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Detection of intermediate protein conformations by room temperature tryptophan phosphorescence spectroscopy during denaturation of *Escherichia coli* alkaline phosphatase

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Abstract

The reversible denaturation of *Escherichia coli* alkaline phosphatase (AP) was followed by monitoring changes in enzymatic activity as well as by measurements of the time-resolved room temperature phosphorescence from Trp 109. It is well known that the denaturants, ethylene diamine tetraacetic acid (EDTA), acid and guanidine hydrochloride (GdnHCl) inactivate AP by different mechanisms as reflected by differences in the time dependence of inactivation. However, further information about structural changes that result during inactivation is obtained by measurement of the phosphorescence intensity and radiative decay rate. Time-resolved tryptophan phosphorescence is exquisitely sensitive to changes in the local environment of the emitting residue, unlike the steady state phosphorescence intensity which is a composite of both the lifetime and concentration of the emitting protein species. The results show that while inactivation in EDTA proceeds by loss of the zinc ion as expected, denaturation in acid or GdnHCl produces a heterogeneous population of AP molecules, detected by a distribution analysis of the phosphorescence lifetime, which may reflect multiple pathways to the final unfolded state. Time-resolved phosphorescence also demonstrates the existence of an enzymatically active but structurally less rigid intermediate state during unfolding. As the rigidity decreases, the susceptibility to further denaturation decreases at lower pH but increases with GdnHCl concentration. The experiments provide new insight into the mechanism of denaturation of AP and demonstrate the sensitivity of time-resolved room temperature phosphorescence to the structural details of intermediate states produced during unfolding of proteins.

Keywords: Protein unfolding; Folding intermediate; Tryptophan phosphorescence; Time-resolved phosphorescence

1. Introduction

The amino acid sequence of a protein contains all the information required to determine the

final three-dimensional (folded) structure. However, the actual mechanism by which this information is used to direct the folding of the protein into its correct conformation remains to be completely determined. Typical rates of protein folding are far greater than would be expected from a model where folding is directed by the random twisting and rotation of a linear chain in solution

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[1]. Much of the current research focuses on the putative presence of various kinetic intermediates in the folding process, on whether there is one unique pathway for folding, and whether all proteins follow the same general folding rules. A closely related process is the unfolding of proteins by various denaturing agents. These agents are believed to inactivate proteins by disrupting the forces which hold them together, and their selective use can yield information on the stability and structure of proteins. In addition, by following various signatures which relate to the degree of protein structure such as activity or luminescence lifetime, it is possible, in principle, to follow folding and unfolding dynamics and provide structural information on the nature of intermediate states.

The folding of proteins is driven largely by hydrophobic interactions among nonpolar amino acids. Other major forces involved in the folding and stabilization of proteins are Van der Waals forces, electrostatic interactions, hydrogen bonds, and covalent bonds between cysteines and between amino acids and prosthetic groups. A direct method for studying the effect of each of these is to study conformational changes in the presence of agents which disrupt the different kinds of bonds. Exposure of a protein to one of these agents while monitoring parameters of the protein (e.g., activity, absorption, or fluorescence) can help determine which forces are most important in the stability of that protein. For proteins whose structure is at least partially known, the observed effects can often be correlated with specific regions of the protein. This information may be used to determine the sequence of events in the process of protein folding.

In the present study the unfolding of *Escherichia coli* alkaline phosphatase (AP) was monitored in the presence of various denaturants. AP is an extremely stable enzyme, resistant to heat denaturation and to denaturation by sodium dodecyl sulfate (SDS). It is a metalloenzyme, containing four zinc atoms and two magnesium atoms per dimer. In addition it has recently been shown to possess an intense, long-lived tryptophan phosphorescence. Alkaline phosphatase has three tryptophans per subunit; however, it has been

determined that the phosphorescence arises from Trp 109, located near the core of the protein [2]. Experiments reported here compare changes in the enzyme activity with changes in the phosphorescence as a function of the concentrations of the different denaturants. As discussed below, these studies yield information about how different domains of the protein are affected by the various denaturing agents. The use of phosphorescence is particularly critical in determining the presence of an *active* unfolding intermediate. The unusual stability of this protein may also serve to understand the forces which stabilize other proteins.

