Hydrogen Exchange at the Core of *Escherichia coli* Alkaline Phosphatase Studied by Room-Temperature Tryptophan Phosphorescence†

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ABSTRACT: The room-temperature tryptophan (Trp) phosphorescence lifetime is sensitive to details of the local environment and has been shown to increase significantly in some proteins following H−D exchange. Careful analysis of the phosphorescence lifetime distribution of Trp 109 in *Escherichia coli* alkaline phosphatase (AP) in solution as a function of time during the H−D exchange shows that this process corresponds to a two-state reaction resulting from the deuteration of a single, specific hydrogen in the core of the protein. The absence of a pH dependence of the exchange rate suggests that the exchange is not an EX2 process, and therefore, a certain degree of unfolding is required for exchange to occur. This discovery opens up the use of phosphorescence-detected hydrogen exchange as a sensitive tool for monitoring the local susceptibility and activation energy for exchange in proteins having a phosphorescent Trp and, for example, for studying the effects of local mutations upon that susceptibility.

Determining the kinetics of hydrogen exchange at specific sites (or residues), both absolute and relative to other residues in the protein, is a powerful tool for measuring both the local and global stability of proteins (*1*−*3*), for determining protein folding mechanisms (*4*−*6*), and for identifying the role played by specific residues in these processes and characteristics (*7*−*9*). It is now understood that the exchange rate of a particular residue is strongly affected by a variety of parameters, including the presence of local charges (*10*), the location of the residue within a particular element of secondary structure (*2*), and, more generally, the degree of protection of the residue from the solvent by hydrogen bonding and its position within the protein (*11*).

The exchange of labile hydrogens (*12*) (hydrogen atoms bound to O, N, or S) of both the amide backbone and that of specific side chains can be monitored with a variety of techniques, including NMR (*9*, *11*), mass spectrometry (*13*), radioisotopic measurements (*14*), and Raman scattering (*15*, *16*). The specific exchange of aromatic residues has been studied before using UV absorbance (*10*, *17*, *18*), NMR (*19*), and UV resonance Raman scattering (*20*, *21*). All these techniques are powerful tools for obtaining general information; however, they do not lend themselves readily to the study of large and complex proteins in solution under physiological conditions and/or conditions where the rate of hydrogen exchange might be changing on a relatively short time scale compared to that at which the exchange rate can be determined (as in the case of protein folding studies).

Previous work in our laboratory (*22*) demonstrated the ability of room-temperature phosphorescence (RTP)[1] to monitor hydrogen exchange occurring nonspecifically within proteins in solution through the effects of this exchange on the RTP decay rate. In this paper, we demonstrate that the exchange process is described by a two-state reaction associated with exchange of a specific single hydrogen within the core of the protein. Given the susceptibility of nitrogen-bound hydrogen to H−D exchange relative to other groups, these hydrogens may belong to the enamine group of Trp 109, the phosphorescent Trp in AP. Interestingly, the mechanism for exchange is not the usual EX2 process observed for most residues in proteins; rather, it appears that local breathing modes are likely responsible for the observed exchange rate. The work demonstrates that under appropriate physiological conditions, RTP is an excellent probe for measuring hydrogen exchange as a function of time occurring at the hydrophobic core of proteins.

EXPERIMENTAL PROCEDURES

The buffer used to dissolve AP for the experiments was 100 mM Tris [pH(D) 8.0 (20 °C)] with additional 1 mM Na_2HPO_4, 0.1 mM $MgCl_2$, and 0.1 mM $ZnCl_2$. Tris base, $MgCl_2$, $ZnCl_2$, and Na_2HPO_4 were all purchased from Sigma Chemical Co. The D_2O was also purchased from Sigma Chemical Co. and was 99.9% pure. The deuterated buffer was titrated to the appropriate pD with DCl (99% pure) and NaOD (99% pure), both of which were purchased from Sigma Chemical Co. All pDs were determined at room

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[1] Abbreviations: Trp, tryptophan; AP, *E. coli* alkaline phosphatase; RTP, room-temperature phosphorescence; WT, wild-type; W220Y, *E. coli* alkaline phosphatase with a tryptophan to tyrosine mutation at position 220; Q320G, *E. coli* alkaline phosphatase with a glutamine to glycine mutation at position 320; H−D exchange, hydrogen and deuterium exchange; PMT, photomultiplier tube.

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temperature using the relationship pD = pH + 0.40 (*23*). Since the pH(D) of Tris buffer is temperature-dependent ($\Delta pK_a/°C = -0.031$) (*24*), all pHs and pDs reported in this paper have been corrected to the appropriate value at the stated temperature.

