POLAROGRAPHIC STUDIES ON RENATURATION OF UREA--DENATURED HUMAN SERUM ALBUMIN

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The renaturation of the two main components of human serum albumin, i.e. of mercaptalbumin and nonmercaptalbumin, was studied polarographically. It has been demonstrated that renaturation of both proteins after 1-min denaturation in 8M urea is reversible. By contrast, renaturation after 200 min denaturation in 8M urea is an irreversible process; the characteristics of renatured mercaptalbumin differ more from the properties of the native protein than the characteristics of nonmercaptalbumin. The studies of the kinetics of renaturation of both proteins have shown that the renaturation can be represented by a two-state model. This means that the existence of stable intermediary products during the renaturation process was not determined polarographically.

Studies on the renaturation of proteins in vitro have been employed as an auxiliary, experimentally available mode of investigation of protein folding in vivo[1]. The problem of the reversibility of protein renaturation after preceding denaturation in various reagents has not been satisfactorily solved as yet. A successful renaturation of denatured and reduced ribonuclease[2,3] seems to suggest that the course of renaturation is reversible yet other results indicate that the reversibility range depends on the conditions of denaturation and renaturation[4,5].

Studies on serum albumin renaturation are necessary for a better understanding of the formation of the native conformation of large globular proteins composed of several domains. It has been evident ever since the time the first studies in this field were carried out that the renaturation of urea denatured serum albumin is an extremely complicated process. Neurath and coworkers[6] were able to show that serum albumin renatured by dialysis consists of two components: 85% of the product is represented by soluble, renatured serum albumin whereas the remaining 15% are an insoluble product resulting from aggregation during dialysis. The soluble fraction is very similar to the native protein in its viscosity and diffusion coefficient yet differs from it in its solubility in ammonium sulfate and in its electrophoretic mobility. Still other studies have shown that the reversibility of this reaction depends on the condi-

tions of denaturation and renaturation (temperature[7], denaturant concentration[6], denaturation time and renaturation mode[8,9]). Taylor and Silver[10] observed a partial restitution of catalytic activity with respect to the Meisenheimer complex after renaturation of two complementary serum albumin fragments. Teale and Benjamin[11,12] used antibodies specific to various parts of the serum albumin molecule to study the independent folding of the individual domains. Similar results supporting the hypothesis of an independent folding of the individual domains in the molecules of globular proteins[13] have been also obtained by Johanson and coworkers[14].

There is a great difference in the course of renaturation of serum albumin with intact disulfide bonds and of the protein whose disulfide bonds have been reduced. The renaturation of reduced serum albumin is a relatively long process (approximately 8 h), which can be accelerated both by the separation of the polymer forms[14] and also by the action of microsomal enzymes catalyzing the conversion of the disulfide bonds[11,12]. By contrast, the renaturation of nonreduced serum albumin is a more rapid process lasting about 15 min[15].

The aim of this study has been to examine by polarographic measurements the reversibility of renaturation of both main serum albumin components, of mercaptalbumin and nonmercaptalbumin, whose behavior differs during urea denaturation.

EXPERIMENTAL

Chemicals: Human serum albumin (HSA) was a product of Immuna, Šarišské Michaľany. The purification of this product, the preparation of mercaptalbumin and nonmercaptalbumin, as well as the recrystallization of urea were described elsewhere[16].

Methods: The polarographic measurements were carried out in Polariter PO_4 (Radiometer, Copenhagen) using the Kalousek vessel and a saturated calomel reference electrode on which all potential values are based. The experimental details of the measurement were described before[17].

Denaturation: A defined quantity of the protein solution was added to a 9M urea solution to yield a final concentration of the protein in the denaturation mixture $7{\cdot}0 \cdot 10^{-5}$ mol l^{-1}. The final urea concentration was 8 mol l^{-1}.

Renaturation: The renaturation was effected by dilution of the reaction mixture with a three-fold volume of distilled water (in case of renaturation by decreasing urea concentration from 8 to 2 mol l^{-1}) or by 2·66M urea solution (when the renaturation was effected by decreasing urea concentration from 8 to 4 mol l^{-1}). The final protein concentration is the same in both cases. When the kinetics of the renaturation was studied samples were withdrawn from the renaturation mixture and added to Brdička solution in which the instantaneous value of current density of Brdička current B of the renaturing protein was measured. The equilibrium values of B of the renatured proteins were measured after 100 min of renaturation when the value of B did not change for a longer period.

All experiments were carried out at 22°C.

