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Bovine Serum Albumin as a Catalyst. III. Conformational Studies

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Abstract: We have used the recently discovered catalytic activity of bovine serum albumin (BSA) as a sensitive probe of the conformational properties of this protein in solution. When the protein is unfolded in 8 *M* urea and the disulfide bonds are broken with mercaptoethanol, as much as 73% of full activity can be regained if the protein is diluted and allowed to slowly air reoxidize and the monomer is isolated. Our evidence indicates that irreversible denaturation of the protein in 8 *M* urea alone is caused by an intermolecular reaction which must involve a disulfide exchange reaction which is facilitated by the free unprotonated sulfhydryl group of the protein. Irreversible thermal denaturation also is strongly aided by the free sulfhydryl group. The results of these and other experiments are in general agreement with recent theories about domain structure in proteins. The data suggest that certain domains in BSA have greater conformational stability than other domains. Our evidence indicates that the catalytic active site of the molecule is part of one domain of relatively high conformational stability.

The plasma albumins have occupied center stage in a number of protein chemistry laboratories throughout the world.[1-6] Plasma albumin is inexpensive and can be obtained in a relatively pure form. It binds a variety of ligands[7] and can be found in a number of different conformational states[1] depending upon such conditions as solvent, temperature, pH, ionic strength, etc. For these reasons there have been a number of studies aimed at characterizing the physical properties of this protein in an effort to obtain information on the physical chemistry of proteins in general.

These physical studies have generated substantial information on the factors which influence the structure of albumin in solution. Unfortunately, many of them have suffered from a lack of an operational definition of the true "native state". In this paper we show that the catalytic activity we have described for bovine serum albumin (BSA)[8] serves as a uniquely sensitive probe of the conformational integrity of the molecule. In fact, our results suggest that this activity is indicative of the degree of "native" conformation of the protein in the vicinity of its active site.

Experimental Section

Bovine Serum Albumin. Three types of protein preparations were used in our studies: IA-BSA (see preceding paper);[8] Sigma Type F defatted BSA (Lot No. 63C 7290), S-BSA; and Armour crystalline BSA (Lot No. L72410 and L72510), which was defatted by the method of Chen[9] as modified by Foster.[10] AR-BSA. In this paper, the initials BSA signify the protein in general rather than one of the specific preparations.

Reagents and Assays. Urea was of ultrapure grade and was obtained from Schwarz/Mann. Mercaptoethanol (ME) was from Pierce Chemical Co. Ammonium sulfate used in the thermal denaturation experiments was ultrapure grade from Schwarz/Mann. Other reagents, activity assays, active site titrations, and pH measurements and data analyses were described in the preceding paper.[8]

Reduction and Reoxidation Experiments. Andersson's[5] procedures for the reduction of BSA in 8 *M* urea and 0.3 *M* ME followed by dilution and air reoxidation were followed. Incubation with ME was extended over a 3-hr period to ensure reduction of all disulfide bonds. After the specified time for air reoxidation, the sample was concentrated and dialyzed in the cold against 0.05 *M* KCl for up to 5 days. If the sample had been reoxidized in the

presence of palmitic acid, it was then charcoal defatted. Turbid solutions were passed through 0.45-μ filters. The monomeric form of the refolded protein was obtained by G-150 Sephadex chromatography as described in the previous paper.[8]

Urea Denaturation Kinetics. To a solution of 1.75 ml of 9 *M* urea in 0.15 *M* buffer (either phosphate or NH_4Cl, depending upon pH) was added 250 μl of a solution of BSA such that the final protein concentration was about 10 mg/ml. Subsequently 100-μl aliquots of this solution (held at room temperature, 23 ± 1°) were assayed for activity as a function of time in the urea solution. The data were analyzed by assuming the observed activity was directly proportional to the concentration of "native" protein remaining in solution. The control consisted of adding the same amount of protein to a buffer solution which had no urea. Within the time scale of these experiments (1–2 hr) the control activity did not change. Direct studies of the effects of urea on activity were performed by examining activity in 0.05 *M* phosphate solutions, pH 8, in which the urea concentration ranged from 0 to 8 *M*.

Stopped-flow experiments were performed on a home-built instrument capable of mixing solutions of either 1:1 or 1:10 ratios. The dead time was determined to be less than 10 msec. The output of the photomultiplier tube was amplified through a logarithmic operational amplifier and, in this way, direct measurements of absorbance changes with time were possible.

