Phosphorescence Reveals a Continued Slow Annealing of the Protein Core following Reactivation of *Escherichia coli* Alkaline Phosphatase†

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ABSTRACT: When *Escherichia coli* alkaline phosphatase (AP) is refolded *in vitro* after extensive denaturation in 6.2 M guanidine hydrochloride, the enzymatic activity reaches its asymptotic value in 1 h at 24 °C. In contrast, the structural rigidity of the hydrophobic core of the protein, monitored by the recovery of the tryptophan phosphorescence lifetime, returns to its characteristic native-like value over several days. Moreover, the protein lability, measured by the rate of inactivation in 4.5 M guanidine hydrochloride, also changes on a time scale much longer than the recovery of activity. These results clearly demonstrate that while the return of enzymatic activity, the traditional measure of the attainment of the native state, indicates that AP has refolded to its final, active conformation, the phosphorescence data indicate otherwise. In the context of the rugged energy landscape model [Frauenfelder, H., et al. (1991) *Science 254*, 1598−1603], the slow annealing of the hydrophobic core is consistent with the presence of high-energy barriers that separate fully active intermediates along the folding pathway. The data suggest that the core of the protein undergoes continued structural rearrangements affecting the rigidity of the protein environment surrounding the emitting tryptophan and the protein lability long after the return of enzyme activity.

During *in vitro* folding of an enzyme, the recovery of enzymatic activity is usually assumed to indicate the return to the native state, the activity generally being regarded as a more sensitive indicator than other measured parameters. There are, however, some reported cases of proteins with different structures exhibiting the same biological activity. Recently, Hattori et al. (1993) reported that while renatured bovine β-lactoglobulin (β-LG)[1] exhibited the same retinol binding activity as the native protein, some specific epitopes in refolded β-LG were not recognized by conformation-specific monoclonal antibodies, suggesting that these epitopes did not return to the native conformation from the denatured state and that this conformational difference between the renatured and native forms has no effect on the biological function of ligand binding. While the native structure did not return in the case of β-LG, phosphoglycerate kinase (PGK) has been shown to exist in fully active, slowly interconverting conformations, leading to more stable forms of PGK without any effect on the enzymatic activity (Rothstein, 1985). These slow transitions were demonstrated both *in vivo* (Hiremath & Rothstein, 1982) and *in vitro* (Yuh & Gafni, 1987; Zúñiga & Gafni, 1988), and while no detailed model for structural changes was reported, the increased stability was suggested to be linked to a process of conformational drift.

The present work reports on the refolding of *Escherichia coli* alkaline phosphatase (AP). AP is a nonspecific phosphomonoesterase of molecular weight ~94 000 (Bradshaw et al., 1981). It is a metalloenzyme containing two Zn^{2+} and one Mg^{2+} ion per monomer, and it exhibits activity and phosphorescence only as a dimer of identical monomers. The essential residue in the active site is thought to be Ser 102 (Coleman, 1992).

This laboratory has previously reported that conformational heterogeneity in *E. coli* AP is evident from the maximum entropy method distribution analysis of the room temperature phosphorescence (RTP) decay (Schlyer et al., 1994) and has demonstrated active intermediates during the unfolding of AP either in guanidine hydrochloride (GuHCl) or at low pH (Mersol et al., 1993).

The room temperature phosphorescence lifetime is known to be exquisitely sensitive to changes in the protein environment surrounding the emitting Trp residue (Strambini & Gonnelli, 1985; Vanderkooi et al., 1987). In extensively deoxygenated solutions, the phosphorescence lifetimes may become exceedingly long (milliseconds to seconds) and are extremely sensitive to the so-called microviscosity determined by the surrounding protein environment (Strambini & Gonnelli, 1985). Native AP has a remarkably long room temperature phosphorescence lifetime (~2 s) (Saviotti & Galley, 1974; Vanderkooi et al., 1987), and while this protein has three fluorescing Trp's, it has been found that the phosphorescence arises from a single residue: Trp 109 (Strambini, 1987; Mersol et al., 1991). Trp 109 is situated close to the active site of the enzyme, with the distance between the C_α atom of Ser 102 and the indole nitrogen being

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[1] Abbreviations: AP, alkaline phosphatase; β-LG, β-lactoglobulin; GuHCl, guanidine hydrochloride; PGK, phosphoglycerate kinase; RTP, room temperature phosphorescence; Trp, tryptophan.

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≈9.5 Å. The structure of the enzyme has been solved by X-ray crystallography and is available from the Protein Data Bank (pdb1alk) (Sowadski et al., 1985).

In this report, we use time-resolved room temperature phosphorescence and enzymatic activity to follow the folding of *E. coli* AP following extensive denaturation in 6.2 M GuHCl.

