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Myelin Basic Protein Binds Heme at a Specific Site near the Tryptophan Residue

Stephen J. Morris,*,‡ Diane Bradley,‡ Anthony T. Campagnoni,§ and Gerald L. Stoner‡

Laboratory of Experimental Neuropathology, National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20892, and Mental Retardation Research Center, University of California at Los Angeles Center for the Health Sciences, Los Angeles, California 90024

Received April 25, 1986; Revised Manuscript Received October 28, 1986

ABSTRACT: Fluorescence of the single tryptophan residue in myelin basic protein (MBP) was excited directly at 295 nm (red-edge excitation) or at 278 nm which allows, in addition, indirect excitation by resonance energy transfer (RET) from any nearby tyrosine residues. Both red-edge excitation and the RET pathway were collisionally quenched by I^- and acrylamide, but not by Cs^+ or Co^{2+}, implying that the fluorophore is in an exposed, positively charged environment. The quenching coefficients (K) for I^- are 12–15 M^{-1} at both excitation wavelengths while coefficients for acrylamide are 15 M^{-1} at 278-nm and 8 M^{-1} at 295-nm excitation. Chloroheme, cyanoheme, and protoporphyrin IX also quench both red-edge excitation and the RET pathway with apparent quenching coefficients which are (2–5) $\times 10^4$-fold higher. This suggests that the mechanism of quenching now includes static in addition to collisional processes and thus that heme has a relatively high affinity for MBP. Scatchard analysis of the quenching suggests that chloroheme binds to MBP at two sites with dissociation constants (K_d) of 1.6×10^{-8} and 2.0×10^{-7} M and stoichiometries of 0.04:1 and 0.16:1, respectively. The hydrophobic fluorescent probe 4,4′-bis[1-(phenylamino)-8-naphthalenesulfonate] [bis(ANS)] binds to MBP less avidly ($K_d = 10^{-7}$ M) and is rapidly displaced by chloroheme ($K_i = 2 \times 10^{-8}$ M). The affinities of bis(ANS) and heme for MBP, along with the fluorescent amino acid quenching data, demonstrate that a subfraction of MBP molecules contain considerable structural specificity, implying stable long-range interactions in the molecule. Resonance energy transfer involving Trp and Tyr suggests a separation of these fluorophores of 10–15 Å or less. The location of the binding site for heme and bis(ANS), both of which require positively charged, hydrophobic environments, should be within the same distance constraints from the sole Trp. The distance requirements are satisfied by the β-sheet model for MBP [Stoner, G. L. (1984) *J. Neurochem. 43*, 433–447] which places Tyr-14 approximately 5 Å from Trp-117 on an adjacent β-strand. The requirement for a hydrophobic site with cationic character would be met if the heme/ANS binding site were located on the predicted β-sheet face near these residues.

The spectral properties of the naturally fluorescent amino acids tryptophan and tyrosine are useful as probes for their immediate environment in the protein polypeptide chain and also as indicators of long-range order in the molecule. For example, the fluorescence emission peak of the fluorophore shifts to lower wavelengths and the quantum yield increases with increasing hydrophobicity of its environment. In addition, two fluorophores with sufficiently high quantum yields and

* Address correspondence to this author.
‡ National Institute of Neurological and Communicative Disorders and Stroke.
§ UCLA Center for the Health Sciences.

0006-2960/87/0426-2175$01.50/0

sufficient overlap between excitation and emission spectra will show resonance energy transfer (RET).[1] The classic example of RET is that of polypeptide hormones containing several possible donors (Tyr) but only one resonance energy acceptor (Trp) (Eisinger, 1969). As the efficiency of resonance energy transfer is related inversely to the sixth power of the distance between fluorophores, it provides a unique measure of the separation between fixed donor and acceptor (Stryer, 1978). Since it is possible to perturb the natural fluorescence of a protein by the binding of ligands which induce a conformational alteration or which bind in the immediate vicinity of the fluorophore, the binding of such ligands can be used to study both binding specificity and ligand affinity (Halfman & Nashida, 1972).

Although most of the early work on myelin basic protein (MBP) pictures this molecule as being highly flexible and in a largely disordered state (Eylar & Thompson, 1969; Krigbaum & Hsu, 1975), experimental evidence has been emerging which suggests that the molecule may, in fact, contain a high degree of structural specificity (Jones & Epand, 1980; Burns & Campagnoni, 1983; Randall & Zand, 1985; Smith, 1985, Stone et al., 1985; Martenson et al., 1985). Recently, a molecular model of MBP structure has been derived which predicts that MBP contains about 25% antiparallel β-sheet (Stoner, 1984). This detailed model generates several testable predictions. First, the β-sheet model of MBP structure places Tyr-14 close to the only Trp residue (Trp-117) and thus predicts RET between Tyr and Trp. The model also predicts that the single Trp residue of the molecule and its Tyr energy donor both exist in a positively charged environment accessible to solvent. Finally, the clustering of hydrophobic amino acid residues in the β-sheet region predicts that hydrophobic probes or other hydrophobic ligands will preferentially bind to the hydrophobic sites on one of the faces of the MBP β-sheet.

There are reports that the fluorescent probes ANS and TNS bind to MBP with a K_d of $(1.1–3.3) \times 10^{-5}$ M (Feinstein & Felsenfeld, 1975; Randall & Zand, 1985). Vacher et al. (1984) have reported that heme derivatives can bind avidly to MBP, supporting earlier work by Liebes et al. (1975). We have reinvestigated the question of ligand binding and report here studies using the spectroscopic properties of MBP and fluorescent probes to confirm the previous reports and to demonstrate binding specificity. We find that both classes of compounds bind to purified 18.5-kilodalton MBP, possibly within the same site. The evidence supports the predictions noted above and suggests that at least a subpopulation of MBP molecules do indeed contain long-range structure.

