We have previously shown that with *Chromatium* FC (Cusanovich & Tollin, 1980) intramolecular electron transfer is quite fast ($k > 10^6$ s^{-1}). The present studies are consistent with this conclusion for both *Chlorobium* and *Chromatium* FC inasmuch as the dzRF results indicate that the rate constant for intramolecular electron transfer must be greater than 3×10^4 s^{-1}.

In summary, the studies reported establish that the mechanism of electron transfer from exogenous flavins is very similar for both *Chromatium* and *Chlorobium* FC, as would be expected for functionally related proteins. Although definitive conclusions are dependent upon acquiring detailed structural information, the available data are consistent with a sequential electron transfer from exogenous reducing agents to the protein-bound flavin moiety, followed by rapid intramolecular electron transfer to the heme.

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Spectroscopic Examination of the Active Site of Bovine Ferrochelatase†

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ABSTRACT: Spectrofluorometric techniques have been employed to examine the active site of the terminal enzyme of the heme biosynthetic pathway, ferrochelatase (protoheme ferrolyase, EC 4.99.1.1). The fluorescence of both endogenous tryptophan and exogenous 2-(4-maleimidylanilino)naphthalene-6-sulfonic acid (MIANS) has been examined. The fluorescence emission of the enzyme's active site bound MIANS is at 428 nm while the enzyme tryptophan(s) yielded a single fluorescence emission maximum at 347 nm. These values are characteristic of a polar environment for tryptophan and a relatively nonpolar environment for the MIANS. The dynamic fluorescence quenching constants for acrylamide of MIANS and tryptophan are 3.00 M^{-1} and 1.85 M^{-1}, respectively. Quenching constants for KI of both fluorescent centers were approximately 1 M^{-1}. These data suggest that both fluorophores are poorly accessible to the external anionic contact quencher but that an unchanged quencher, while larger, is still better able to penetrate the enzyme's active site. The extrapolated anisotropies (r_0) for ferrochelatase-bound MIANS and tryptophan are 0.198 and 0.307. The dissociation constant (K_D) determined by fluorescence anisotropy of protoporphyrin was 1.5 μM with the calculated number of porphyrin binding sites as 1.0 per 40 000 daltons. A model is presented for the active site of ferrochelatase based upon the data presented here and previously. This model proposes that the active site is a hydrophobic pocket similar in nature to the heme binding crevices found in many hemoproteins.

The terminal step of the heme biosynthetic pathway is the insertion of ferrous iron into the porphyrin macrocycle to form protoheme IX. Ferrochelatase (protoheme ferrolyase, EC 4.99.1.1), the enzyme that catalyzes this step, is bound to the inner mitochondrial membrane in eukaryotes (Jones & Jones, 1969; Harbin & Dailey, 1985) and to the cytoplasmic membrane of prokaryotes (Dailey, 1982). Recently, the enzyme has been purified to homogeneity from a variety of sources (Taketani & Tokunaga, 1981, 1982; Dailey, 1982; Dailey & Fleming, 1983; Hanson & Dailey, 1984). With the availability of pure enzyme, the kinetic mechanism (Dailey & Fleming, 1983) and the role of enzyme sulfhydryl residues (Dailey,

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1984) have been described for bovine liver ferrochelatase. The data obtained thus far suggest that ferrochelatase possesses a porphyrin binding site that is highly selective for the presence and position of both the two porphyrin carboxylates (Honeybourne et al., 1979) and the substituents at the 2- and 4-positions (Dailey & Fleming, 1983; Dailey & Smith, 1984).

This high degree of specificity and the known structure of many hemoproteins have led us to investigate further the nature of the substrate binding site of ferrochelatase. Among heme binding proteins whose structures are known, a fairly common feature is the presence of a heme binding "pocket" (Argos & Rossman, 1979). In general, it is found that the propionate side chains extend outward into the solute while the actual tetrapyrrole ring is sequestered in a relatively hydrophobic pocket. Since ferrochelatase may also be viewed as a heme or porphyrin binding protein, it was of interest to determine if its porphyrin binding site is similar in properties to the heme pocket or if it is a more exposed site.