Time-resolved phosphorescence is a particularly powerful tool in studies of protein folding and denaturation and has a number of advantages over fluorescence. Indeed, it has been shown only relatively recently that most proteins phosphoresce at room temperature in solution if oxygen levels are reduced to subnanomolar levels [3], resulting in phosphorescence lifetimes as long as several seconds. Moreover, the phosphorescence lifetime is very sensitive to the structural rigidity associated with the residue, increasing with increasing viscosity [4]. For this reason, the number of tryptophans which phosphoresce in most proteins is significantly smaller than the number which fluoresce since tryptophan residues located near the surface are in a relatively "soft" environment and do not phosphoresce. This is an especially noteworthy observation in the study of AP, whose two nonphosphorescent tryptophans are at the protein surface, hence changes in fluorescence lifetimes could be due to interactions between these surface tryptophans and denaturant which are unrelated to unfolding. Because of the sensitivity of the phosphorescence lifetime to the environment, changes in this lifetime provide a means to detect structural changes in the region of the corresponding tryptophan. This is facilitated by the fact that in contrast to fluorescence, most tryptophans which phosphoresce have been found to have a nearly monoexponential radiative decay. In addition, the extreme sensitivity of the tryptophan triplet state to its local environment makes the range of lifetimes of phosphorescence in multityryptophan proteins much greater than

that of fluorescence. These two facts make assignment of decay components to individual tryptophans a more straightforward task than for fluorescence.

The unusual stability of AP has led many workers to study the forces which keep this protein folded. Early work addressing AP inactivation by EDTA [5] established that the Zn ions were essential to the protein's activity. The effect of the metal ions on AP stability was further studied by Cioni et al. [6], who monitored the room-temperature phosphorescence of this protein when the metal ions were removed and then replaced by Cd or Co. The almost complete loss of phosphorescence demonstrates the importance of the metal ion for maintaining the rigidity of the protein interior. Other studies on the stability of AP have focused on the effects of external denaturing agents; in particular sodium dodecyl sulfate [7], formamide [8], high pH [9], and urea [10]. Early work on the effect of acid on AP activity showed that incubation at low pH caused a loss of activity in a biphasic process, with the rate of loss faster at lower pH. It was also noted that 6 M GdnHCl completely inactivated the protein [11].

Room-temperature tryptophan phosphorescence has previously been used as a probe for denaturation of several proteins. Strambini et al. [12] used phosphorescence to detect fragments of glutamate dehydrogenase in the presence of GdnHCl. They noted drops in the phosphorescence lifetime with increasing concentrations of GdnHCl which were attributed to transition from hexamer to trimer, trimer to monomer, and finally the unfolding of the monomer. Berger and Vanderkooi [13] have unfolded α -crystallin in high concentrations of urea and observed a continued presence of room temperature tryptophan phosphorescence (with a lifetime of 7 ms, whereas that without denaturant is \approx 40 ms) indicating that some structure remains in this protein even in the presence of this harsh denaturant.

In each of the above experiments, the unfolding of the protein was allowed to proceed to equilibrium before phosphorescence lifetimes were measured. Although valuable information was obtained about the final conformation of the

protein, no information as to the mechanism of the unfolding (e.g. the presence of any intermediate states) was sought. Strambini and Gonnelli [14] examined the denaturation of equine liver alcohol dehydrogenase (LADH) in urea and GdnHCl by following the phosphorescence and enzymatic activity of the protein in the presence and absence of denaturant. They found a decrease in the protein phosphorescence lifetime in the presence of even small amounts of GdnHCl, however the curve of activity vs. concentration of GdnHCl showed gradual inactivation indicating that the effect is more likely due to an interaction between protein and denaturant than to the presence of unfolding intermediates. In the presence of urea, phosphorescence intensity and enzymatic activity disappeared at the same rate, indicating a simple two-state unfolding process.

The present study correlates the phosphorescence lifetime and activity of AP both during the unfolding process and after equilibrium has been reached. The appearance of shortened lifetimes, compared to that of native AP, reveals the presence of transient unfolding intermediates in concentrations of GdnHCl above 4 M or at pH values below 6.0. These are true intermediates, which form less than a few seconds after addition of denaturant, completely unfold before equilibrium has been reached and are therefore not detectable in equilibrium experiments.

2. Materials and methods

Escherichia coli alkaline phosphatase was obtained from Sigma Chemical Co. as a crystalline suspension in 2.5 M ammonium sulfate solution. Ultrapure GdnHCl was obtained from Calbiochem Corporation. All other chemicals used were of reagent grade. Denaturation was achieved by mixing aliquots of AP in buffer (50 mM Tris at pH 7.5) with appropriate amounts of concentrated solution of either EDTA or GdnHCl. For denaturation at low pH, the buffer was a mixture of 10 mM Tris and 10 mM sodium acetate at the appropriate pH. The final concentration of AP was 0.05 mg/ml. The activity of AP was measured by adding 50 μ l of incubated protein-de-

denaturant solution to 1.50 ml of 1 mM *p*-nitrophenyl phosphate solution in 1.5 M Tris-HCl at pH 8.0, and the change in absorption at 410 nm was followed. Concentration of GdnHCl was determined by measurements of the refractive index of its solution as described by Nozaki [15].