The protein used in these experiments was expressed in *Escherichia coli* (SM547 strain) and purified according to the procedure outlined by Kantrowitz (*25*). SM547 used as the host strain for the expression of alkaline phosphatases was prepared from the plasmid−strain combination pEK154−SM547. Following the harvesting of the cells, the pellet was washed and osmotically shocked; the periplasmic proteins were precipitated from the wash via the addition of ammonium sulfate. This suspension was then dialyzed against TMZP buffer [10 mM Tris, 1 mM $MgCl_2$, 0.1 mM Na_2HPO_4, 0.01 mM $ZnSO_4$, and 0.5 mM NaN_3 (pH 7.4)] and purified by ion exchange chromatography using Q-Sepharose Fast Flow resin. The enzyme was eluted from the column using a gradient of NaCl, and the fractions were identified by electrophoresis on a 10% polyacrylamide denaturing gel. The protein concentration was determined spectrophotometrically at 278 nm; 1 mg/mL samples have absorbances of 0.72 (WT) and 0.603 (W220Y).

Because Trp RTP in proteins is strongly quenched by molecular oxygen, care must be taken to extensively deoxygenate all samples and to ensure that oxygen does not leak back into the sample chamber after deoxygenation. The latter proved to be especially troublesome at elevated temperatures. To solve these problems, we designed a quartz cuvette consisting of 2 in. of 10 mm OD quartz tubing, with a flat, sealed bottom fused to $^3/_4$ in. of 6 mm OD quartz tubing. The 6 mm OD "neck" of the cuvette was attached to a Whitey stainless steel "O series" integral bonnet needle valve (purchased from H. E. Lennon, Inc.) by a Cajon stainless steel Ultra-Torr brand compression fit adapter (also purchased from H. E. Lennon, Inc.). The other port on the valve was then attached to a solenoid manifold (manufactured by Bürkert and purchased from Cole-Parmer) which exposes the cuvette alternately to purified nitrogen gas (purchased from Metro Welding, Detroit, MI) and to moderate vacuum. Adequate deoxygenation of our samples requires 70 min, which includes more than 4000 oscillations between gas and vacuum. After thoroughly deoxygenating our samples and closing the valve, we find that the samples can maintain their phosphorescence lifetime to within 1% of its initial value even after 24 h at 70 °C.

For the deuterium exchange experiments, 7 μL of a concentrated protein solution and 250 μL of the exchange solution were deoxygenated simultaneously in the same cuvette, but physically separated from each other. The protein and buffer solutions were kept separated until both were thoroughly deoxygenated and equilibrated to the temperature of the experiment. At that point, they were mixed (final protein concentration between 0.2 and 0.5 mg/mL) and the phosphorescence decays were subsequently monitored as a function of time. Fully deuterated and fully protonated samples were prepared by mixing 10 μL of the concentrated protein solution and 990 μL of the exchange solution; this mixture was then incubated at 60 °C for 8 h to ensure that final equilibrium was reached. Phosphorescence measurements were then taken on 250 μL samples of this final solution.

The triplet state of Trp is populated from the first excited singlet state by intersystem crossing. The latter state is populated by excitation with a short pulse of laser radiation at 280 nm. A detailed discussion of the system is presented elsewhere (*26*). The frequency-doubled pulses of a Spectra-Physics model DCR-11 Nd:YAG laser with a wavelength of 532 nm and a pulse width of 8 ns (fwhm) are used to pump a Spectra-Physics model PDL-3 dye laser emitting at 560 nm. This light is then frequency-doubled in a BBO crystal to produce 280 nm radiation. The excess 560 nm light is removed by a Schott glass UG-11 filter, and the remaining 280 nm light is directed into the sample. The phosphorescence signal is passed through an interference filter centered at a wavelength of 450 nm with a fwhm of 25 nm before being detected by a Hamamatsu R3550 PMT. Pulses from the PMT are sent into a Pacific Instruments model AD6 amplifier/discriminator whose output is collected by an ACEMCS multichannel scaler from EG&G Ortec, capable of collecting counts at up to 100 MHz. In comparison with more standard time-resolved fluorescence data, the quality of the data is excellent, with several million photons collected in a few minutes and a dynamic range spanning three decades.

The phosphorescence decays were analyzed in two ways, a detailed explanation of which appears in the literature (*27*). The first method is a discrete components analysis which assumes that the decay is composed of a sum of a small number of exponential components, and the second method applies the maximum entropy method (MEM) (*28*) algorithm. The primary advantage of the MEM method over the discrete components method is that while MEM does not assign precise lifetimes to a multiexponential decay, the analysis is far more robust and relatively insensitive to the total number of photons collected (*29*).

