Identification of the Iron Entry Channels in Apoferritin. Chemical Modification and Spectroscopic Studies

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ABSTRACT: The knowledge of the route through which iron can enter and leave the apoferritin shell is a prerequisite for the understanding of ferritin's function. The involvement of the hydrophilic 3-fold channels in the iron uptake process has been studied by taking advantage of the reactivity of specific residues that line such channels, i.e., glutamic acid-127 and aspartic acid-130, the major Cd(II) binding sites, and cysteine-126. ^{113}Cd NMR experiments have provided direct evidence for the competition between Fe(II) and Cd(II) binding to major Cd(II) binding sites on the protein and for a higher affinity of Fe(II) for these sites, in line with the well-known inhibitory effect of Cd(II) on iron uptake. Further evidence for the use of the 3-fold channels in the iron entry process has been obtained by means of chemical modification of Cys-126 with different mercurials. In particular, the introduction of the additional carboxylate carried by *p*-(chloromercuri)benzoate near Asp-127 and Glu-130 increases the initial rate of iron uptake and affects the coordination geometry of the metal in the Fe(III)–apoferritin complex as indicated by optical absorption and EPR data. The assignment of these effects to the carboxylate moiety of *p*-(chloromercuri)benzoate is brought out by the observation that the introduction in the 3-fold channel of the benzene ring only by means of phenylmercuric acetate has no effect on the initial iron uptake kinetics and on the spectroscopic properties of the Fe(III)–apoferritin complex.

Ferritin is the iron storage protein utilized by most living cells since it can maintain iron in a soluble, nontoxic, and metabolically accessible form. A ferritin molecule, which consists of a hollow symmetrical shell assembled from 24 subunits, can store up to 4500 iron atoms as a polymeric ferrihydrite core (Ford et al., 1984). The iron storage role involves the control of two opposite processes, namely, iron incorporation into the protein cavity and iron release therefrom. Hence, the understanding of ferritin's function at a molecular level requires the knowledge of how iron can enter and leave the protein shell.

The packing of the subunits by *432* symmetry produces 2 types of channel around the 4-fold and 3-fold axes of the apoferritin molecule. The first are lined with hydrophobic residues (12 leucines), while the latter are hydrophilic and contain on the outer surface 3 histidines, 6 serines, and 3 cysteines (Cys-126) that are followed by 3 glutamic acid (Glu-130) and 3 aspartic acid (Asp-127) residues toward the middle of the channel (Rice et al., 1983; Harrison et al., 1986a). The existence of channels of such different nature immediately suggests that they may be used in a different way during the iron uptake and release processes. The hydrophilic channels have been proposed as the possible route through which iron enters the protein shell on the basis of a variety of studies. Thus, in apoferritin crystals, two Cd(II) or two Zn(II) ions are bound within these channels, the metal ligands being provided by the carboxylates of the symmetry-related Asp-127 and Glu-130 residues. In turn, both chemical modification of carboxyl residues (Bryce & Crichton, 1973; Wetz & Crichton, 1976) and Cd(II) or Zn(II) binding (Niederer, 1970; Macara et al., 1973) have long been known to inhibit iron incorporation. More recent experiments have provided further evidence for a competition between the binding of iron and that of several metal probes, like Tb(III), Mn(II), Zn(II), and VO(IV) (Chasteen & Theil, 1982; Stefanini et al., 1983; Treffry & Harrison, 1984; Wardeska et al., 1986), and for the involvement of carboxylate groups in the initial binding of Fe(II) (Chasteen & Thiel, 1982; Harrison et al., 1986a,b). The highly invariant Asp-127 and Glu-130 residues appear as the most likely candidates to carry out this binding process which is then followed by oxidation of the metal to Fe(III) and by its movement inside the apoferritin molecule (Harrison et al., 1986a,b). However, the use of metal probes alone to arrive at the identification of the iron entry ports suffers from an intrinsic ambiguity since some metal binding elsewhere on the protein may and does occur (Harrison et al., 1986a).