Because it is a very effective quencher of triplet states, molecular oxygen must be removed from aqueous solution in order to detect room temperature tryptophan phosphorescence. Deoxygenation methods used in these experiments were based on those described by Englander et al. [16]. For experiments requiring a lifetime measurement only, samples were deoxygenated by the addition of a solution of sodium dithionite to a final concentration of 2.5 mM (this is a tenfold excess of dithionite over dissolved oxygen) and the lifetime quickly measured. This method has the advantage of rapidly removing oxygen, but may also damage the protein over time. For experiments where the phosphorescence intensity was measured over an extended period of time, a dithionite free deoxygenation technique was used. A 500 μ l AP solution was placed in an airtight vacuum cuvette equipped with two sidearms, one of which can be opened to allow for exchange of air and argon and the other is used for holding the denaturant separate from the sample while allowing for its simultaneous deoxygenation. Deoxygenation was achieved by blowing ultrapure argon through the cuvette for 2 hours. The phosphorescence lifetime of the AP was then measured, the cuvette inverted to mix in the denaturant, and the lifetime of the AP was remeasured at various time intervals after mixing.

It should be noted that while the protein phosphorescence lifetime was measured in the presence of denaturant, the activity was measured in a 30-fold dilution of denaturant. This leaves open the possibility that some partial refolding of the protein occurred during the assay, giving an artificially high value for the activity. Refolding is detected by an increasing rate of substrate consumption during the assay. This effect was corrected for, when necessary, by subtracting the additional change in absorption due to the refolding.

The instrument for measurement of time re-

solved phosphorescence lifetimes has been described in detail earlier [2]. Briefly, a Spectra-Physics DCR:11 Nd:YAG laser was used to pump a dye laser running at 560 nm. These pulses were sent through a second harmonic generating crystal, and the resulting 280 nm pulses were used to excite the sample. Emission was sent through a band-pass filter centered at 450 nm and the pulses detected by a photomultiplier tube operating in the photon counting mode. Phosphorescence intensity measurements were recorded by triggering the laser system at 10 Hz for 5 seconds and counting the number of photons detected. A mechanical shutter was triggered by the laser to close for 2 ms and block the protein fluorescence and stray laser light. A potential complication in phosphorescence intensity measurements is the photodestruction by the excitation beam of trace amounts of residual oxygen which may have escaped the deoxygenation process. This would result in an increase in the phosphorescence lifetime and in the steady-state intensity level. To insure that this was not a significant effect in these experiments, the intensity of phosphorescence over the five seconds interval was monitored for any monotonic increase. The lifetime of the sample was also checked immediately after each intensity measurement.

Phosphorescence decay curves were analyzed by one of two methods: in the first case, a multi-exponential decay was fit using a standard Marquardt χ^2 algorithm [17] which determined both decay constants and associated exponential prefactors. In the second case the decay curves were fit using a distribution analysis of the decay based on the maximum entropy method [18]. In this case, exponential decay constants were set at equally spaced intervals and only the prefactors were allowed to vary.

3. Results

Figure 1 shows the activity and phosphorescence intensity of AP during incubation in 5 mM EDTA. The activity decreases monoexponentially over time with a rate constant of 0.036/min, and

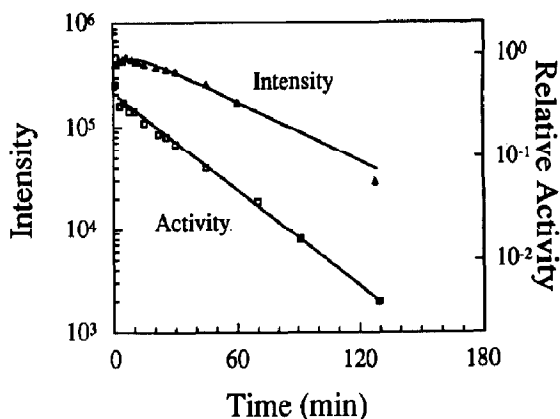


Fig. 1. The enzymatic activity and phosphorescence intensity of AP during incubation in 5 mM EDTA. The activity is lost monoexponentially, however, the intensity changes in two steps, with an initial increase followed by a slower decrease.