Materials and Methods

ANS, TNS, and bis(ANS) were purchased from Molecular Probes, Junction City, OR, and used without further purification. All other chemicals were of the highest purity obtainable. Glass-distilled, deionized water of essentially null fluorescence background was used for all solutions.

Preparation of Myelin Basic Protein. Bovine brain myelin basic protein was prepared by a modification of the method previously described (Burns & Campagnoni, 1983). The highly purified (>95%) lyophilized material was stored at −80 °C until used. Stock solutions of 1 mg/mL were prepared in 20 mM sodium acetate buffer, pH 4.5, and were kept on ice until used. All experiments were performed in this buffer.

MBP preparations have been reported to self-aggregate as the pH approaches 7 (Smith, 1980). Vacher et al. (1984) noted that cyanoheme precipitated MBP at pH 7. Anderson (1971) reported that bis(ANS) aggregated one of the lactate dehydrogenase isozymes. Preliminary experiments with the lyophilized protein dissolved in 20 mM HEPES, pH 7.0, showed some self-precipitation with time which was greatly hastened by addition of hemin. For these reasons, we performed the studies presented here at pH 4.5. At this pH, no changes in turbidity were noted when either ANS or heme derivatives were added. A possible solution to this problem may be found in recent preliminary observations. If the lyophilized protein is first dissolved at the lower pH and then titrated to pH 7, it will stay in solution. One experiment suggested that neither heme nor ANS titrations would produce aggregation.

Fluorescence measurements were carried out in either a Perkin-Elmer Model 650-10S or Perkin-Elmer Model MPF-44B recording spectrofluorometer thermostated at 25 °C. Results on both instruments were identical. Stern–Volmer quenching constant determinations were made by adding small volumes of concentrated material sequentially to the cuvette containing the MBP solution and noting the reduction in fluorescence. Titrations with ANS and TNS were performed by diluting a 10 mM stock solution in ethanol with buffer and then adding microliter volumes directly to the cuvette. Final ethanol concentrations in the titrations did not exceed 0.5%.

The Stern–Volmer quenching coefficient, K, was determined as follows: the initial fluorescence, F_0, from a sample of 5.4×10^{-6} M MBP was determined at excitation/emission wavelengths of 278/350 nm to allow Tyr → Trp RET or at 295/350 nm to excite Trp alone (red-edge excitation). A small volume of highly concentrated solution of a collisional quencher was added, and the new, lower fluorescence emission noted as F_c. K was determined from the relationship $F_0/F_c = K[Q] + 1$, as the slope of a plot of F_0/F_c vs. the concentration of the quencher, [Q].

As noted by Eftink and Ghiron (1976), acrylamide absorbs in the UV and can contribute an appreciable inner filter effect. At the concentrations used for this study, no correction for this effect was necessary for excitation at 295 nm. The data for excitation at 278 nm were corrected by the method described by Parker (1968).

Heme Titrations. For heme binding studies, heme solutions were prepared as described by Vacher et al. (1984) in 0.1 N NaOH and filtered through a Millipore 0.22-μm filter, and the absorption of the filtered material was determined. Concentrations of the stock solutions were calculated by using the value of 5.0×10^4 in 0.1 N NaOH as the molar extinction coefficient at 385 nm for chloroheme (hemin) and cyanoheme. The equivalent value for protoporphyrin IX was 2.62×10^5 at 408 nm.

Heme derivatives also may contribute inner filter effects. Preliminary experiments with tryptophan in solution established that at 278 nm, chloroheme > cyanoheme > protoporphyrin IX for quenching the fluorescence of tryptophan in pH 4.5 buffer. Due to the greatly reduced quantum yield of tryptophan incorporated into proteins, these effects become less important (Cantor & Timasheff, 1982). For chloroheme and MBP, corrections according to Parker (1968) showed these effects to be negligible (<5%) below about 60 μM. Data at higher hemin concentrations are not presented. Evidence against other possible inner filter effects is discussed in more detail below.

[1] Abbreviations: MBP, myelin basic protein; RET, resonance energy transfer; ANS, 1-anilino-8-naphthalenesulfonate; bis(ANS), 4,4′-bis-[1-(phenylamino)-8-naphthalenesulfonate]; TNS, 2-(*p*-toluidinyl)-naphthalene-6-sulfonate; kDa, kilodalton(s); HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; CPK, Corey–Pauling–Koltun.

Bis(ANS) Titrations. A 2.0 mM solution of bis(ANS) in dimethylformamide was prepared (E_{385} = 16790 M^{-1} cm^{-1}), always kept at 0–4 °C, and renewed weekly. Dilutions to lower concentrations were made in the acetate buffer. These solutions were added in microliter increments to MBP in acetate buffer, and the increases in fluoroescence at 460 nm (excitation 355 nm) were noted. The final dimethylformamide concentration in the titration did not exceed 0.5%.

Calculations of bis(ANS) bound at each concentration of total ligand were made essentially by the method described by Halfman and Nishida (1972). Briefly, the increment in bis(ANS) fluorescence at low bis(ANS) concentrations (33.3–166 nM) upon binding to MBP at high (1.08×10^{-4} M) protein concentration was measured. This produced a straight line as would be expected if all the bis(ANS) were bound. The slope of these data was used to transform the change in fluorescence to the amount of bis(ANS) bound at any total bis(ANS) addition. Free fluorophore was then calculated as the difference (total − bound), and the data were plotted according to the method of Scatchard. A problem encountered with this approach was imprecision in the estimation of free bis(ANS) and therefore of the bound:free ratio at very low concentrations of bis(ANS). The binding constant (K_d) was determined by graphical analysis. Constants from duplicate titrations varied by factors of 1.5–2; therefore, the precision of the data presented cannot be considered to be greater than this range.

The apparent inhibition constant for chloroheme, K_i, was calculated from the relationship $K_i = [I]/[(K_{app}/K_d) - 1]$, where K_{app} is the apparent binding constant in the presence of chloroheme inhibitor at concentration [I] (Dixon & Webb, 1964).