In the present study, two different approaches have been used to probe the channels around the 3-fold axes. On the one hand, the binding of Fe(II) to the major Cd(II) binding sites at Asp-127 and Glu-130, which are also the putative Fe(II) binding sites, has been followed directly by means of ^{113}Cd NMR. On the other, Cys-126, which is located on the outer surface of the channels, has been modified in such a way as to introduce a further carboxylate residue near Asp-127 and Glu-130. To this end, *p*-(chloromercuri)benzoate (PMB) has been used, and its effect on the iron uptake kinetics at low iron/protein ratios and on the spectroscopic properties of the Fe(III)–apoferritin complex has been compared with that produced by phenylmercuric acetate (PMA) which does not carry a negative charge. In addition, given the proximity of the residues involved, the reciprocal effects of Cd(II) binding and of the Cys-126 modifications have been studied. All the experimental data obtained provide independent evidence for the entry of iron into the apoferritin shell through the 3-fold channels.

Materials and Methods

Horse spleen ferritin was prepared as described previously

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Table I: Iron Uptake by Chemically Modified Apoferritin at Low Fe/Protein Ratios[a]

protein	$t_{1/2}$ (s)	absorbance at 420 nm of reconstitution product	iron atoms (per subunit) added	iron atoms (per subunit) incorporated
apoferritin	75 ± 13 (5)	0.040 ± 0.006	2.2 ± 0.20	2.1 ± 0.25
PMB–apoferritin	15 ± 5 (4)	0.015 ± 0.003	2.2 ± 0.14	2.2 ± 0.08
PMA–apoferritin	85 ± 14 (2)	0.039 ± 0.005	2.2 ± 0.14	2.0 ± 0.12
Cd(II)–apoferritin	430 ± 50 (3)	0.047 ± 0.002	2.4 ± 0.10	2.3 ± 0.04
PMB–Cd(II)–apoferritin	220 ± 55 (3)	0.016 ± 0.0009	2.2 ± 0.10	2.1 ± 0.12
apoferritin	65 ± 15 (5)	0.037 ± 0.002	2.1 ± 0.03	nd[b]
PMB–apoferritin	17 ± 2 (5)	0.017 ± 0.004	2.1 ± 0.03	nd
PMA–apoferritin	55 ± 12 (5)	0.033 ± 0.002	2.1 ± 0.03	nd

[a] The experiments were carried out with 1 mg/mL aporferritin solutions at 20 °C in (upper part of table) 20 mM MOPS–NaOH, pH 6.5, and (lower part of table) 20 mM imidazole pH 7.0. Cd(II)–apoferritin was obtained by adding 2 Cd(II)/subunit. The amounts of iron incorporated were determined as the Fe(II)–α,α'-dipyridyl complex after reduction with dithionite (Bothwell & Mallet, 1955). The number of experiments is given in parentheses. [b] nd, not determined.

(Stefanini et al., 1982); apoferritin was obtained by reduction of iron with sodium dithionite and chelation with α,α'-bipyridyl (Stefanini et al., 1975). The quality of the apoferritin preparations was checked by means of their CD spectra in the near-UV region (Leach et al., 1976); their iron content was determined as the α,α'-bipyridyl complex at 520 nm (Bothwell et al., 1955) and was found to correspond to about five iron atoms per molecule. The apoferritin concentration was calculated from the absorption at 280 nm by using the extinction coefficient $E^{1\%}_{1cm}$ = 9.0 (Bryce & Crichton, 1973) or by the Bradford method (Bradford, 1976).

Modification of the reactive sulfhydryl groups at Cys-126 with PMB and PMA (both commercial products of Sigma) was achieved by addition of the mercurial (1.1 equiv/subunit) to apoferritin solutions at 1–2 mg/mL in the desired buffer. The extent of modification was checked by means of sulfhydryl group titrations performed according to Boyer (1954). The concentration of PMB was determined spectrophotometrically at 232 nm and pH 7.0 using the molar extinction coefficient 1.65×10^4; the concentration of PMA was determined at 260 nm using the molar extinction coefficient 2.64×10^2.

