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Photoaffinity Labeling of Human Serum Vitamin D Binding Protein and Chemical Cleavages of the Labeled Protein: Identification of an 11.5-kDa Peptide Containing the Putative 25-Hydroxyvitamin D_3 Binding Site†

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ABSTRACT: In this paper, we describe photoaffinity labeling and related studies of human serum vitamin D binding protein (hDBP) with 25-hydroxyvitamin D_3 3β-3′-[*N*-(4-azido-2-nitrophenyl)amino]propyl ether (25-ANE) and its radiolabeled counterpart, i.e., 25-hydroxyvitamin D_3 3β-3′-[*N*-(4-azido-2-nitro-[3,5-^{3}H]phenyl)amino]propyl ether (^{3}H-25-ANE) (Ray et al., 1986, 1991). We have carried out studies to demonstrate that (1) 25-ANE competes with 25-OH-D_3 for the binding site of the latter in hDBP and (2) ^{3}H-25-ANE is capable of covalently labeling the hDBP molecule when exposed to UV light. Treatment of a sample of purified hDBP, labeled with ^{3}H-25-ANE, with BNPS-skatole produced two Coomassie Blue stained peptide fragments, and the majority of the radioactivity was associated with the smaller of the two peptide fragments (16.5 kDa). On the other hand, cleavage of the labeled protein with cyanogen bromide produced a peptide (11.5 kDa) containing most of the covalently attached radioactivity. Considering the primary amino acid structure of hDBP, this peptide fragment (11.5 kDa) represents the N-terminus through residue 108 of the intact protein. Thus, our results tentatively identify this segment of the protein containing the binding pocket for 25-OH-D_3.

Vitamin D binding protein (DBP), identical with the group-specific component (Gc), is an abundant serum protein, which contains a single polypeptide chain and 0-4% neuraminidase-sensitive sialic acids (Cooke & Haddad, 1989). It is well established that the primary function of DBP is to act as a plasma carrier for vitamin D and its metabolites, a property manifested by its high-affinity binding to metabolites of vitamin D, of which one of its highest affinities is toward 25-hydroxyvitamin D_3 (25-OH-D_3), the major circulatory form of vitamin D_3 (Francheschi et al., 1981; Daiger et al., 1975; Haddad & Walgate, 1976). Recently high-affinity binding of DBP by a variety of other substrates, which include monomers of actin (Van Baelen et al., 1980; Haddad, 1982), polyunsaturated fatty acids (Williams et al., 1988; D. Z. Xiang and R. Bouillon, unpublished results), and membrane immunoglobulins of lymphocytes (Petrini et al., 1983, 1985), has been described. The full-length primary amino acid structure of hDBP has been determined recently, which shows remarkable homology with albumin and α-fetoprotein (Schoentgen et al., 1986; Cooke & David, 1985; Yang et al., 1985a).

The photoaffinity labeling technique has been used extensively to probe proteins and other biomolecules for their organelle distribution, polymorphic behavior, ligand interaction, and ligand-binding-site structure (Bayley & Knowles, 1977; Sweet & Murdock, 1987). For the past several years we have been interested in obtaining information about the ligand-binding site in DBP. To this effect, we reported the synthesis of a photoaffinity analogue of 25-OH-D_3 and successful labeling of rat serum DBP with this analogue (Ray et al., 1986). However, instability of this analogue in basic reaction conditions prompted us to develop a second-generation photoaffinity analogue of 25-OH-D_3. Recently, we reported the synthesis of 25-hydroxyvitamin D_3 3β-3′-[*N*-(4-azido-2-nitro-[3,5-^{3}H]phenyl)amino]propyl ether (^{3}H-25-ANE), a

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hydrolytically stable photoaffinity analogue of 25-OH-D_3, and described the photoaffinity labeling of rat serum DBP with this analogue (Ray et al., 1991). In this paper, we report the results of our studies involving photoaffinity labeling of purified hDBP with ^{3}H-25-ANE, site-specific cleavages of the labeled protein, and analysis of the peptide fragments to identify the putative 25-OH-D_3-binding domain in hDBP.