Materials and Methods

Materials. Protoporphyrin IX was obtained from Porphyrin Products, Logan, UT. Porphyrin solutions were prepared fresh prior to each experiment as described previously (Dailey & Lascelles, 1974). Ferrochelatase was assayed as described by Dailey (1982) with deuteroporphyrin as the porphyrin substrate. 2-(4-Maleimidylanilino)naphthalenesulfonide (MIANS) was obtained from Molecular Probes, Junction City, OR, and 50 mM solutions were freshly prepared for each experiment. All other reagents were of the highest quality available.

Bovine liver ferrochelatase was prepared from freshly slaughtered beef liver obtained from Welborn's Abattoir, Danielsville, GA, as described previously (Dailey & Fleming, 1983) and stored at −20 °C. Unless otherwise noted, all experiments were performed in 10 mM tris(hydroxymethyl)aminomethane (Tris)–acetate, pH 8.1, 1.0% (w/v) sodium cholate, and 0.1 mM dithiothreitol (DTT). Since ferrochelatase is stored in buffer containing 20% glycerol and phenylmethanesulfonyl fluoride, the protein was passed through a Sephadex G-25 column equilibrated with the above buffer prior to each experiment. For reaction with 2-(4-maleimidylanilino)naphthalene-6-sulfonic acid (MIANS), the DTT was eliminated from the buffer.

Absorption spectra were determined with a Cary 219 spectrophotometer. Fluorescence measurements were performed with a Perkin-Elmer 650-40 spectrofluorometer with a thermostated cell holder. All fluorescence measurements were done in the ratio mode to compensate for variation in source lamp intensity, and slit widths are listed for each experiment. All samples had an absorbance of less than 0.1 at the wavelength examined to ensure against inner filter effects.

Quenching Measurements. All quenching experiments were carried out at 24 °C with a slit width of 2 nm. For tryptophan fluorescence, the excitation wavelength was 308, and the emission was 347 nm. For MIANS, the excitation wavelength was 330 nm, and the emission was 428 nm. Freshly prepared solutions of acrylamide and KI were used. Potassium chloride at concentrations equivalent to those of KI used was found to have no effect on the spectra. Data are shown as Stern–Volmer plots. Since all data, except that obtained with KI quenching of MIANS, yield linear plots, the Stern–Volmer constant (Stern & Volmer, 1919) is calculated as

$$F_0/F = 1 + K_{SV}[Q]$$

In this equation, F_0 and F are the fluorescence intensities in the absence and presence of quencher Q. K_{SV} is the Stern–Volmer quenching constant. Acrylamide and iodide are both excellent contact quenchers with quenching efficiency of 1.0 (Eftink & Ghiron, 1981).

Determination of Binding Constants. The dissociation constant, K_D, of bovine ferrochelatase was deteremined fluorometrically for protoporphyrin IX. This was accomplished by following the association of the porphyrin, which is fluorescent, with the enzyme by determining the anisotropy as a function of added protoporphyrin. The experimentally observed anisotropy can be expressed by

$$r = f_F r_F + f_B r_B$$

where f_F and f_B are the fractions of the total fluorescence and r_F and r_B are the anisotropies of the free and bound protoporphyrin, respectively. Assuming

$$K_D = \frac{[\text{free enzyme}][\text{free porphyrin}]}{[\text{enzyme porphyrin}]}$$

it can be shown that for the experimentally measured anisotropy, r, then

$$f_B = \frac{r - r_F}{r_B - r_F}$$

Since the equation is valid only if the fluorescence yield of protoporphyrin does not change upon binding [see Lakowicz (1983)], the effect of binding upon protoporphyrin was determined by adding an excess of enzyme to a free protoporphyrin solution. Even in the presence of a 10-fold excess of ferrochelatase, there was no detectable change in the fluorescence yield of the porphyrin under the conditions we employed. This may be attributable to the fact that the porphyrin is kept in a detergent solution to prevent dimer formation and the porphyrin in the porphyrin–detergent micelle may be in an environment similar to that found in the porphyrin–enzyme complex. The quantity r_B was determined by measuring the anisotropy of porphyrin (10 nM) in the presence of excess ferrochelatase (1 μM).

