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Evidence for the Existence of an Unfolding Intermediate of Thyroglobulin during Denaturation by Guanidine Hydrochloride

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Abstract—The unfolding of bovine thyroglobulin (Tg) in guanidine hydrochloride (GuHCl) solution was studied by following the fluorescence and circular dichroism. With increasing GuHCl concentrations, the emission maximum of the intrinsic fluorescence clearly red-shifted in two stages. At concentrations of GuHCl less than 1.2 M or more than 1.6 M, the red shift showed a cooperative manner. At concentrations of GuHCl between 1.2 and 1.6 M, an unfolding intermediate was observed. It was further characterized by the increased binding of the fluorescence probe 1-anilinonaphthalene-8-sulfonic acid (ANS). No significant changes of the secondary structure were indicated by CD spectra at the concentrations of GuHCl between 1.2 and 1.6 M. The conformation of this state has properties similar to those of a molten globule state which may exist in the folding pathway of the protein. Further changes in fluorescence properties occurred at concentrations of denaturant higher than 1.6 M with a significant red shift of the emission maximum from 340 to 347 nm and a marked decrease in ANS binding. This *in vitro* study gave a clue to understand the biochemical mechanism for the occurrence of aggregation and molecular chaperone binding during Tg maturation *in vivo*.

Key words: thyroglobulin, guanidine hydrochloride, unfolding, intermediate

Thyroglobulin (Tg), the major secretory product of the thyroid epithelial cell, is an extremely large glycoprotein that makes up 13% of total thyroid proteins [1] and serves as the matrix for thyroid hormone synthesis and iodide storage [2]. Tg mRNA has been found to encode a single 2750 amino acid polypeptide [2]. It is well known that correct folding and assembly of nascent secretory proteins are important both for intracellular transport and for performance of biological function [3]. Therefore, to assume its mature type, a 660 kD homodimer, it must undergo a complicated assembly process and extensive modification during maturation. Despite the obvious complexity and technical difficulty of working with this protein, Tg provides several advantages that make it possible to study glycoprotein folding *in vivo* [4].

Previous studies have shown that in thyrocytes, before transport to the Golgi [4], newly synthesized Tg proceeds through a series of discrete folding states, including protein aggregates, unfolding free monomers, folded monomers, and finally dimers [5, 6]. This conformational maturation is concerned with many factors including protein disulfide isomerase, calnexin, calreticulin, BiP, GRP94, Erp72, GRP170, and ER60 that have all been implicated in interaction with immature form of Tg [7-11]. However, relatively little is known about conformational information of Tg, based on which all the events during maturation might occur, especially aggregation and molecular chaperone binding.

Our experiments were designed to study the unfolding state of Tg during denaturation by GuHCl *in vitro*, so as to describe the conformational properties of Tg unfolded to various degrees. The result shows that under our experimental conditions an unfolding intermediate exists in the presence of 1.2-1.6 M GuHCl, which has conformation characters similar to those of a molten globule state that may exist in the folding pathway of the protein.

Abbreviations: ANS) 1-anilinonaphthalene-8-sulfonic acid; GuHCl) guanidine hydrochloride; Tg) thyroglobulin.

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MATERIALS AND METHODS

Thyroglobulin, GuHCl, ANS, and Tris were Sigma (USA) products and the other reagents were local products of analytical grade.

Protein was dissolved in 20 mM Tris-HCl, pH 7.0, and concentration was measured with the Pierce BCA Protein Assay kit (USA).

The intrinsic fluorescence emission spectra were measured with a Hitachi F-2500 (Japan) spectrofluorometer using 1-cm pathlength cuvettes with excitation wavelength 295 nm. For ANS fluorescence emission spectra, the excitation wavelength was 380 nm.

CD spectra were acquired on a Jasco J-715 spectrophotometer over a wavelength range of 200-250 nm with a 2-mm pathlength cell. Each spectrum was the result of eight scans obtained by collecting data at 0.5-nm intervals with an integration time of 0.5 sec.

All experiments were carried out at 25°C.

RESULTS

Changes in the fluorescence emission spectra during unfolding of Tg in GuHCl of different concentrations. The conformational changes of Tg during unfolding in different concentrations of GuHCl were studied by intrinsic fluorescence spectra with excitation wavelength 295 nm. Changes in the fluorescence emission spectra of Tg after denaturation for 4 h in GuHCl solutions of increasing concentrations are shown in Fig. 1. Increasing the GuHCl concentration caused the maximum emission to red-shift. Figure 2 shows the plot of the emission maximum red shift versus the GuHCl concentration. As shown by the plot, increasing GuHCl concentrations caused significant changes in the red shift of the emission maximum, which occurred in two stages. At the GuHCl concentration less than 1.2 M, increasing GuHCl concentration caused a cooperative red shift of the emission maximum (from 330 to 339 nm). The emission maximum also cooperatively red-shifted from 340 to 347 nm in the GuHCl concentrations more than 1.6 M. However, between about 1.2 and 1.6 M GuHCl, there was little change in the emission maximum. The data of the intrinsic fluorescence spectra implied that unfolding of Tg during GuHCl denaturation under the experimental conditions included an intermediate state.