The uptake of iron by apoferritin was followed at 20 °C by measuring the change in absorbance at 420 nm. Ferrous ammonium sulfate was used as the source of iron in the presence of oxygen as oxidant. An anaerobic stock solution about 10 mM in Fe(II) was prepared, and its concentration was determined by the bipyridyl method both before and after reduction with dithionite in order to verify the oxidation state of the metal [the amount of Fe(III) was always below 5%]. Typically, very low amounts of iron corresponding to one to two Fe(II) atoms per subunit were added to a 1 mg/mL protein solution in the desired buffer. At the end of the kinetic experiments, the reaction was stopped by addition of sodium citrate (at a final concentration corresponding to the iron added); subsequently, any nonspecifically bound iron was removed by extensive dialysis against 20 mM MOPS–NaOH at pH 6.5 or 20 mM imidazole hydrochloride at pH 7.0 containing citrate and finally by dialysis against buffer alone. In the experiments involving the use of chemically modified apoferritin, the chemical modification was carried out just before the addition of iron. The effect of Cd(II) on the iron uptake process was studied after the addition of cadmium sulfate (Merck) corresponding to 2 equiv/subunit.

The ultracentrifuge experiments were carried out in a Spinco Model E ultracentrifuge at 42 000 rpm and 10 °C.

X-Band EPR spectra were measured with a Varian E9 spectrometer at liquid nitrogen temperature. The samples were prepared by addition of appropriate aliquots of the anaerobic Fe(II) stock solution. In the kinetic experiments, the first spectrum was taken about 30 s after the addition of iron.

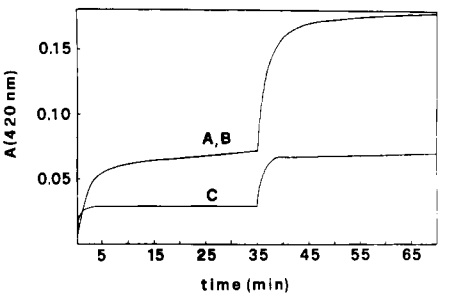

FIGURE 1: Effect of chemical modification on the kinetics of iron uptake by apoferritin. Protein: (A) unmodified; (B) PMA-reacted; (C) PMB-reacted. Conditions: 1.9 mg/mL apoferritin in 20 mM MOPS–NaOH buffer at pH 6.5. Two successive additions of 2 Fe(II)/subunit were carried out.

The ^{113}Cd NMR spectra were obtained at 56.55 MHz on a homemade UL 6T spectrometer equipped with specifically designed solenoid-type probes. The spectra were obtained by accumulating 25 000 free induction decays using a 10-s pulse length (45° flip angle), a spectral width of 20 kHz, an acquisition time of 0.10 s, and a repetition time of 0.7 s. A stock solution of 0.1 M $Cd(ClO_4)_2$ was prepared by dissolving CdO (95% isotopically enriched in ^{113}Cd; Oak Ridge National Laboratories, Oak Ridge, NC) in $HClO_4$ and adjusting the pH to neutrality by addition of 1 M NaOH. In the competition experiments between Cd(II) and Fe(II), the iron was maintained in the reduced state by addition of a few grains of sodium dithionite. Control experiments showed that the presence of dithionite did not affect the ^{113}Cd NMR spectra.

Results

PMB- and PMA-Reacted Apoferritin. The addition of PMB or PMA to apoferritin leads to modification of 1.05 ± 0.05 SH group/subunit; this residue can be identified as Cys-126, the major PMB binding site of the apoferritin molecule in the high-resolution X-ray structure (Banyard et al., 1978; Harrison et al., 1986a). The effect of this modification on the initial rate of iron incorporation and on the spectroscopic properties of the reconstitution product has been studied at pH 6.5–7.0 in two buffer systems characterized by different iron chelating properties, namely, 20 mM MOPS–NaOH or imidazole hydrochloride (Pâques et al., 1980).