Heme binding was calculated in an analogous fashion, but using the quenching of fluorescence emission at 350 nm with excitation at 278 nm as the measure of binding.

Results and Discussion

Natural Fluorescence of Myelin Basic Protein. The fluorescence spectrum obtained for myelin basic protein is shown in Figure 1. As can be seen, the excitation peak is near 280 nm with the peak falling off rapidly around 295–300 nm. The emission spectrum shows a peak at about 350 nm. This behavior is reminiscent of tryptophan in water with its excitation maximum around 288 nm and emission at about 350 nm. As the polarity of the solvent decreases, tryptophan fluorescence shows a characteristic blue shift of the emission peak. [Fluorescence properties of proteins are reviewed by Cantor and Timasheff (1982).] The lack of a significant blue shift of the tryptophan fluorescence in the MBP emission spectrum indicates that the tryptophan residue is not confined within a highly hydrophobic environment, in agreement with earlier fluorescence liftime studies (Munro et al., 1979; Lakowicz et al., 1983). Tyrosine in water excites at 276 nm and fluoresces at about 305 nm. There is a pronounced shoulder in the MBP spectrum at 305 nm which becomes more prominent if excitation is lowered to 276 nm. This peak can be attributed to unquenched Tyr emission. It is possible that another portion of the Tyr emission may be transferred to Trp and emitted at around 350 nm. Tryptophan can be excited exclusively by choosing a wavelength at the red edge of its excitation peak (295 nm). This also produces an emission peak at 350 nm (Figure 1c). These results are in agreement with MBP spectra previously published (Feinstein & Felsenfeld, 1975; Jones & Rumsby, 1975; Jones & Epand, 1980).

Resonance energy transfer occurs when the emission spectrum of the donor fluorophore overlaps with the absorption

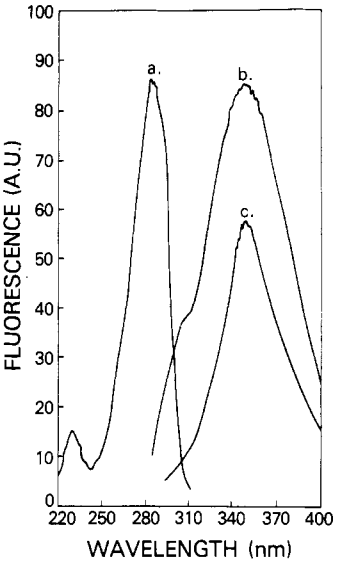

FIGURE 1: Excitation and emission spectra of 5.4×10^{-6} M myelin basic protein in 20 mM sodium acetate buffer, pH 4.5. (a) Excitation spectrum with emission at 350 nm. (b) Emission spectrum with excitation at 280 nm. (c) Emission spectrum with excitation at 295 nm. Excitation and emission slits were 2 and 5 nm, respectively. Temperature = 25 °C.

spectrum of an acceptor which is close by. In proteins containing a single tryptophan residue and multiple tyrosine residues, RET may go in either direction depending on the pH: at acidic and neutral pH, Tyr may transfer energy to Trp, while at alkaline pH, Trp may transfer energy to the tyrosinate anion. It has already been shown that MBP exhibits resonance energy transfer from Trp to at least one of the four Tyr residues as a tyrosinate anion (Tyr^-) at high pH (Jones & Epand, 1980). The interresidue distance was estimated to be about 10 Å. RET from Tyr to Trp is more difficult to demonstrate because the absorption spectra of Tyr and Trp overlap, and any wavelengths which excite Tyr also excite Trp (Lakowicz, 1983). Thus, excitation at 278 nm, which is near the tyrosine excitation peak, elicits emission at 350 nm in the tryptophan. This could be due to RET from Tyr, as well as to direct excitation of Trp, and the contribution of each is difficult to assess. However, excitation at 295 nm (red-edge excitation) excites primarily Trp alone and largely eliminates RET. Therefore, we refer to excitation at 278 nm, which permits resonance energy transfer, as the RET pathway, to distinguish it from excitation at 295 nm which eliminates RET.

As shown by Förster (1951), the quantum efficiency of transfer, E, is $E = R^6/(R^6 + R_0^6)$ where R is the actual separation distance between the donor and acceptor and R_0 is the Förster critical transfer distance at which transfer is half-maximal. Since the R_0 value for Tyr → Trp is 10–11 Å (Eisinger, 1969), this evidence for RET would imply that at least one of the tyrosine residues is within approximately this distance from the single Trp. The presence of a detectable Tyr peak at 305 nm shows that not all of the Tyr fluorescence is quenched or donated to the Trp residue.

While it is theoretically possible to determine transfer efficiencies from excitation spectra, the method is not without its difficulties (Eisinger et al., 1969), and, in the case of MBP, is complicated by the possibility that only a fraction of the molecules are in an active conformation (see below). Therefore, we sought indirect evidence for RET by looking for differential quenching effects on the RET pathway and red-edge excitation. A group of experiments described in the next section places both the RET donor and acceptor in highly