this rate was found to increase at higher concentrations of EDTA and at higher temperatures. In contrast to the effect on activity, EDTA results in an initial 10–20% increase in the phosphorescence intensity followed by a monotonic decrease in intensity as a function of time. The rate of decline in phosphorescence gradually increases and appears to approach the rate of inactivation. The changes in intensity were not accompanied by any change in the phosphorescence lifetime or by a shift in the phosphorescence emission or excitation spectra (data not shown). EDTA inactivates AP by chelating the Zn ions which are necessary for activity. The initial rise in the phosphorescence intensity may reflect an effect of Zn removal above and beyond the inactivation of the protein such as an increase in the efficiency of intersystem crossing. However, another explanation is that the biphasic change in phosphorescence intensity is the result of a two-step process involving a first order reaction prior to the loss of the structural Zn. This reaction could reflect the loss of the non-structural Zn, or perhaps extraction by EDTA of the Mg ion. The solid line through the phosphorescence intensity data in Fig. 1 is a fit to a biexponential with a negative prefactor. The decay times obtained are 5.7 min and 50 min with prefactors -1.8×10^5 and 5.8×10^5 , respectively. It is interesting to note that no change in phosphorescence lifetime is observed

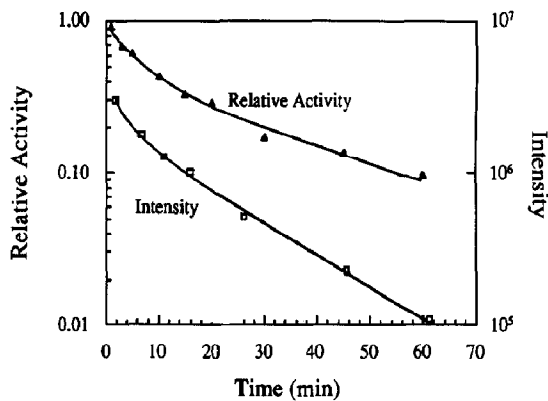


Fig. 2. The loss over time of enzymatic activity and phosphorescence intensity of AP in solution of pH 4.0. Both the activity and phosphorescence intensity are lost in a biphasic process.

as the phosphorescence intensity and activity are lost. This suggests that removal of the metal ions results in a major structural change near Trp 109 causing a reduced local rigidity, loss of phosphorescence and a corresponding loss of activity.

Figure 2 shows the time dependent activity and phosphorescence intensity of AP at pH 4.0 while Fig. 3 shows the time dependence of these parameters in 5.7 M GdnHCl. Unlike EDTA which affects only metal containing proteins, acid and GdnHCl are general denaturants. Acid is believed to unfold by disrupting electrostatic bonds which stabilize the native conformation while GdnHCl binds to exposed amino acid

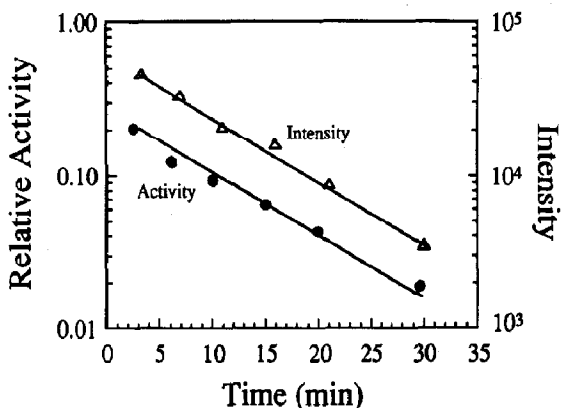


Fig. 3. The enzymatic activity and phosphorescence intensity vs. time of AP in 5.7 M GdnHCl. The rate of loss is approximately the same for both quantities.

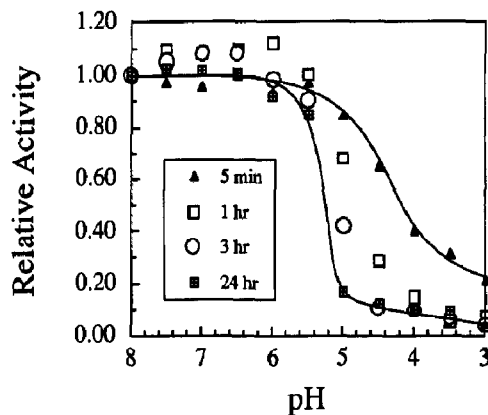


Fig. 4. The activity of AP during incubation in decreasing pH for several different times. After 5 min one transition is apparent below pH 6. Equilibrium is reached after ≈ 24 h, with no further transition regions observed.

residues and stabilizes the unfolded protein. Figure 2 shows that as in the case of EDTA, the time course of the loss of phosphorescence and activity is different for AP in acid and moreover, the loss of activity and phosphorescence are both clearly nonexponential. However, at a high concentration of GdnHCl, Fig. 3 shows that both parameters follow an exponential decay with the same lifetime and suggests a simple two state unfolding process. An examination of the phosphorescence lifetime described below shows that the problem is more complex.