RESULTS

The renaturation processes were studied with denatured proteins which were prepared by the action of 8M urea for 1 or 200 min. These states are designated in the case of mercaptalbumin as D^1_{SH} and D^{200}_{SH} and in the case of nonmercaptalbumin as D^1_{NSH} and D^{200}_{NSH}.

When D^1_{SH} and D^1_{NSH} was renatured by a decrease in urea concentration from 8 to 2 mol l^{-1} no changes in Brdička current were observed. When the urea concentration was decreased from 8 to 4 mol l^{-1} the value of Brdička current after 1 min of renaturation was the same as the value obtained with the native proteins, then an increase of Brdička current occurred corresponding to denaturation in 4M urea.

When D^{200}_{SH} was renatured by a decrease of urea concentration from 8 to 2 mol l^{-1} the value of Brdička current dropped to a value corresponding approximately to denaturation by 4M urea. The renaturation of D^{200}_{SH} by a decrease of urea concentration from 8 to 4 mol l^{-1} yielded a *B* value which was slightly higher than the value of *B* obtained by denautration of mercaptalbumin in 4M urea after 24 h of its action. When renaturation of D^{200}_{NSH} is effected by a decrease of urea concentration from 8 to 4 mol l^{-1} the resulting *B* value is identical to the value observed after denaturation in 4M urea. The renaturation of D^{200}_{NSH} by a decrease of urea concentration from 8 to 2 mol l^{-1} yielded a value of *B* which is slightly higher than the value obtained by the denaturation of nonmercaptalbumin in 2M urea. The results of the renaturation experiments with D^{200}_{SH} and D^{200}_{NSH} are shown in Fig. 1.

The reduction currents of native, 1 min denatured in 8M urea and renatured (100 min by decreasing urea concentration from 8 to 2 mol l^{-1} proteins after 1-min denaturation), R^1_{SH} and R^1_{NSH} are identical. The reduction currents of D^{200}_{SH} and mercaptalbumin after 200-min denaturation and of renatured (100 min by de-

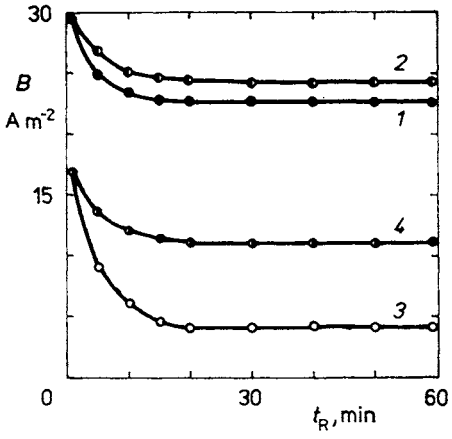

FIG. 1

Renaturation of D^{200}_{SH} and D^{200}_{NSH} by decrease of urea concentration from 8 mol l^{-1} to 4 and 2 mol l^{-1} resp. Curves: *1* renaturation of D^{200}_{SH} by decrease of urea concentration from 8 to 2 mol l^{-1}; *2* renaturation of D^{200}_{SH} by decrease of urea concentration from 8 to 4 mol l^{-1}; *3* renaturation of D^{200}_{NSH} by decrease of urea concentration from 8 to 2 mol l^{-1}; *4* renaturation of D^{200}_{NSH} by decrease of urea concentration from 8 to 4 mol l^{-1}

creasing urea concentration from 8 to 2 mol l^{-1}) protein, i.e. R_{SH}^{200} are markedly higher than the reduction current of the native protein. In the case of nonmercaptalbumin are the reduction currents of D_{NSH}^{200} and R_{NSH}^{200} lower than the corresponding reduction currents of mercaptalbumin; at the same time the value of the reduction

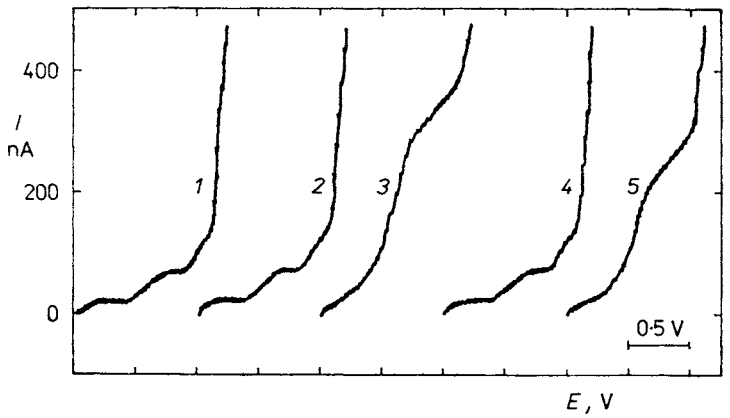

FIG. 2

Reduction currents of various conformational states of mercaptalbumin in the basic electrolyte containing 0·1M-NH_4Cl and 0·1M-NH_4OH. Curves: *1* native mercaptalbumin, *2* D_{SH}^{1}, *3* D_{SH}^{200} *4* R_{SH}^{1}, *5* R_{SH}^{200}

