Biophysical Journal Volume 71 October 1996 2138–2143

Detection of a pH-Dependent Conformational Change in Azurin by Time-Resolved Phosphorescence

John E. Hansen,* Duncan G. Steel,*§ and Ari Gafni*#
The University of Michigan, *Institute of Gerontology, #Department of Biological Chemistry, and §Department of Physics, Ann Arbor, Michigan 48109-2007 USA

ABSTRACT Azurin, a blue copper protein from the bacterial species *Pseudomonas aeruginosa*, contains a single tryptophan residue. Previous fluorescence measurements indicate that this residue is highly constrained and unusually inaccessible to water. In the apoprotein this residue also possesses a long-lived room-temperature phosphorescence (RTP), the nonexponential decay of which can be resolved into two major components associated with lifetimes of 417 and 592 ms, which likely originate from at least two conformations of the protein. The relative weights of these two decay components change with pH in good correlation with a change in protonation of His-35, which has been studied in Cu(II) azurin. Interestingly, the structural changes characterized in earlier work have little effect on the fluorescence decay and appear to occur away from the tryptophan residue. However, in the present work, the two RTP lifetimes suggest conformations with different structural rigidities in the vicinity of the tryptophan residue. The active conformation that predominates below a pH of 5.6 has the shorter lifetime and is less rigid. Phosphorescence decays of several metal derivatives of azurin were also measured and revealed strong similarities to that of apoazurin, indicating that the structural constraints upon the metal-binding site are imposed predominately by the protein.

INTRODUCTION

Azurins are categorized as type I blue copper proteins, a class of redox proteins incorporating a single copper atom. Azurin from the bacterium *Pseudomonas aeruginosa* (azurin Pae) contains a single tryptophan residue at position 48, which is highly shielded from solvent and surrounded by hydrophobic residues, causing this residue to display fluorescence with the smallest Stokes shift known for a tryptophan residue in any protein (Finazzi-Agro et al., 1970; Szabo et al., 1983). Furthermore, the motion of this tryptophan residue appears to be greatly restricted, as time-resolved fluorescence-anisotropy measurements indicate no detectable motion of the indole ring with respect to the protein backbone on either the subpicosecond, picosecond, or nanosecond time scale (Hansen et al., 1990b, 1992; Petrich et al., 1987). The measured fluorescence decay of apoazurin Pae also fits well to an exponential function with a lifetime near 5 ns (Petrich et al., 1987; Hansen et al., 1990a), which suggests minimal heterogeneity for the local environment of the indole moiety.

The unique features of this tryptophan residue (being sequestered from an aqueous environment, restricted local motion, and minimal heterogeneity of the local environment) also lead to strong room-temperature phosphorescence (RTP) (Vanderkooi et al., 1987; Strambini and Gabellieri, 1991; Klemens and McMillin, 1992). RTP decay times of internal tryptophan residues in proteins have been correlated with the local flexibility of their domains (Papp and Vanderkooi, 1989). Out-of-plane distortions of the indole moiety, which depend on the rigidity of the chromophore's microenvironment, are predicted to enhance coupling to nonradiative modes, resulting in shorter lifetimes (Lower and El-Sayed, 1966). Strambini and Gonnelli (1985), using various tryptophan derivatives, have demonstrated that indole phosphorescence lifetimes decrease by two orders of magnitude over a viscosity range of 10^9 to 10^4 poise. As pointed out by Vanderkooi et al. (1987), tryptophan room-temperature phosphorescence provides an ideal probe for structural studies of protein cores and can yield important information regarding protein stability.

In the present study we document a correlation between changes in the phosphorescence lifetime of azurin and structural changes relevant to protein function. Early measurements characterized the RTP decay of this protein as a single exponential function (Vanderkooi et al., 1987; Strambini and Gabellieri, 1991). The high quality of data (determined by the total photon count and dynamic range) and the resulting improved signal-to-noise ratio allow us to show that the phosphorescence decay of apoazurin is multiexponential and can be well described by two major components. Because azurin is a single-domain protein containing only one tryptophan residue, interpretation of the phosphorescence decay is not complicated by either interdomain interactions or ambiguity regarding the emitting residue. Hence, the origin of the nonexponential decay can be attributed to the existence of at least two conformational states, which interconvert on a time scale longer than the lifetimes of the major decay components. Furthermore, the two phosphorescence decay components of apoazurin are pH dependent, and the change in their relative proportions correlates with the change in protein function known to occur over the same

Received for publication 11 April 1996 and in final form 20 June 1996.