Further insight into the nature of AP unfolding in acid and in GdnHCl is gained by comparing phosphorescence lifetime and activity as a function of pH or GdnHCl concentration and as a function of time as equilibrium is approached. It is under these conditions that intermediate states would be detected. Figures 4 and 5 show relative AP activities as a function of pH and GdnHCl concentration, respectively, for different times of incubation. In acid, AP does not show the clear two step inactivation pattern seen in GdnHCl (most clearly at 3 and 24 hours). When equilibrium is reached, the inactivation curves show a single transition apparent above 1.8 M GdnHCl and below pH 6 in acid.

At early times during denaturation, the phosphorescence lifetimes show the presence of conformational changes that are not directly observ-

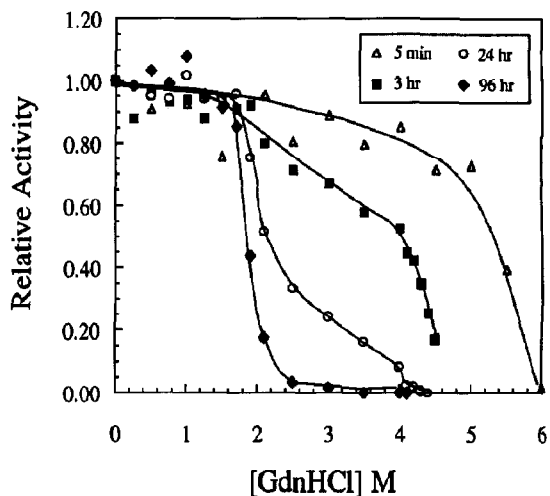


Fig. 5. The activity of AP during incubation in increasing GdnHCl concentration for several incubation periods. After 5 min there is one transition apparent above 4.5 M GdnHCl. After 3 h a second transition is apparent at ≈ 1.8 M GdnHCl, and both transitions remain after 24 h. Equilibrium is not reached until 96 h, when only the transition at the lower concentration of GdnHCl is left.

able in the activity. Both acid (Fig. 6) and GdnHCl (Fig. 7) show step-like changes in the phosphorescence lifetime that do not correspond to changes in the activity, demonstrating the presence of enzymatically active unfolding intermediate states. For example, comparing Fig. 5 and Fig. 7 for GdnHCl shows the presence of an *active intermediate state* around 4.5 M GdnHCl. In addition, at

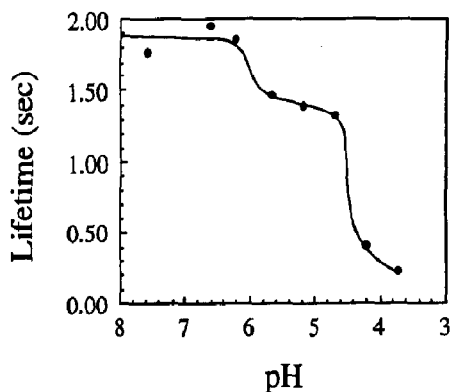


Fig. 6. The phosphorescence lifetime of AP after 3 to 6 min in low pH. There is a slight drop in the lifetime when the pH drops below 6, followed by a more dramatic drop for pH values below 4.5.

6 M GdnHCl most of the activity is lost within five minutes, however, the time resolved phosphorescence shows that the region around Trp 109 remains significantly intact. Similarly in acid, Fig. 4 shows that loss of activity appears to be characterized by a simple two state process. At equilibrium this behavior is followed by phosphorescence; however, at early times in the unfolding, Fig. 6 shows that changes in the phosphorescence lifetime indicate the presence of at least one transition at pH 4.5 where there is no corresponding change in activity. In addition there is a possible second transition in the phosphorescence below pH 6.5, the same pH range where the loss of activity occurs at long times.