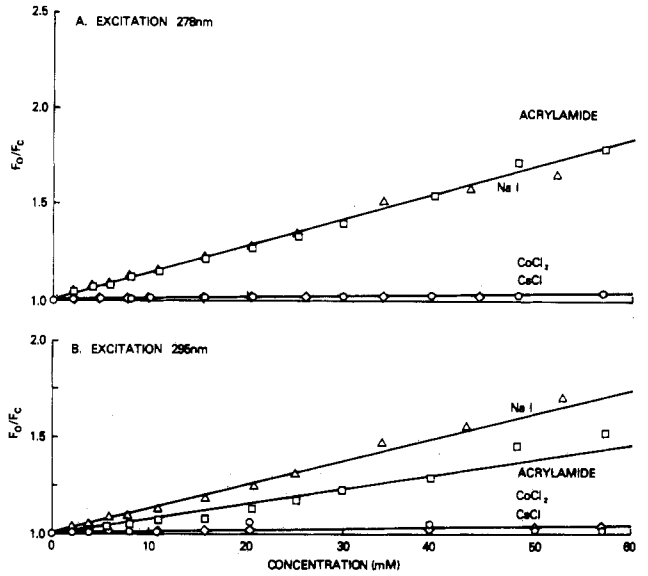

FIGURE 2: Collisional quenching of myelin basic protein fluorescence. (A) Excitation at 278 nm (with possible Tyr → Trp RET) and (B) direct excitation of Trp at 295 nm. Samples were titrated as described under Materials and Methods and results expressed as the increase in quenching of tryptophan fluorescence emission at 350 nm (F_0/F_c). The Stern–Volmer quenching coefficients for resonance energy transfer were 0.5 M^{-1} for Cs^+ and Co^{2+} and 15 M^{-1} for I^- and acrylamide. Quenching coefficients for direct excitation were 0.7 M^{-1} for Cs^+ and Co^{2+}, 12.5 M^{-1} for I^-, and 7.7 M^{-1} for acrylamide.

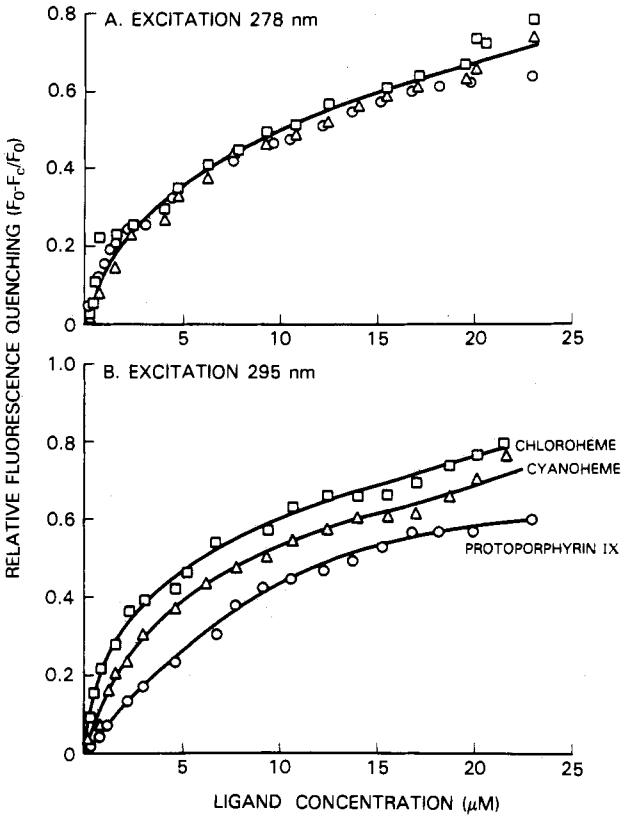

FIGURE 3: Quenching of myelin basic protein fluorescence by heme compounds. (A) Excitation at 278 nm (with possible Tyr → Trp RET). (B) Direct excitation of Trp at 295 nm. Measurements were made as described under Materials and Methods. Results are presented as the fractional increase in quenching of tryptophan fluorescence $[(F_0 - F_c)/F_0]$ vs. the concentration of added ligand.

positively charged environments with similar, but not identical, quenching characteristics.

Collisional Quenching of the Tryptophan and Tyrosine Residues. Quenching of fluorophores can take place by collisions with other ions or molecules in their immediate surroundings. This so-called Stern–Volmer or collisional quenching has been well characterized. In water solution, the classic quenchers are negatively or positively charged ions, such as I^- or Co^{2+} and Cs^+, as well as neutral molecules such as the acrylamide monomer. Figure 2 shows the effect of these quenchers upon (A) the fluorescence from excitation at 278 nm allowing Tyr → Trp RET or upon (B) the fluorescence from the tryptophan excited directly at the red edge (295 nm) with little or no energy transfer from Tyr. In both cases, the results for the positively charged cobalt and cesium ions are quite clear. These ions have no ability to quench either Tyr → Trp resonance energy transfer or direct Trp excitation. Thus, the immediate environment of the tryptophan is positively charged, and the Co^{2+} and Cs^+, usually good collisional quenchers, cannot approach the Trp or its Tyr energy donor because of the positive charge in their surroundings. On the other hand, the iodide, being negatively charged, is not repelled and in fact may be drawn into this region. Iodide is a fairly good collisional quencher for both red-edge excitation and the RET pathway, with a Stern–Volmer constant, K, in the range of 12–15 M^{-1}. The MBP β-structural model suggests that positively charged residues near Trp-117 would include Lys-13 and His-89 on adjacent β-strand numbers 0 and 2, respectively (Stoner, 1984). Also of interest is the fact that acrylamide, a neutral molecule with the ability to penetrate into hydrophobic as well as hydrophilic domains (Eftink & Ghiron, 1976), is as effective a tryptophan quencher as iodide when excitation is through the RET pathway (K = 15 M^{-1}). However, acrylamide is much less effective for quenching of direct excitation of Trp at 295 nm (K = 8 M^{-1}), while the effect of I^- (K = 15 M^{-1}) is unchanged. The differential quenching of the two pathways by acrylamide may be related to the relative exposure to solvent of the two fluorophores or to the solubility of the quencher in their immediate environments.

A second line of evidence suggests that the tryptophan residue is in a positively charged environment. The trivalent ion Tb^{3+} is able to act as a resonance energy acceptor for tryptophan fluorescence (Gafni & Steinberg, 1974). This phenomenon can be used to detect negatively charged binding sites in the vicinity of the Trp (Morris & Schober, 1977). Addition of Tb^{3+} to MBP solutions, at concentrations high enough to detect low-affinity binding ($K_d \sim 10^{-2}$–10^{-4} M) or resonance energy transfer by collision, produced no Trp → Tb^{3+} resonance energy transfer (data not shown), again supporting a positively charged environment for the fluorophore.

