DIFFERENT RATES OF FORMATION OF SECONDARY AND TERTIARY STRUCTURE DURING RENATURATION OF UREA-DENATURED HUMAN SERUM ALBUMIN

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Received November 11, 1988
Accepted December 16, 1988

A comparison of the results of our polarimetric measurements with the polarographic experiments reported earlier shows that the restoration of the secondary structure during the renaturation of human serum albumin is a process which is faster than the formation of the tertiary structure. These results, which are in agreement with the data on the kinetic control of protein folding, are discussed from the viewpoint of the importance of the individual types of interactions which take place during the formation and stabilization of three-dimensional protein structures. We have been able to demonstrate the great importance of electrostatic and hydrophobic interactions which together with the disulfide bonds are essential for the reversibility of the denaturation phenomena. The discussion also shows the essential role which evolution processes play in the selection of the mode of protein folding.

One of the key problems molecular biology is facing to date is the proper understanding of the physical forces and mechanisms which affect the formation of three-dimensional protein structures[1]. Efforts in this field have been aimed at the finding of methods capable of predicting three-dimensional structures of proteins from their amino acid sequences. Studies on protein denaturation and renaturation in vitro have afforded many important findings necessary for the solution of this problem.

The denaturation and renaturation processes which undergo small globular proteins can be usually represented by a two-state model[2] yet this model is insufficient for large multi-domain protein molecules[3]. The deviations of the process of denaturation of human serum albumin from the two-state model have been demonstrated experimentally[4-6]. In contrast we have observed in our previous study[7] that, according to the results of our polarographic measurements, the renaturation of urea-denatured human serum albumin (HSA) corresponds to the two-state model. None of the methods by itself, however, can provide us with sufficient information on the conformational change since each physicochemical magnitude can reflect a part of the conformational change only (the polarographic measurements characterize the accessibility of cystine residues for the electrode reaction). The renaturation

of HSA was therefore measured by polarimetry which reflects mainly changes in the secondary structure and thus characterizes the conformational changes which parallel HSA renaturation from a different viewpoint than the polarographic measurements.

EXPERIMENTAL

Chemicals. Human serum albumin was a product of Imuna, Šarišské Michal'any. The purification of this preparation, the preparation of mercaptalbumin and nonmercaptalbumin and the recrystallization of urea were described earlier[6].

Methods. The optical rotary dispersion measurements (ORD) were done in Model ORD/UV-5 Jasco instrument equipped with a cell of 1 mm optical path. The change in ORD was read at 233 nm (minimum of Cotton effect). The optical rotation measurments at 589 nm were carried out in ETL-NPL Automatic Polariter type 143A (British Physical Laboratories Radlett).

Denaturation. A defined quantity of the protein solution was placed in 9M urea to obtain a final protein concentration in the denaturation mixture of $7{\cdot}0 \cdot 10^{-5}$ mol l^{-1} and a urea concentration of 8 mol l^{-1}. The renaturation studies were carried out with proteins denatured in 8M urea for 1 or 200 min. These preparations are marked as D_{SH}^{1} and D_{SH}^{200} in the case of mercaptalbumin and as D_{NSH}^{1} and D_{NSH}^{200} in the case of nonmercaptalnumin.

Renaturation. The renaturation was effected by dilution of the reaction mixture with a triple volume of distilled water or 2·66M urea. The kinetics of renaturation was examined in the renaturation mixture.

RESULTS

A great change in specific rotation $[\alpha]_D^{22}$ was observed already before the first measurement possible (1 min after dilution of the denaturation mixture). The values of specific rotation did not undergo additional changes in the case of renaturation of D_{SH}^{1} and D_{NSH}^{1} and were identical to the values corresponding to the specific rotation or proteins denatured in urea of the same concentration as was the final urea concentration in the denaturation mixture (4 or 2 mol l^{-1}, resp.). The initial big change observed during the renaturation of D_{SH}^{200} and D_{NSH}^{200} was followed by a smaller change in specific rotation (the extent of this change was larger with D_{SH}^{200} than with D_{NSH}^{200}). Renaturation arrived in the equilibrium state as estimated by polarimetry after about 3 min in the case of D_{NSH}^{200} and after 6 min in the case of D_{SH}^{200}. The final values of specific rotation were identical in the case of D_{NSH}^{200} to the values obtained by denaturation in urea of the same concentration yet a certain difference between these values was observed in the case of D_{SH}^{200}. The results of polarimetric examination of the renaturation of mercaptalbumin and nonmercaptalbumin at 589 nm are shown in Figs 1 and 2.