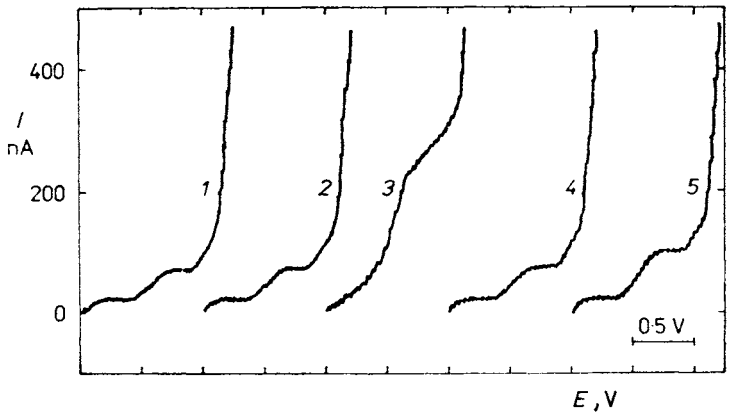

FIG. 3

Reduction currents of various conformational states of nonmercaptalbumin in basic electrolyte containing 0·1M-NH_4Cl and 0·1M-NH_4OH. Curves: *1* native nonmercaptalbumin, *2* D_{NSH}^{1}, *3* D_{NSH}^{200}, *4* R_{NSH}^{1}, *5* R_{NSH}^{200}

current of R_{NSH}^{200} approximates the value of the reduction current measured with native nonmercaptalbumin. The results of the measurements of the reduction currents are shown in Figs 2 and 3.

DISCUSSION

The existence of Brdička currents is conditioned on the presence of cysteine o cystine side chains in protein molecules[18] and on their accessibility to the electrode reaction[19]. The current density of the Brdička current *B* is directly proportional to the number of cysteine and cystine residues liberated during the process of denaturation. Additional information on the location of the cystine residues in proteins can be provided by the reduction curents of the disulfide groups.

The denatured states D_{SH}^{1} and D_{NSH}^{1} cannot be distinguished polarographically from the native states N_{SH} and N_{NSH}. Likewise the behavior and properties of renatured proteins R_{SH}^{1} and R_{NSH}^{1} are polarographically indistinguishable from native proteins; it may thus be stated that the renaturation of states D_{SH}^{1} and D_{NSH}^{1} is reversible.

Unlike with the partially unfolded denatured states D_{SH}^{1} and D_{NSH}^{1} the renaturation of completely unfolded states D_{SH}^{200} and D_{NSH}^{200} is substantially more complicated. As can be seen in Fig. 1, the resulting values of Brdička current are approximately the same for both cases of renaturation of D_{SH}^{200} effected by a decrease of urea concentration from 8 to 4 or 2 mol l^{-1}. This means that approximately the same number of catalytically active groups of both renatured forms participate on the electrode reaction whereas the numbers of catalytically active groups accessible for the electrode reaction considerably differ when the denaturation is carried out in 4 or 2M urea[16]. It follows from this finding that the conformation of the polypeptide chain is not transformed from the random coil to the native conformation during renaturation of D_{SH}^{200} yet rather to an incorrectly folded conformation containing more polarographically active groups accessible for the electrode reaction; this is also evidenced by the values of the reduction currents (see Fig. 2). The results of the kinetic test[20] show (Fig. 4) that, judging by the polarographic behavior, the renaturation of D_{SH}^{200} is a two-state process.