Quenching of MBP Fluorescence by Heme Derivatives. We have investigated reports that several heme compounds bind to MBP. Vacher et al. (1984) have demonstrated that addition of cyanoheme to MBP or certain MBP peptides both increases heme absorption and quenches the natural fluorescence of MBP. Our preliminary experiments verified both spectroscopic observations for intact MBP, confirming the suggestion that heme binds to a site on MBP. In order to localize this putative binding site on the MBP molecule, we tested for differential quenching by various heme analogues of both the direct excitation of tryptophan fluorescence and the energy transfer from one or more Tyr residues to Trp-117 (Figure 3). The results showed that the three porphyrins are equally potent quenchers of the pathway allowing RET from tyrosine to the tryptophan residue. However, with red-edge excitation of tryptophan, chloroheme quenches better than cyanoheme which, in turn, quenches better than protoporphyrin IX, suggesting the possibility of some differential binding spe-

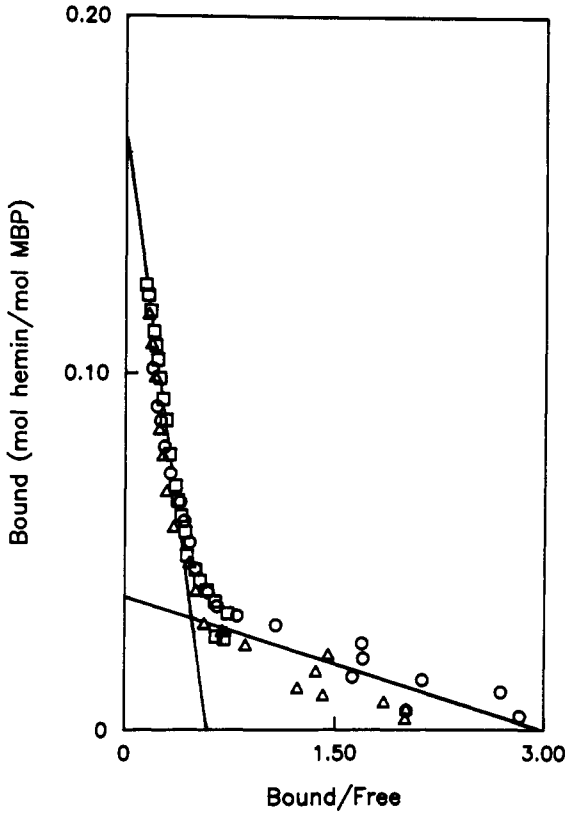

FIGURE 4: Scatchard plot of hemin (chloroheme) binding to MBP. (○) 3.06×10^{-6} M MBP; (□) 1.06×10^{-5} M MBP; (△) 1.06×10^{-4} M MBP. Binding constants and stoichiometries were determined graphically by the method of Dixon and Webb (1964).

cificity. If the quenching were due to inner filter effects alone, one would not expect to see such large differential effects at the higher but not the lower wavelength.

A second observation (not shown) argues strongly against inner filter effects accounting for the heme quenching of the natural fluorescence of MBP. Upon the addition of the small volume of highly concentrated heme solution to the cuvette, the reduction of fluorescence is not "instantaneous" on the hand-mixing time scale as would be expected for an inner filter quencher. Rather, the changes require several minutes to relax to their new value. This strongly suggests a binding phenomenon.

The results of Figure 3 can also be analyzed in Stern–Volmer fashion (Lakowicz, 1983). The apparent constants obtained (K_{app}) are on the order of (2–5) $\times 10^4$ times higher for the heme derivatives than for iodide and acrylamide (data not shown). The large values for K most likely reflect the specific binding of the heme molecules in the region near Trp (and any donor Tyr), thus dramatically increasing the local heme concentration and the efficiency of quenching. This effect can be compared to the enhanced ability of cobalt ions to quench fluorescent-labeled head groups of phospholipid probes embedded in negatively charged phospholipid bilayers to which Co^{2+} binds, compared to the same fluorophores located in neutral bilayers to which Co^{2+} does not bind (Morris et al., 1985b). It should be noted that to definitively distinguish the contributions of static and dynamic processes to the quenching by heme derivatives will require measurements of fluorescence lifetimes (Lakowicz, 1983).

If such binding sites exist, they should be amenable to analysis by accepted methods. As described under Materials and Methods, the quenching of fluorescence at 278 or 295 nm can be used to measure the binding of heme derivatives to MBP (Halfman & Nashida, 1972). From the data of Figure 3, chloroheme (hemin) appeared to have the strongest binding to the MBP molecule and was chosen for further investigation. A Scatchard-type plot of the binding data obtained from quenching of the RET pathway is presented in Figure 4. The combined data from three experiments, ranging in MBP concentration from 3.06 to 106 μM, suggest that two classes of binding sites exist with apparent K_d's of approximately 1.6×10^{-8} M and 2.0×10^{-7} M and with heme:MBP stoichiometries of 0.04:1 and 0.16:1, respectively.

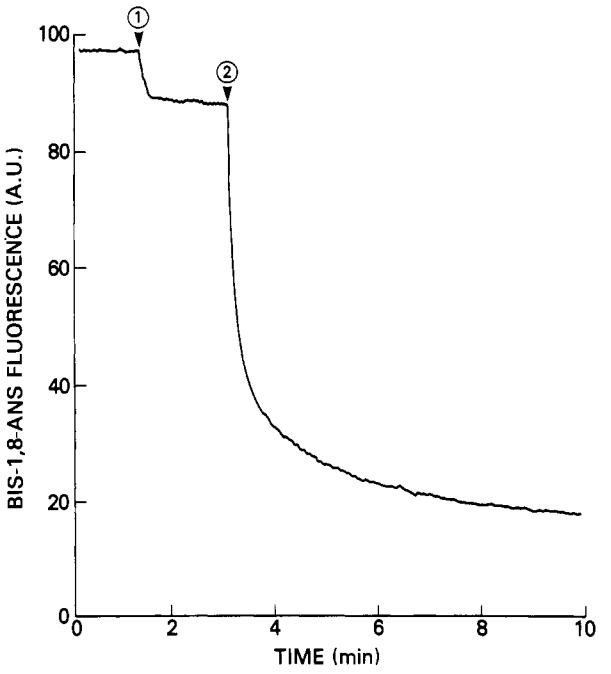

FIGURE 5: Time courses of chloroheme displacement of bis(ANS) from MBP. A cuvette containing 1.08×10^{-5} M MBP + 1.1×10^{-5} M bis(ANS) in acetate buffer at 25 °C was placed in the fluorometer (excitation/emission: 355/460 nm). At arrow 1, 1.8×10^{-9} M chloroheme was added. A second addition of 1.1×10^{-6} M chloroheme was made at arrow 2. Note the slow relaxation of the fluorescence which argues against inner filter effects as the cause for the reduction in signal.