ANS binding. The fluorescence emission of ANS is known to increase when the dye binds to hydrophobic regions of a protein [12]. Here, ANS binding was used as another criterion to identify the unfolding intermediate of the protein. The results in Fig. 3 showed that increasing the GuHCl concentration caused the fluorescence emission intensity of ANS bound by denatured protein to increase. The fluorescence emission intensity increased in magnitude to a maximum value between 1.2-1.4 M GuHCl, indicating that the formation of the hydrophobic core occurs (Fig. 4). Further increase in GuHCl concentrations caused the intensity of the fluorescence emission to decrease and the emission maximum to significantly red-shift, implying

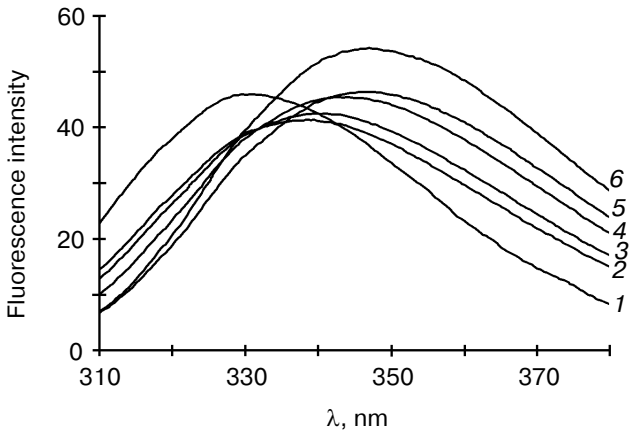

Fig. 1. Changes of fluorescence emission spectra of Tg denatured in various concentrations of GuHCl. The final Tg concentration was 0.34 mg/ml. The protein was preincubated for 4 h in 20 mM Tris-HCl buffer, pH 7.0, containing the indicated concentrations of GuHCl and then the emission fluorescence was measured with excitation wavelength of 295 nm. The original fluorescence spectra (curves *1* to *6*) were measured in different GuHCl concentrations of 0, 1.0, 1.6, 2.6, 4.0, and 5.0 M, respectively.

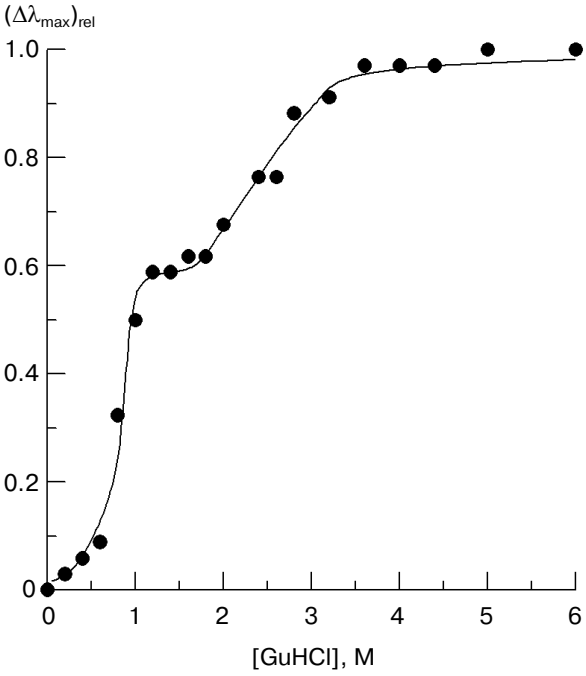

Fig. 2. Relative red shift of the fluorescence emission. Points indicated the changes of emission maximum derived from the original fluorescence spectra measured in various GuHCl concentrations from 0 to 6.0 M.