Address reprint requests to Dr. Art Gafni, Institute of Gerontology, University of Michigan, 300 N. Ingalls, Ann Arbor, Mi 48109-2007. Tel.: 313-936-2156; Fax: 313-936-2116; E-mail: arigafni@umich.edu.

0006-3495/96/10/2138/06 $2.00

range of pH values (Silvestrini et al., 1981, 1986; Adman et al., 1982; Pettigrew et al., 1983).

Decays of several metal derivatives of azurin were also studied and compared with that of apoazurin. From this comparison we were able to determine the effect of metal binding on protein stability and conformation.

EXPERIMENTAL

Apoazurin Pae (from the bacterial strain ATCC 19429) was generously provided by Professor Graham Fleming (University of Chicago) and was used with no further purification. Purity of the apoazurin and its structural integrity were assessed from its ability to bind Cu(II), which was determined from the ratio of the absorption at 625 nm to that at 280 nm after reconstitution with Cu(II). This ratio, which is customarily taken as the criterion of purity for native azurin, was found to be 0.52 ± 0.01, in good agreement with the value of 0.54 determined for this ratio from absorption measurements of reconstituted Cu(II) azurin Pae by Blaszak et al. (1983). Methods employed in this study for the preparation of metalloazurins were previously described elsewhere (Hansen et al., 1990a). All chemicals used in this study were reagent grade. To prepare a protein sample at a given pH between 4.0 and 6.6, or between 7.0 and 8.0, the sample was equilibrated (by dialysis) against a 5 mM sodium citrate buffer or a 5 mM HEPES buffer, respectively, at the appropriate pH. Samples ready for RTP study were placed in 1-cm quartz cuvettes and deoxygenated by purging with high-purity argon (according to the method of Vanderkooi et al., 1987) over a period of 4 h.

Phosphorescence decays were collected at 20° ± 1°C from samples that had an optical density at 280 nm of 0.3–0.4, by using a time-resolved phosphorescence spectrometer described in detail elsewhere (Mersol et al., 1991). Samples were excited with the second harmonic (280 nm) output of a PDL-3 dye laser/amplifier (Spectra-Physics, Freemont, CA), using rhodamine 6G as the gain medium. The dye was pumped with the second harmonic (532 nm) of a DCR-11 Nd:YAG laser (Quanta-Ray) with a temporal pulse width of 8 ns (FWHM). The UV excitation pulse was passed through a UG-11 filter (Schott Glaswerke, Mainz, Germany) and focused into the sample. Residual excitation light was removed from the emission using a 3 M potassium nitrite solution with a 1-cm optical path. Emission was collected at 450 nm using an HR-320 monochromator (Instruments-SA, Edison, NJ) and detected by a R928P photomultiplier tube (Hamamatsu Photonic Systems Corp., Bridgewater, NJ). Signals were then sent to an amplifier-discriminator (Pacific Instruments), and the output was collected by an ACEMCS multichannel scaler (EG&G Ortec, Oak Ridge, TN). Both laser and multichannel scaler were simultaneously and externally triggered. Decays were collected into 512 channels with a time scale of 10 (or 5) ms/channel and contained 3×10^5 to 5×10^5 counts in the peak channel, except for the Cu(II) derivative, which contained 3×10^4 counts in the peak channel.

Parameters for the phosphorescence decays were determined by global lifetime analysis (Knutson et al., 1983) and single decay-curve analysis performed on an IBM PC using an iterative fitting procedure based on the Levenberg-Marquardt algorithm (Levenberg, 1944; Marquardt, 1963). Global analysis allows several decays collected as a function of a particular variable to be fit simultaneously (Knutson et al., 1983; Beechem et al., 1985a,b). During this fitting procedure parameters assumed to be common to these decays are linked, thus constraining and overdetermining those parameters. Software programs used for these analyses were part of the LS-100 Fluorescence Lifetime Analysis Modules obtained from Photon Technology International (London, ON, Canada).

RESULTS

RTP decays of apoazurin were collected to over 3×10^5 counts in the peak channel, and the data covered a dynamic range of four decades. When these decays were fit to a functional form, they were found to be clearly nonexponential (see Fig. 1). Assuming the functional form to be a sum of exponentials ($\Sigma\alpha_i \exp[-t/\tau_i]$), we found a three-component fit adequate, as presented in Table 1. The two major components had lifetimes that differed by less than 30%, whereas the third, shorter-lived component was minor (less than 1% of the total phosphorescence) and we believe that it arises from a protein impurity. The lifetimes of the two major components are sufficiently close that to adequately resolve them from fitting analysis requires the large dynamic range in the data we collected.

