and Baldwin, 1990). It has been suggested recently that unfolding occurs in two stages, and the changes from the native to the molten-globule state, and from the latter to the completely unfolded state, are both all-or-none processes (Bychkova and Ptitsyn, 1993). It appears that the two-state model holds only under specific conditions where the breaking of most of the secondary bonds responsible for the maintenance of the native conformation occurs in a highly cooperative manner.

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