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STOPPED-FLOW STUDIES OF THE N ⇌ F TRANSITION IN SERUM ALBUMIN

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Stopped-flow studies of the refolding of iodoacetamide-blocked bovine serum albumin from the acid unfolded "F" state have been performed. If the protein is incubated with low concentrations of perchlorate anion then the refolding kinetics follow a simple first-order process. The dependence on pH of both the amplitude of the observed transients and the measured rate constants indicates that the N ⇌ F transition is highly cooperative. The results are consistent with the postulated multidomain structure of albumin which has been developed as a result of both sequence work and a variety of physical studies.

1. Introduction

Detailed studies of the well-known acid induced N ⇌ F transition in serum albumin indicate that an accurate description of the physical chemistry of the process depends upon a number of factors [1–7]. The particular physical variable being monitored, the ionic strength and pH of the solution, and the degree of heterogeneity of the protein [8] all influence the nature of the observed transition. Brown's elegant sequence work [9] and the results of other physical studies [10] have revealed that albumin is folded as a multidomain protein and it is clear that the interaction between domains (or lack of it!) can also strongly influence any cooperative transition in the protein.

Recently we have reported that an unusual enzyme-like activity of BSA [11,12] can be used as a sensitive probe of the conformational dynamics [13] and equilibria of the protein. For example, we have used this activity in an assay to demonstrate that isolated domains of the protein will refold independently with high fidelity to their "native" states from the unfolded, reduced (random coil) structures [14]. In addition, we have reported that when the pH of the protein is rapidly changed from the inactive unfolded F form (pH 3.5) to the folded, active N form (pH 8.0) full catalytic activity is restored in less than 10 ms [13]. In this paper we have examined the kinetics of refolding of the protein from low pH by studying a different variable, its intrinsic fluorescence. Our results indicate that the refolding process is not as fast when this parameter is studied. By carefully controlling the pH and ionic strength we have been able to isolate a region in which the refolding process is simple and follows first order kinetics and in which the cooperative nature of the transition is clearly revealed by the pH profiles of the transition parameters.

2. Materials and methods

IA-BSA* was prepared as described by Taylor et al. [12]. IA-HSA was prepared in an analogous manner. All reagents and buffers were of the highest purity available. Phenol red (from Sigma) was recrystallized three times according to the procedures of Orndorff and Sherwood [15].

Stopped-flow experiments were performed with an instrument built at the University of Minnesota, with a cell capable of measuring both fluorescence and absorbance. Protein fluorescence was monitored with a Xenon-Mercury lamp as an excitation source (excita-

* Abbreviations used: IA-BSA, iodoacetamide-blocked, defatted monomeric bovine serum albumin; IA-HSA, human serum albumin treated the same way.

tion wavelength = 290 nm, band width = 3 nm) and a cut-off filter which let through light at wavelengths greater than approximately 305 nm. With the special cell the dead time of the instrument was determined to be between 18 and 20 ms. Data were recorded on a Biomation Model 802 transient recorder and then displayed on a "Y-time" recorder. In a typical experiment IA-BSA at a concentration of approximately 2×10^{-6} M in 0.01 M acetate, pH 3.5 in the presence or absence of 0.02 M perchlorate (see below) was mixed with 0.2 M acetate, pH of ca. 5.6, and the increase in protein fluorescence with time was monitored. Both the rate of the reaction *and* the amplitude of the transient were measured. For this "standard experiment" ca. 40% of the expected transient (based simply on steady-state values) was observed. This is in reasonably good agreement with the dead time of the instrument and the observed rate constant for the refolding reaction (see below).

Rate constants for the first order reactions were determined by semilogarithmic plots (cf. fig. 2). Typically 3 or 4 independent transients were obtained for a given reaction. The reproducibility in observed rate constants was generally about ± 5% or better. The curves describing the pH dependence of the amplitude and rate constants for the transients (figs. 6 and 7) were fit by the Gauss–Newton method [16]. Essentially identical kinetics (as seen in fig. 1) were obtained for our typical experiment if acetate was not present on the *protein side* before mixing or if an excitation wavelength of 280 or 295 nm was used instead.