Preparation of MIANS-Labeled Ferrochelatase. The procedures for reacting ferrochelatase with the fluorescent sulfhydryl-specific reagent 2-(4-maleimidylanilino)-naphthalene-6-sulfonic acid (MIANS) are the same as those previously described for reacting the active site sulfhydryl group of ferrochelatase with *N*-ethylmaleimide (NEM) (Dailey & Fleming, 1983) except that MIANS was employed in place of NEM. The kinetics of inactivation of ferrochelatase by MIANS were idential with those previously reported for NEM. It was not possible to follow the rate of reaction by monitoring the increase in fluorescence that usually accompanies MIANS reactions (Andley et al., 1982; Gupte & Lane, 1979) since 1.0% (w/v) sodium cholate, which is present in all solutions, causes high background fluorescence. Free dye was removed prior to determinations by centrifugal filtration through a Sephadex G-25 column (Penefsky, 1977) that had been equilibrated with 10 mM Tris–acetate, pH 8.1, 1.0% (w/v) sodium cholate, and 0.1 mM dithiothreitol.

Anisotropy Measurements. The fluorescence anisotropy of both ferrochelatase tryptophanyl residues and protein-bound MIANS was measured with a Perkin-Elmer 650-40 spectrofluorometer using Polacoat filters. Corrections were made for wavelength-dependent artifacts by the following formula:

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}}$$

where r is the measured anisotropy, I_{VV} is the intensity of emitted light with both filters aligned parallel, and I_{VH} is the intensity of emitted light with the exciting light polarized in

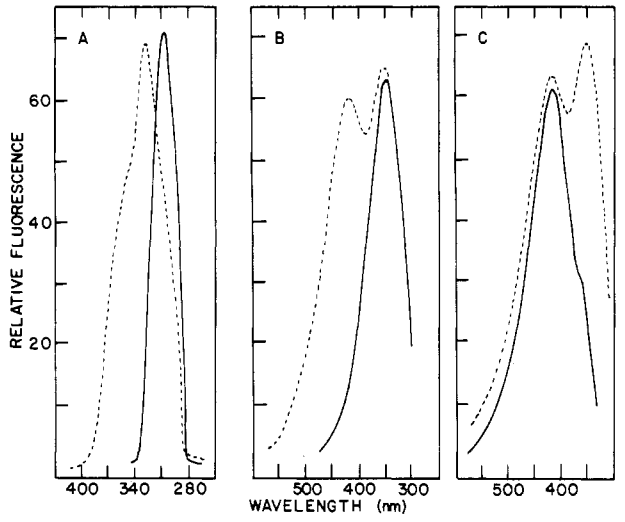

FIGURE 1: Fluorescence emission and excitation spectra for native ferrochelatase and MIANS-inactivated ferrochelatase. (A) Fluorescence emission (--) and fluorescence excitation (—) spectra for native bovine ferrochelatase in buffer containing 1.0% sodium cholate. See the text for details. (B) Fluorescence emission spectrum for MIANS-reacted bovine ferrochelatase. The emission spectra of both modified (--) and unmodified (—) enzyme are shown with excitation by 308-nm light. (C) Fluorescence emission spectrum for MIANS-modified ferrochelatase excited with 308- (--) and 330-nm (—) light.

the vertical and emitted light observed polarized to the horizontal or perpendicular direction. G is a wavelength-dependent correction factor for the sensitivity of the detection system (Lakowicz, 1983) where

$$G = I_{HV}/I_{HH}$$

RESULTS

Fluorescence Spectra of Bovine Ferrochelatase. The fluorescence emission spectrum of purified ferrochelatase in detergent solution is shown in Figure 1A. There is a single peak at 347 nm in the native enzyme. The excitation spectrum of the enzyme (Figure 1) shows a maximum at 308 nm, which is unusually high for a protein tryptophanyl residue.