In analogy to optical rotation measurements the results of optical rotary dispersion measurements have demonstrated that already before the first measurement possible (after 1 min of renaturation) a considerable change in the mean residual rotation

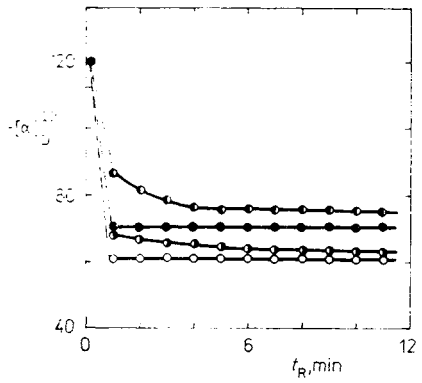

FIG. 1

Time profile of specific rotation $[\alpha]_D^{22}$ during renaturation of mercaptalbumin denatured in 8M urea. Curves: ○ 1-min denaturation, renaturation by decreasing urea concentration from 8 to 2 mol l^{-1}; ◑ 200-min denaturation, renaturation by decreasing urea concentration from 8 to 2 mol l^{-1}; ● 1-min denaturation, renaturation by decreasing urea concentration from 8 to 4M; ◐ 200-min denaturation, renaturation by decreasing urea concentration from 8 to 4 mol l^{-1}

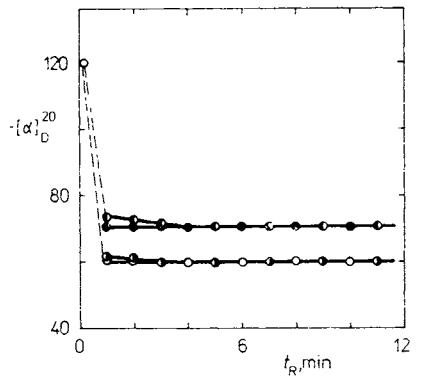

FIG. 2

Time profile of specific rotation $[\alpha]_D^{22}$ during renaturation of nonmercaptalbumin denatured in 8M urea. The curves are designated as in Fig. 1

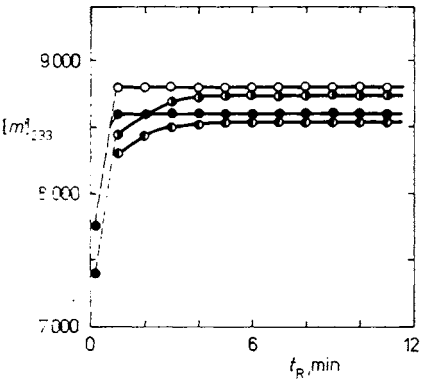

Fig. 3

Time profile of mean residual rotation $[m']_{233}$ during renaturation of mercaptalbumin denatured in 8M urea. The curves are designated as in Fig. 1

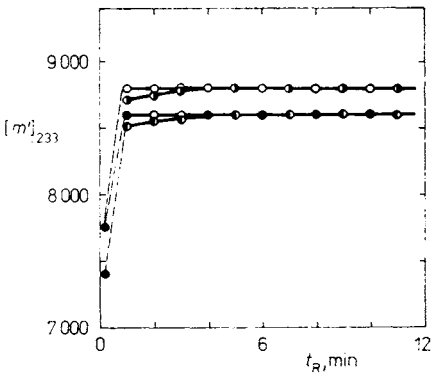

FIG. 4

Time profile of mean residual rotation $[m']_{233}$ during renaturation of nonmercaptalbumin in 8M urea. The curves are designated as in Fig 1

$[m']_{233}$ had taken place. The mean residual rotation did not change further during renaturation of D^1_{SH} and D^1_{NSH} whereas another small change still followed during renaturation of D^{200}_{SH} and D^{200}_{NSH} (see Figs 3 and 4). When D^1_{SH} and D^1_{NSH} and D^{200}_{NSH} were renaturated by decreasing urea concentration from 8 to 4 or 2 mol l^{-1}, respectively the values of mean residual rotation were identical to the values obtained when the proteins were denatured in 4 or 2M urea, resp. In contrast, the values of $[m'']_{233}$ measured during the renaturation of D^{200}_{SH} differ from the values obtained when the protein was denatured in 4 or 2M urea, resp. (cf. Fig. 3).