RESULTS

When samples of alkaline phosphatase solutions are diluted into H_2O buffer, the phosphorescence decays that are observed are nearly monoexponential as seen in Figure 1A [see Schlyer et al. (*27*) for detailed analysis of AP phosphorescence and the structural heterogeneity reflected in the finite width of the distribution]. As expected, the associated lifetimes remain constant over time. In contrast, when the protein sample is diluted into D_2O buffer, the resulting RTP decays are initially clearly not monoexponential (see Figure 1B), and furthermore, the average RTP lifetime increases as a function of time following the mixing, eventually reaching a constant value, typical of the RTP lifetime of fully deuterated AP. More significantly, at all early times, both MEM and discrete analysis of the nonexponential decays yield a two-component distribution function as shown in Figure 1B for data taken 10 min after exchange into D_2O buffer at 60 °C (Independent simulation of the data and corresponding MEM analysis shows that the apparent broadening of the distribution is an artifact of the MEM analysis fitting a two-component distribution. The slight widening is due to the Poisson noise in the data, and does not necessarily reflect a true broadening of the distribution). The peak associated with the shorter of the two decay components in the distribution corresponds to the lifetime of AP in H_2O buffer (see Figure 1A). At long times, as the exchange is complete and equilibrium is established, the

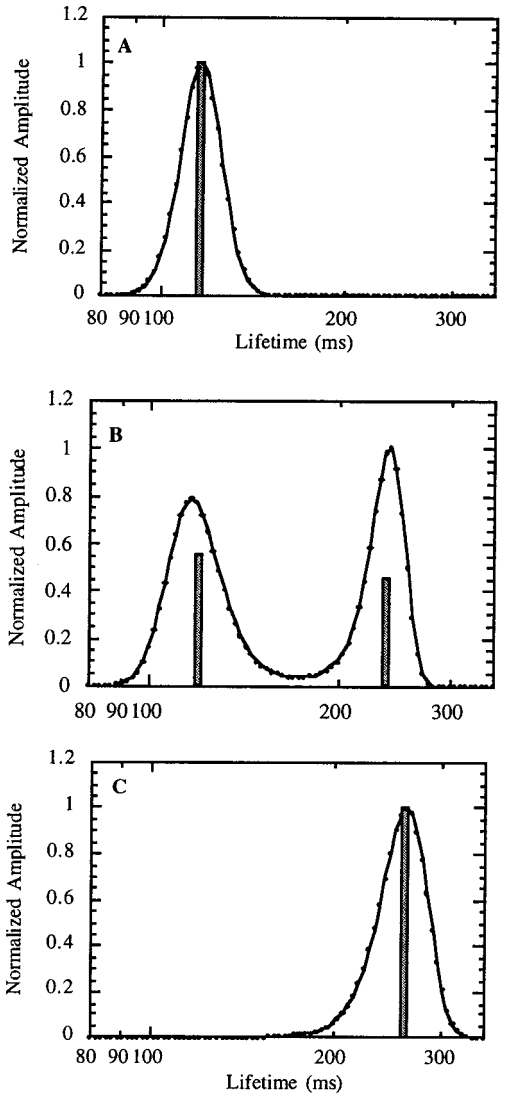

FIGURE 1: (A) MEM distribution analysis of the phosphorescence decay of WT AP in H_2O at 60 °C. Also shown by a vertical line is the result of the discrete analysis. (B) MEM distribution analysis of the phosphorescence decay of WT AP in D_2O at 60 °C. The data were collected 10 min after mixing the protein into the D_2O buffer. Also shown by vertical lines are the results of the discrete analysis. (C) MEM distribution analysis of the phosphorescence decay of WT AP in D_2O at 60 °C. Also shown by a vertical line is the result of the discrete analysis.

MEM analysis shows a distribution similar to that prior to exchange, but centered at the longer decay time (Figure 1C). The normalized widths of the distributions in panels A and C of Figure 1 are nearly identical. While the relative fractions and lifetimes at any given time depend on temperature, the simple two-component distribution is observed at all temperatures at early times following the initiation of exchange. In addition, the mean of each distribution always corresponds to the lifetime of AP Trp 109 phosphorescence in H_2O and the fully deuterated form with the shorter lifetime component corresponding to AP in H_2O and the long component corresponding to fully deuterated AP.