The phosphorescence decay curves of native AP as well as of enzyme samples incubated for 3 min in 4.5 M Gdn:HCl or at pH 4.5 were also analyzed assuming a distribution of lifetimes and the results are presented in Fig. 8. In each of these three cases a broad distribution of lifetimes is evident. It is important to note that even a monoexponential decay curve, when analyzed by the lifetime distribution model, will show an envelope with a finite width due to the fundamental noise in the data. However, when the distribution of

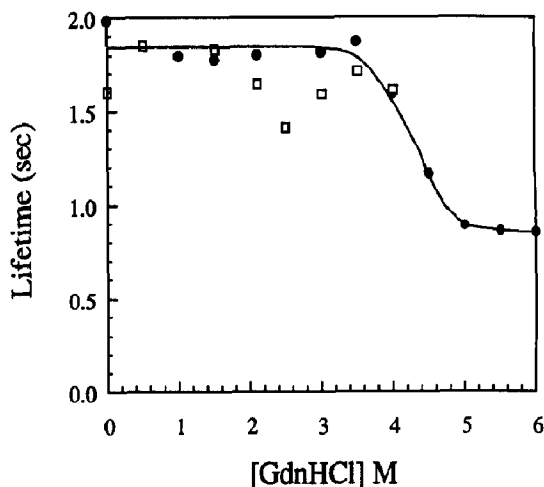


Fig. 7. The phosphorescence lifetime of AP in increasing concentrations of GdnHCl after 5 min (●) and after 100 h (□). The steady loss of phosphorescence intensity in increasing concentrations of GdnHCl resulted in no phosphorescence being detected for concentrations of GdnHCl above 4 M after 100 hours.

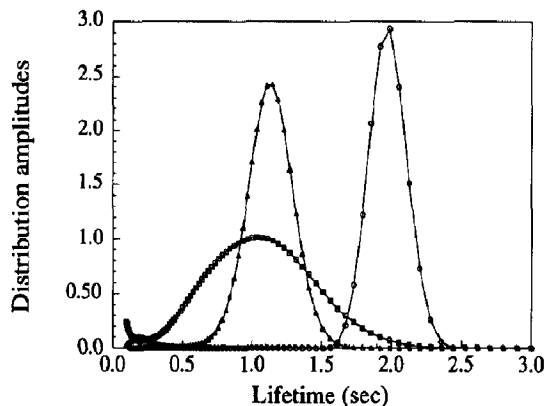


Fig. 8. The distribution of phosphorescence lifetimes of AP. Circles are native AP, triangles are AP in 4.5 M GdnHCl (3 min) and the squares are AP in pH 4.5 (3 min) The area under each distribution envelope was normalized to one.

AP lifetimes is compared with that of monoexponentially decaying model compounds, with the data collected to the same statistical significance, the distribution of AP decay times is strikingly broader, clearly demonstrating a heterogeneity of emitting protein species [22]. As seen in Fig. 8, the width of this distribution increases as the enzyme is incubated in GdnHCl and is more dramatically increased at low pH. The implications of these observations will be addressed in the Discussion.

4. Discussion

A number of theories attempt to account for the mechanisms by which proteins fold. The simplest model assumes that the protein exists in two forms: folded and unfolded, with a single transition between the two. The rate equation analysis for this model is simple and measurements need only be taken on the initial and final states. Gonnelli and Strambini [19] concluded that this was the probable mechanism for the unfolding of LADH by urea, since no change was seen in the phosphorescence lifetime and the loss of phosphorescence intensity proceeded at the same rate as the loss of enzymatic activity.

Other models depict the unfolding of proteins as passing from the folded state through one or

more short lived intermediate states before the final unfolded state is reached. Such intermediates are often difficult to detect, however, and are not always revealed by measurements of activity or luminescence intensity. For example, a change in intensity could be due to a change in lifetime of all the protein molecules in the sample or reflect a complete loss of luminescence in a subpopulation of molecules. Luminescence lifetime measurements are extremely useful in this regard since different populations will as a rule have different lifetimes. Indeed, it is the objective of this work to examine the inactivation and unfolding of AP in three different denaturants and use time resolved phosphorescence to show that a simple two state unfolding model is inadequate for the description of this process.

The monoexponential decrease with time in the enzymatic activity of AP in EDTA is consistent with previous reports [5], and can be explained by the removal of the Zn ions necessary for the protein activity. Such removal, however, is known to result in a nonphosphorescent form of AP and does not explain the initial rise in AP phosphorescence observed. A change in phosphorescence intensity can be due to a change in the phosphorescence lifetime or in the emission and excitation spectra, but these were measured (as was the difference spectrum) and found not to have changed. A plausible explanation for our observations is that EDTA causes a change in the intersystem crossing rate from the first excited singlet state to the first triplet state. In principle this could be detected by a decrease in fluorescence intensity and lifetime. Unfortunately, there are three tryptophans in each AP monomer and the phosphorescence results from intersystem crossing by only a relatively small percentage of only one of the tryptophans, thus making a quantitative measurement difficult. More likely, however, is the proposal that the rise in phosphorescence is due to an initial reaction that precedes the transition causing the loss of phosphorescence. A simple picture is that a single Zn ion is responsible for the rigidity in AP near the phosphorescing tryptophan. However, the chelating process may result in extraction of the other metal ions, a reaction that would result in loss of

activity but not phosphorescence. Further extraction of metal ions from this new state of AP would result in the loss of the critical Zn and the necessary rigidity for phosphorescence.