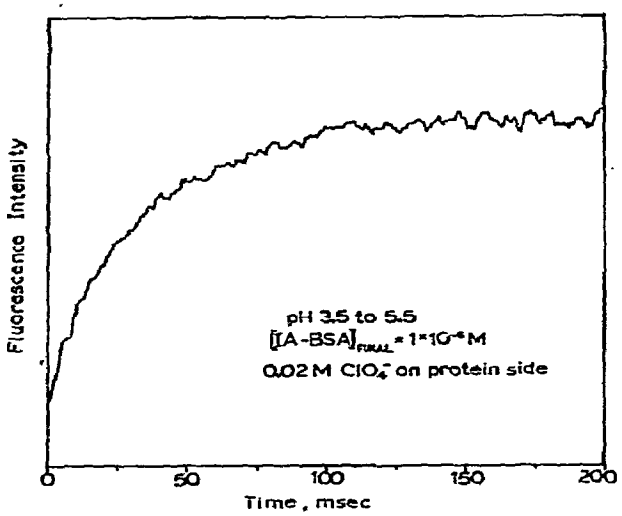

Fig. 1. Increase in fluorescence intensity with time at 25°C upon rapid mixing of equal volumes of a solution of 2×10^{-6} M IA-BSA in 0.02 M sodium perchlorate, pH 3.5 with a solution of 0.2 M acetate, pH 5.6.

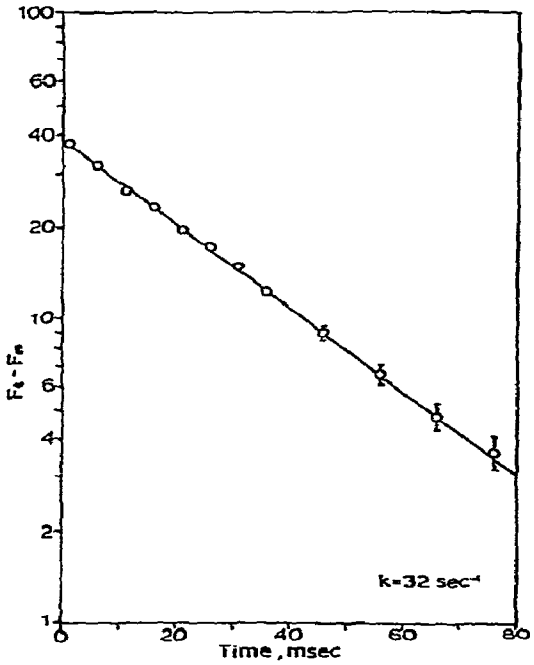

Fig. 2. First order plot of the transient in fig. 1.

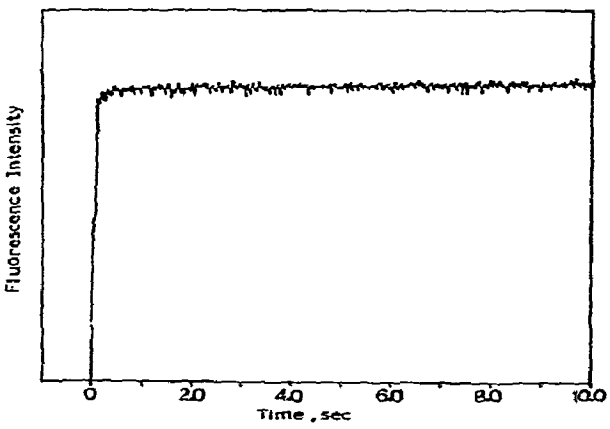

Fig. 3. Same reaction as fig. 1, except over a longer time frame.

A few preliminary experiments on the phenol red-IA-BSA system (see below) were performed with an absorbance cuvette with an instrumental dead time of 2 ms and the same transients were observed for this system as were seen when the cell with the 18 ms dead time was used. All experiments were performed at 25.0°C.