Protein phosphorescence at room temperature in solution is typically emitted from Trp residues deeply buried in the hydrophobic environment of the protein core and is well-known to be strongly temperature dependent, reflecting a thermal deactivation component due to the nonradiative

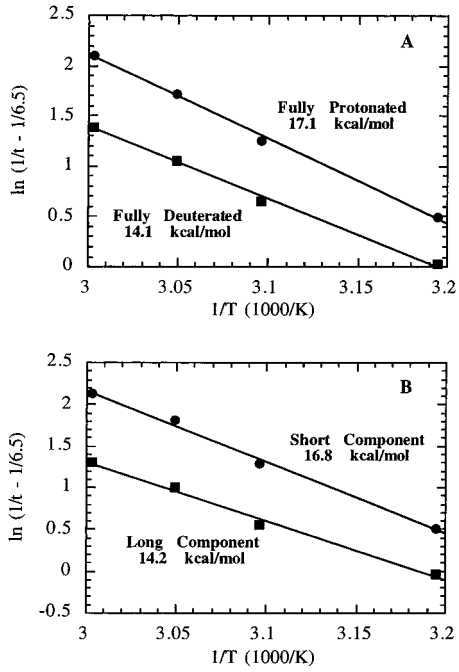

FIGURE 2: (A) Arrhenius plot of the phosphorescence lifetimes of fully deuterated (■) and fully protonated (●) WT AP. (B) Arrhenius plot of the long (■) and short (●) components of the phosphorescence lifetime observed for WT AP during D_2O exchange experiments [Trp's limiting low-temperature lifetime of 6.5 s (*53*) has been subtracted in both panels A and B].

triplet state quenching (*30*). Measurement of the temperature dependence of the RTP lifetime for AP in H_2O and when fully deuterated reveals two activation energies of 6.4 and 5.0 kcal/mol, respectively, at low temperatures (<15 °C) (data not shown) and 17.1 and 14.1 kcal/mol, respectively, at high temperatures (>30 °C, Figure 2A). Measurement of the temperature dependence of the average lifetimes from the H−D exchange experiments yields similar activation energies as seen in Figure 2B, where E_a for the short component is 16.8 kcal/mol and for the long component 14.2 kcal/mol. Further analysis of the temperature-dependent lifetime data is given Table 1 which compares the lifetime in H_2O (D_2O) as a function of temperature to the average lifetime of the shorter (longer) lifetime observed during H−D exchange.

While the lifetimes measured during H−D exchange do not change with time, the relative fraction associated with each lifetime is strongly time dependent. Figure 3A shows the relative percentages associated with the long and short lifetime components as a function of time after diluting into D_2O buffer at 50 °C. The data show the reaction is well described by first-order kinetics reflecting a two-state system; the data depicted in Figure 3B are similar except they show the behavior following exchange into a 50/50 H_2O/D_2O mixture. The exchange rates are observed to be a strong function of temperature as shown in Figure 4. Over the temperature range that was examined, the temperature dependence of the exchange rate follows an Arrhenius equation with an activation energy of 22.6 kcal/mol and is independent of the final D_2O concentration.

As discussed below, depending upon the details of the hydrogen exchange process, a strong dependence of k_{ex} (the exchange rate) upon pD may be expected. It is, therefore, noteworthy that we detected no change between the exchange

Table 1: Comparison of the Short and Long RTP Lifetime Components of AP Exchanged into D_2O Buffer to the RTP Lifetime of Fully Protonated and Fully Deuterated Forms, Respectively, as a Function of Temperature[a]

temp (°C)	lifetime (ms) in H_2O buffer	short component in D_2O buffer (ms)	ratio	lifetime (ms) in D_2O buffer	long component in D_2O buffer (ms)	ratio
40	550	560	1.02	900	850	1.06
50	265	275	1.04	530	485	1.09
55	160	175	1.09	350	330	1.07
60	116	120	1.03	260	240	1.08

[a] At early times, as shown in Figure 1B, the lifetime distribution has two well-resolved components. In this table, a comparison (as a function of temperature) of the short (long) component lifetime to the lifetime of fully protonated (deuterated) AP shows both RTP lifetimes are nearly the same.