In stopped-flow urea dilution experiments, 1 vol of IA-BSA (about 11 mg/ml) in 6 *M* urea, pH 9.0, was mixed with 10 vol of buffered substrate (0.05 *M* Tris-HCl, pH 9.0, 80 m*M* substrate) and the absorbance at 478 nm was monitored after the flow stopped. In pH jump experiments 1 vol of an unbuffered solution of IA-BSA at either pH 3.0 or 11.0 was mixed with 1 vol of buffered substrate (0.05 *M* phosphate, pH 8.0) and similar procedures were followed.

Thermal Denaturation Experiments. The general procedures described by Warner and Levy[11] were followed. In a typical experiment, to 3 ml of a solution of 0.05 *M* phosphate in 0.05 *M* KCl (pH 7.66 at room temperature) in a centrifuge tube equilibrated for 15 min at 66.8 ± 0.3° was rapidly (within 5 sec) added 500 μl of a solution of BSA (70 ± 10 mg/ml). After a measured time period (between 15 sec and 20 min) the tube was removed from the bath and immediately immersed in an ice bath. One milliliter of the solution was removed and saved for activity assays. Then 7.5 ml of a solution of $(NH_4)_2SO_4$ (2.8 *M*) in acetate buffer (1.06 *M*, pH 5.5) (called the "high salt" precipitating buffer by Warner and Levy)[11] was added. The tube was stored at 4° for 12–15 hr and then centrifuged. The protein remaining in the supernatant was determined and compared with the amount of protein remaining in a "control" which had not been heated at 66.8° but was otherwise treated in the same manner. The amount of protein *remaining* in solution was assumed to be "*native*". The absorbances of the 1-ml aliquots withdrawn before addition of the precipitating solution served as a check on the accuracy of these procedures. The activity in the precipitates was determined as follows. They were dissolved in a minimum of water, reprecipitated in the high salt stopping buffer, and centrifuged. This reprecipitated material was then dissolved in a minimum of water and dialyzed exhaustively vs. 0.05 *M* KCl in the cold to remove excess salt and then assayed for activity.

Subfractionation Experiments. Our procedures were modeled after Foster's[12] general methods for isolation of different subfractions of BSA and are based on a difference in their solubilities in 3 *M* KCl in the region of the N-F transition. A 1% solution of the protein in 3 *M* KCl was adjusted to a pH in the upper range of this transition and after ~15 min the precipitate and supernatant were separated through centrifugation. After isolation of the supernatant, the pH was again lowered. Precipitates from each step were individually redissolved in 0.2 *M* sodium acetate buffer, pH 5.5, exhaustively dialyzed in 0.05 *M* KCl, and then assayed for activity. Controls were also performed in which the protein was never precipitated but was still dialyzed (to remove KCl) or was totally precipitated in one step and subsequently dissolved and dialyzed.

Results

Effects of Urea. Unfolding of BSA in urea results in the complete loss of catalytic activity (Figure 1). The unfolding process is complete in 6 *M* urea and is instantaneous; no initial activity could be measured when the protein was first added to the cuvettes. It is difficult to compare these results with earlier studies[13] because there are significant time-dependent changes in this protein's structure which accompany its unfolding in urea (see below). Earlier investigations[13] were not "instantaneous" measurements with respect to initial response of the protein to urea. The kinetics of *refolding* of IA-BSA out of 6 *M* urea are rather rapid (Figure 2). Our stopped-flow experiments indicate that recovery of activity must be completed within a time of less than 10 msec when the protein is rapidly diluted from 6 to $\frac{6}{11}$ *M* urea. Over longer time periods the trace (Figure 2) was still linear. Control samples of IA-BSA which were not incubated in urea gave similar initial linear behavior, as the experiment in effect samples a small time window of the *initial rate* of catalysis. The calculated catalytic rate constant from the trace was 0.83 sec^{-1}, which is in good agreement with the value expected for instantaneous (i.e., ~10 msec or less) regeneration of recoverable activity if we correct for (a) ca. 15% inhibition due to the presence of $\frac{6}{11}$ *M* urea, (b) loss of ca. 20% of the activity of IA-BSA which is irreversibly lost on incubation in concentrated urea [i.e., 1.30 sec^{-1} (k_c at pH 9) × 0.85 × 0.8 = 0.88 sec^{-1}], and (c) the lower temperature. Studies of the absorbance and fluorescence properties of the protein itself should serve as further indices of the refolding process and these studies are in progress to independently test this observation.