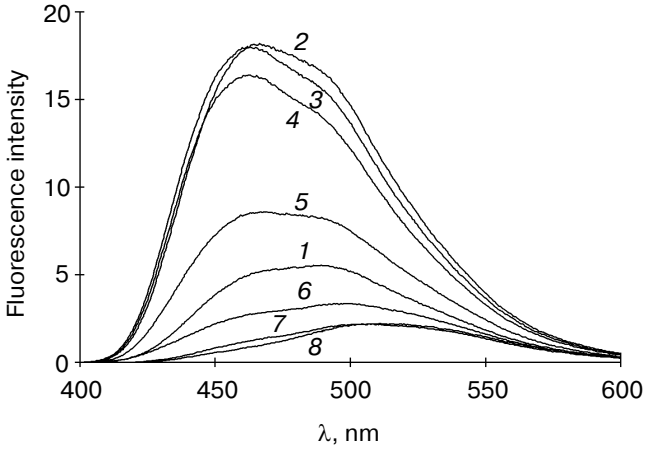

Fig. 3. ANS binding characteristics of Tg in various GuHCl concentrations. Curves *1* to *7* were original spectra for Tg incubated in GuHCl of different concentrations, 0, 1.0, 1.4, 1.6, 2.0, 3.2, and 4.0 M, respectively. Curve *8* was for that of ANS in buffer, pH 7.0, without protein. Experimental conditions were as for Fig. 1 except that the excitation wavelength was 380 nm.

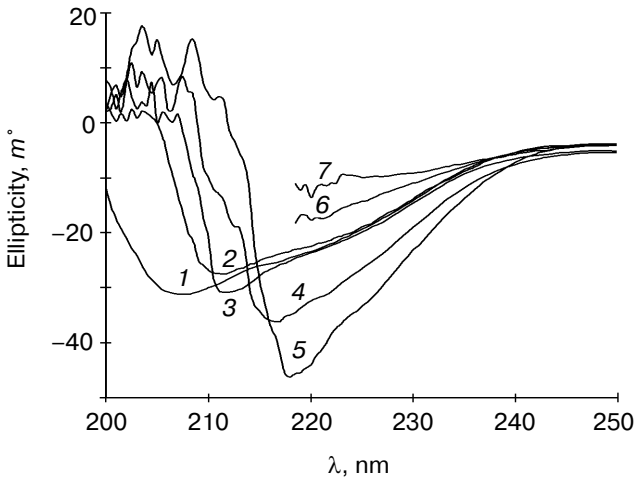

Fig. 5. Changes of CD spectra of Tg denatured at various concentrations of GuHCl. The conditions were as for Fig. 1. Curves *1* to *7* were original spectra for Tg incubated in GuHCl of different concentrations, 0, 1.0, 1.4, 2.0, 2.6, 4.0, and 5.0 M, respectively.

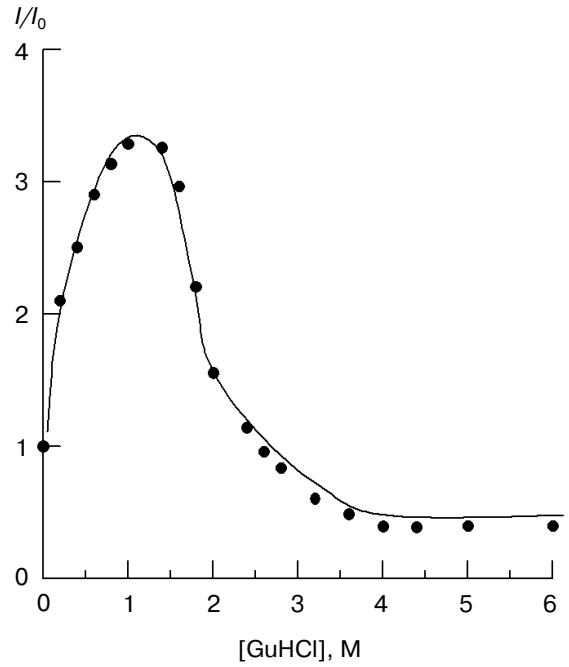

Fig. 4. Relative intensity of ANS fluorescence. Points indicated the relative intensity of the original fluorescence spectra measured in various GuHCl concentrations from 0 to 6.0 M.

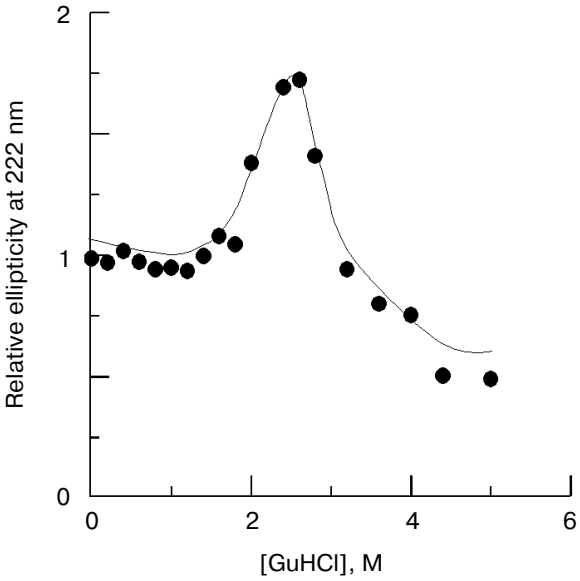

Fig. 6. Relative ellipticity of Tg denatured in various concentrations of GuHCl at 222 nm. Points indicated the relative ellipticity at 222 nm of the original CD spectra measured in various GuHCl concentrations from 0 to 5.0 M.