Binding of ANS Derivatives to MBP. ANS and several related compounds are sparingly soluble and almost completely quenched in water solutions. When these compounds move into hydrophobic environments upon binding to proteins, they show the characteristic increase in quantum yield as well as the characteristic blue shift in the emission fluorescence peaks. Preliminary experiments established that ANS, TNS, and bis(ANS) (a dimer of ANS) all show these changes in fluorescence properties in the presence of MBP. It had previously been reported that TNS and ANS bind to myelin basic protein. A single site with a binding constant of about 1.1×10^{-5} to 2.1×10^{-5} M has been determined for TNS binding (Feinstein & Felsenfeld, 1975; Randall & Zand, 1985), and the stoichiometry was estimated as 1:1 by Randall and Zand (1985). In an earlier study, Feinstein and Felsenfeld (1975) reported a 1:1 ratio for ANS binding to MBP, with $K_d = 3.3 \times 10^{-5}$ M.

We chose bis(ANS) for further study because it has higher affinity for many proteins than do ANS or TNS, and because some of the previous reports of ANS binding may have been due to the contamination of the ANS by bis(ANS) (Rosen & Weber, 1969; York et al., 1978). We found that bis(ANS) binds avidly to myelin basic protein. Figure 5 demonstrates that this binding can be reversed by the addition of hemin. Once again, the low hemin concentration used and the relatively slow rate of reversal rule out inner filter effects. No

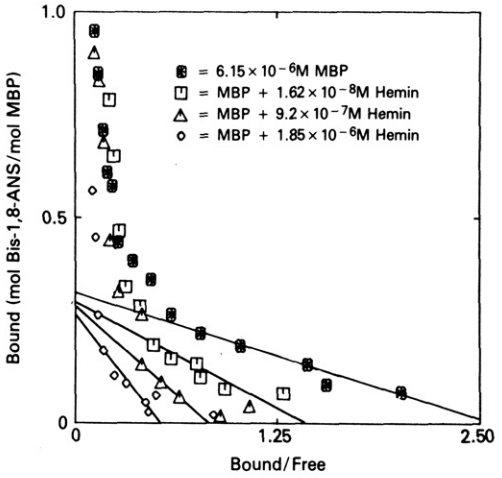

FIGURE 6: Scatchard plots of bis(ANS) binding to 6.15×10^{-6} M MBP in the absence and presence of various concentrations of hemin (chloroheme) as identified on the graph. The apparent K_i's calculated from these three latter inhibition experiments were 0.62×10^{-8}, 2.2×10^{-8}, and 3.1×10^{-8} M, giving a mean (±SEM) of $2.0 (\pm 1.3) \times 10^{-8}$ M.

attempt was made to quantitate the reversal rates.

Because of its very low background fluorescence, it is possible to quantitate bis(ANS) binding to MBP as described (see Materials and Methods). Scatchard analysis of these data again suggests two classes of binding sites. Four titrations over a concentration range of 1.06×10^{-6} to 1.06×10^{-5} M MBP produced a site with a K_d of approximately $1.0 (\pm 0.2) \times 10^{-7}$ M (mean ± SEM) and a class of nonspecific low-affinity sites with a stoichiometry greater than one site per MBP molecule [data similar to Figure 6 (encircled asterisks) not shown]. The latter type of site is typical of nonspecific binding of ANS derivatives to proteins (Rosen & Weber, 1969). The former site appears to be competitively inhibited by hemin with an apparent $K_i \sim 2 \times 10^{-8}$ M (Figure 6). At the hemin concentrations used (1.6×10^{-8} to 1.8×10^{-6} M), inner filter effects are negligible. Also, the slow reduction in bis(ANS) fluorescence presented in Figure 5 argues against both the inner filter effect and bis(ANS) → hemin RET as the cause. The stoichiometry for the high-affinity bis(ANS) site (0.25:1) is approximately the same as the high- and low-affinity heme binding sites together.

Ideally, the type of binding study presented in Figure 6 should be performed with the MBP concentration approximately equal to the K_d, since large errors in the apparent binding constant will occur when protein is in large excess. Titrations at 10^{-7} M MBP were very noisy due to the imprecision in estimating bound and free bis(ANS) using the fluorescence enhancement method. However, the precision of the dissociation measurement at higher MBP concentrations gives us confidence that, although the K_i's measured in Figure 6 may have a severalfold absolute error, they serve to qualitatively demonstrate the tight binding of bis(ANS) to MBP and its easy displacement by hemin.

We find it interesting that binding of two different classes of hydrophobic, negatively charged molecules to MBP can be demonstrated. For both classes, the binding stoichiometries are low, implying that only a fraction of the total MBP molecules may possess binding activity; the greater portion may be partially denatured and inactive. Alternatively, the active molecules may differ with respect to one or more posttranslational modifications, e.g., phosphorylation (Martenson et al., 1983).