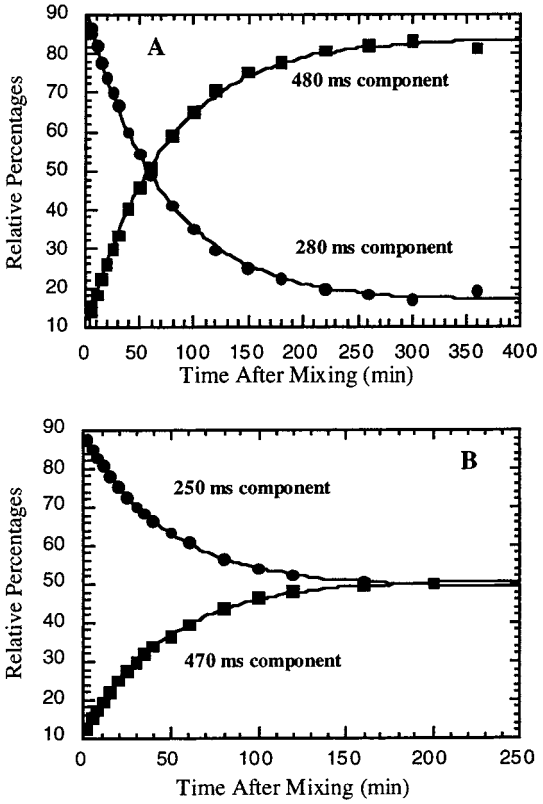

FIGURE 3: (A) Time evolution of the relative percentages of long (■) and short (●) lifetime components in the RTP lifetime of D_2O-exchanged WT AP at 50 °C. The phosphorescence decay of WT AP under these conditions is best fit by two exponentials: a 280 ms component and a 480 ms component. (B) Time evolution of the relative percentages of long (■) and short (●) lifetime components in the RTP lifetime of WT AP at 50 °C exchanged into a buffer consisting of 50% H_2O and 50% D_2O as a function of time. The phosphorescence decay of WT AP under these conditions is best fit by two exponentials: a 250 ms component and a 470 ms component.

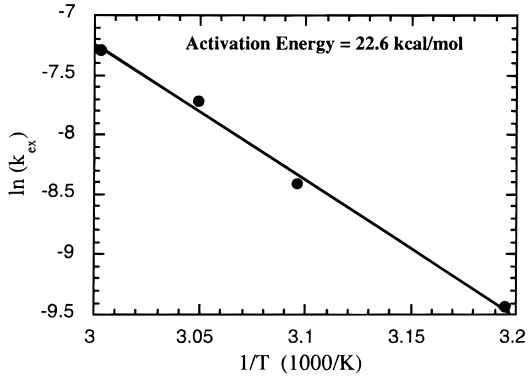

FIGURE 4: Plot of the natural log of the rate of the H−D exchange process for WT AP. The activation energy was calculated from the slope of a linear fit to the data, using $\Delta G' = -RT \ln(k_{ex})$.

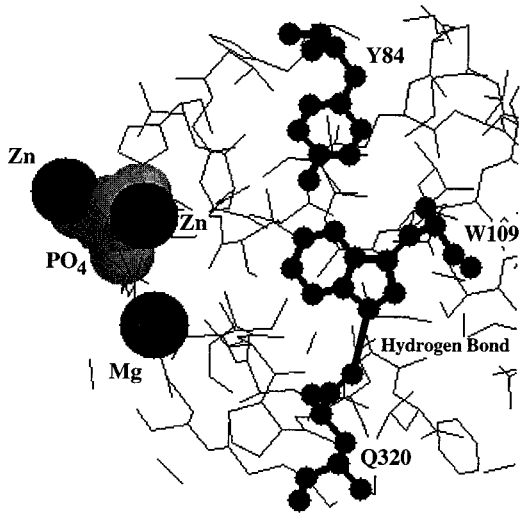

FIGURE 5: Hydrophobic core of AP centered on W109 depicting the location of Q320, which is attached to W109 by a hydrogen bond, Y84, the bound phosphate ion, and the bound Zn and Mg metals.

rates when WT was mixed at 60 °C into Tris buffer at pD 6.76 ($k_{ex} = 0.0409 \pm 0.0009$ min^{-1}) and pD 7.76 ($k_{ex} = 0.041 \pm 0.001$ min^{-1}).

DISCUSSION

AP, a nonspecific phosphomonoesterase, is a dimer with a molecular mass of approximately 94 kDa (*31*). It is a metalloenzyme which binds two Zn^{2+} ions and one Mg^{2+} ion per monomer. AP has three Trp residues per monomer; however, it is known that only Trp 109 phosphoresces at room temperature (*26*, *32*). Trp 109 is buried deep within the hydrophobic core of AP (*26*), and a stable H-bond is formed between its indole NH and a neighboring glutamine residue (Q320) as shown in the local structure presented in Figure 5. This H-bond stabilizes the core of the protein by linking together an α-helix and a β-sheet.