It was found that upon denaturation of the enzyme by freezing and thawing, a second emission peak would appear at 328 nm and that denaturation of the protein by 1% sodium dodecyl sulfate (SDS) would yield an emission spectrum with a major peak at 328 nm and a shoulder around 347 nm. When ferrochelatase is denatured in 1% SDS, the excitation spectrum has a peak at 295 nm, rather than the 308-nm peak found in the native enzyme. For the studies reported below, only native enzyme with the single peak at 347 nm was examined.

The fluorescence emission spectra of MIANS-modified ferrochelatase are shown in Figure 1B,C. Two excitation peaks, 308 and 330 nm, are found with the major excitation peak at 330 nm due to the MIANS moiety on the protein (data not shown). The emission spectrum has a peak from the ANS moiety at 427 nm when excited with 330-nm light, and excitation of the modified protein with 308-nm light gives an emission spectrum with both tryptophan (347 nm) and MIANS (427 nm) contributions.

Quenching of Protein Trp and MIANS Fluorescence. The data obtained for steady-state quenching of ferrochelatase tryptophan residues and bound MIANS are shown in Figure 2. In the case of quenching by KI of tryptophan, the data yield a straight line suggesting only a single class of fluorescent tryptophan residues with a quenching constant (K_{SV}) of 1.05 M^{-1}. The quenching fluorescence of MIANS by KI shows

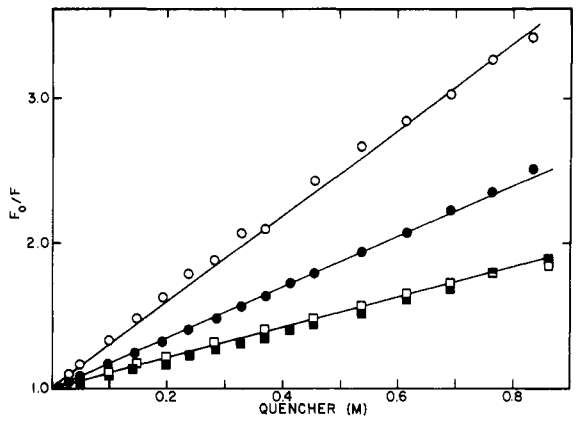

FIGURE 2: Fluorescence quenching of bovine ferrochelatase by KI. Experimental details are in the text. Quenching of tryptophan fluorescence and enzyme-bound MIANS is shown plotted according to Stern & Volmer (1919). F_0 is fluorescence without quencher; F is fluorescence in the presence of quencher at the stated concentration. Quenching by KI of tryptophan (□) and enzyme-bound MIANS (■) are shown in the lower portion of the figure. Acrylamide quenching of tryptophan (○) and enzyme-bound MIANS (●) are the upper two lines.