We find that BSA containing a free sulfhydryl group suffers *irreversible* time-dependent losses of activity in the pH range of 7.0–10 (Figure 3). On the other hand, IA-BSA under identical conditions suffers an instantaneous loss of about 20% activity (Figure 3) and is subsequently *stable* over the time scale of the experiment. The loss of 20% activity cannot be due to the low concentration (0.26 *M*) of urea introduced into the assay solutions. Control experiments indicate that this concentration of urea causes only about 5% inhibition. Our results are in agreement with the studies of other workers[10] who noted the enhanced stability in 8 *M* urea of BSA which previously had its sulfhydryl group blocked.

At the concentration range under investigation after an initial brief period the inactivation of AR-BSA follows second-order kinetics (Figure 3). In this case we mean that the rate-determining step for loss of activity involves attack of one molecule by a second. The mechanism could involve dimerization, but we have no specific evidence here. We would expect that a plot of the reciprocal of the concentration of residual active protein vs. time would be linear for such kinetics. We have used relative activity as a convenient index of the residual native protein concentration. The average zero-time intercept for our second-order kinetic curves gave a reciprocal relative activity of 1.3 ± 0.1, which was *independent* of pH. Therefore, second-order rate constants, k_2, for all data were calculated assuming the zero-time intercept was exactly 1.3 each time. If the intercept was not held fixed, but was determined by a least-squares "best fit" approach, and then used in the calculation of k_2, large random errors appeared in k_2. Therefore, rate constants were calculated as described. The differences in the *slopes* of the calculated lines for the procedures (that is, either the zero-time intercept was held fixed or it was allowed to vary) were small (about 3% or less). These results, along with the pH dependence of the reaction (Figure 4), indicate that an intermolecular disulfide interchange (one possibility includes dimerization) catalyzed by the free *unprotonated* sulfhydryl group of the protein must be involved in the rate-determining step for irreversible denaturation and inactivation.[14] The pH dependence of the second-order rate constant for this reaction indicates a base (presumably the sulfhydryl group) of pK_a = 8.85 must be unprotonated for irreversible

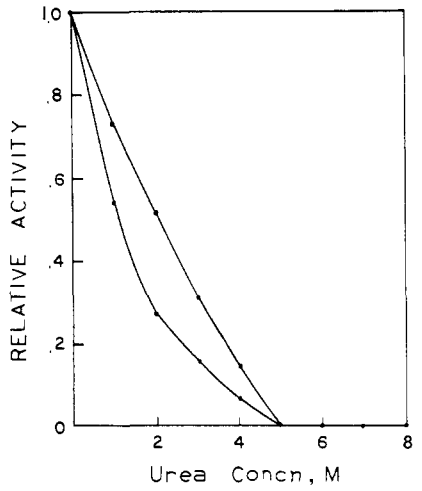

Figure 1. Relative activity of samples of IA-BSA (upper curve) and AR-BSA (lower curve) as a function of urea concentration, 0.05 *M* phosphate, pH 8.0, 25.0°. Both curves are normalized to the activity of each protein respectively in the absence of urea.

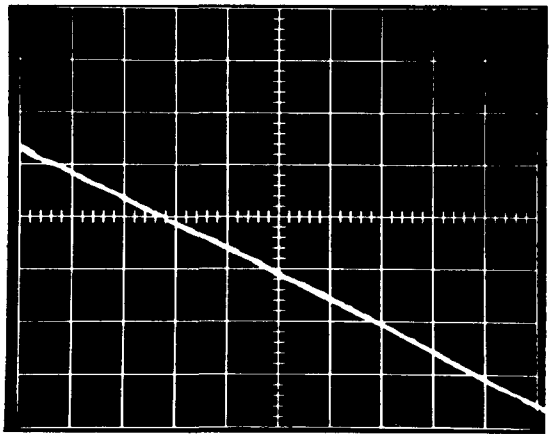

Figure 2. Stopped-flow experiment illustrating the return of activity when IA-BSA is diluted from 6 to $^6/_{11}$ *M* urea, at pH 9, 0.05 *M* Tris-HCl, 23°. Each horizontal division corresponds to 20 msec, and each vertical division corresponds to 0.01 absorbance units. λ = 478 nm. The final concentration of IA-BSA was 1.0 mg/ml. Ten parts of a solution of buffered substrate was mixed with 1 part IA-BSA in 6 *M* urea.