The question of whether the same fraction of molecules and the same binding site are binding both the heme derivatives and bis(ANS) naturally arises. We found that heme compounds, even at very low concentrations, readily displace bis(ANS) (Figures 5 and 6). The apparent K_i of 2×10^{-8} M for the high-affinity site (Figure 6) is consistent with the values measured for direct chloroheme binding (Figure 4). It is clear

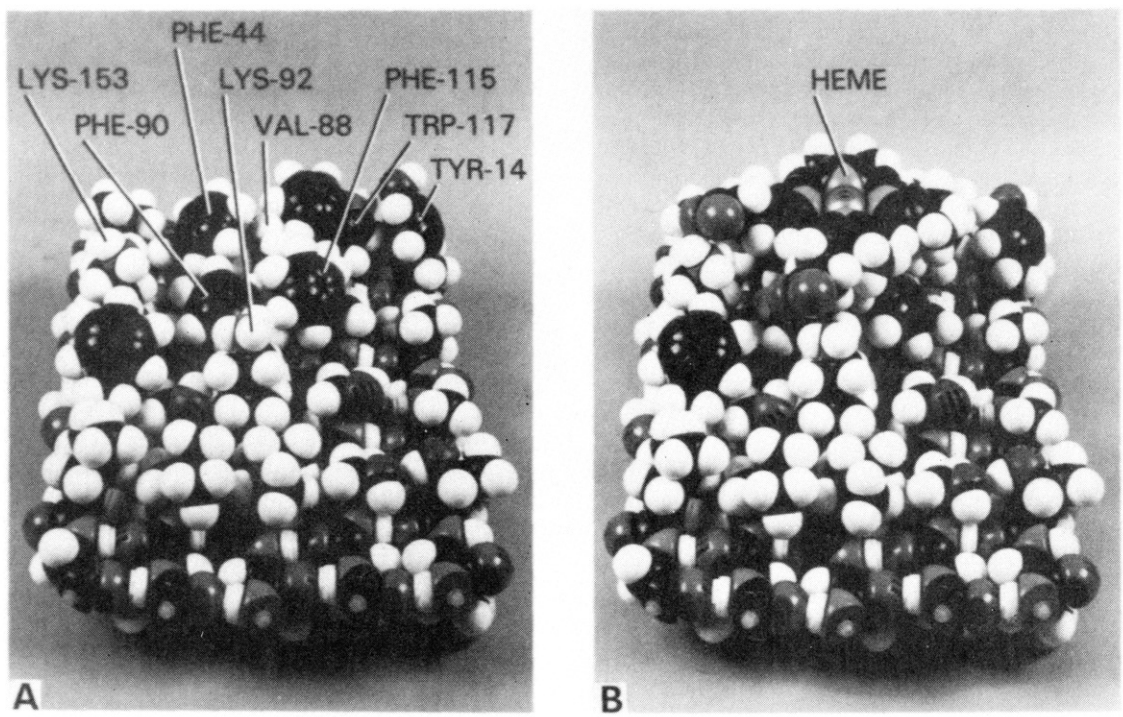

FIGURE 7: Space-filling CPK model of proposed heme binding site in the β-sheet region of MBP. (A) Proposed heme binding site in β-sheet face with contact residues identified. [For identification of all residues, see Figure 7 in Stoner (1984).] Hydrophobic residues in position to interact with the porphyrin ring include Trp-117 and Tyr-14 (the proposed RET pair), Phe-44, Val-88, Phe-90, and Phe-115. Lys-92 and Lys-153 contribute cationic side chains for electrostatic interaction with the carboxymethyl groups of the heme moiety. (B) Same site with bound heme oriented for electrostatic interaction with Lys-92 and Lys-153.

from Figure 6 that hemin and bis(ANS) are binding to the same subpopulation of MBP molecules. The displacement of bis(ANS) bound to MBP is most likely explained by the binding of heme to the same site or to an overlapping site. However, we cannot formally exclude the possibility that heme binding induces a conformational change in MBP which displaces bis(ANS) from a distant site. Studies utilizing ANS quenching of Trp fluorescence (Weber & Daniel, 1966) might provide further information on this question.

The affinities of bis(ANS) and heme for MBP demonstrate that MBP contains considerable structural specificity. The evidence from the fluorescent amino acid quenching data suggests that the binding site involves the single Trp and, possibly, one or more of the four Tyr residues. The efficient RET from Trp to Tyr^- at high pH found in MBP by Jones and Epand (1980) suggests that these fluorophores are about 10 Å or less apart. If so, the binding site for the heme derivatives should be near the Trp/Tyr energy transfer pair. It is likely that both the heme derivatives and bis(ANS) binding sites have some hydrophobic character with nearby positive charge (Perutz, 1979; Weber et al., 1979; Madsen et al., 1983). Together, the energy transfer evidence and the character of the binding site argue strongly for some type of long-range structure in at least a subpopulation of MBP molecules. The β-sheet model provides a useful conceptual framework. The requirement for an interresidue Trp–Tyr distance of about 10 Å or less is satisfied by the β-sheet model which places Tyr-14 as close as 5 Å from Trp-117 on the adjacent β-strand (Figure 7). Further studies utilizing MBP from a species whose sequence lacks Tyr-14, i.e., the chicken protein (Mendz et al., 1982), should help to confirm the identity of the donor Tyr. The requirement for a nearby positively charged, hydrophobic environment also would be met if the heme/bis(ANS) binding site were located on the β-sheet near these residues. In addition to the Trp and Tyr side chains, three Phe and a Val residue are positioned for hydrophobic interaction with the porphyrin ring, and two Lys side chains contribute cationic character to the proposed binding site (Figure 7). The reverse side of the β-sheet, with the same number of hydrophobic residues and four cationic side chains, could provide additional binding sites of lower affinity [see Figure 7 in Stoner (1984)].