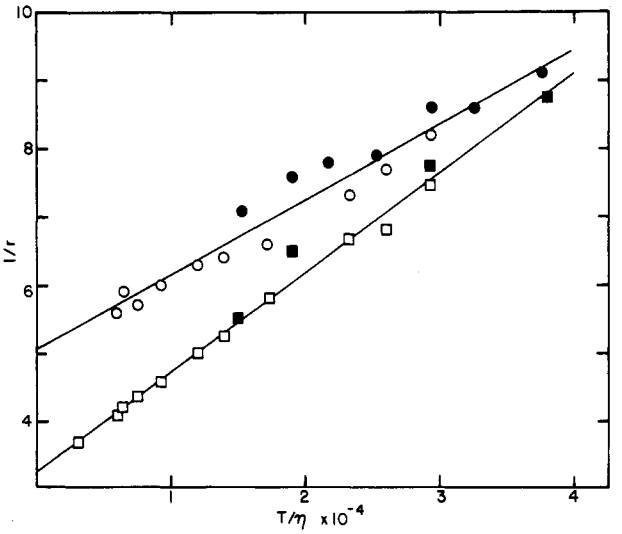

FIGURE 3: Fluorescence anisotropy of bovine ferrochelatase. Data were obtained by varying both temperature (solid markers) and viscosity (open markers). The square markers are data obtained for tryptophan fluorescence, and the circles are for enzyme-bound MIANS.

a slight upward deflection at high KI concentrations indicative of some static quenching by this ion. The quenching constant determined from the linear portion of the quenching curve is 0.96 M^{-1}.

Quenching by the uncharged molecule acrylamide yielded significantly different data than that obtained with the anionic quencher iodide. The data for both tryptophan and MIANS yielded linear Stern–Volmer plots. The quenching constants were determined to be 3.00 and 1.85 M^{-1} for MIANS and tryptophan, respectively. In all instances, the fluorescence emission spectra were found to be unaltered by either KI or acrylamide. Given the lifetime (τ) of protein bound MIANS to be around 4 ns (Andley et al., 1982), the calculated bimolecular rate constants for quenching (k_q) are approximately 7.5×10^7 M^{-1} s^{-1} and 2.5×10^7 M^{-1} s^{-1} for acrylamide and iodide quenching, respectively. These values are characteristic of buried or poorly accessible residues (Eftink & Ghiron, 1976).

Fluorescence Anisotropy of Ferrochelatase. The anisotropies of both ferrochelatase tryptophanyl residues and pro-

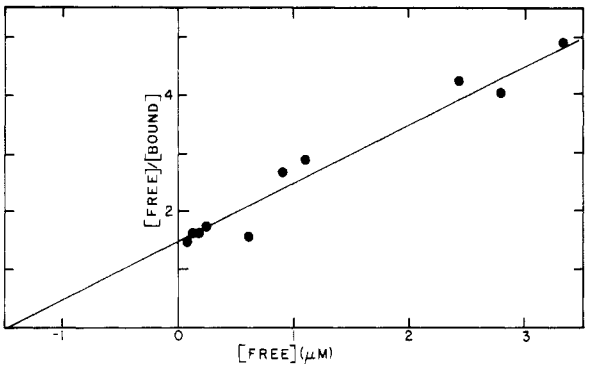

FIGURE 4: Dissociation constant of ferrochelatase for protoporphyrin as determined by fluorescence anisotropy. Experimental details are in the text. The slope is 1.0, and the x intercept is at 1.45 μM.

tein-bound MIANS are shown in Figure 3. The anisotropy, r, was determined for various temperatures (T) and viscosities (η). A plot of $1/r$ vs. T/η then yields r_0. For protein-bound MIANS this was 0.198, and for ferrochelatase tryptophan it was 0.307. The slopes of the lines are 1.093 and 1.470 $(T/\eta \times 10^{-4})^{-1}$ for MIANS and tryptophan residues, respectively.

Dissociation Constant for Protoporphyrin. Since one substrate, protoporphyrin, is strongly fluorescent, it was possible to determine its binding constant via measured anisotropies. The data are shown in Figure 4. The calculated K_D is 1.5 μM and the number of porphyrin binding sites is 1.0 per 40 000 daltons. This correlates with the previous finding that reaction of a single sulfhydryl group per enzyme molecule inactivates ferrochelatase (Dailey, 1984) and that one molecule of the tight binding competitive inhibitor N-methylprotoporphyrin binds per M_r 40 000.