DISCUSSION

The physical character of optical rotation is very complicated and the interpretation of its changes in terms of structure is difficult. In analogy with the results of measurements of synthetic polypeptides the changes in optical rotation can be interpreted in terms of changes in the secondary structure of proteins[8], which in the case of HSA means in terms of changes of its α-helical structure[9].

The results of our measurements show that the restoration of the secondary structure of mercaptalbumin and nonmercaptalbumin is a rapid process. In experiments with 1-min denaturation the renaturation is completed already before the first measurement is even possible, i.e. in less than 1 min (most likely during a much shorter period[10]). The values of optical rotation measured during the renaturation are in such a case identical to the values obtained in denaturation experiments using a concentration of urea identical to its final concentration in the renaturation mixture. Practically the same results were obtained also with D^{200}_{NSH} (the small changes after 1 min of renaturation are comparable to the error of the measurement). These results show that the denaturation is reversible in the case of D^1_{SH}, D^1_{NSH} and D^{200}_{NSH}, in agreement with our electrophoretic and chromatographic measurements[6].

The situation is more complicated with D^{200}_{SH}, unlike with the results discussed above. Even in this case, however, the changes in optical rotation are over earlier than the changes in the Brdička current measured during polarographic investigation of the renaturation. This shows that the denaturation of D^{200}_{SH} is irreversible and that the reversibility of denaturation therefore depends on the length of the period of urea treatment. This phenomenon is likely caused by the release of only SH group (Cys 34) and following SH–SS interchange reactions.

A comparison of the results of polarographic measurements on the one side and of the polarimetry data on the other indicates a difference in the rate of renaturation of D^{200}. Whereas the polarimetric characteristics of D^{200} markedly change within a period shorter than that during which the first measurement can be made (in the case of D^{200}_{SH} this great change is followed by a small change accounting for about 20% of the total change for 5 min), the polarographic activity of D^{200} gradually decreases during 15 min. This shows that the renaturation of D^{200} proceeds via an

intermediary state which cannot be picked up polarographically. The renaturation mechanism of D_{SH}^{200} can then be described by the following scheme:

$$D_{SH}^{200} \rightarrow Y_{SH}^{200} \rightarrow R_{SH}^{200} \neq N_{SH},$$

where Y_{SH}^{200} represents the intermediary state characterized by the polarimetric properties and R_{SH} represents an incorrectly folded conformation whose polarographic, chromatographic and electrophoretic[6] properties markedly differ from the native conformation of N_{SH}.

The renaturation of D_{NSH}^{200} can be expressed by an analogous scheme except that R_{NSH}^{200} is similar in its properties to the native protein:

$$D_{NSH}^{200} \rightarrow Y_{NSH}^{200} \rightarrow R_{NSH}^{200} \sim N_{NSH}.$$

These results point to the importance of hydrophobic interactions and disulfide bonds for HSA folding. Unless the action of the denaturant results in a marked disturbance of the hydrophobic regions and in changes in disulfide pairing it is likely that the conformational change will be reversible. If hydrophobic regions are unfolded or the pairing of disulfide bonds changed the change in HSA conformation will be irreversible.

The different rates of renaturation of D^{200} as established by different assay methods reflect differences in formation of secondary and tertiary structure. The results of polarimetric measurement indicate a very rapid restoration of the secondary structure (mainly of the α-helix in the case of HSA) whereas the refolding of the three-dimensional structure examined polarographically from the viewpoint of the accessibility of the cystine residues (i.e. of residues which of all amino acid residues of proteins are the least accessible for the solvent[11]) is markedly slower. The renaturation time of D^{200} corresponds to the kinetic control of protein folding since the time necessary for a thermodynamic selection of all the conformations possible would be by about 60 orders longer than the time of protein folding observed both in vivo and in vitro[12-14]. It is therefore likely that certain nucleation sites[15] are formed during the first phase of HSA renaturation; as a result the number of pathways along which folding proceeds from the unordered polypeptide chain to the native conformation is limited. Naturally only those nucleation sites which can overcome the energetic barrier involved in the necessary decrease in configuration entropy of the polypeptide chain by mutually stabilizing interactions of amino acid residues can play a role in the formation of the native structure of the protein[16]. The most important stabilizing factors to be considered are electrostatic and hydrophobic interactions. This shows us that essentially two types of nucleation sites can exist. The first type is represented by segments of secondary structure α-helix, β-bend and β-sheet, refs[17,18]) and of the second type are hydrophobic clusters[19]. It is im-

possible to decide at present which type of nucleation sites will develop earlier or whether there is a combination of both types. This question cannot be answered unambiguously by our results either, nevertheless a rapid formation of the secondary structure[10] seems to favor the first type of nucleation sites.