The phosphorescence decay of apoazurin shows a clear, systematic dependence on pH (see Fig. 2). Fig. 3 displays how the amplitudes of the two major decay components (α_1 and α_2 with lifetimes of 417 ms and 592 ms, respectively) depend on pH. It is apparent that α_1 decreases, whereas α_2 increases, with increasing pH. The solid line in Fig. 3 presents the ratio [A]/([A] + [AH^+]) versus pH, calculated using the Henderson-Hasselbalch equation for the deprotonation of an acid (AH^+) to form the conjugate base (A), with a pK_a value of 5.6. The excellent fit between the experimentally derived data ratios and the theoretical curve supports our conclusion that the phosphorescence decay amplitudes reflect the titration of a group with a pK_a close to 5.6.

These decay parameters were determined from a nonlinear least-squares fitting procedure applying global analysis (Beechem et al., 1985a,b). This analysis offers the practical advantage of determining more precisely the fitting parameters of the two major decay components, which have lifetimes that do not differ greatly. This is especially useful at those pHs where the relative amplitudes of these two decay components are nearly equal, making it difficult to determine a unique set of fitting parameters from single decay fits. The global fit for the phosphorescence decays of apoazurin collected as a function of pH has a global χ^2 of 1.27.

Phosphorescence decays were also collected for various metal derivatives of azurin. Fitting parameters for these

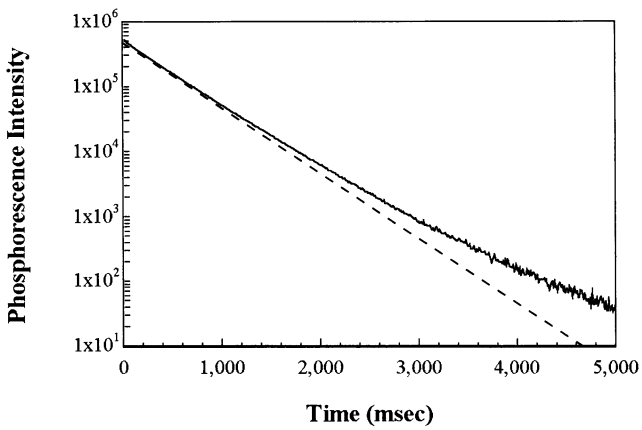

FIGURE 1 Time-resolved phosphorescence of apoazurin measured at pH 5.0. The dashed line depicts a monoexponential decay curve and highlights the deviation of the experimentally observed decay from a single exponential function.

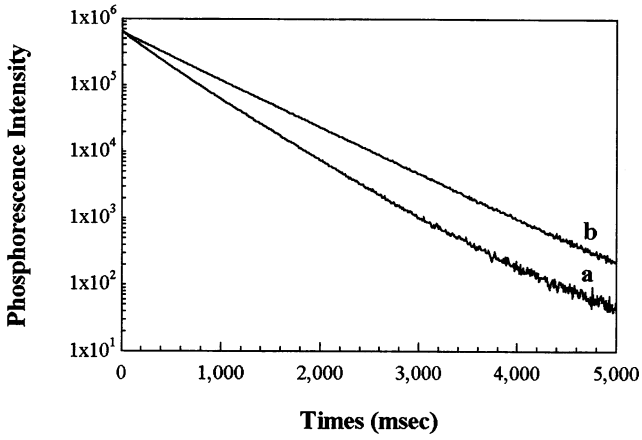

FIGURE 2 Time-resolved phosphorescence traces of apoazurin at pH 4.6 (*a*) and pH 7.4 (*b*).

decays collected at pH 4.3 and 7.4 are listed in Table 1. Parameters for the phosphorescence decays of Zn(II), Cd(II), and Hg(II) were determined from global analysis. Lifetimes for components of the decay collected at pH 4.3 were linked to those of the decay collected at pH 7.4. For these metal derivatives the relative amplitudes of the two major decay components are affected by pH in a similar fashion, as was found for the decay components of apoazurin. Furthermore, the lifetimes determined for these decay components are also similar to those determined for the decay components of apoazurin.