Discussion

Among proteins that bind heme, whose tertiary structures are known, a feature common to many is the so-called heme pocket that appears to have been conserved among a wide diversity of proteins [see Argos & Rossman (1979)]. In its most basic form, this structure can be viewed as a relatively hydrophobic pocket or groove into which the protoheme moiety fits snugly with the two propionate side chains extending outward into the aqueous exterior. While this model is general, it is not universal. It appears from suicide inactivation reactions that some cytochrome P-450's may have a surface of their heme exposed for reaction with substrates (Kunze et al., 1983). Ferrochelatase catalyzes the insertion of ferrous iron into protoporphyrin IX to form protoheme, so while it cannot be considered to be a true hemoprotein, it does contain a porphyrin/heme binding site. In this study we have examined the substrate binding site of ferrochelatase in an effort to determine its relative exposure.

The porphyrin binding site on ferrochelatase exhibits a high selectivity with regard to the porphyrins that it will use. Only dicarboxylate porphyrins of the IX isomer serve as substrates (Honeybourne et al., 1979). Alteration in the position of even one of the propionate groups results in loss of activity with that porphyrin. Allowable substituents on the other two rings are also limited. The replacement of the vinyl groups of protoporphyrin with either hydrogens or ethyl or hydroxyethyl groups is allowed (although with decreased affinities), but substitution with a larger or charged moiety yields an unacceptable porphyrin (Dailey & Fleming, 1983; Dailey & Smith, 1984). The suggestion that the active site may be an enclosed area or pocket, rather than an open porphyrin binding site, comes from inhibition studies with N-alkylprotoporphyrins. The substituted porphyrin with an N-methyl group is a strong inhibitor of the enzyme, while N-ethyl is a very weak inhibitor (DeMatteis et al., 1980).

To further define the nature of the active site of ferrochelatase, we have examined the fluorescence properties of protein tryptophan residues and the enzyme modified with the fluorescence sulfhydryl reactive probe MIANS. Previously, it has been shown that one of two active site sulfhydryl groups involved in iron binding is rapidly modified by a variety of hydrophobic sulfhydryl reagents (Dailey, 1984). The fluorescenct reagent monobromobimane reacted with bovine ferrochelatase and yielded a blue-shifted emission spectrum. However, these reagents are less well characterized than the ANS type. Fluorescent probes with an ANS moiety have received wide usage as environmental probes of protein structure (Hudson & Weber, 1973; Andley et al., 1982; Gupte & Lane, 1979). Binding of MIANS to a hydrophobic or nonpolar portion of a protein results in an emission spectrum that is blue shifted to just below 420 nm from about 440 nm for the probe in aqueous solution. In ferrochelatase it was found that MIANS inactivates the enzyme and that iron protects against inactivation as was found for other maleimides (Dailey, 1984). The emission spectrum of enzyme-bound MIANS was shifted to 427 nm. This is similar to that reported for MIANS in butanol (429 nm) by Gupte & Lane (1979). If the emission maxima reported by Stryer (1965) for ANS in a series of alcohols are used, this would, by comparison, make the binding site on ferrochelatase less polar than the heme pocket of *Aplysia* myoglobin but more polar than that of human hemoglobin (Anderson et al., 1970). Attempts to use Acrylodan, a thiol-selective, polarity-sensitive probe (Prendergast et al., 1983), proved unsuccessful since the reagent reacted very poorly with the sulfhydryl group of ferrochelatase.

The contact quenching of ferrochelatase-bound MIANS by acrylamide and KI yielded quenching constants of approximately 3 and 1 M^{-1}, respectively. Both of these numbers are considerably less than those found for exposed residues on proteins and suggest that the MIANS moiety is relatively protected. The differential quenching by these two quenchers, whose quenching efficiencies are both 1.0 (Eftink & Ghiron, 1981), once again suggests that the environment of MIANS is nonpolar since the smaller, charged I^- is less efficient that the larger but uncharged acrylamide molecule. However, the 427-nm emission along with the reported quenching constants does suggest that the MIANS is not entirely shielded from access by bulk water. This would be expected since Fe^{2+}, one of the two substrates, must gain access to the active site along with the nonpolar protoporphyrin macrocycle.