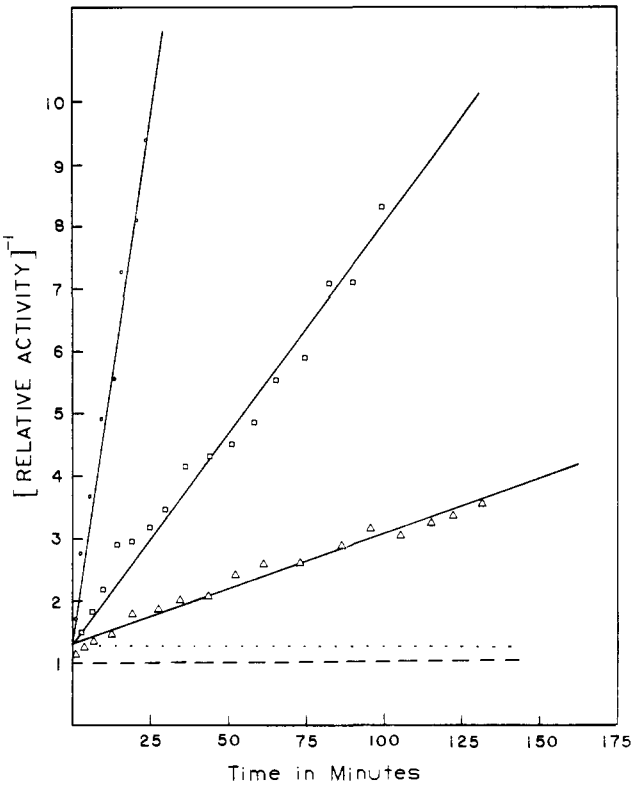

Figure 3. Second-order kinetic plots illustrating loss of activity in AR-BSA in 8 *M* urea (top three lines) at different pH's, 23°. Top line, pH 9.8; next line, pH 8.22; third line, pH 7.39. The fourth line from the top demonstrates that the activity of IA-BSA remains constant (approximately 80% of the control) in 8 *M* urea, pH 9.5. Bottom line, control. In each case 100 μl of the solution of the protein in 8 *M* urea was added to a 3-ml cuvette which contained substrate (ca. 74 μM) and 0.05 *M* phosphate, pH 8, and the remaining activity was determined. Data were analyzed for second-order kinetics by assuming that the concentration of residual "native" protein was proportional to the remaining activity.

denaturation to occur. This result is in good agreement with other reported values of the pK_a of sulfhydryl groups in denaturing media.[15] The fact that the initial intercept in the curves is too high (Figure 3) (i.e., the lines extrapolate to too low an activity) suggests that initially there may be a unimolecular unfolding leading to a modified structure of slightly reduced activity. A similar disparity between the control activity and the activity at zero time is observed for IA-BSA, despite a lack of any activity changes thereafter. Subsequently, the *intermolecular* mechanism dominates giving second-order kinetics and eventually all activity is lost.

As expected, we have observed that if the initial protein concentration in the urea is lowered (to about 4 mg/ml) the rate of denaturation decreased, and the results still gave approximate second-order kinetics.[16] The fact that denaturation *must* be an intermolecular process at the concentration range under investigation is proven in the following experiment. A solution of IA-BSA (about 7.5 mg/ml) was allowed to stand in urea at pH 9.5 for 5 hr. The activity remained approximately constant (approximately 80% of the control) over this time period. However, in a solution containing approximately equal amounts of AR-BSA and IA-BSA (each at about 7.5 mg/ml) the activity decayed to zero over the same time period. If an intramolecular denaturation mechanism was prevalent, we would have expected the activity to level off at about 40% of the control. As the activity did go to zero (although more slowly, as less free sulfhydryl groups were available to effect denaturation) we can be sure that the IA-BSA (normally *stable* under these conditions) was inactivated by reaction with the free sulfhydryl groups of AR-BSA. In retrospect this also explains why the activity in AR-BSA decays to zero despite the fact that the sulfhydryl titer of this protein is only about 0.7.[1b] We are confident that IA-BSA would also be irreversibly inactivated if it was incubated in 8 *M* urea in the presence of mercaptoethanol. This is essentially our "redox" experiment (see below). Unfortunately, mercaptoethanol reacts with the substrate and, therefore, we cannot assay the system directly.