Materials and Methods

The photoaffinity analogue of 25-OH-D_3, i.e., 25-hydroxyvitamin D_3 3β-3′-[*N*-(4-azido-2-nitrophenyl)amino]-propyl ether (25-ANE), and its radiolabeled counterpart, i.e., 25-hydroxyvitamin D_3 3β-3′-[*N*-(4-azido-2-nitro-[3,5-^{3}H]-phenyl)amino]propyl ether (^{3}H-25-ANE) (specific activity 50 Ci/mmol), were synthesized according to the reported procedure (Ray et al., 1991). As a general rule, exposure to light was kept as low as possible during operations with ^{3}H-25-ANE. [26(27)-^{3}H]25-OH-D_3 (specific activity 20.6 Ci/mmol) and EN^{3}HANCE, the autoradiography enhancer, were obtained from Amersham Corp. (Arlington Heights, IL) and Du Pont Co. (Boston, MA), respectively. Irradiation of the samples was carried out on ice with a preequilibrated Can Rad-Hanovia low-pressure mercury arc lamp (254-nm wavelength) (Ace Glass Co., Vineland, NJ) from a height of 1–2 cm from the samples, which were kept in small petri dishes on ice. In the case of the labeling experiment on a larger scale, the sample was stirred gently with a magnetic spin bar during photolysis. Liquid scintillation counting was carried out on a Taurus liquid scintillation counter (ICN Micromedics Systems, Huntsville, AL).

DBP was obtained from pooled human serum and was purified by the published method (Van Baelen & Bouillon, 1986). Competitive binding assays were carried out with serum obtained from a norman human male according to published procedure (Skinner & Wills, 1977) with slight modifications.

Competitive Binding Assays of 25-OH-D_3 and 25-ANE with hDBP. A total of 1 mL of human serum was stirred at 4 °C with 100 mg of Norit GSX adsorbing charcoal (Hopkins & Williams Ltd., Essex, U.K.) for 30 min followed by centrifugation (3000 rpm, 4 °C, 15 min). The supernatant was diluted 5000 times with sodium barbital buffer containing BSA (Pierce Chemical Co., Rockford, IL), pH 8.6 (Chen et al., 1990). Various concentrations of either 25-OH-D_3 (0.125–8.0 nmol) or 25-ANE (0.4–161.3 nmol) in 20 μL of ethanol were added in triplicate to solutions (0.5 mL) of human serum. A solution of ^{3}H-25-OH-D_3 (2000 cpm) in 10 μL of ethanol was added to each tube. The solutions were incubated in the dark at 4 °C for 18 h followed by the addition of 200 μL of an ice-cold suspension of dextran-coated charcoal (prepared in the assay buffer without BSA). The mixtures were incubated on ice for 15 min and then centrifuged at 3000 rpm for 15 min. The supernatant from each tube was mixed with 10 mL of Instagel (Packard Instruments Co., Chicago, IL) and counted for radioactivity.

Photoaffinity Labeling Studies of hDBP with ^{3}H-25-ANE. Three tubes, each containing hDBP (20 μg in 50 μL of PBS, pH 7.4), were incubated in the dark at 4 °C with ethanolic solutions (10 μL) of samples a and b (^{3}H-25-ANE alone, 100 000 cpm) and sample c (^{3}H-25-ANE, 100 000 cpm, and 25-OH-D_3, 1 μg). After 20 h of incubation, samples b and c were irradiated on ice, with magnetic stirring, with a preequilibrated Hanovia low-pressure mercury arc lamp for 2 min, while sample a was kept in the dark. After the irradiation, electrophoresis sample buffer (30 μL), containing DTT, was added to each sample and they were heated on boiling water for 2 min. Finally, the samples, along with ^{14}C-labeled standard molecular weight marker proteins (Amersham Corp.), were electrophoresed on a 12% SDS gel according to Laemmli (Laemmli, 1970). After the run, the gel was fixed, treated with EN^{3}HANCE, dried, and exposed to Kodak XOMAT AR film at −78 °C for 4 days.

Photoaffinity Labeling of hDBP with ^{3}H-25-ANE on a Semipreparative Scale. A solution of ^{3}H-25-ANE (1 μCi, 20 nM, in 20 μL of ethanol) was added to a solution of purified hDBP (0.5 mg, 9.8 μM) in 0.5 mL of salinated phosphate buffer (PBS, pH 7.2), and the resulting solution was incubated in the dark at 4 °C for 20 h. After this period, the sample was irradiated on ice for 1 min from a height of 2 cm. The irradiated sample was diluted with 0.5 mL of PBS and 3 mL of ethanol, and the protein was precipitated by the addition of an ice-cold, concentrated solution of trichloroacetic acid (20% total concentration). After incubation on ice for 3 h, the protein was pelleted out by centrifugation. The amount of radioactivity associated with the labeled protein (0.31 μCi, 31% of the total radioactivity) was determined by dissolving it in 70% formic acid, mixing an aliquot with scintillant, and radioactive counting.