The nucleation centers already formed control the subsequent formation of the native structure so that it may follow the fastest pathway[15]. A series of hypotheses have been adduced to explain this phase of the folding. Levinthal assumes that the mode of protein folding is a result of evolution and that the final product of the folding is the most thermodynamically stable state[20]. According to Ptitsyn and Rashin[21] the mode of the folding is determined by the relative stabilities of intermediates, multiple folding modes involve rapid reversible steps in each folding cycle of the folding process and each subsequent step is determined by mutual thermodynamic stabilities of all states accessible. According to the "diffusion-collision model"[14,22] the independent folding of short segments to microdomains is followed by their stabilization via subsequent diffusion, collision and coalescence. Harrison and Durbin[23] assume that protein folding is analogous to putting together a jig-saw puzzle and that there is no predetermined initial state, hence the pathway along which folding will proceed depends on the selection of the initial state (out of a number of possible initial states). In contrast Nall[24] was able to show the existence of a predetermined pathway of folding of yeast iso-2 cytochrome c. According to the model presented by Eisenberg and coworkers[25] the force driving the folding is a tendency to attain the minimal potential energy possible.

It is likely that hydrophobic interactions play the key role in the formation of microdomains during this phase of the folding. Whereas a correlation of the hydrophobic properties of the amino acids with their preference for certain types of secondary structure is only partial (β-structures, ref.[26]) the hydrophobic interactions play an important role in the stabilization of the secondary structure and in the formation of supersecondary and tertiary structure[11,27,28]. The small number of structure motifs found in globular proteins is also in agreement with the kinetic control of protein folding[29,30].

The disulfide bonds are another factor stabilizing protein structures since the formation of a disulfide bond markedly decreases the configuration entropy of the polypeptide chain[31]. This is probably also the case with HSA as follows from a comparison of the renaturation of reduced and nonreduced serum albumin in vitro and its formation in vivo[7].

The changes in the secondary structure (polarimetric measurements) and in tertiary structure (polarographic measurements[7]) during the subsequent renaturation phase can be caused also by the isomerization of proline residues[32]. During this isomerization, which is regarded as the slowest phase of protein folding[16], both local changes in secondary structure and changes in the localization of various amino acid residues in the three-dimensional structure of the protein occur.

Interesting information on the formation and stability of secondary and tertiary structures afforded studies on homologous proteins. It has been shown[33,34] that a relatively small identity (even 16% only) in primary structures of proteins is sufficient for the formation of closely related secondary and tertiary structures to occur. In contrast it has been known that short identical sequences of polypeptide chains of nonhomologous proteins can develop completely different conformations[35,36]. These studies show that rather evolutionary than physicochemical reasons may be responsible for the maintaining of a certain secondary or tertiary structure in spite of the drastic changes the sequence or the organization of the gene may have undergone[37].

Evolutionary reasons play no doubt an important part also in the kinetics of the formation of the secondary and the tertiary structure of HSA. It is likely that during the first phase of renaturation of unreduced HSA α-helical segments are formed in those parts of the polypeptide chain which contain a higher number of α-helix-preferring amino acid residues. Interactions between these segments during the subsequent phase lead to the formation of more extensive structure units mainly stabilized by hydrophobic interactions. Nonpolar groups become buried in the interior of the domain formed during this stage. The establishment of the renatured molecules is completed during the final stage of the process by interactions of the domains leading to minimalization of the surface of HSA molecules; according to the results of our measurements[6,7] the renatured molecules are entirely identical to the native molecules in the case of D^1_{SH} and D^1_{NSH}, very similar to native nonmercaptalbumin in the case of D^{200}_{NSH}; the properties of the renatured molecule differ, however, from those of native mercaptalbumin in the case of D^{200}_{NSH}.