Prolonged dialysis against 0.05 *M* KCl of a solution of BSA which had been completely inactivated by incubation in 8 *M* urea at pH 9 did not regenerate any activity. However, when this improperly folded protein was completely unfolded and reduced with mercaptoethanol in 8 *M* urea and then reoxidized, substantial activity was regenerated (see below). We conclude that the thermodynamically most stable disulfide pairing for the protein is not the same in 8 *M* urea as in 0.05 *M* KCl. The free sulfhydryl group in AR-BSA facilitates disulfide interchange in 8 *M* urea (and presumably formation of different conformational states and aggregates via different disulfide bonding arrangements) and structures are formed which cannot readily dissociate and refold to the "native" configuration when the urea is di-

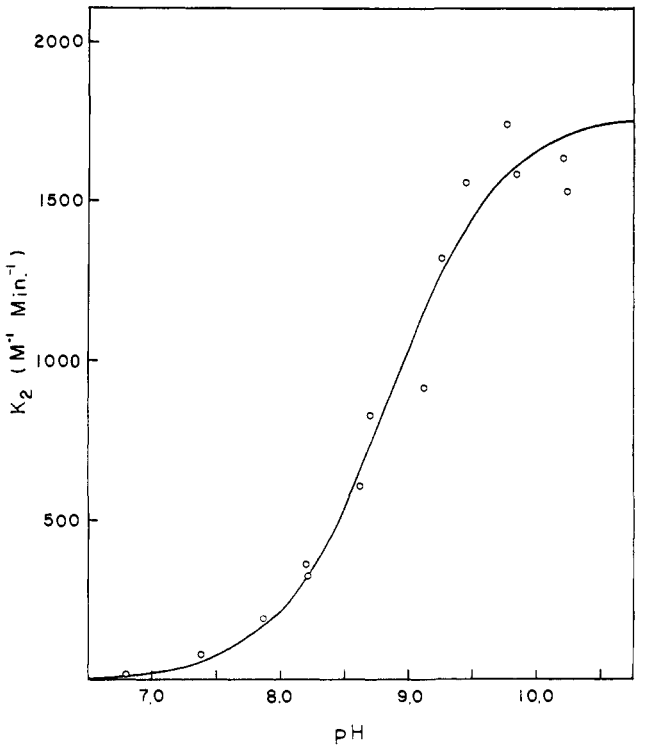

Figure 4. pH dependence for the second-order rate constants (k_2) obtained for irreversible inactivation of AR-BSA in 8 *M* urea, 23°. The solid line represents the least-squares fit to the data which indicates a group of $pK_a = 8.85 \pm 0.02$ must be unprotonated for inactivation to occur. The data were fit to the equation, $k_2 = k_2 \max/(1 + H^+/K_a)$; k_2 max was calculated to be 1770 M^{-1} min^{-1}.

luted out. Finally, we note that these results are in accord with the predictions and discussion set forth by Kauzmann and Douglas[17] 20 years ago on the urea induced denaturation of BSA which they studied by entirely different methods.

We find that disulfide interchange mechanisms are operative in AR-BSA (i.e., containing a free sulfhydryl group) even at lower pH values in 4 *M* guanidinium hydrochloride. Incubation of defatted AR-BSA for 4 hr in 4 *M* guanidinium hydrochloride at pH 5.2 (0.2 *M* acetate buffer) followed by prolonged dialysis against 0.05 *M* KCl resulted in a loss of 29% activity. IA-BSA treated under identical conditions was perfectly stable; the activity loss was less than 1%. These results are in excellent agreement with Foster's[10] comparisons of the conformational motility of BSA in which the sulfhydryl group is either blocked or free.

Air Reoxidation of the Unfolded, Reduced Species. Reduction of the 17 disulfide bonds in BSA followed by air reoxidation results in the regeneration of as much as ~70% of full activity, depending upon conditions (Table I). As noted by Andersson,[5] reoxidation in the presence of fatty acids helps to improve the final yield, but it should be noted that substantial activity is restored even in the absence of fatty acids. Peters and Goetzl[18] as well as Andersson[5] have indicated that the physical and antigenic properties of the "refolded" monomer obtained in the *absence* of fatty acids are significantly different from those of the native monomer. Experiments are currently in progress to compare the physical properties of our refolded active species and starting material.

Some aspects of the refolding experiment are particularly noteworthy. Though most samples were allowed to air reoxidize for 5 days, it is clear that the initial refolding process must be complete in not more than 1 day. The absolute yield of the monomer is considerably better if the free sulfhydryl group on the protein is *not blocked*. This demonstrates the importance of the free sulfhydryl group in acting as an internal catalyst to help the protein seek out its best disulfide bond arrangement. Dimers[19] and polymers due to intermolecular disulfide bond formation are presumably not favored thermodynamically but are probably less easily dissociated if the free sulfhydryl group is blocked.