Fitting parameters for the phosphorescence decay of Cu(II) azurin were determined from analyses of a single decay (see Table 1). The decay rates obtained show that the phosphorescence from Cu(II) azurin is strongly quenched, such that the contribution by the short-lived component (which is believed to represent an impurity) becomes prominent. A 300-ms decay component comprises most of the remaining phosphorescence decay of Cu(II) azurin Pae.

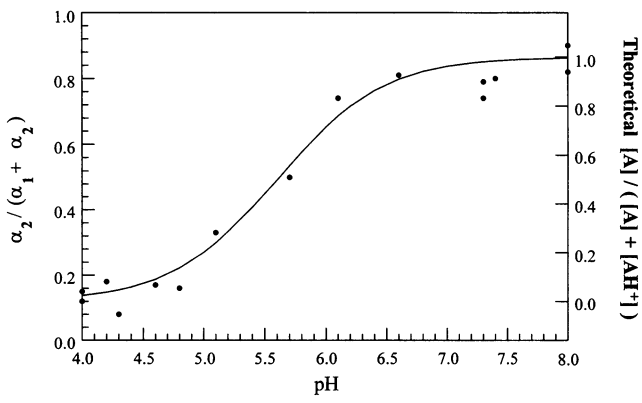

FIGURE 3 The fraction of the phosphorescence decay amplitude of apoazurin associated with the 590-ms decay component plotted against pH. The functional form of the decay is described as a sum of exponentials (Σ_i α_i exp$[-t/\tau_i]$), and the fraction is defined as the ratio $\alpha_2/(\alpha_1 + \alpha_2)$, where α_1 and α_2 are the preexponential terms for the 417-ms and 592-ms decay components, respectively. The solid curve represents the ratio [A]/([A] + [AH^+]) versus pH as calculated for an acid, AH^+, with a pK_a of 5.6, employing the Henderson-Hasselbalch equation.

DISCUSSION

Nonexponential decay of apoazurin room-temperature phosphorescence

The single tryptophan residue of azurin Pae is buried in a hydrophobic core of a β-barrel (Adman and Jensen, 1981)—a location that severely limits the possible orientations available to this residue. Molecular dynamics simulations of azurin obtained from the bacterium *Alcaligenes denitrificans* (highly homologous to azurin Pae) predict only very small-amplitude motions for this tryptophan (Chen et al., 1988). Temporally resolved fluorescence depolarization measurements of the tryptophan residue in azurin Pae on time scales ranging from subpicoseconds (Hansen et al., 1992) to nanoseconds (Petrich et al., 1987) reveal only motion that correlates with the rotational diffusion of the whole protein. Furthermore, the measured fluorescence decay of apoazurin fits well to a single exponential function (Petrich et al., 1987; Hansen et al., 1990a,b), indicating minimal heterogeneity of the local environment of the indole moiety. Thus, previous fluorescence data provide a model that is consistent with that expected from crystallographic data, in which the tryptophan residue is constrained by the protein matrix to adopt a singular configuration.

In contrast to the monoexponential fluorescence decay, we find the phosphorescence to clearly decay nonexponentially. Whereas nonexponential phosphorescence decays have been observed from a number of proteins (Schlyer et al., 1994), azurin is, so far, the only single tryptophan-containing protein exhibiting nonexponential decay kinetics.

In a homogeneous environment tryptophan phosphorescence is expected to decay exponentially, a notion that is supported by the single exponential decay of tryptophan in solid media at 77°K (Longworth, 1971). Nonradiative deactivation of the triplet state by quenchers is a major factor that determines the rate of phosphorescence decay of tryptophan residues in proteins (Papp and Vanderkooi, 1989), the quenching rate of which is diffusion limited. RTP from proteins is observed only after drastically lowering the oxygen concentration (Vanderkooi et al., 1987), as we have done in this study. Another factor believed to govern the rate of phosphorescence decay is the occurrence of out-of-plane vibrations of the indole ring, which allow for coupling to nonradiative modes (Lower and El-Sayed, 1966). When the oxygen concentration is adequately lowered, the phosphorescence lifetime thus reveals the structural rigidity of a protein in the vicinity of the tryptophan residue (Strambini and Gonnelli, 1985) with longer lifetimes associated with greater local rigidity.