that the transfer of the ANS molecules from a hydrophobic to hydrophilic environment takes place.

Changes of the secondary structure of Tg at different GuHCl concentrations. Figure 5 shows the CD spectra of Tg during denaturation in GuHCl solutions of different concentrations. The secondary structure changed little at GuHCl concentrations between 0 to 1.6 M. With the GuHCl concentration further increasing, the secondary structure of Tg changed markedly, especially the α-helix. Figure 6 is the plot of the relative ellipticity at 222 nm versus GuHCl concentration, which shows that the α-helix content changes little at GuHCl concentrations between

0 to 1.6 M but sharply increases in 1.6 to 2.4 M GuHCl. Further increasing GuHCl concentrations caused a cooperative decrease in α-helix.

DISCUSSION

It is generally accepted that protein denaturation is a highly cooperative process that for small globular proteins may be approximated by a two-state model and no significant intermediates exist during the transition from native state to unfolded state [13]. However, for many proteins investigated in recent years some intermediates are present during the unfolding process. In the present study, the effects of sugar groups in Tg were ignored for simplicity. An unfolded intermediate was found in the denaturant concentration region between 1.2 and 1.6 M GuHCl. This unfolding intermediate is similar to the molten globule state in the folding pathway of proteins. Both a slight red shift of the tryptophan fluorescence emission maximum in this state and shape of ANS fluorescence spectra indicate the formation of a hydrophobic core that is much more mobile than the native state. Furthermore, the amount of α-helix in this state is close to that of the native protein. The increase of α-helix in 1.6 to 2.4 M GuHCl indicates another unfolding intermediate may exist in this denaturant region. However, it needs further study to provide more evidence for its existence.

All the results indicate that this unfolding intermediate has properties very similar to those of the molten globule [14, 15]. The evidence for the existence of an unfolding intermediate implies that there may be a similar state existing during Tg folding *in vivo*, which at least partly counts for the aggregation, molecular chaperone binding, and assembly during its maturation. Further research will focus on the interaction of this unfolding intermediate with molecular chaperones and its aggregation behavior *in vitro*.

REFERENCES

1. Wagar, G. (1974) *Acta Endocrinol.*, **77**, 64-70.
2. Dunn, J. T., Kim, P. S., and Moore, R. C. (1985) in *Thyroglobulin: The Prothyroid Hormone* (Eggo, M. C., and Burrow, G. N., eds.) Raven Press, New York, pp. 33-42.
3. Hurtley, S. M., and Helenius, A. (1989) *Annu. Rev. Cell Biol.*, **5**, 277-307.
4. Kim, P. S., and Arvan, P. (1991) *J. Biol. Chem.*, **266**, 12412-12418.
5. Kim, P. S., Bole, D., and Arvan, P. (1992) *J. Cell Biol.*, **118**, 541-549.
6. Kim, P. S., Kim, K.-R., and Arvan, P. (1993) *Am. J. Physiol.*, **265**, C704-711.
7. Kim, P. S., and Arvan, P. J. (1993) *J. Biol. Chem.*, **268**, 4873-4879.
8. Kuznetsov, G., Chen, L. B., and Nigam, S. K. (1994) *J. Biol. Chem.*, **269**, 22990-22995.
9. Kim, P. S., and Arvan, P. J. (1995) *Cell Biol.*, **128**, 29-38.
10. Kuznetsov, G., Bush, K. T., Zhang, P. L., and Nigam, S. K. (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 8584-8589.
11. Kuznetsov, G., Chen, L. B., and Nigam, S. K. (1997) *J. Biol. Chem.*, **272**, 3057-3063.
12. Stryer, L. (1965) *J. Mol. Biol.*, **13**, 482-495.
13. Aune, K. C., and Tanford, C. (1969) *Biochemistry*, **8**, 4586-4590.
14. Goto, Y., and Fink, A. L. (1989) *Biochemistry*, **28**, 945-952.
15. Goto, Y., Calciano, L. J., and Fink, A. L. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 573-577.