Table I. Recovery of Activity in Unfolded, Reduced BSA upon Air Reoxidation

Starting material	% act. recovered	% act. recovered in monomer
IA–BSA	22	73[a]
AR–BSA	60	72[b]
Armour defatted monomer	37	61[c]
Armour defatted monomer[d]	55	68[e]
Armour crystalline (not defatted)[f]	15	*g*
X–BSA[h]	16	*g*

[a] Yield of monomer was 12%. [b] Yield of monomer was 42%; recovery of activity is calculated relative to the activity of the defatted monomer. [c] Yield of monomer was 47%. [d] Sample was reoxidized in the presence of 2×10^{-5} *M* palmitate and then charcoal defatted. [e] Absolute yields of monomer could not be calculated because protein is lost on charcoal defatting. [f] Identical results were obtained for either 1 or 4 days of air reoxidation. [g] Not done. [h] Sample was Armour crystalline BSA which had been previously completely inactivated by overnight incubation at pH 9 in 8 *M* urea. A "control" in which the urea was simply dialyzed out had *no activity*.

This result provides an interesting contrast with the results obtained from incubation of AR-BSA in 8 *M* urea. In that case the free SH group acts as an agent to *denature* the protein, because presumably other disulfide bonding arrangements are thermodynamically more stable in the denaturing medium. In the refolding experiments the free SH group apparently helps refold the protein back to its "native conformation". We cannot tell if the SH group acts on an intra- or intermolecular basis during refolding, but considering the fact that much lower protein concentrations are used, the intramolecular mechanism seems more reasonable. If higher protein concentrations were used during refolding it is likely that there would have been more polymerization due to formation of disulfide bonds between partially refolded intermediates.

Finally, as we have noted, BSA which was previously irreversibly inactivated through a disulfide interchange in 8 *M* urea can also be *reactivated* if the improper disulfide pairing is eliminated through massive unfolding and reduction of the molecule followed by air reoxidation. This experiment provides strong evidence indicating that the catalytic activity of BSA serves as a sensitive probe of the thermodynamic forces which influence the conformation and integrity of its catalytically active site. Furthermore, it tells us that urea denaturation is not thermodynamically irreversible; rather, the kinetic barriers to proper disulfide rearrangements are too high in solvents in which the protein should be native.

Other Conformational Transitions. We can detect little activity for BSA below pH 6. The protein is known to undergo large conformational transitions at both low (about 4)[20] and high (about 10.5)[21] pH and our experiments indicate the conformational isomers obtained in these regions are not active. However, our "pH jump" experiments indicate that activity is restored in less than 10 msec when an unbuffered solution of the protein at either pH 3 or 11 is mixed with buffered substrate at pH 8. It will be desirable to see if other measures of the protein's folding (such as tryptophan absorbance)[22] give similar rapid refolding rates.

Microheterogeneity. Subfractionation of albumin using Foster's[12] 3 *M* KCl precipitation technique indicates that there are small but reproducible differences in activity be-

Table II. Correlation of Subfractionation of BSA with Catalytic Activity

Sample	pH of fraction	% of total	Rel act.[a,b]
IA–BSA[c]	4.35	6.4	0.72
	4.25	3.8	0.94
	4.15	66.0	1.09
	4.00	23.0	1.03
	3.24	0.8	*d*
Armour crystalline BSA	4.10	21.9	1.15
(not defatted)	3.94[e]	*d*	1.02
	3.89	47.9	0.91
	3.25	30.2	0.84

[a] In both cases a dialyzed control (not precipitated at all) had about 10% more activity than a sample which was totally precipitated. [b] Relative activity refers to the activity in a given subfraction vs. the activity obtained from a sample which was precipitated at pH 3.25 without prior removal of "early" subfractions. [c] Results are the average of two independent experiments. [d] Not done. [e] This pH was examined during a different experiment.