There are several reasons for protein phosphorescence to follow nonexponential decay kinetics, the most trivial rea-

son being that the protein contains several emitting tryptophan residues (Schauerte et al., 1992). The different locations of the residues either make them more accessible to quenchers or expose them to different local rigidities. In an example of nonexponential decay kinetics in which the phosphorescence is believed to originate from a single tryptophan, Strambini and co-workers (Strambini, 1987; Strambini et al., 1990) have reported that ligand binding induces biphasic decay kinetics in glutamate dehydrogenase and in liver alcohol dehydrogenase. This has been interpreted as arising from structural changes in protein conformation. Recent work (Schlyer et al., 1994) has also described two proteins (liver alcohol dehydrogenase and alkaline phosphatase) where the phosphorescence decays nonexponentially. This has been attributed to an inherent conformational heterogeneity among protein molecules.

The phosphorescence decay of azurin Pae unambiguously originates from the single tryptophan present in this protein, and yet is nonexponential. We are able to fit the decay to a sum of exponential terms and interpret the two major decay components as originating from two (or more) conformations differing in structural rigidity. These differences in the constellation of residues about the tryptophan residue are subtle, as indicated by the fact that the fluorescence decays monoexponentially. The greater sensitivity of RTP lifetime to the tryptophan environment, relative to the fluorescence lifetime, is vividly displayed here. In a previous study Strambini and Gabellieri (1991) reported that RTP decay of apoazurin fit to a single exponential function; however, the dynamic range of those decays covered only a decade and a half of counts, and the accuracy of fitting the decay curves to a functional form was limited. As mentioned above, the dynamic range our data covers four decades, making the resolution of components with lifetimes that are not vastly different much more feasible. By calculating the average lifetime ($\Sigma\alpha_i\tau_i/\Sigma\alpha_i$) for our multiexponential decay obtained for apoazurin, we derive a value similar to the single lifetime reported by Strambini and Gabellieri (1991) determined at the same pH and temperature.

Effects of pH on the room-temperature phosphorescence of azurin derivatives

Strambini and Gabellieri (1991) measured the phosphorescence decay of apoazurin at pH 5.0 and 7.2, and reported single exponential fits with the decay measured at pH 5.0 that were somewhat shorter than those measured at pH 7.2. No discussion of this pH dependence of the lifetimes was provided. By analyzing our data in terms of the two major components discussed above, we find that at the lower pH the shorter lifetime component makes up a significantly greater fraction of the decay than at a higher pH. Interpreting these two decay components as arising from two different conformations provides a model consistent with the biological function of this protein.

The effect of pH on the relative weights of the two major decay components (see Fig. 3) suggests that a change in conformation is induced by a change in pH. Specifically, a plot of $\alpha_2/(\alpha_1 + \alpha_2)$ against pH reveals the titration of a group with a pK_a around 5.6. Strong support for this conclusion is provided by the excellent fit to the data by a theoretical curve (*solid line* in Fig. 3) calculated using the Henderson-Hasselbalch equation for the titration of an acid with a pK_a of 5.6. This pK_a falls in the range of values associated with histidine residues. Four histidine residues (at positions 35, 46, 83, 117) are conserved among the azurins. Two of these residues, His-48 and His-117, are involved with metal binding. It is unlikely that the pH effects we observe in the phosphorescence of apoazurin originate in a histidine at the metal-binding site, because we observe the same pH effects in the phosphorescence of Hg(II), Zn(II), and Cd(II) azurin, where these histidines are ligated to metal cations and are unavailable for protonation.

NMR measurements (Hill and Smith, 1979) indicate that only His-35 and His-83 in native azurin Pae are titratable between pH 4 and 9. His-83 was found to have a pK_a of 7.6 (Hill and Smith, 1979), whereas Corin et al. (1983) report a pK_a of 5.9 $\pm$ 0.4 for His-35 in Cu(II) azurin Pae, a value similar to the one reported here from the titration curve derived from our phosphorescence measurements for the apoprotein. It thus seems likely that the phosphorescence measurements are sensitive to the degree of protonation of His-35. Gonnelli and Strambini (1995) reported that His is an effective dynamic quencher of Trp phosphorescence and found the quenching rate constant to increase about 50-fold upon protonation of the imidazole ring. These authors, however, determined the quenching to be short-range and to require contact between the imidazole and indole rings. Because in azurin the distance between His-35 imidazole and Trp-48 indole is above 12 Å, direct quenching appears unlikely. We therefore conclude that the shortened phosphorescence lifetime reflects the fact that when His-35 is protonated, azurin adopts a conformation with less structural rigidity in the vicinity of the trypotphan residue. Because azurin is a small, single-domain protein, this may reveal a softer structure for the entire protein.