At low iron to protein ratios (about 2 Fe/subunit), the half-time for the incorporation reaction is decreased in the PMB-reacted protein, but not in the PMA-reacted one with respect to the control (Figure 1). At the specific wavelength used to monitor iron uptake, the presence of PMB also affects the molar absorptivity of the reconstitution product (Table I). In contrast, at high iron to protein ratios (40–60 Fe atoms/subunit), all the reconstitution products have the same ab-

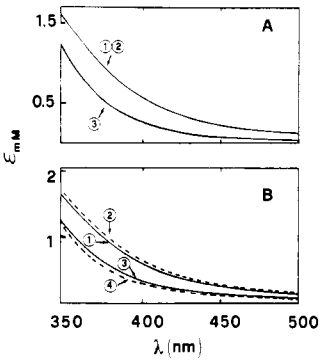

FIGURE 2: Absorption spectra of the Fe(III)–apoferritin complex at 4 iron atoms/subunit as a function of chemical modification and Cd(II) binding. Protein: (A) unmodified (1), PMA-reacted (2), and PMB-reacted (3); (B) unmodified (1), containing 2 Cd(II)/subunit (2), PMB-reacted (3), and PMB-reacted containing 2 Cd(II)/subunit (4). Conditions: 1 mg/mL apoferritin in 20 mM MOPS–NaOH buffer at pH 6.5.

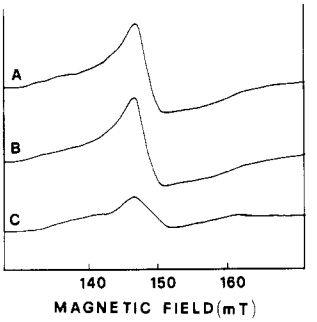

FIGURE 3: Effect of chemical modification on the $g' = 4.3$ EPR signal of the Fe(III)–apoferritin complex obtained upon addition of 2 Fe(II) atoms/subunit and subsequent oxidation by air. Protein: (A) unmodified, (B) PMA-reacted, (C) PMB-reacted. Conditions: 1 mg/mL apoferritin in 20 mM MOPS–NaOH buffer at pH 6.5. Instrument settings: modulation amplitude, 1.5 mT; microwave power, 50 mW; frequency, 9.13 GHz; temperature, 110 K.

sorptivity within 5–10%; moreover, they display the same molecular weight distribution of the iron micelles in sedimentation velocity experiments (data not shown).

In view of these results, the optical absorption spectra of the reconstituted ferritins containing around 4 Fe atoms/subunit have been measured over the wavelength range 330–500 nm. In the PMB-reacted protein, the absorptivity of the Fe(III)–protein complex is decreased over the whole spectral range analyzed with respect to the PMA-reacted protein and the control (Figure 2A).

In order to further characterize the spectroscopic properties of the various reconstitution products, a series of EPR experiments has been performed at liquid nitrogen temperature in 20 mM MOPS–NaOH of pH 6.5. The reconstitution products of the control and PMA-reacted protein containing 1–2 Fe atoms/subunit display the same spectrum with the characteristic $g' = 4.3$ signal due to the small amount of mononuclear Fe(III) present in the complex (Chasteen et al., 1985), while PMB-reacted apoferritin containing the same amount of iron displays a broader and less intense $g' = 4.3$ signal (Figure 3).

In a further series of EPR experiments, the $g' = 4.3$ signal was used to monitor the kinetics of iron uptake after presenting apoferritin with 2 Fe(II) atoms/subunit. Figure 4 shows that in the control and in the PMA-reacted protein the intensity of the $g' = 4.3$ signal decreases as a function of the time elapsed after the addition of iron and reaches a constant value in 45–60 min. In contrast, the broad signal typical of PMB-reacted apoferritin appears within 30 s and does not change