While most proteins with buried Trp residues have been shown to phosphoresce after extensive deoxygenation (*33*), Trp 109 in AP gives rise to the longest Trp RTP lifetime reported, ∼2 s (*33*). In contrast, Trp in solution at low concentrations is reported to have a lifetime of ∼1 ms (*34*). More recent measurements on Trp (*30*) and NATA (*34*) in solution show a correlation between an increase in phosphorescence lifetime and increasing solvent viscosity. Although these results are controversial (*35*) and may result from changes in the diffusion of triplet state quenchers in the solution (*36*), it is generally accepted (*37*, *38*) that a rigid environment is necessary for long phosphorescence lifetimes

since Trp RTP is quenched by out-of-plane motion of the indole ring, leading to intersystem crossing and decay (*38*). In the absence of quenchers (either intrinsic like disulfide bonds or extrinsic like O_2), a decrease in lifetime is interpreted as a reduction in local rigidity. Indeed, the RTP lifetime describes molecular plasticity in a manner similar to that of X-ray temperature factors, but with much greater sensitivity. Furthermore, compared to the short-lived fluorescence which usually arises from all the Trps and is relatively insensitive to structural changes, most proteins have only one or two Trps with long-lived RTP which makes RTP a very site specific probe of structural changes.

Previous hydrogen exchange work with AP in our laboratory by Schlyer et al. (*22*) reported a nonspecific process which resulted in an increase in the average phosphorescence lifetime of Trp 109. Because of metal binding inhomogeneity in the protein sample that was used, the true two-state nature of the exchange event was not resolved. Inhomogeneity in the binding of phosphate and/or metals (magnesium and zinc) has been shown to affect the activity (*39*) and phosphorescence lifetime (*40*) of AP, and in the case of magnesium binding, this inhomogeneity has been shown to dramatically affect tritium exchange kinetics (*41*) in AP. In all cases, it was theorized that the inhomogeneity in the metal and/or phosphate binding could be correlated with inhomogeneity in the structure of the enzyme. In the case of the preliminary hydrogen exchange experiments presented in ref *22*, we theorize that the presence of many inhomogeneous structures, each with a different H−D exchange rate caused by the inhomogeneity in metal and phosphate binding, gave rise to multistate exchange kinetics which we could analyze only as a general increase in the average lifetime.

In the study presented here, we achieved a more homogeneous protein sample by including 0.1 mM $ZnCl_2$, 0.1 mM $MgCl_2$, and 1 mM Na_2HPO_4 in all buffers and stock solutions to saturate the enzyme's binding sites. Under these conditions, the level of inhomogeneity is reduced and the two-state kinetics of the exchange are apparent as demonstrated by analysis of the data in Figure 3A. From the fact that the average lifetimes observed during H−D exchange correspond well to the lifetimes of fully protonated and fully deuterated AP, we conclude that these two states correspond to unexchanged and exchanged protein populations. This conclusion is further supported by the data in Table 1 which compares the lifetimes of the short and long components observed during H−D exchange to the values in fully protonated and fully deuterated AP as a function of temperature. Within experimental error, the lifetimes are the same and are time-independent. Furthermore, Figure 3A shows that the relative amplitudes of the two components follow a simple exponential as expected from the corresponding coupled rate equation for a two-state system.

It is interesting to mention that during the kinetic experiments the final population of "exchanged" protein does not regularly reach 100% (as would be expected for a completely homogeneous population), although equilibrium measurements on fully deuterated samples reveal only one, long lifetime population. This discrepancy is an artifact of the methodology which we attribute to the deoxygenation procedure. As mentioned earlier, during kinetic H−D exchange experiments, the protein solution and buffer are deoxygenated separately. The relatively small volume of protein solution used easily drys out during deoxygenation and, when mixed into the exchange buffer, gives rise to a small subpopulation of extremely slowly exchanging proteins (resulting from incomplete metal binding and/or partial unfolding or misfolding). Since we do not deoxygenate small volumes of protein for our equilibrium measurements, we do not see this discrepancy in population distribution occurring during these experiments.

In the original work (*22*), an initial jump in lifetime at early times was attributed to an osmolyte effect, a conclusion confirmed by a similar increase in lifetime when various polyols were used. The effect in polyols has been reproduced by us, but the initial jump seen in the original analysis is absent and was likely an artifact of the lifetime analysis in the strongly inhomogeneously distributed system that was used.

It is possible, on the basis of the above data alone, to assume that the phosphorescence lifetime in the fully deuterated form of AP reflects the fully deuterated state of many residues, not just one residue, or even that the difference in the lifetimes observed in D_2O and H_2O results from a general solvent effect; i.e., the protein is more rigid in one solvent than the other. However, the data depicted in Figure 3B performed with 50% H_2O/50% D_2O buffer suggest that, in fact, it is the exchange of a single hydrogen that is affecting the phosphorescence lifetime of Trp 109.