3. Results

3.1. Kinetics of the F ⇌ N transition in IA-BSA

The refolding of IA-BSA from the unfolded F form follows simple first order kinetics if a solution of the protein in this unfolded form in the pH range of 3.0 to 4.2 also contains 0.02 M perchlorate (figs. 1–3) before the pH jump. However, if perchlorate is not present on the protein side, the refolding reaction is considerably more complicated; multiple transients are observed (figs. 4,5). For this reason, in all experiments reported in this paper solutions of the unfolded F form of the protein always contained 0.02 M perchlorate. We have noted that if the high pH buffer syringe (pH 5.6) contained the perchlorate *instead* of the protein side, transients such as those depicted in figs. 4, 5 were obtained. Thus, it appears the perchlorate must affect the conformation of the *unfolded* F *form* of the protein in such a way that refolding

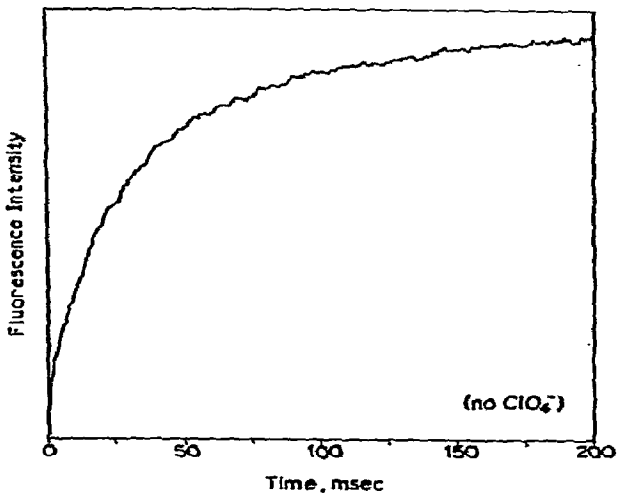

Fig. 4. Same reaction as fig. 1, except in this case the protein solution did not contain any perchlorate.

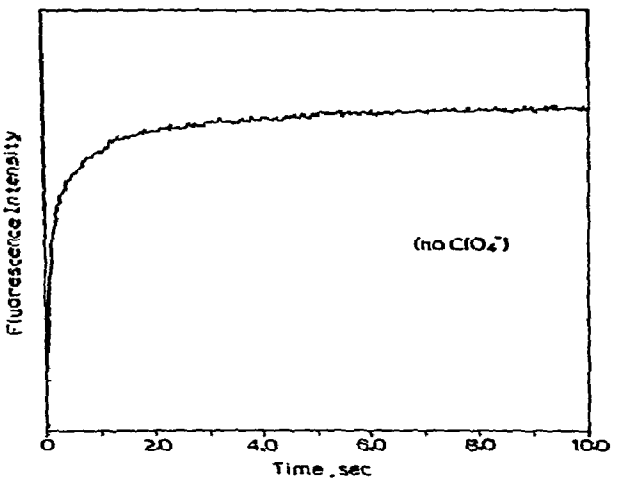

Fig. 5. Same reaction as fig. 4, except over a longer time frame.

occurs through a simple first order pathway. As expected the rate of the refolding transition depicted in figs. 1–3 was *independent* of the protein concentration in the range of ca. 5×10^{-7} M to 5×10^{-6} M. The kinetics we have studied are not affected by albumin microheterogeneity. Four different subfractions of albumin isolated by precipitation with 3 M KCl [17] in the pH range of 4.4 to 4.0 gave the same kinetics for the experiment as the unfractionated material.

The *rate* of the observed refolding reaction (for a *final pH* of anywhere between 5.5 and 8) is independent of the *initial pH* of the protein solution in the pH range of about 3.0 to 4.2. If the initial pH of the unfolded protein was less than 3.0 considerably more complex kinetics were seen. It is well known [1,2] that the protein unfolds further below pH 3.0 and we have therefore focused our studies on the unfolded form of the protein which exists *above* pH 3.0. The *amplitude* of the transient is strongly pH dependent (fig. 6); the results suggest that a cooperative unit containing 6 protons which are titrated together must be involved in the *unfolding* transition. This is because the amplitude of the transient is, in effect, directly proportional to the fraction of protein which is initially in the unfolded F state. Many workers have shown that this state is characterized by a lowered tryptophan fluorescence [4,5,18];

The fact that the observed amplitude of the transient (see experimental section) is in reasonable agree-