MATERIALS AND METHODS

Escherichia coli alkaline phosphatase type III was obtained from Sigma Chemical Co. as a crystalline suspension in 2.5 M ammonium sulfate solution. Concentrated AP (10 mg/mL) was denatured in a 6.2 M solution of ultrapure GuHCl (Calbiochem) for 24 h. Aliquots of the denatured protein were rapidly diluted at 24 °C with 100 mM Tris-HCl buffer at pH 7.5 to a final protein concentration of 0.1 mg/mL. Complete and rapid mixing was accomplished by vortex mixing the mixture. An aliquot was removed for activity measurements, and the rest of the protein was reserved for the phosphorescence measurements. The recovery of activity was measured by a standard colorimetric assay procedure (Garen & Levinthal, 1960) monitoring the change in absorption at 410 nm as *p*-nitrophenyl phosphate is hydrolyzed to *p*-nitrophenol. Note that all activity values are normalized to the activity of native protein at the same concentration.

Since molecular oxygen is an extremely effective quencher of the room temperature phosphorescence, great care was taken to extensively deoxygenate the sample by alternating between partial vacuum and a stream of ultrapure argon gas for 15 min, after which the sample was kept sealed at room temperature without further deoxygenation for the remainder of the experiment. The phosphorescence lifetime obtained in deoxygenated solution prior to denaturation was used to define the native state. The value of ≈1.8 s measured is comparable to those measured by Vanderkooi *et al.* (1987) and Strambini (1987). All decays were collected to the same level of precision.

The triplet state from which tryptophan phosphorescence occurs is populated by intersystem crossing from the first excited singlet state. The latter state was excited by a 5 ns FWHM laser pulse at 280 nm. Briefly, the experimental configuration consists of a Spectra-Physics Model DCR-11 Nd:YAG laser emitting pulses at a wavelength of 532 nm to pump a Spectra-Physics Model PDL-3 dye laser operating at 560 nm. The 560 nm light is externally frequency doubled in a nonlinear crystal to produce 280 nm light. Phosphorescence was detected at 440 nm using an Instruments SA HR 320 single monochromator. Data were collected using an EG&G Ortec ACEMCS multichannel scaler card, with a minimum dwell time of 2 μs per channel, installed in an IBM personal computer. Phosphorescence decays were analyzed using PTI fluorescence decay software, which applies a Marquardt χ^2 algorithm. Deconvolution of the instrument response is not necessary since the laser pulse is contained entirely in the first channel of the data.

The status of the renatured protein was assessed through its lability, i.e., the rate of inactivation in 4.5 M GuHCl compared to the rate of inactivation of native protein. To accomplish this, aliquots of renatured protein were removed at various times after renaturation was initiated and incubated in 4.5 M GuHCl. The activity of the protein was measured as a function of time as noted earlier.

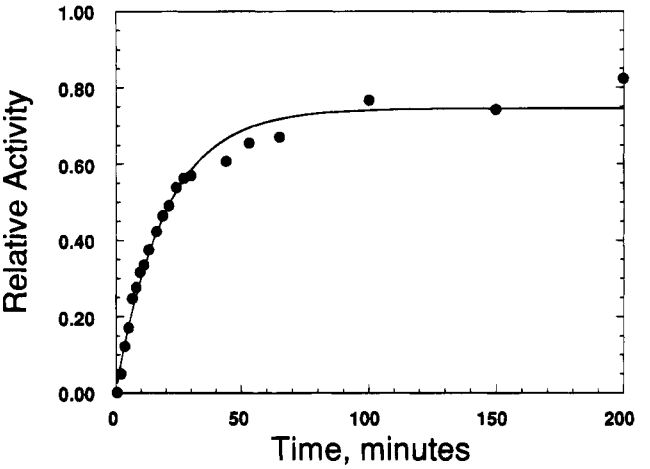

FIGURE 1: Recovery of activity of AP that has been denatured in 6.2 M GuHCl for 24 h. Aliquots of the denatured protein were diluted at 24 °C with 100 mM Tris-HCl buffer (Sigma) at pH 7.5 to a final protein concentration of 0.1 mg/mL. The assay is as described in the text. The solid line is a nonlinear least-squares fit of the data to a first-order reaction, with a limiting recovery value of 75% and a time constant of 19.6 min.

RESULTS

The recovery of enzymatic activity of denatured AP is depicted in Figure 1. The solid line is a nonlinear least-squares fit of the data to a first-order reaction, with a limiting recovery value of 75% and a time constant of 19.6 min. The activity shows no evidence of further recovery beyond the limiting value, even when measured after several days (data not shown), indicating that the 25% of the protein that does not reactivate is most likely permanently withheld from the refolding reaction.