Examination of the fluorescence properties of ferrochelatase tryptophan residues yields unusual but interesting results. The emission maximum occurs at 347 nm, which is typical of an exposed residue, but the quenching data (K_{SV} = 1 and 1.8 M^{-1} for KI and acrylamide, respectively) are more typical of a buried residue. A possible explanation for this is that the fluorescent residue(s) is (are) present in the protected pocket, surrounded by trapped water, but is (are) poorly accessible to the external bulk solvent. The finding that some energy transfer from the tryptophan to the bound MIANS occurs would also support this model. The higher quenching constant with acrylamide of MIANS might be attributable to the ANS moiety extending farther out of the pocket than the tryptophan's indol ring.

In a general model of the active site of bovine ferrochelatase based upon data presented here and previously (Dailey & Fleming, 1983; Dailey, 1984), the substrate binding region is

shown as a pocket that encloses the porphyrin macrocycle on all sides except one. Since iron binding occurs via vicinal sulfhydryls prior to porphyrin binding, it should be possible for both substrates to freely associate. The observation that the size of substituents at the 2- and 4-positions is limiting supports a binding area enclosed or restricted at that end, and the lack of inhibition by N-substituted porphyrins with alkyl groups larger than two carbons suggests that a region surrounding the top and bottom of the porphyrin ring exists. The tryptophan residue(s) is (are) present in the pocket, exposed to surrounding water, but poorly accessible to the bulk solvent phase and is (are) in proximity to the MIANS. The presence of a porphyrin binding pocket rather than a more open, hydrophilic site is compatible with the physical properties of protoporphyrin, which is poorly soluble in aqueous solution and readily partitions into hydrophobic solvents. The properties described above for ferrochelatase are also similar to those detailed for heme binding pockets in hemoproteins. It is interesting to speculate about the similarities between ferrochelatase and hemoproteins since all bind tetrapyrroles and may share common structural features. Complete evaluation of this, however, must await further structural studies including the amino acid sequence of ferrochelatase.

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Increased Stability of the Higher Order Structure of Chicken Erythrocyte Chromatin: Nanosecond Anisotropy Studies of Intercalated Ethidium

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ABSTRACT: Internal motion of the DNA in chicken erythrocyte chromatin fibers was studied by measurement of the fluorescence anisotropy decay of ethidium intercalated in the linker region. A comparison of the decay curves of the dye in chicken erythrocyte chromatin with those of calf thymus chromatin [Ashikawa, I., Kinosita, K., Jr., Ikegami, A., Nishimura, Y., Tsuboi, M., Watanabe, K., Iso, K., & Nakano, T. (1983) *Biochemistry 22*, 6018–6026] revealed greater suppression of nucleosome movement in chicken erythrocyte chromatin. Furthermore, the transition of this chromatin to the compact (solenoidal) structure occurred at lower solvent concentrations of Na^+ or Mg^{2+} than those for calf thymus chromatin. These results demonstrated increased stability of the higher order structure (the solenoid) of chicken erythrocyte chromatin, which may be related to the reduction of nuclear activity in the chicken erythrocyte cell. In addition to intact chicken erythrocyte chromatin, we studied the structural transitions of H1-depleted and H1,H5-depleted chromatins. The result indicated that histone H5 of this chromatin stabilizes the higher order structure in the presence of magnesium (or divalent) cation and did not induce the transition in the solution containing only sodium cation.

Internal motion of DNA in solution has been investigated recently by NMR (Early & Kearns, 1976; Hogan & Jardetzky, 1979, 1980; Bolton & James, 1980; Opella et al., 1981), fluorescence depolarization (Wahl et al., 1970; Thomas et al., 1980; Millar et al., 1980, 1982), ESR (Robinson et al., 1980), and triplet anisotropy decays (Hogan et al., 1982,

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