Several groups (Silvestrini et al., 1981, 1986; Adman et al., 1982; Pettigrew et al., 1983) have correlated the kinetics of electron transfer between azurin Pae and other redox proteins with the degree of protonation of His-35. When this histidine is protonated, azurin Pae participates more efficiently in the electron transfer pathway (the more active form of the protein). It has also been shown (Pettigrew et al., 1983) that a change in the reduction potential of azurin is correlated with the protonation of this residue. NMR studies reveal several conformational changes and alteration of the coordination shell of the copper cation in azurin Pae with a change in protonation of His-35 (Adman et al., 1983). Adman et al. (1982) propose that this transition involves a shuttling motion of the imidazole ring of His-35 from a position where it is accessible to water molecules to a position inaccessible to water, but available for intraprotein hydrogen bonding. It has been suggested that the change in the position of the imidazole ring of His-35 distorts the

coordination shell of the copper (Adman et al., 1983). Changes in the position of ligands about the copper have been shown to affect the reduction potential of azurin (Gray and Malmstrom, 1983). The proximity of His-35 to His-47 (one of the copper ligands) has also led to the suggestion (Adman et al., 1982) that these histidines together play a role in electron transfer—possibly through a hydrogen bond relay between the two residues (Adman et al., 1982).

The conclusion, based on our RTP data, that the protein conformation that predominates at lower pH values is the less rigid one, is significant biologically. It is to be expected that azurin in the active form should require structural flexibility to allow for more efficient protein-protein interactions with the appropriate cytochrome, the latter ligand inducing further conformational change at the binding site of a protein that allows for complementary interaction.

Strong similarities among the phosphorescence decays were revealed for several metal derivatives (Hg(II), Zn(II), Cd(II)) of azurin and that of apoazurin, indicating that the presence of a metal cation in the binding site does not affect the rigidity of the tryptophan domain. Strambini and Gabellieri (1991) also reported that the phosphorescence decay measured for Cd(II) azurin is nearly identical to that of apoazurin. This supports the suggestion (Baker, 1988), based on crystallographic data, that structural constraints upon the metal-binding site are imposed by the protein (rather than by the presence of a metal cation), making it the most rigid and well-ordered part of the structure. Even when a metal cation as heavy as Hg(II) is located in the metal-binding site, there is little difference in the phosphorescence decay compared to that measured for the apoprotein. This seems to further emphasize the significance of the positions of the metal-binding ligands, which are determined by the protonation state of His-35.

The presence of Cu(II) cation in the binding site greatly affects the phosphorescence; however, this is accounted for by factors not associated with changes in protein structure. The phosphorescence quantum yield of Cu(II) azurin Pae is very small for two reasons: the singlet state is known to be extremely short lived—the fluorescence of Cu(II) azurin is quenched 51-fold compared to that of apoazurin (Petrich et al., 1987; Hansen et al., 1990a,b), making population transfer to the triplet state, by intersystem crossing, much less efficient; and the triplet state is expected to be very strongly quenched as a result of electronic energy transfer to metal-ligand charge transfer transitions (there is extensive spectral overlap between the tryptophan phosphorescence and the absorption due to these charge transfer transitions). Consequently, the minor 100-ms component, found in the phosphorescence decay of apoazurin and attributed to an impurity, becomes prominent in the phosphorescence decay of Cu(II) azurin.

The present study provides additional insight into the time-resolved RTP of tryptophan residues in proteins. Measuring the time-resolved room-temperature phosphorescence of azurin, a single tryptophan-containing protein, we find the tryptophan phosphorescence to decay nonexponentially. The decay fits to a sum of exponentials. The two major decay components we interpret as arising from two conformations of differing structural rigidity, which is not reflected in the fluorescence decay. The relative fractions of the two major decay components are pH dependent, which suggests a pH-induced transition from one conformational state to the other. This transition has a pK_a of 5.6, similar to the pK_a measured (Corin et al., 1983) for His-35 in Cu(II) azurin. Protonation of this residue has previously been associated with a transition from a less to a more active form of the protein. The more active form of the protein, corresponding to a protonated His-35, is the less rigid conformer and may provide the flexibility in protein-protein interactions necessary for efficient electron transfer. Furthermore, the phosphorescence decays measured for several metalloazurins are very similar to that measured for apoazurin, supporting the view that the structural rigidity of the native protein and the position of the metal ligands are not affected by the presence of a metal cation. This work demonstrates the added information provided by time-resolved phosphorescence that is not available from fluorescence measurements.