Table III. Effects of Thermal Denaturation at 66.8° on BSA[a]

Sample	Time in bath, sec	% denaturation[b]	% act. lost[c]	% act. in precipitate
AR–BSA[d]	15	34 ± 1	37 ± 2	4 ± 2
	60	66 ± 4	66 ± 1	6 ± 2
	120	80 ± 1	79 ± 1	6 ± 2
IA–BSA[e]	180	10 ± 2	0	24 ± 4
	300	20 ± 1	5 ± 4	39 ± 7
	420	37 ± 2	22 ± 3	41 ± 9
	600	56 ± 4	41 ± 4	30 ± 10
	1200	80 ± 1	66 ± 4	20 ± 4

[a] All data were taken at a protein concentration of about 8–10 mg/ml. At an initial concentration of 4–5 mg/ml similar results were obtained for denaturation of AR–BSA, in agreement with the general observations of Warner and Levy.[11] [b] Denaturation is defined as loss of solubility in the high salt buffer of Warner and Levy.[11] [c] Activity was measured in our "standard assay".[8] [d] Based on two independent experiments. [e] Based on three or four independent experiments.

tween different subfractions (Table II). It is interesting to note that for IA-BSA the first subfraction has the lowest relative activity, but in *unpurified* Armour crystalline BSA the earliest subfraction has the highest relative activity. Presumably this can be related to the effects of bound impurities and dimer in the "unpurified" BSA. The observation of different activities for different subfractions of both samples provides strong independent proof of the validity of the microheterogeneity model in protein conformation in serum albumin proposed independently by Foster[23a] and Stokrova.[23b]

Thermal Denaturation Experiments. Incubation of albumin at elevated temperature in the neutral pH range is known to cause conformational unfolding and aggregation.[11] We find that the activity of AR-BSA is rapidly abolished under these conditions (Table III). In addition, there is an excellent correlation between the extent of denaturation (measured by the amount of protein which is not precipitated by the "high salt" buffer) and the extent of inactivation determined by measuring the residual catalytic activity after the high-temperature incubation (Figure 5). As expected, when the denatured precipitates were redissolved and the excess salt was dialyzed away the recovered protein had very low activity (approximately 5%). The fact that *any* activity is seen in the precipitates indicates that the inactivation may be slightly reversible under the conditions of the experiment. This experiment offers additional evidence that the catalytic activity we study is, in fact, a sensitive probe of the conformational state of the protein. Within the time scale of our experiment, the *rate* of inactivation of the protein (i.e., loss of catalytic activity) is identical with the rate

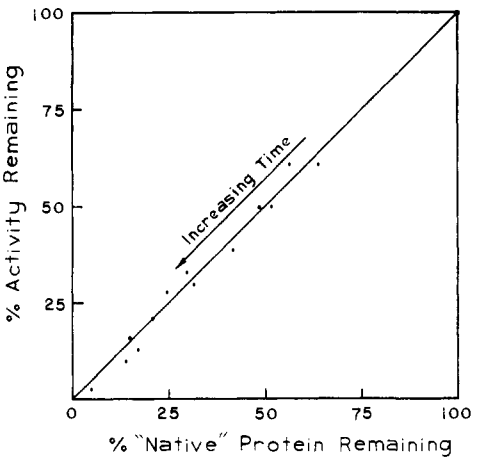

Figure 5. Illustration of the equivalence of denaturation and inactivation (see Table III for definitions) on heating AR-BSA at 66.8°. The points range in time from 15 (approximately 60% activity remaining) to 300 sec (approximately 3% activity remaining).

of denaturation (i.e., loss of solubility in the high salt buffer) and therefore the two processes may have a common rate-determining step. *Instantaneous* assays of both activity and extent of denaturation after heating would have to be performed to prove this point.

The results with IA-BSA were considerably more complicated. In order to obtain significant precipitation in the high salt buffer longer incubation periods were required (Table III). In addition, there was a poor correlation between the rate of loss of catalytic activity and the rate of denaturation measured by loss of solubility (Table III). Apparently in IA-BSA the protein "denatures" more rapidly than it loses catalytic activity. This was in fact confirmed when it was found that redissolved and dialyzed precipitates of IA-BSA had substantial activity (Table III). Errors in the data are too large to allow for quantitative comparison of the observed activity in the precipitates with that expected based on the ratios of percent denatured to percent activity lost. However, we note that there is considerably more activity in these precipitates than is found when AR-BSA is examined (Table III). The results cannot be due to any artifacts introduced by the high salt buffers. Samples of both IA-BSA and AR-BSA were incubated in the high salt buffer for 12 hr. Subsequently, the solutions were dialyzed exhaustively against 0.05 *M* KCl and then the proteins were assayed for activity. In each case full activity was observed.