In contrast to EDTA, which inactivates metal binding proteins by chelating the metal ions, acid and GdnHCl are general denaturing agents. Low pH is believed to denature proteins by disrupting the electrostatic forces that contribute to the stability of the tertiary structure. GdnHCl binds to exposed amino acid groups and assists in protein denaturation by stabilizing the unfolded protein.

An examination of the time evolution of the denaturation curves shows that the pathways for denaturation for AP at low pH and for AP in GdnHCl are quite different. Figure 3 demonstrates that AP loses activity in GdnHCl in a single step while the multiexponential time dependence of the loss of activity in acid in Fig. 2 shows a more complex pathway. There is a similar behavior in the phosphorescence intensity curves¹. However, measurements of the activity and phosphorescence lifetime as a function of denaturant strength and time for both denaturants show that the pathway to denaturation is even more complex

Figure 4 depicts the loss of activity of AP as a function of pH at different times. There is a clear transition centered at pH 5.5 at long times when the system approaches equilibrium. However, the nonexponential inactivation as a function of time (Fig. 2, measured at pH 4) is related to the behavior seen in the time dependent inactivation as a function of pH (Fig. 4.) At short times, Fig. 4 shows a clear transition near pH 4. However, this transition moves to higher pH at longer times

¹ Analysis of the unfolding of AP by acid is somewhat complicated since the activity was measured at pH 8.0, while the phosphorescence was measured at the lower pHs. It is possible that there was some instantaneous refolding of AP upon dilution of the acid solution into the assay mixture, which would result in an activity measurement which does not reflect the actual behavior of AP at low pH. By comparing phosphorescence and activity we are then assuming that the activity measured at pH 8.0 is a direct reflection of the amount of protein that remains active after incubation in low pH.

since the inactive state is reached more quickly at lower pH. The time dependence in Fig. 2 reflects the “motion” of this transition. Figure 6 shows the phosphorescence lifetime at early times, presenting clear evidence for an *active* intermediate state at pH 4.5 which is part way between the two transition points in Fig. 4 and which also corresponds to the isoelectric point [20]. In addition the phosphorescence lifetimes give indication of a second transition near pH 6 which corresponds to the equilibrium pH value for loss of activity.

Figure 5 shows a similar complex behavior for the loss of activity in GdnHCl. At long times, the transition between the active and inactive states occurs near 2 *M*. This is paralleled by the phosphorescence whose intensity decreases with increasing GdnHCl concentration and falls below the noise above 4 *M*. As seen in Fig. 7, however, for concentrations below 4 *M*, the lifetime is a constant and this is consistent with the simple picture suggested by Fig. 5 that for long times, AP is in equilibrium between folded and unfolded states. However, at short times, additional structure is clearly seen in the activity plots of Fig 5 at higher concentrations of GdnHCl. Examining the phosphorescence lifetime shows that at short times, the higher concentrations of GdnHCl induce the formation of an active unfolding intermediate of AP characterized by a phosphorescence lifetime of 800 ms.

We note in comparing the nature of the intermediate states of AP in acid and in GdnHCl that both states are associated with a decrease in structural rigidity or “softening” of the Trp 109 domain. This softening is the result of a decrease in the viscosity around the residue [4]. However, it is interesting to note that in the case of GdnHCl, the change in viscosity only results in a relatively small drop in phosphorescence lifetime while in the case of acid, the phosphorescence become highly quenched. In both cases, the activity of the enzyme is sustained though at a reduced level.

While the above data suggests that the simplest model for unfolding in these two denaturants requires only the addition of one unfolding intermediate between the initial folded state and final unfolded state, the phosphorescence decay spectra show that this is not adequate to describe