The time evolution of the room temperature phosphorescence decay kinetics is dramatically distinct from the recovery of activity and is shown in Figure 2. It is immediately apparent from the change in the slopes of the decay curves that the phosphorescence lifetime increases only very slowly, over many days, and reflects an apparently continuous change in the rigidity of the protein environment surrounding Trp 109. The initial phosphorescence intensity (the decay amplitude) of the raw data is somewhat decreased with respect to that of a sample of the native protein at the same concentration and is consistent with the 25% loss in activity upon renaturation.

The phosphorescence decays presented in Figure 2 are clearly multiexponential and were fit (arbitrarily, since no model was assumed) to two exponential components. The evolution of each of the two decay times is shown as a function of time in Figure 3. As in the raw decay curves, the slowly increasing phosphorescence lifetime after denaturant dilution indicates an increase in rigidity of the core of AP surrounding Trp 109, approaching the original value only after several days. The prefactors corresponding to each of the two decay components are also time dependent and show that the fractional contribution of the long decay component of the phosphorescence increases to a value over 90% at long times. Even in this simplified, two-component picture, the data clearly show an active folding intermediate that is characterized by conformational heterogeneity affecting the

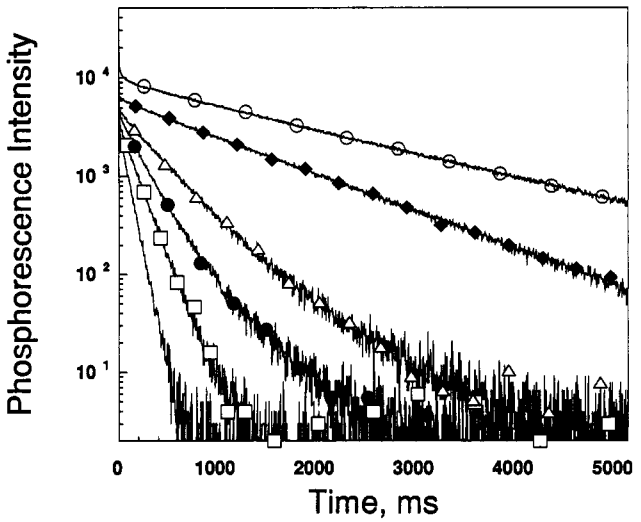

FIGURE 2: Recovery of the phosphorescence of AP denatured in 6.2 M GuHCl for 24 h. The dramatic increase in phosphorescence is apparent from the change in the slope of the decay curves with time: —, 28 min after initiation of renaturation; □, 64 min; ●, 72 h; △, 168 h; ◆, 360 h; ○, native AP. All decays were collected to the same level of precision. The curve marked native was obtained for a sample of untreated AP at the same concentrations of protein and GuHCl as the renatured samples: 0.1 mg/mL AP and 0.062 M GuHCl.

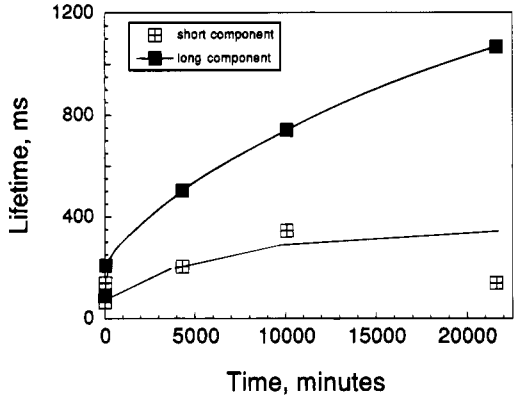

FIGURE 3: Slow increase in the short and long phosphorescence lifetime components, based on a two-exponential component fit to the RTP decay data in Figure 2.

rigidity of the hydrophobic core and leading to the highly multiexponential phosphorescence decay. A long term (i.e., on a time scale of days) conformational transition transforms this fully active, "soft" protein to native-like conformations where the rigidity, as reflected in the phosphorescence lifetime, approaches the value of the native state.

A less rigid protein core is also expected to be accompanied by an increase in the lability of the protein, a characteristic reflected in the rate of unfolding in denaturant. To determine the lability of the reactivated state of AP relative to the native protein, we monitored the kinetics of inactivation of native and renatured AP in 4.5 M GuHCl as a function of reactivation time. Figure 4 depicts a clearly biphasic inactivation curve for freshly reactivated protein, with a fast component representative of a more labile state and a slow, native-like component. The relative contribution of the latter component increases with reactivation time (similar to the fraction of the long lifetime component in the phosphorescence decay in Figure 2), indicating that the resistance to denaturant of the reactivated