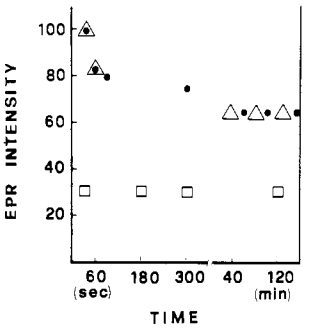

FIGURE 4: Relative intensity of the $g' = 4.3$ EPR signal as a function of time after addition to native and chemically modified apoferritin of 2 Fe(II)/subunit and subsequent oxidation by air. Protein: (●) unmodified; (△) PMA-reacted; (□) PMB-reacted. Experimental conditions as in Figure 2. The intensity is normalized with respect to the most intense signal observed in the unmodified and PMA-reacted apoferritin 20 s after the metal addition.

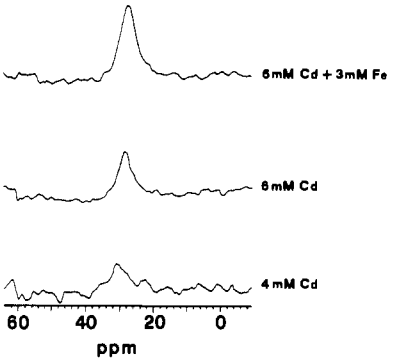

FIGURE 5: ^{113}Cd NMR spectra of apoferritin in the absence and presence of Fe(II). Cd(II) concentration: (A) 4 mM, (B) 6 mM, (C) 6 mM in the presence of 3 mM Fe(II). Protein concentration: 32 mg/mL apoferritin in 10 mM Tris-HCl at pH 7.0.

in intensity as a function of time.

Cd(II)-Reacted Apoferritin. As mentioned above, the major Cd(II) binding sites have been located within the 3-fold channels on the basis of recent high-resolution X-ray data (Harrison et al., 1986a) and binding studies in solution (Wardeska et al., 1986). They consist of the three symmetry-related carboxyl groups of Asp-127 and Glu-130. In turn, their functional relevance has been brought out by the observation that Cd(II) binding results in a marked decrease in the rate of iron uptake (Niederer, 1970).

In order to obtain direct information on the competition between the two metal ions, advantage has been taken of the fact that ^{113}Cd is amenable to NMR studies. The ^{113}Cd spectra were recorded upon successive addition of a 0.1 M Cd(II) solution to a 1.8 mM apoferritin subunit solution. No ^{113}Cd resonance is observed at Cd(II) concentrations up to 2 mM. At 4 mM Cd(II), a signal at 20 ppm is apparent whose intensity increases and line width decreases with increase in Cd(II) concentration. The addition of 3 mM Fe(II) to the apoferritin solution containing 6 mM Cd(II) produces a significant increase in the intensity of the ^{113}Cd resonance, but no other changes are observed upon a further addition of 3 mM Fe(II) (Figure 5). In parallel with the ^{113}Cd NMR experiments, the kinetics of iron incorporation have been followed spectrophotometrically. As observed by Niederer (1970), with respect to the control, a significant increase in the half-time of the reaction is observed when 2 Cd(II)/subunit are added to apoferritin before iron, although the amount of iron incorporated is the same (Table I). Table I also shows that the effect of Cd(II) on the kinetics of iron incorporation by PMB-reacted apoferritin is smaller than in unmodified

apoferritin. However, in both proteins containing 4 Fe(III)/subunit, the presence of Cd(II) does not affect the molar absorptivity of the reconstitution product (Figure 2B).

Discussion

The data presented in this paper provide direct evidence for the entry of iron into the apoferritin shell through the hydrophilic channels around the 3-fold axes of the molecule. These channels have been probed by taking advantage of the reactivity of specific residues, namely, Cys-126, which can be modified easily with mercurials, and Asp-127 and Glu-130, which furnish the carboxylate ligands for the major Cd(II) binding site and hence can be studied by means of ^{113}Cd NMR. On the basis of a number of binding studies with a variety of metal probes (Harrison et al., 1986b; Wardeska et al., 1986), the carboxylate residues located at the 3-fold channels have been proposed recently as the putative sites at which the initial binding and oxidation of Fe(II) occur.