We wish to thank Professor Graham Fleming (University of Chicago) for the generous gift of apoazurin Pae.

This work was supported by ONR grant N00014-91-J-1938 and by NIA grant AG09761.

REFERENCES

Adman, E. T., G. W. Canters, H. A. O. Hill, and N. A. Kitchen. 1982. The effect of pH and temperature on the structure of the active site of azurin from *Pseudomonas aeruginosa*. *FEBS Lett.* 143:287–292.

Adman, E. T., G. W. Canters, H. A. O. Hill, and N. A. Kitchen. 1983. The redox properties of azurin from *Pseudomonas aeruginosa* as studied by high frequency proton NMR. *Inorg. Chim. Acta.* 79:127–128.

Adman, E. T., and L. H. Jensen. 1981. Structural features of azurin at 2.7 Å resolution. *Isr. J. Chem.* 21:8–12.

Baker, E. N. 1988. Structure of azurin from *Alcaligenes denitrificans* refinement at 1.8 Å resolution and comparison of the two crystallographically independent molecules. *J. Mol. Biol.* 203:1071–1095.

Beechem, J. M., M. Ameloot, and L. Brand. 1985a. Global and target analysis of complex decay phenomena. *Anal. Instrum.* 14:379–402.

Beechem, J. M., M. Ameloot, and L. Brand. 1985b. Global analysis of fluorescence decay surfaces—excited-state reactions. *Chem. Phys. Lett.* 120:466–472.

Blaszak, J. A., D. R. McMillin, A. T. Thornton, and D. L. Tennet. 1983. Kinetics of copper(II) uptake by apoazurin in complexing media. *J. Biol. Chem.* 258:9886–9892.

Canters, G. W., H. A. O. Hill, N. A. Kitchen, and E. T. Edman. 1984. The assignment of the ^1H nuclear magnetic resonance spectrum of azurin. *Eur. J. Biochem.* 138:141–152.

Chen, L. X.-Q., R. A. Engh, A. T. Brunger, D. T. Nguyen, M. Karplus, G. R. Fleming. 1988. Dynamics simulation studies of apoazurin of *Alcaligenes denitrifcans*. *Biochemistry.* 27:6908–6921.

Corin, A. F., R. Bersohn, and P. E. Cole. 1983. pH dependence of the reduction-oxidation reaction of azurin with cytochrome c-551: role of histidine-35 of azurin in electron transfer. *Biochemistry.* 22:2032–2038.

Finazzi-Agro, C., G. Rotilio, K. Avigliano, P. Guerrieri, V. Botti, and B. Mondovi. 1970. Environment of copper in *Pseudomonas fluorescens* azurin—fluorometric approach. *Biochemistry.* 9:2009–2018.

Gonnelli, M., and G. B. Strambini. 1995. Phosphorescence lifetime of trypotphan in proteins. *Biochemistry.* 34:13847–13857.

Gray, H. B., and B. G. Malmstrom. 1983. On the relationship between protein-forced ligand fields and the properties of blue copper centers. *Comments Inorg. Chem.* 2:203–208.

Hansen, J. E., J. W. Longworth, and G. R. Fleming. 1988. Electronic energy transfer in metalloazurins. *Photochem. Photobiol.* 47(Suppl.): 12S.

Hansen, J. E., J. W. Longworth, and G. R. Fleming. 1990a. Photophysics of metalloazurins. *Biochemistry.* 29:7329–7338.

Hansen, J. E., S. J. Rosenthal, and G. R. Fleming. 1990b. Subpicosecond fluorescence anisotropy measurements of tryptophanyl residues in proteins. *In* Ultrafast Phenomena VII. C. B. Harris, E. P. Ippen, G. A. Mourou, A. H. Zewail, editors. Springer-Verlag, Berlin. 548–550.

Hansen, J. E., S. J. Rosenthal, and G. R. Fleming. 1992. Subpicosecond fluorescence depolarization studies of tryptophan and tryptophanyl residues of proteins. *J. Phys. Chem.* 96:3034–3040.

Hill, H. A. O., and B. E. Smith. 1979. Characteristics of azurin from *Pseudomonas aeruginosa* via 270-MHz ^{1}H nuclear magnetic resonance spectroscopy. *J. Inorg. Biochem.* 11:79–93.

Klemens, F. K., and D. R. McMillin. 1992. Room-temperature phosphorescence from azurin derivatives. Phosphorescence quenching in oxidized native azurin. *Photochem. Photobiol.* 55:671–676.