Digestion of the Photoaffinity Labeled hDBP with BNPS-skatole and Analysis of the Digest by SDS–PAGE and Autoradiography. To a solution of labeled hDBP (30 600 cpm, 50 μg) in 50 μL of 70% acetic acid was added 10 mg of BNPS-skatole (Pierce Chemical Co.) dissolved in 10 μL of glacial acetic acid. The resulting solution was flushed with argon and was incubated at 37 °C for 44 h. After this period, acetic acid was removed under argon, and the residue was analyzed on a 17.5% SDS–polyacrylamide gel. A sample of labeled hDBP (10 000 cpm, 16.3 μg) and molecular weight marker (Bethesda Research laboratory, Beltsville, MD) were also run on the gel along with the BNPS-skatole-treated sample. After the electrophoresis, the gel was fixed, stained with Coomassie Blue, destained, treated with EN^{3}HANCE and exposed to a Kodak XOMAT AR film at −78 °C for 20 days followed by developing.

Digestion of the Photoaffinity Labeled hDBP with CNBr and Analysis of the Digest by SDS–PAGE and Autoradiography. A solution of photoaffinity labeled hDBP (75 000 cpm, 123 μg) in 100 μL of 70% formic acid and 10 mg of CNBr (Aldrich Chemical Co., Milwaukee, WI) in 20 μL of 70% formic acid was incubated in the dark at 25 °C for 20 h. After this period, the solvent was removed under argon and the resulting digest was electrophoresed on a 15–24% gradient SDS–polyacrylamide gel along with ^{14}C-Rainbow molecular weight markers (Amersham Corp.). After the dye front reached the bottom of the gel, electrophoresis was stopped, the gel was fixed, stained with Coomassie Blue, destained, treated with EN^{3}HANCE, and finally dried by standard procedures. The dried gel was exposed to Kodak XOMAT AR film at −78 °C for 7 days.

Results and Discussion

Stoichiometric studies have demonstrated that DBP, which serves as the transporter of vitamin D sterols, contains one vitamin D sterol-binding site per molecule of the protein (Haddad & Walgate, 1976). Considering the normal concentration of DBP in serum (5×10^{-6} M) and circulatory concentration of 25-OH-D_3 (5×10^{-8} M), its most important ligand, it is clear that only 1–2% of the available vitamin D sterol-binding sites in DBP are occupied by vitamin D sterols at any given time. This observation has led to the speculation that DBP may serve as a reserve for vitamin D sterols to prevent their rapid tissue delivery. Alternatively, the large excess of binding sites may be occupied by less-favored ligands.

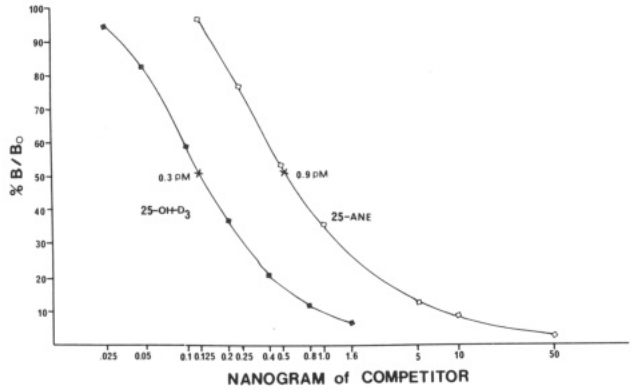

FIGURE 1: Competitive radioligand binding assays for 25-OH-D_3 and 25-ANE with hDBP. Various amounts of 25-OH-D_3 (◆) and 25-ANE (◇) were incubated in the dark with ^{3}H-25-ANE and human serum in the assay buffer. Following the incubation, unbound sterols were removed by treatment with Dextran-coated charcoal, and the supernatants were counted for radioactivity. Concentrations of 25-OH-D_3 and 25-ANE, required to displace 50%-bound ^{3}H-25-OH-D_3 from its binding site in DBP, are noted on the curves.

This is an attractive theory in light of the fact that several additional high-affinity ligands for DBP have recently been discovered. It has been observed that DBP, in conjunction with plasma gelsolin, depolymerizes potentially dangerous actin polymers, which are associated with injury and cell lysis (Lees et al., 1984). On the other hand, interaction between DBP and cell membranes has been detected in a variety of cells (Cooke & Haddad, 1989, and references cited therein). It has been speculated that DBP may be an integral part of the cytoskeleton and involved in a unique cell-surface signaling process.