Binding of two classes of compounds to MBP has been examined by two different methodologies, one measuring a property of the ligand [bis(ANS) fluorescence] and the second a property of the protein (quenching of natural fluorescence by heme derivatives). As noted, displacement of bis(ANS) by chloroheme implies competition of the two ligands for the same or overlapping binding sites on the same subpopulation of molecules. The possible significance of displacement of bis(ANS) by heme is pointed up by an early study by Stryer (1965), who showed that apohemoglobin and apomyoglobin both bound ANS with binding constants similar to those reported for MBP (Feinstein & Felsenfeld, 1975). Furthermore, heme rapidly and completely displaced bound ANS with renaturation of both hemoglobin and myoglobin. A similar competition has been reported for the heme binding site of horseradish peroxidase (Rosen, 1970). However, whether the competing interactions of heme and bis(ANS) with MBP in solution as reported here have any physiological significance is unknown. The proposed heme binding site in MBP (Figure 7) bears little resemblance to the heme binding pocket of globins (Perutz, 1979), and there is no evident functional analogy between MBP and the O_2 binding globins or the heme-containing electron carriers such as the cytochromes.

Registry No. ANS, 82-76-8; bis(ANS), 63741-13-9; TNS, 7724-15-4; chloroheme, 16009-13-5; cyanoheme, 25513-04-6; protoporphyrin IX, 553-12-8; heme, 14875-96-8; tryptophan, 73-22-3.

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An Engineered Disulfide Bond in Dihydrofolate Reductase†

Jesus E. Villafranca,*,‡ Elizabeth E. Howell,‡ Stuart J. Oatley,§ Nguyen-huu Xuong,§ and Joseph Kraut§

The Agouron Institute, La Jolla, California 92037, and Department of Chemistry, University of California, San Diego, La Jolla, California 92093

Received August 18, 1986; Revised Manuscript Received December 12, 1986

ABSTRACT: Substitution of cysteine for proline-39 in *Escherichia coli* dihydrofolate reductase by oligonucleotide-directed mutagenesis positions the new cysteine adjacent to already existing cysteine-85. When the mutant protein is expressed in the *E. coli* cytosol, the cysteine sulfur atoms are found, by X-ray crystallographic analysis, to be in van der Waals contact but not covalently bonded to one another. In vitro oxidation by dithionitrobenzoate results in formation of a disulfide bond between residues 39 and 85 with a geometry close to that of the commonly observed left-handed spiral. Comparison of 2.0-Å-refined crystal structures of the oxidized (cross-linked) and reduced (un-cross-linked) forms of the mutant enzyme shows that the conformation of the enzyme molecule was not appreciably affected by formation of the disulfide bond but that details of the molecule's thermal motion were altered. The disulfide-cross-linked enzyme is at least 1.8 kcal/mol more stable with respect to unfolding, as measured by guanidine hydrochloride denaturation, than either the wild-type or the reduced (un-cross-linked) mutant enzyme. Nevertheless, the cross-linked form is *not* more resistant to thermal denaturation. Moreover, the appearance of intermediates in the guanidine hydrochloride denaturation profile and urea-gradient polyacrylamide gels indicates that the folding/unfolding pathway of the disulfide-cross-linked enzyme has changed significantly.

Disulfide bonds are a common feature of many extracellular proteins, where they presumably serve to stabilize the native conformation by lowering the entropy of the unfolded form (Anfinsen & Scheraga, 1975). This stabilizing property has, prospectively, made disulfide bond cross-linking an attractive strategy for engineering additional conformational stability into proteins by site-directed mutagenesis (Villafranca et al., 1983; Perry & Wetzel, 1986). However, criteria for selecting the appropriate locus for disulfide cross-linking in a given protein have not been firmly established. Detailed analyses of disulfide bonds in proteins of known high-resolution X-ray structure have shown that, although many disulfides can be described simply as left-handed spirals or right-handed hooks, disulfide bridges in fact adopt a wide range of conformations and cross-link most kinds of secondary structure present in proteins (Richardson, 1981; Thornton, 1981). Thus, the set of geometric parameters on which one might base an effective disulfide cross-linking strategy is rather weakly constrained.

Another pertinent consideration for a disulfide-based stabilization strategy is the mechanism of formation of disulfide bonds. Disulfide-cross-linked cystosolic proteins are extremely rare, presumably because the intracellular environment is too reducing to allow cysteine oxidation to the disulfide state (Ziegler & Poulsen, 1977; Creighton et al., 1980). In those cases where cystosolic proteins do contain disulfide bonds, the proteins themselves are able to catalyze their formation (i.e., glutathione reductase and thioredoxin reductase). In vitro studies have shown that disulfide bond formation occurs under oxidizing conditions through disulfide interchange reactions that are driven to the final, correctly cross-linked species by protein folding (Haber & Anfinsen, 1962; Creighton, 1978). In vivo, this interchange reaction may be catalyzed by an enzyme, protein disulfide-isomerase, during the secretory posttranslational translocation process (Freedman & Hillson, 1980). Thus it appears that in vivo secretion may be required to produce a disulfide-cross-linked engineered protein without an additional chemical oxidation step.

To explore these concerns, we have engineered a disulfide bridge in the cystosolic enzyme dihydrofolate reductase (DHFR,[1] EC 1.5.1.3) from *Escherichia coli*. A mutation, proline-39 → cysteine, produced by site-directed mutagenesis, was designed to form an intramolecular disulfide bond between Cys-85 and Cys-39, thus cross-linking the N-terminus of α-helix E to the start of β-strand B (Villafranca et al., 1983). We report here on the effect of this disulfide cross-link on the mutant enzyme's structural stability and compare the high-resolution X-ray structures of the reduced (un-cross-linked) and oxidized (cross-linked) forms of this mutant enzyme, which we refer to as Cys-39 DHFR. Our results indicate that the disulfide bridge does not form spontaneously in the cytosol of

† This work was supported by Office of Naval Research Contract N0014-85-K-0663 and National Institutes of Health Contract GM-10928, both awarded to J.K.

* Author to whom correspondence should be addressed.
‡ The Agouron Institute.
§ University of California, San Diego.

[1] Abbreviations: DHFR, dihydrofolate reductase; DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetate; Gdn-HCl, guanidine hydrochloride.

0006-2960/87/0426-2182$01.50/0