To consider the implications of Figure 3B more carefully, we note that hydrogen exchange is a dynamic, reversible process; therefore, deuterated sites can become reprotonated, depending upon the relative concentrations of protons and deuterons in the buffer solution. In the experiments whose results are depicted in Figure 3A, the final concentrations of D_2O and H_2O in the sample buffer were 97 and 3%, respectively; hence, when equilibrium is established, we would expect 97% of the proteins' hydrogens to be exchanged. Changing the relative concentrations of protons and deuterons in the solution will naturally affect the fraction of a given proton being replaced by a deuteron. If the exchange of a single hydrogen were responsible for the change in the phosphorescence lifetime, we would expect at equilibrium to see two decay components whose relative percentages correspond to the concentrations of H_2O and D_2O in the solution. If n hydrogens needed to be exchanged to affect the lifetime then, when equilibrium is established, the percentage of proteins exhibiting a long phosphorescence lifetime would be inversely proportional to the relative concentration of D_2O raised to the nth power. The distribution would also be highly multiexponential. If the change in lifetime resulted from some general solvent effect, we would expect to see a single lifetime centered between the lifetimes observed for 100% H_2O and 100% D_2O solutions.

When we conduct our experiments with a buffer containing 50% H_2O and 50% D_2O, the equilibrium phosphorescence lifetime of the sample is still fit best by two exponentials (with the same lifetimes that are observed in the 100% H_2O and 100% D_2O solutions, respectively) with the relative percentages of each of these two lifetime components being 50% (Figure 3B). Since two (and no more than two) lifetimes are present at equilibrium with this buffer and each lifetime accounts for 50% of the observed phosphorescence, it is clear that it is the exchange of a single, specific hydrogen which is affecting the phosphorescence lifetime.

Since a molecule the size of AP has a large number of labile hydrogens, the determination of the specific hydrogen responsible for the change in phosphorescence lifetime is, naturally, problematic. Since the exchange rate is very slow, even at elevated temperatures, we conclude that the hydrogen must be deeply buried (*1*, *4*) in the protein [and perhaps involved in a hydrogen bond (*11*)]. We eliminated the possibility that the deuteration of a neighboring tyrosine residue (Y84; see Figure 5) was affecting the ability of that residue to quench the phosphorescence of Trp 109 since mutants lacking this tyrosine also demonstrated a change in lifetime following deuteration. Although it is possible that any of the remaining labile hydrogens in the core of AP might be the origin of the increase in the RTP lifetime, the importance of out-of-plane motions of the indole ring in determining the phosphorescence lifetime of tryptophan (*38*, *42*, *43*) and the effect of deuteration of the enamine of tryptophan upon these motions (*44*, *45*) suggest that the critical labile hydrogen is likely to be the enamine hydrogen of Trp 109. This is certainly plausible given that deuteration has been shown to modify the phosphorescence lifetime in other aromatic compounds (*42*, *46*, *47*). Direct confirmation remains to be demonstrated, but measurements in our laboratory on the Q320G mutant of AP, which is anticipated to lack the enamine hydrogen bond, show a 5-fold increase in the magnitude of the effect of deuteration on the RTP lifetime. A similar correlation between the extent of hydrogen bonding and the extent of deuteration was proposed by Lim and Yu (*42*) to explain the phosphorescence of heteroaromatic molecules and its dependence upon solvent.

The importance of the absence of a pD dependence of the rate of exchange is highlighted by the fact that hydrogens in proteins continually exchange with solvent hydrogens in a reaction catalyzed by solvent acids and bases, especially the D^+ and OD^- ions. The accepted model of hydrogen exchange in proteins as a two-step process was described in detail by Hvidt and Nielsen (*12*) and has been subsequently developed (*3*, *48*). There are two limiting cases for this model. If the rate-limiting step in the reaction is the actual exchange event, then the process is said to follow EX2 kinetics and the observed exchange rate will depend linearly on pD as long as the experiment is conducted away from the minimum pD [typically between 3 and 5 (*3*)] for intrinsic exchange of a particular residue or peptide hydrogen. In the other limit, EX1, the rate-limiting step in the process is the opening of the protein domain (i.e., the exposure of the residue to the solvent), and there should be no significant dependence on pD as long as the protein's stability is not pD-dependent.