Discussion

Our activity assay provides a direct test of the conformational integrity of BSA. Those parameters which are known to induce conformational changes in the protein have the expected effect on measured activities. The active sites of proteins (be they enzymes, antibodies, or molecular carriers) are generally produced by the juxtaposition of amino acids which are often "far apart" with regard to primary structure.[24] One of the chief functions of the protein structure is to maintain the active site in the proper configuration. It is not surprising, therefore, that conformational changes which disrupt protein conformation lead to loss of biological activity. We find that a similar situation obtains for the BSA activity we have studied.

The regeneration of significant activity from the unfolded reduced protein on air reoxidation even in the *absence* of fatty acids might be somewhat surprising in view of the results of other investigations.[5,18] This suggests that despite the fact that some physical properties of the protein changed, the structural integrity of the *active site* was regenerated to a great extent. One possible explanation of

these results is that some improper disulfide pairings occurred which, although they affected certain physical properties of the protein, did not significantly damage the active site. That is, perhaps the driving force for refolding of the protein in the vicinity of its active site is greater than the forces which govern refolding of other parts of the molecule. Put another way, it suggests that the active site may be in a domain of greater conformational stability than other parts of the molecule.

Our thermal denaturation experiments which compare the changes occurring in IA-BSA with AR-BSA on heating provide additional evidence for these hypotheses. In AR-BSA we find that there is an excellent correlation between the rate of denaturation and the rate of loss of activity. This suggests that thermal denaturation in AR-BSA must be a cooperative process which both destroys the active site and unfolds enough of the molecule to cause it to precipitate in the high salt buffer. The free SH group of the protein serves as an efficient agent to facilitate rapid disulfide interchanges (probably both inter- and intramolecular) and this can *couple* the unfolding processes of the various domains.

In IA-BSA, on the other hand, there must be a partial *uncoupling* of denaturation from loss of activity. This is because we isolate species which appear to be denatured by one criterion (insoluble in the high salt buffer) but still have significant activity. Presumably the first step for IA-BSA involves unfolding and possible aggregation of the protein; these steps must principally involve domains outside the region of the catalytic active site as the protein is more "denatured" than it is catalytically inactive. Subsequently, further unfolding and aggregation must occur which serve to block or disturb and therefore destroy the catalytic active site of the protein. The important point to recognize is that these results provide strong evidence for the existence of a *partially unfolded intermediate* in IA-BSA in which the active-site region must still maintain more "native" structure than other domains. This is further evidence for the enhanced stability of the active site as opposed to other regions of the molecule.

To summarize, blocking the free SH group in BSA with iodoacetamide prevents disulfide interchange between domains and thus uncouples different domains of the BSA molecule during thermal denaturation. Subsequently if certain domains in IA-BSA are less stable than the active site domain, they will unfold first and the protein will appear denatured by certain criteria despite the fact that the active site is intact and activity is maintained to a significant extent.

The rapid return of activity observed in our urea dilution and pH jump experiments is most intriguing. It seems reasonable that the 17 disulfide bonds in BSA preserve much structure when the molecule unfolds and therefore provide substantial direction to facilitate rapid refolding. However, the fundamental question is whether *all* of the molecule rapidly refolds, or does the active site refolding "lead the way", in a sense, and proceed more rapidly than the refolding of other domains. If in fact the active site is thermodynamically more stable, this might be manifested in a kinetics experiment as well. Physical measurements which examine the rate of refolding by monitoring chromophores associated with *other* domains on the protein should answer these questions.

It is likely that these results can be extended to include other proteins which contain multiple domains;[25] the immunoglobulins are the best example. Rowe and Tanford[26] have shown that the unfolding and refolding of a homogeneous immunoglobulin light chain does not follow a simple "two state" picture. The results are most consistent with the progressive refolding of independent domains. The question of the degree of interaction between domains in these proteins remains to be answered, although it is known that the antibody binding region can bind hapten even when the F_c region and additional portions of the constant regions of the H and L chains are removed enzymatically.[27] Whether or not the various domains undergo other conformational transitions (e.g., initial folding after synthesis) independently of each other is not known but must be considered a possibility.

A potential way to extend our studies of domains includes examining the conformational characteristics of protein domains which can be isolated by limited proteolytic cleavage reactions. Large molecular weight fragments corresponding to different domains have been obtained for both BSA[2] and the immunoglobulins.[27] If, in fact, different domains can fold and unfold in an "uncoupled" independent fashion in the intact molecule, it should be possible to examine analogous conformational transitions in the isolated domains. Such experiments will be most useful in extending our understanding of the forces which stabilize proteins in solution.

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