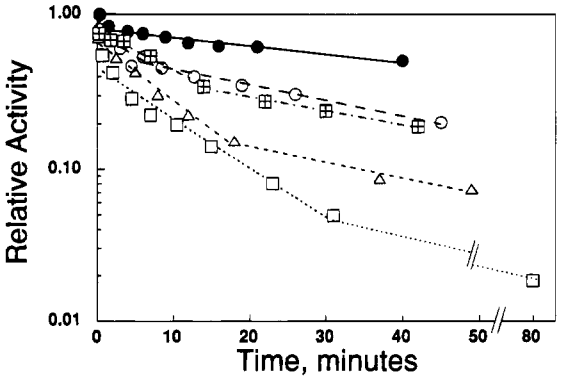

FIGURE 4: Kinetics of inactivation of native and renatured AP samples upon incubation in 4.5 M GuHCl and 50 mM Tris-HCl (pH 7.5). The activity of the following samples was measured as a function of time: ●, native AP; □, 1 h into renaturation; △, 2 h into renaturation; ○, 24 h into renaturation; ⊞, 48 h into renaturation. The different rates of inactivation reflect upon the relative lability of the intermediate and native-like components of the refolded protein.

protein approaches native levels over a time scale that, while faster than the return of phosphorescence, significantly lags reactivation.

DISCUSSION

The inactivation of AP, as for most enzymes, is a cooperative process, which implies that the time evolution of unfolding in denaturant probes the rate of loss of *global structure* of the protein. The time dependent change in the unfolding rate of the reactivated form of the AP molecule, shown in Figure 4, can be interpreted in a two-state model, where a fully active but more labile intermediate state that is initially formed when the protein refolds is transformed into the native, and of course fully active, state. In contrast, the time-resolved room temperature phosphorescence is a *local* probe that reports on the rigidity of the environment of a single amino acid residue in the protein, namely, Trp 109 (Figure 2).

The recovery of the phosphorescence clearly shows a gradual increase in the rigidity of the protein structure around Trp 109, a process akin to annealing, and does not fit to a two-state model involving inactive and active states. The biphasic lability data in Figure 4 are suggestive of at least two *active* states, with the more labile states slowly converting to the native-like state.

It is tempting to associate the longer (shorter) lived phosphorescence components in Figure 2 with the less labile (more labile) active states in Figure 4. However, the time scales for recovery of these two measures of the conformational state clearly are different. Moreover, the phosphorescence data are suggestive of a continued conformational drift from the initial active folding intermediates through a number of folding trajectories to the more native-like state. In terms of the rugged energy landscape model (Frauenfelder et al., 1991), the long time scale associated with these transitions shows that the potential energy surface that determines the folding trajectory is characterized by high-energy barriers relative to k_BT. This is in agreement with reports in the literature of high activation barriers involved in refolding to the native state of α-lytic protease (Baker & Agard, 1994) and bacterial luciferase (Clark et al., 1993).

Possible molecular mechanisms that characterize the annealing process include the disruption of incorrectly formed hydrogen bonds or unfavorable van der Waals contacts in the hydrophobic core of the protein, followed by reformation of the correct contacts. Since the out of plane vibrations of the indole rings of tryptophan residues significantly contribute to triplet state quenching (Lower & El-Sayed, 1966), it is also possible that damping of these vibrations due to a slow relaxation of the indole ring of Trp 109 to its lowest energy state also plays a role in the observed increase in RTP lifetime. Other mechanisms could include the slow release of an entrapped guanidine hydrochloride molecule from the core (this appears very unlikely since the resulting steric interference with the core side chain packing would likely disrupt the active site as well and cause a loss of enzymatic activity). It is also possible that slight misfolding in the core with *local* effects results in an interaction with a specific residue that quenches the RTP decay, and subsequent bond rearrangement mitigates the effect of the interaction, leading to an increase in the RTP lifetime. Slow steps in refolding have previously been attributed to the cis−trans isomerization of proline residues (Brandts et al., 1975); however, in the present case we believe this mechanism to be highly unlikely since the protein is already maximally active when the phosphorescence measurements are made.

We note that *E. coli* AP is a secretory protein with a high degree of thermal stability (Torriani, 1968). Conceivably, the origin of the quasi-stability of the intermediate states lies in the high potential barriers that also limit the rate of conformational drift and give rise to the rigid environment in the core.

In summary, we have compared the time evolution of the return of activity with the return of core rigidity and global protein stability. The freshly reactivated state of AP continues to undergo structural changes long after achieving the final value of activity. Our results provide direct evidence for the existence of two or more distinct, fully active states with extremely long interconversion times and are contrary to the traditional expectation that the final reactivated conformation of a protein is identical to the native conformation. Further experiments to characterize the molecular mechanism of the annealing process are underway.

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