Hydrogens that exchange following EX2 kinetics are usually solvent-exposed in the native state of the protein or require only small structural fluctuations to expose them. In contrast, hydrogens exchanging with EX1 kinetics are generally deeply buried and require a more global fluctuation in the structure of the protein to be exposed to the solvent. Determining which limit best describes the exchange of a particular hydrogen can be a powerful tool for determining the relative location of that hydrogen within the protein, i.e., how deeply buried or protected it is from the solvent. The observation that there is no dependence of the exchange rates on the pD of the solution indicates that the exchange observed here is not following EX2 kinetics, and is quite possibly determined by EX1 kinetics associated with exchange of a residue buried deeply in the core of AP. This observation is also consistent with our assignment of this hydrogen to the enamine nitrogen of Trp 109.

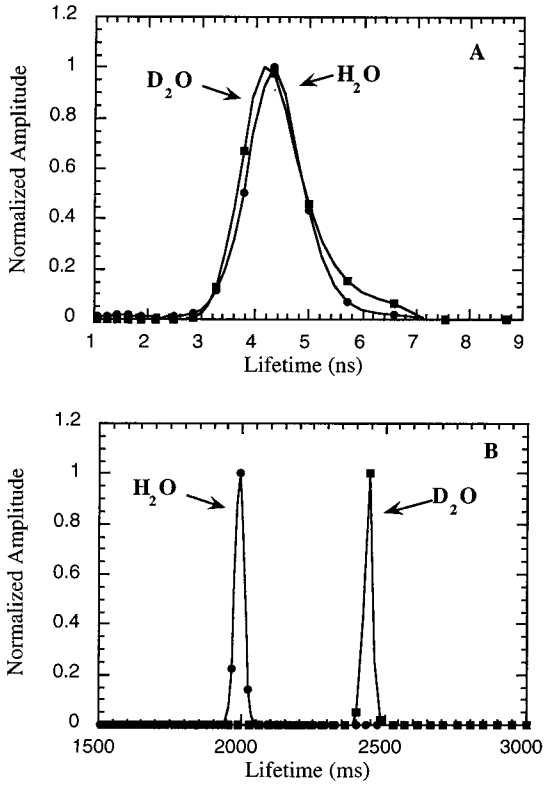

FIGURE 6: (A) MEM distribution analysis of the fluorescence lifetime of W220Y in H_2O (■) and D_2O (●) at 20 °C. The fluorescence lifetime of the protein in both buffers is the same. (B) MEM distribution analysis of the phosphorescence of W220Y in H_2O (■) and D_2O (●). at 20 °C. The lifetime in D_2O is 23% longer than the lifetime in H_2O.

It is important to note, however, that given the unusual rigidity of AP compared to other proteins, as inferred by its long phosphorescence lifetime, the exchange process may in fact be more complicated than EX1. Indeed, given that the activation energy we measure for H−D exchange is 22.6 kcal/mol and that the ΔG for unfolding is 120 kcal/mol (*49*), it is clear that the protein is not completely unfolding during the exchange process. Much of the ΔG of unfolding is due to metal binding, and hence, it may be that the "breathing" motions leading to exchange do not include release of the metals. However, it may also be that the exchange process is mediated by diffusion of water through channels that open and close. Indeed, work by Cioni and Strambini (*50*) suggests that acrylamide quenching of the phosphorescence of W109 in AP is mediated by the migration of the quencher through the protein to within interaction distance of the residue.

Finally, we note that while the phosphorescence lifetime is remarkably sensitive to H−D exchange, fluorescence reveals no detectable effect. To demonstrate this, we note that AP has three Trps, but the fluorescence from Trp 268 is expected to be heavily quenched by a nearby cysteine group. To study the fluorescence from Trp 109, we replaced Trp 220 with tyrosine; the fluorescence lifetimes of WT AP and the W220Y mutant are very similar. To examine the affect of deuteration on the fluorescence lifetime of W109, we diluted 10 μL of concentrated W220Y into 1 mL of H_2O or D_2O buffer and then incubated both solutions at 60 °C

for 8 h. The samples were then allowed to incubate overnight at room temperature before we measured their fluorescence and phosphorescence lifetimes. The results, shown in Figure 6A, validate the claim made by Nakanishi (*51*) and Kirby and Steiner (*52*) that deuteration of the indole NH has no effect upon the fluorescence lifetime of Trp other than bulk solvent effects. The corresponding phosphorescence lifetime for W220Y is shown in Figure 6B which shows a behavior similar to that discussed above for WT AP.

This paper demonstrates that the Trp phosphorescence-detected H−D exchange in AP is adequately described by a simple two-state model and that it provides a sensitive diagnostic for detection of H−D exchange in a very local region of AP. This approach opens up the possibility of detecting these H−D exchange events with considerably more sensitivity than other optical methods with the advantage that the presence of the exchange can be detected on a time scale that is short compared to those of some slower folding reactions, enabling these reactions to be followed as a function of time with this methodology.

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