Knutson, J. R., J. M. Beechem, and L. Brand. 1983. Simultaneous analysis of multiple fluorescence decay curves—a global approach. *Chem. Phys. Lett.* 102:501–507.

Levenberg, K. 1944. A method for the solution of certain nonlinear problems in least squares. *Q. Appl. Math.* 2:164–168.

Longworth, J. W. 1971. Luminescence of polypeptides and proteins. *In* Excited States of Proteins and Nucleic Acids. R. F. Steiner and I. Weinryb, editors. Plenum Press, New York. 319–484.

Lower, S. K., and M. A. El-Sayed. 1966. Triplet state and molecular electronic processes in organic molecules. *Chem. Rev.* 66:199.

Marquardt, D. W. 1963. Algorithm for least squares estimation of nonlinear parameters. *J. Soc. Ind. Appl. Math.* 11:431–441.

Mauk, A. G., R. A. Scott, and H. B. Gray. 1980. Distances of electron transfer to and from metalloprotein redox sites in reactions with inorganic complexes. *J. Am. Chem. Soc.* 102:4360–4363.

Mersol, J. V., D. G. Steel, and A. Gafni. 1991. Quenching of tryptophan phosphorescence in *Escherichia coli* alkaline phosphatase by long range transfer mechanisms to external agents in the rapid-diffusion limit. *Biochemistry.* 30:668–675.

Papp, S., and J. M. Vanderkooi. 1989. Tryptophan phosphorescence at room temperature as a tool to study protein structure and dynamics. *Photochem. Photobiol.* 49:775–784.

Petrich, J. W., J. W. Longworth, and G. R. Fleming. 1987. Internal motion and electron transfer in proteins: a picosecond fluorescence study of three homologous azurins. *Biochemistry.* 26:2711–2721.

Pettigrew, G. W., F. A. Leitch, and G. R. Moore. 1983. The effect of iron-hexacyanide binding on the determination of redox potentials of cytochromes and copper proteins. *Biochim. Biophys. Acta.* 725: 409–416.

Schauerte, J. A., A. Gafni, and D. G. Steel. 1992. Time-resolved circularly polarized protein phosphorescence. *Proc. Natl. Acad. Sci. USA.* 89: 10154–10158.

Schlyer, B. D., J. A. Schauerte, D. G. Steel, and A. Gafni. 1994. Time-resolved room temperature protein phosphorescence: nonexponential decay from single emitting tryptophans. *Biophys. J.* 67:1192–1202.

Silvestrini, M. C., M. Brunori, M. Tegoni, M. Gervais, and F. Labeyrie. 1986. Kinetics of electron transfer between two *Hansenula anomala* flavocytochrome b_2 derivatives and two simple copper proteins (azurin and stellacyanin). *Eur. J. Biochem.* 161:465–472.

Silvestrini, M. C., M. Brunori, M. T. Wilson, and V. M. Darley-Usmar. 1981. The electron transfer system of *Pseudomonas aeruginosa*—a study of the pH-dependent transitions between redox forms of azurin and cytochrome-C551. *J. Inorg. Biochem.* 14:327–338.

Strambini, G. B. 1987. Quenching of alkaline phosphatase phosphorescence by O_2 and NO. *Biophys. J.* 43:127–130.

Strambini, G. B., and E. Gabellieri. 1991. Phosphorescence from Trp-48 in azurin: influence of Cu(II), Cu(I), Ag(I), and Cd(II) at the coordination site. *J. Phys. Chem.* 95:4352–4356.

Strambini, G. B., and M. Gonnelli. 1985. The indole nucleus triplet-state lifetime and its dependence on solvent microviscosity. *Chem. Phys. Lett.* 115:196–200.

Strambini, G. B., M. Gonnelli, and W. C. Galley. 1990. Room temperature phosphorescence of Trp-314 as a monitor of subunit communications in alcohol dehydrogenase from horse liver. *Biochemistry.* 29:203–208.

Szabo, A. G., T. M. Stepanik, D. M. Wayner, and N. M. Young. 1983. Conformational heterogeneity of the copper-binding site in azurin—a time-resolved fluorescence study. *Biophys. J.* 41:233–244.

Vanderkooi, J. M., D. B. Calhoun, and S. W. Englander. 1987. On the prevalence of room-temperature protein phosphorescence. *Science.* 236: 568–569.