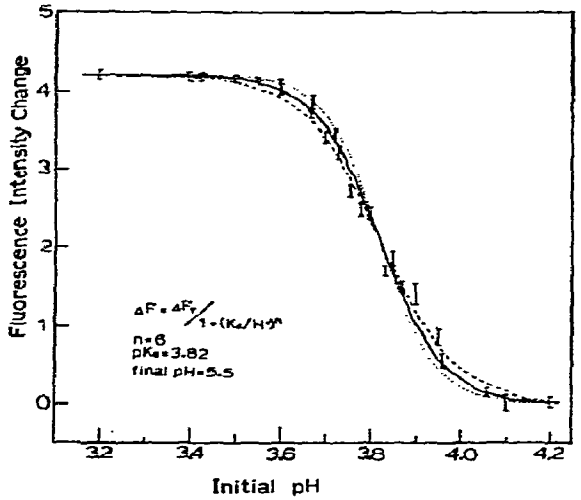

Fig. 6. The dependence on the *amplitude* of the observed transient (see fig. 1) as a function of the initial pH of the protein. The protein side contained 2×10^{-6} M IA-BSA in 0.02 M perchlorate, and the pH varied between 3.0 and 4.2. The buffer side contained 0.2 M acetate, pH 5.6. The solid line drawn through the data is a theoretical curve for a cooperative transition involving 6 protons with a midpoint at pH 3.82. The dashed and dotted lines represent the same transition but with either 7 or 5 protons, respectively, in the cooperative unit.

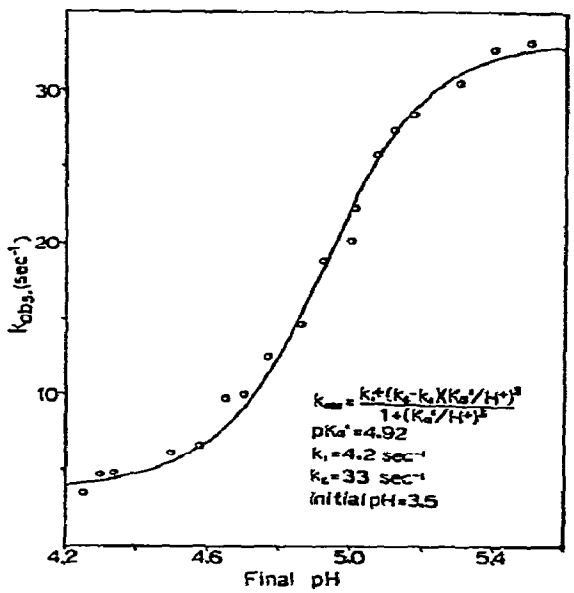

Fig. 7. Dependence of the first order rate constant for the transient (see fig. 1) on the *final* pH of the solution. In each case IA-BSA at 2×10^{-6} M in 0.02 M perchlorate, pH 3.5, was mixed with a solution of 0.2 M acetate, where the final pH of the sample after mixing varied between 4.2 and 5.5. The rate constant for a final pH between ca. 6 and 8 was identical to that seen at 5.5.

ment with that expected based on the dead time of the instrument and the rate constant of the reaction indicates that quenching by the *protonated* carboxyl groups of the protein is probably not a significant quenching mechanism in the F state. This is because the carboxyl groups should be deprotonated immediately after the pH jump (in considerably less than 20 ms) and yet considerable quenching of tryptophan fluorescence is still evident *immediately* after the pH jump. The transient then presumably is indicative of a refolding of the protein from its previous F state conformation into its final N state conformation in which the tryptophans move from a region in which they fluoresce weakly into one in which they fluoresce more strongly.

Some cooperativity is also seen in the refolding kinetics; whereas the amplitude of the transient is independent of pH in the range of 5.1 to 8 (for an initial pH of 3.5), the *rate* of refolding shows a marked pH dependence (fig. 7). The results suggest that 3 protons must be involved in the cooperative unit during refolding, and it should be noted that the midpoint of the titration curve in fig. 7 is considerably higher than the midpoint of the transition characterizing the *unfolding* reaction (fig. 6).