The existence of common binding sites for Cd(II) and Fe(II) on the apoferritin molecule and for a direct competition in the binding of the two metal ions has been demonstrated for the first time, to our knowledge, by the ^{113}Cd NMR experiments. The ^{113}Cd NMR spectra can be interpreted as follows: the absence of a signal up to Cd(II) concentrations around 2 mM under the conditions used indicates that the signal of bound Cd(II) is broadened out due to either chemical exchange or a short correlation time. In turn, the changes in intensity and line width of the ^{113}Cd signal apparent at higher concentrations of the metal show that the observed signal is due to "free" Cd(II) ions; the broadening of the "bound" ^{113}Cd resonance is caused by exchange between "free" and protein-bound ions with an exchange rate on the order of 10^3 s^{-1}. On this basis, if one assumes that the observed changes are due to purely local effects, the significant increase in intensity of the ^{113}Cd resonance observed upon addition of Fe(II) can be accounted for in terms of a direct competition between Cd(II) and Fe(II) for the same binding sites on the protein. The affinity of Fe(II) for these sites appears to be higher than that of Cd(II). The ^{113}Cd NMR data therefore strongly suggest that Asp-127 and Glu-130, the major Cd(II) binding sites, correspond to the sites where the initial binding of Fe(II) occurs. As expected on this basis, the introduction of an additional carboxylate near Asp-127 and Glu-130 upon reaction of Cys-126 with PMB results in a perturbation of the iron binding process. Thus, the velocity of iron uptake at low Fe/protein ratios shows a significant increase in the PMB-reacted protein with respect to unmodified apoferritin. The accelerating effect produced by PMB can be ascribed solely to the presence of a carboxylate since the iron uptake kinetics are not affected by reaction of Cys-126 with PMA, which introduces in the channel the benzene ring only. It may be envisaged that due to the additional carboxylates in the 3-fold channel there is a stronger electrostatic interaction for the positively charged Fe(II) ion, which in turn allows a faster oxidation of the metal. Further, the additional carboxylates produce a structural perturbation of the initial Fe(III)–protein complex as indicated clearly by the spectroscopic properties of the PMB-reacted protein. Thus, at low Fe/protein ratios where the contribution from the iron in the channels dominates the experimental situation, the absorption spectrum in the visible region of PMB-apoferritin is significantly lower than that of the control and of the PMA-reacted protein although the amount of iron incorporated is the same. The EPR spectra likewise point to a different coordination geometry of the metal ion in PMB-reacted apoferritin in which the well-known EPR signal at $g' = 4.3$, characteristic of the mononuclear Fe(III)–apoferritin complex, becomes broader and less intense; in contrast, the $g' = 4.3$ signal is unaffected after reaction of the protein with PMA (Figure 3). The presence of the new negative group, namely, the carboxylate carried by the PMB molecule, creates a structural rearrangement of the iron binding site resulting in different spectral features of the EPR line. Such spectral differences indicate that the hydrophilic 3-fold channels contain the primary sites for Fe(II) complexation and oxidation and hence can be identified as the entry route of the metal into the protein shell.

The kinetics of iron incorporation followed by EPR are consistent with the data just discussed. Addition of iron to the unreacted protein in an amount corresponding to 2 Fe atoms/subunit gives rise to a $g' = 4.3$ signal that decreases to a constant value within 45 min. This result suggests that iron first binds as Fe(II), is oxidized in a mononuclear complex, and then moves slowly toward a polynuclear site that, at this low Fe/protein ratio, is likely to be a precursor of the ferrihydrite core crystallite as indicated by recent EXAFS studies (Yang et al., 1987). On the other hand, iron reconstitution with the PMB-modified apoprotein gives rise within 30 s to the same low-intensity EPR signal at $g' = 4.3$ observed at equilibrium, whose intensity does not vary with time. It may be envisaged that due to the faster rate of iron uptake by PMB-reacted apoferritin with respect to the unmodified protein (Table I), the changes in intensity of the EPR signal are too fast to be observed in the accessible time range.