the system. In the analysis of decay spectra for Figs. 6 and 7, the data was reasonably well fit with a single exponential and the lifetime reported represents the effective lifetime. However, it has recently been shown that protein phosphorescence does not decay monoexponentially as previously thought, but that these decays are better analyzed using distributions [21]. Using a numerical algorithm based on the maximum entropy method, we analyzed the phosphorescence decay spectra of AP at neutral pH in the absence of GdnHCl and at 4.5 *M* GdnHCl or at pH 4.5 for the case of acid. Figure 8 shows the (un-normalized) lifetime distributions for these three cases. We first note that even for native AP, the distribution analysis reveals clear heterogeneity. This lifetime distribution broadens in 4 *M* GdnHCl and an even larger broadening is observed at low pH. As explained under Section 3, these observations reflect increases in the heterogeneity of AP forms in solution and show that at high denaturant concentrations, the unfolding of AP proceeds initially by the formation of numerous slowly interconverting conformational states. The activity is concomitantly reduced which may reflect either an overall reduced activity for these conformations or possibly be the result of heterogeneity (i.e., that some states remain completely active while other states are inactive). We also note that the presence of a distribution in phosphorescence lifetime for a pure protein may be somewhat surprising since it could be expected that such states interconvert on a much shorter time scale. Similar behavior in the phosphorescence decay to that described here has been recently observed in the time-resolved phosphorescence of liver alcohol dehydrogenase [21]. The presence of a distribution corresponds to a potential energy surface with many local minima separated by high energy barriers, the latter being required in order to assure the inter-conversion rate is sufficiently slow. Indeed, a slow interconversion rate has also been proposed by Cioni et al. [22] to explain the multi-exponential decays in phosphorescence from tryptophan in yeast phosphoglycerate kinase.

Two models are considered to explain these observations. The first assumes that AP exists in

only two major conformations (each containing a distribution of substates); the native (long lifetime) and a transient (soft) state which is active but with a reduced lifetime. The long lifetime conformation would predominate for concentrations of GdnHCl below 4 M or pH > 4 with a transition at higher concentrations or lower pH, leading to the region of reduced lifetime. In the transition region for this model, there would be rapid conversion (relative to the phosphorescence lifetimes) between each of the initial states and the corresponding different soft states. The result would be a distribution of lifetimes corresponding to the average values between the initial states and soft states. The lifetimes in the distribution would depend on the relative populations of the native and soft states which of course depends on the concentration of GdnHCl or pH.

The second model to account for these data assumes a continuous softening of AP with increasing denaturant concentration where at a given level of denaturant, AP exists in a well defined state of heterogeneity. In this case however, there would not be a rapid conversion between the soft state and the native state.

To examine these possibilities, experiments were performed using GdnHCl as the denaturant. A sample of AP was placed in 4.5 M GdnHCl (with a phosphorescence lifetime between that of the native state and 800 ms) for ca. 5 minutes. The GdnHCl was then diluted to low concentration and the phosphorescence lifetime was monitored for any changes. The results show that the lifetime does not immediately return to that of the native state. This argues against the first model and supports the second model, since in the first case rapid interconversion between the folded and unfolded state at high GdnHCl concentration would have been expected to produce the longer lifetime as soon as the denaturant concentration was reduced by dilution. In the second case, however, time is needed for the softened state to return to the native state and hence the lifetime would remain initially unchanged upon removal of the denaturant, in agreement with the data.

We also note that the time resolved phosphorescence and activity data provide information on

the reactivity of the intermediate state. More specifically, the susceptibility of the intermediate state to further denature is different for the two denaturants. This is seen by noting that the rate of loss of activity increases at higher GdnHCl concentrations (i.e., the slope in Fig. 5 above 5 M) while the rate of loss decreases in acid. Hence, the intermediate state in GdnHCl is more susceptible to denaturation while the intermediate state at low pH is less susceptible. This may reflect the fact that not all of the states associated with the heterogeneity seen in Fig. 8 at low pH are stabilized by electrostatic forces.

It is pertinent to mention that previous reports have suggested that the end result of the denaturation of AP by acid and GdnHCl is the same unfolded monomeric form of the protein [11]. AP is reported to dissociate into its monomeric subunits at low pH or in the presence of high concentrations of GdnHCl [11]. The monomeric form exhibits no phosphorescence [6], and possesses no enzymatic activity [9], hence these two quantities are unable to detect any differences between the final states of AP in the two denaturants. However, the different phosphorescence behavior of AP during denaturation, shows that the end product of incubation in acid or GdnHCl is created by different unfolding pathways.

The recognition of the importance of intermediate conformations in the unfolding and refolding of proteins has highlighted the need for methods of unambiguous detection of such conformations. Room-temperature tryptophan phosphorescence is a useful tool for such measurements since it is extremely sensitive to changes in the local protein structure. Since phosphorescence arises almost entirely from interior tryptophans, it reflects on residues which are less susceptible to interaction with denaturant molecules in ways which are unrelated to unfolding. Through the use of time-resolved phosphorescence from AP in solution, we are able to demonstrate the presence of transient active intermediate states in its unfolding through changes in the phosphorescence lifetime. While these changes clearly show alterations in the rigidity of the domain in which the phosphorescent tryptophan resides, complementary methodologies will provide additional de-

tailed information on the structural modifications in the unfolding intermediates. Current work in this area is underway in our laboratory.

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