3.2. Results with phenol red

The binding of phenol red to albumin is known to cause a perturbation in the absorption spectrum of this dye [19]. We have used phenol red to probe the rate of refolding of IA-BSA by measuring the change in absorbance at 560 nm with time when a solution of IA-BSA (4×10^{-5} M) and phenol red (3.0×10^{-5} M) in 0.02 M perchlorate, pH 3.4, are mixed with 0.15 M phosphate, pH 7.8. A single first order transient of rate constant ca. 32 s^{-1} is observed, thus indicating that the phenol red must be bound in a domain which refolds at the same rate as the regions of the protein which contain its tryptophans. We have noted that the direct binding of phenol red to native IA-BSA at pH 7.8 occurs considerably *faster* than 18 ms, if the pH jump is omitted.

3.3. Preliminary results with IA-HSA

Human albumin contains only 1 tryptophan but its overall three dimensional structure is believed to be very similar to bovine albumin. Our standard pH jump experiment with IA-HSA (8×10^{-6} M protein in 0.02 M perchlorate, pH 3.5, rapidly mixed with 0.2 M acetate, pH 5.5) also reveals a single first order transient of rate constant ca. 5 s^{-1}.

3.4. Studies of N → F transition

The unfolding of IA-BSA occurs considerably faster than the refolding reaction. Experiments in which a pH jump down from 5.5 to 3.5 was performed indicate that greater than 95% of the decrease in tryptophan fluorescence occurs in less than 20 ms for IA-BSA. A similar very rapid unfolding of BSA for a pH jump down from 7 to 2 has previously been reported by Brewer and Desa [20].

3.5. Studies within the region of the N ⇌ F transition

Stopped-flow experiments on IA-BSA where the final pH is between 3.0 and 4.2 indicate the kinetics of the interconversion of the N and F forms within this region are rather complicated. For example, if the pH of a solution of IA-BSA originally at pH 4.5 is rapidly lowered to 3.8 a small transient (half-life of about 0.2 sec) of decreasing fluorescence is observed. However, if the pH of the protein is rapidly *raised* from 3.3 to 3.8 greater than 90% of the increase in fluorescence apparently occurs faster than 18 ms. This suggests there may be intermediates along the kinetic pathway within the N ⇌ F transition and we are currently investigating this possibility.

3.6. Comparison with a recent study

Recently Rudolph et al. [7] reported on stopped flow studies of the N ⇌ F transition in albumin using protein fluorescence. Their results are considerably more complicated than ours; over a considerably wider range of pH than we examined they detected transients containing multiple exponentials. Where comparisons can be made between our data and theirs the agreement is not very good. There are some possible explanations for this fact. First, they did not use perchlorate in their system, and, in addition, it is not clear if they took precautions with albumin after it was defatted. Foster has shown that a number of isomerizations can occur in the protein after it is defatted if the free sulfhydryl group is not blocked. This is the reason we always block the sulfhydryl group *before* the protein is defatted.

4. Discussion

Recent work by Sogami et al. [5,6] and earlier studies by Leonard and Forster [3] have shown that by appropriate manipulation of ionic strength it is possible to *separate* the N ⇌ F transition into discrete phases which involve the formation of intermediate states. Measurement of protein fluorescence, optical rotation, or fluorescence polarization and lifetime reveal discrete breaks in curves characterizing the transition; these breaks suggest the presence of intermediate forms of unfolded protein. Our kinetic analysis confirms these steady state analyses. We have detected an unfolded F state for IA-BSA which by our *kinetic criteria* behaves as a well defined entity. Simple first order kinetics are observed over a relatively wide range if the protein is incubated with perchlorate in the unfolded state. In the absence of perchlorate the kinetics are considerably more complicated. Under these latter conditions it is likely that a number of unfolded states exist and the kinetics presumably monitor the refolding transition for these various states.