The mutual effects of Cd(II) binding and the modification of Cys-126 on the iron uptake kinetics and spectroscopic properties of the various apoferritin samples are in line with the picture emerging from the data discussed above. Thus, binding of Cd(II) to PMB-reacted apoferritin slows down the initial rate of iron uptake albeit to a smaller extent than in the unmodified protein. Moreover, due to the competition between the binding of Cd(II) and Fe(II) and of the higher affinity of the latter metal ion for apoferritin, Cd(II) does not affect the spectral properties of the initial Fe(III)–protein complex.

In conclusion, the two independent approaches employed in the present work to probe the 3-fold channels, namely, ^{113}Cd to study the major Cd(II) binding site and the modification of Cys-126, both provide direct evidence for the use of these channels as the iron entry ports into the apoferritin shell.

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Registry No. Fe, 7439-89-6; L-Cys, 52-90-4; L-Glu, 56-86-0; L-Asp, 56-84-8.

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Fluorescence Lifetime and Solute Quenching Studies with the Single Tryptophan Containing Protein Parvalbumin from Codfish†

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ABSTRACT: The fluorescence decay of cod parvalbumin (both its Ca^{2+}-loaded and Ca^{2+}-depleted forms) is found to be a nonexponential process. The decay data can be fitted either by a double-exponential decay law or by a distribution of decay times. To try to distinguish between the double-exponential and distribution fits, we have collected frequency domain and steady-state fluorescence data as a function of temperature and concentration of the quencher acrylamide. We argue that the correct decay law (i.e., double exponential or distribution) must be consistent with all the data collected as a function of temperature and quencher concentrations. We employ a global analysis procedure to simultaneously fit multiple data sets that are linked by an Arrhenius or Stern-Volmer relationship. For the Ca^{2+}-loaded form of parvalbumin, the distribution model provides a consistent and reasonable fit for all of the frequency domain and steady-state data. The double-exponential model requires more fitting parameters, and some of these assume unreasonable values when this model is fitted to all of the data. For the Ca^{2+}-depleted form of the protein, it is not clear whether the double-exponential or distribution model is superior. For our steady-state solute quenching studies we present a novel analysis in terms of a distribution of quenching constants.

The intrinsic fluorescence of tryptophan residues in proteins provides a sensitive probe to study the conformational dynamics of the microenvironment of these residues. For a number of years it has been clear that the fluorescence of individual tryptophan residues may not be homogeneous. More often than not, the fluorescence decay of such residues is nonexponential (Grinvald & Steinberg, 1976; Beechem & Brand, 1985). Examples of single tryptophan residues where this nonexponentiality has been observed are ribonuclease T_1 (Chen et al., 1987; Eftink & Ghiron, 1987), holoazurin (Szabo et al., 1983; Petrich et al., 1987), melittin (Lakowicz et al., 1986), adrenocorticotropin (Ross et al., 1981a), nuclease (Grinvald & Steinberg, 1976; Brochon et al., 1974; Lakowicz et al., 1986), and phospholipase A_2 (Ludescher et al., 1985). Even the amino acid tryptophan, in water, displays a nonexponential decay (Beddard et al., 1980; Szabo & Rayner, 1980; Petrich et al., 1983).

The traditional practice has been to try to describe such decays in terms of the minimum number (i.e., two or three) of exponential decay time. The question then becomes one of trying to relate these various decay times to different conformational states and/or different kinetic processes. For example, the two decay times found for tryptophan in water have been interpreted in terms of emission from different rotamers (Szabo & Rayner, 1980; Petrich et al., 1983), or dual emission from the L_a and L_b bands of the excited indole ring (Rayner & Szabo, 1978), or as being a result of the $t^{1/2}$ term for diffusional quenching by side chains (Beddard et al., 1980). Likewise for proteins, analogous interpretations, involving two conformational states or excited-state reactions, have been offered (Beechem & Brand, 1985).

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