REFERENCES

1. Schellman J. A.: Annu. Rev. Biophys. Chem. *16*, 115 (1987).
2. Lumry R., Biltonen R., Brandts J.: Biopolymers *4*, 917 (1966).
3. Baldwin R. L.: Adv. Enzymol. *43*, 453 (1975).
4. Neurath H., Cooper G. R., Erickson J. D.: Phys. Chem. *46*, 203 (1942).
5. Aoki K., Murata M., Hiramatsu K.: Anal. Biochem. *59*, 146 (1974).
6. Chmelík J., Anzenbacher P., Chmelíková J., Matějčková M., Kalous V.: Collect. Czech. Chem. Commun. *53*, 411 (1988).
7. Chmelík J., Anzenbacher P., Kalous V.: Collect. Czech. Chem. Commun. *54*, 536 (1989).
8. Lumry R., Eyring H.: J. Phys. Chem. *58*, 110 (1954).
9. Peters Th. jr: Adv. Protein Chem. *37*, 161 (1985).
10. Kuwajima K., Yamaya H., Miwa S., Sugai S., Nagamura T.: FEBS Lett. *221*, 115 (1987).
11. Janin J.: Nature *277*, 491 (1979).
12. Levinthal C.: J. Chim. Phys. *65*, 44 (1968).
13. Wetlaufer D. B.: Proc. Natl. Acad. Sci. U.S.A. *70*, 697 (1973).
14. Karplus M., Weaver D. L.: Nature *260*, 404 (1976).
15. Baldwin R. L. in: *Protein Structure, Folding and Design 2*, (D. L. Oxender, Ed.), p. 313. Alan R. Liss, Inc., New York 1987.
16. Jaenicke R.: Prog. Biophys. Mol. Biol. *49*, 117 (1987).

17. Dolgikh D. A., Gilmanshin R. I., Brazhnikov E. V., Bychova V. E., Semisotnov G. V., Venyaminov S. Y., Ptitsyn O. B.: FEBS Lett. *136*, 311 (1981).
18. Kim P. S., Baldwin R. L.: Annu. Rev. Biochem. *51*, 459 (1982).
19. Matheson R. R. jr, Scheraga H. A.: Macromolecules *11*, 819 (1978).
20. Levinthal C.: cited in ref.[15].
21. Ptitsyn O. B., Rashin A. A.: Biophys. Chem. *3*, 1 (1975).
22. Weaver D. L.: Biopolymers *21*, 1275 (1982).
23. Harrison S. C., Durbin R.: Proc. Natl. Acad. Sci. U.S.A. *82*, 4028 (1985).
24. Nall B. T.: Biochemistry *25*, 2974 (1986).
25. Eisenberg D., Wilcox W., Eshita S. M., Pryciak P. M., Ho S. P., DeGrado W. F.: Proteins: Struct. Funct. Genet. *1*, 16 (1986).
26. Chmelík J.: *Thesis*. Charles University, Prague 1982.
27. Kanehisa M. I., Tsong T. Y.: Biopolymers *19*, 1617 (1980).
28. Ptitsyn O. B.: Proc. Int. Symp. Biomol. Struct. Interact., Suppl. J. Biosci. *8*, 1 (1985).
29. Levitt M., Chothia C.: Nature *261*, 552 (1976).
30. Richardson J. S.: Methods Enzymol. *115*, 341 (1985).
31. Schaffer S. W., Ahmed A. K., Wetlaufer B. D.: J. Biol. Chem. *250*, 8483 (1975).
32. Brandts J. F., Halvorson H. R., Brennan M.: Biochemistry *14*, 4953 (1975).
33. Lesk A. M., Chothia C.: J. Mol. Biol. *136*, 225 (1980).
34. Bajaj M., Blundell T.: Annu. Rev. Biophys. Bioenerg. *13*, 453 (1984).
35. Kabsch W., Sander C.: Proc. Natl. Acad. Sci. U.S.A. *81*, 1075 (1984).
36. Wilson I. A., Haft D. H., Getzoff E. D., Tainer J. A., Lerner R. A., Brenner S.: Proc. Natl. Acad. Sci. U.S.A. *82*, 5255 (1985).
37. Pain R. H.: Nature *320*, 216 (1986).

Translated by V. Kostka.