Similarly, in case of renaturation of D_{NSH}^{200} the polarographic characteristics of the renatured molecule of R_{NSH}^{200} are not entirely identical to the properties of native nonmercaptalbumin even though the agreement is significantly better than in the case of mercaptalbumin. In both native proteins N_{SH} and N_{NSH} 3 disulfide groups are reduced on the mercury drop electrode (the same also holds for renatured states R_{SH}^{1} and R_{NSH}^{1}); however, 11 and 5 disulfide groups, respectively, are reduced in states R_{SH}^{200} and R_{NSH}^{200}. The results of kinetic studies of the renaturation of R_{NSH}^{200} (Fig. 1) show that the resulting values of Brdička current differ depending whether the renaturation was effected by a decrease of urea concentration from 8 mol l^{-1} either to 4 or to 2 mol l^{-1}. In the former case the value of B of renatured nonmer-

captalbumin is approximately identical to the value measured with the protein denatured in 4M urea, in the latter case the value of B of the renatured protein is higher than that obtained with the protein denatured in 2M urea. The kinetic test (Fig. 4) shows that the renaturation of D^{200}_{NSD} corresponds to the model of a two-state process.

Unlike with the renaturation of states D^1 the renaturation of states D^{200} is not reversible from the viewpoint of polarographic measurements; there is a greater difference between the properties of native and renatured mercaptalbumin than in the case of nonmercaptalbumin.

The conditions of in vitro renaturation of a denatured protein differ from the conditions under which proteins are folded in vivo and this may result in differences in conformation of the native and the renatured protein[21]. In our experiments the external conditions of renaturation of denatured conformations D^1 and D^{200} are the same yet different forms of renatured HSA also arise from renaturation. The results of our experiments unambiguously show that the reversibility of renaturation of urea-denatured HSA depends both on the time the denaturant is allowed to act on native HSA, i.e. on the original conformation of HSA used for the renaturation experiments and also on the presence of the SH-group. The main difference between conformations D^1 and D^{200} is that most of the hydrophobic regions of the native molecule are retained in conformation D^1 whereas D^{200} represent entirely unfolded conformations. Another difference follows from the changes in the pairing of disulfide bonds caused by SH—SS interchange reactions is unfolded conformation D^{200}_{SH}, whereas in conformations D^1, in which no SH— or S—S groups are liberated, and in conformation D^{200}_{NSH} (not containing an SH-group) native disulfide bonds are retained.

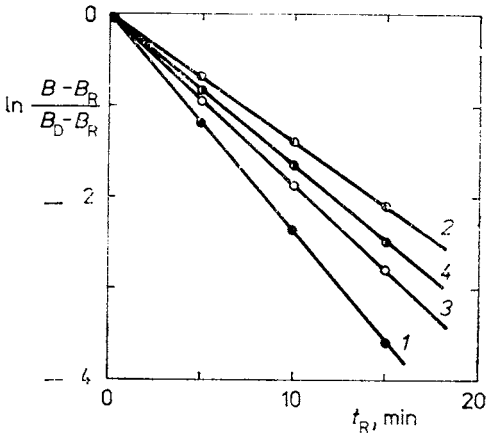

FIG. 4

Kinetic test of renaturation of D^{200}_{SH} and D^{200}_{NSH}. The designation of the individual renaturation processes is the same as in Fig. 1. B_D stands for the current density of Brdička current of the denatured protein, B_R for the current density of the protein after 100 min renaturation and B for the instantaneous value at the given renaturation time

The conformations of denatured states D^1 and D^{200} were described in detail in our preceding paper[16], conformations D^1 correspond to state X_1 and conformations D^{200} to states D.

The facts shown point to the essential role of hydrophobic interactions and disulfide bonds in HSA folding. Unless the hydrophobic regions are considerably disordered and the pairing of disulfide bonds changed during the conformational changes of HSA it is probable that the changes will be reversible. If, however, the hydrophobic regions are unfolded and the native disulfide bonds changed the conformational changes become irreversible.

Our results are in agreement with the domain structure of HSA and provide additional experimental support for the concept of independent folding of the individual domains[22]. A comparison of the times necessary for the renaturation of reduced and nonreduced serum albumin and of the mode of pairing of the native disulfide bonds offers an interesting conclusion on the formation of disulfide bonds in vivo. It is likely that native disulfide bonds are formed already during the synthesis of the polypeptide chain or during its transport from the liver to plasma. The disulfide bonds form loops on the polypeptide chain thus enabling the formation of domains and enhancing the folding of the polypeptide chain.

This finding is in agreement with the observed ability of serum albumin to bind antibodies already during its synthesis and during the intracellular phase of transport and with the results of experiments with labeled amino acids which have demonstrated that the time necessary for the appearance of labeled serum albumin in plasma is about 20 min; of this time about 2 min are necessary for the synthesis of the polypeptide chain[23]. The time required for the renaturation of reduced serum albumin is several hours whereas the time necessary for the renaturation of nonreduced serum albumin is comparable to the time required for the formation of the native conformation in vivo.

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