Leonard and Foster [3] were the first investigators to show that low concentrations of anions such as perchlorate and thiocyanate could be used to control the structure of the acid unfolded forms of albumin. These anions are generally considered to *denature* proteins [21]; that is, they are on the "salting in" or "destabilizing" side of the Hofmeister series with respect to their interaction with proteins. It is likely that these anions act because of their enhanced ability to bind to a protein relative to other anions in the series [22]. However, in the case of albumin the binding of perchlorate would appear to prevent further unfolding. Presumably in the present case the perchlorate acts to decrease disruptive interactions between regions of high positive charge in albumin at pH 3.5. There is in fact a precedent for this observation. Peggion et al. [23] have shown that high concentra-

tions of perchlorate anion can be used to cause the *protonated* form of poly-L-lysine to refold from a random coil to a helical structure. In this case the anion must be acting to decrease the unfavorable interactions among neighboring positive charges in the helix. A number of investigators have suggested that multiple forms of an *unfolded* state of a protein influence its *refolding* kinetics [24–26]; an impressive array of evidence indicates this may be the case for the kinetics associated with thermal unfolding reactions in small globular proteins [25,26]. What the specific mechanism is that generates these multiple forms of the unfolded state for albumin (e.g., trans⟷cis isomerization about prolines [26]) remains to be determined.

The number of protons involved in the apparent cooperative unit in the N → F transition is striking. The best titration data available, that of Tanford [27], suggests that approximately 18-21 protons are titrated in albumin between pH 4.2 and 3.5. The fact that only about 6 "show up" in the transition (fig. 6) may be explained in terms of the 3 domain structure of albumin which Brown [9] has postulated on the basis of his sequence studies of the protein. It is reasonable based on Wetlaufer's arguments that each domain unfolds independently [28]; there are sequence homologies in each domain [9] and it is possible that *each* domain unfolds in an independent cooperative manner upon loss of about 6-7 protons. Thus, although the total number of protons involved in titrating the protein is about 20, based on this mechanism we would expect only 6-7 to appear in the unfolding transition. It should be recalled that in 1960 Foster [29] also suggested on the basis of preliminary data on the N ⇌ F transition that albumin must contain multiple domains which are folded independently.

We recognize that we are measuring protein fluorescence *only*, and as there are 2 tryptophans on BSA, and they are located on different domains, it is possible at least that the entire transition involves only 1 tryptophan in 1 specific domain. This is unlikely, however. Numerous other studies using a variety of other physical criteria have generated rather sharp transition curves similar to that seen in fig. 6, although the number of protons involved in the transition was generally not determined in these earlier studies [1–6]. We emphasize that the work of Leonard and Foster [3] using optical rotation and that of Sogami et al. [5,6] using fluorescence all suggest that in the presence of low concentrations of perchlorate a sharp transition occurs in the protein in the pH range of about 4.2 to 3.5, and our results are in excellent agreement with these observations and strongly support the domain theory of structure for albumin. We also note that a transient of the same rate constant was observed when the absorbance of an IA-BSA-phenol red complex was used to monitor refolding.

The nature of the cooperative unit in the *kinetics* of refolding from the F state apparently must involve different protonation sites from those involved in the unfolding reaction. There is a high degree of cooperativity in the transition. Though the protein apparently refolds completely (as judged by steady-state criteria) if the final pH is above 4.2, it can be seen that the refolding *rate* is faster if 3 groups in a cooperative unit are unprotonated, where the midpoint of transition is pH 4.9. Titration data indicate that in the pH range of 4.2 to 5.4 about 18 groups are titrated [27]. As only 3 protons directly influence the kinetics of refolding in this region it is likely we are monitoring a considerably different process (on the molecular scale) in the refolding kinetics than what is observed in the unfolding transition. We note that these results (i.e., the number of protons in a cooperative transition) do not contradict the principles of microscopic reversibility because the transition *kinetics* for refolding (F → N, fig. 7) occur in a completely different pH range from that in which the *amplitude* of the transition for unfolding (N → F, fig. 6) was monitored.

Though our results indicate there are cooperative processes involved in the N ⇌ F transition in albumin, obviously many of the molecular details remain to be unraveled. It would be most interesting to determine how the interaction between domains and within a domain affects the processes we see. One possible approach to understanding this problem would involve preparing fragments of albumin [30,31] which contain intact domains and then studying their individual physical characteristics both with respect to the N ⇌ F transition and with respect to the reassociation of the fragments. Preliminary studies on the reassociation behavior of certain fragments have already been reported by us and others [11,14,30,32], and it is likely that further studies on these fragments along the lines discussed in this present work will lead to a clearer understanding of the specific interactions which govern the structure and stability of albumin.

Acknowledgement

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