Our interest in probing the ligand-binding domains of vitamin D binding proteins has led us to develop several photoaffinity labeling reagents and methods for DBP (Ray et al., 1986, 1991) and the 1,25-dihydroxyvitamin D_3 receptor (Ray et al., 1985a,b; Ray & Holick, 1988). The synthesis and application of another photoaffinity analogue of 25-OH-D_3 have also been reported (Link et al., 1987). In this paper, we describe the utility of 25-ANE, the second-generation photoaffinity analog of 25-OH-D_3, in covalently labeling the 25-OH-D_3-binding domain in human serum DBP.

Competitive radioligand binding assays of 25-ANE and 25-OH-D_3 with hDBP demonstrated that both 25-OH-D_3, the actual substrate, and 25-ANE, a substrate analogue, were capable of displacing ^{3}H-25-OH-D_3 from the binding site of the protein in a dose-dependent manner (Figure 1). Furthermore, on a molar basis, the amount of 25-ANE required to displace 50% of the specifically bound ^{3}H-25-OH-D_3 from the binding site of hDBP was approximately 3 times higher than that required for 25-OH-D_3 (Figure 1).

We have demonstrated the photolabile nature of ^{3}H-25-ANE in a recent publication (Ray et al., 1991). We have also described the dependence of covalent labeling (of rat DBP) on the duration of UV exposure. In the case of hDBP, incubation of a sample of the protein with ^{3}H-25-ANE in the dark followed by UV exposure resulted a single labeled band (M_r 51) as shown in lane 2 of Figure 2, while no labeling was observed in the absence of UV light (lane 1, Figure 2). On the other hand, covalent labeling was drastically reduced, but not totally obliterated, in the presence of a large excess of 25-OH-D_3 (lane 3, Figure 2). The presence of residual radioactivity in the protein band even in the presence of an excess of 25-OH-D_3 (lane 3, Figure 2) is, however, not unexpected and is probably caused by the weak hydrophobic interaction between the protein and ^{3}H-25-ANE molecules, displaced by

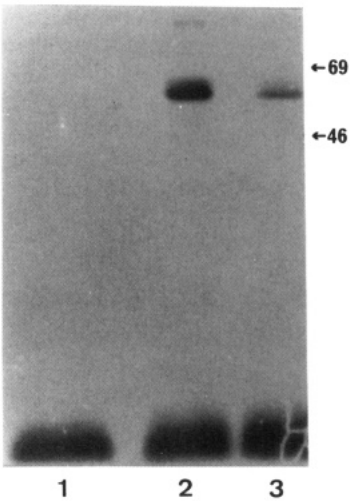

FIGURE 2: SDS–PAGE and autoradiographic analysis of samples of hDBP photoaffinity labeled with ^{3}H-25-ANE. Samples of hDBP were incubated in the dark with either ^{3}H-25-ANE alone (samples a and b) or with ^{3}H-25-ANE and a large excess of 25-OH-D_3 (sample c). Sample a was kept in the dark, while samples b and c were irradiated for 2 min. The samples were then electrophoresed and autoradiographed. The lanes represent (1) sample a, (2) sample b, and (3) sample c. Positions of standard molecular weight marker proteins are marked on the right.

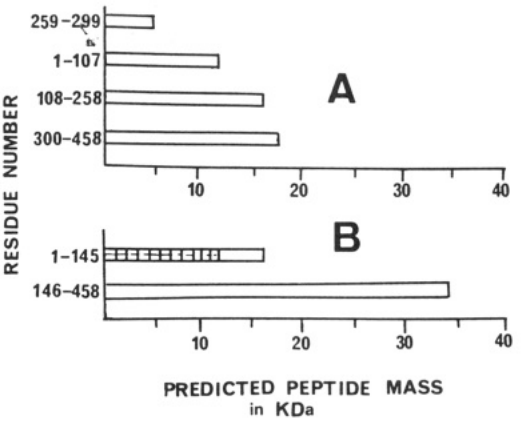

FIGURE 3: Predicted points of cleavage and lengths of the peptide fragments obtainable from CNBr (A) and BNPS-skatole (B) cleavages of hDBP, based on its amino acid sequence. The shaded area represents the overlap in the amino acid sequences of fragments 1–145 (B) and 1–107 (A).

25-OH-D_3 from its binding pocket. These results strongly suggest that ^{3}H-25-ANE, an analogue of 25-OH-D_3, was capable of covalently labeling the 25-OH-D_3-binding site in hDBP.

Our next task was to locate the particular region in the DBP molecule where the majority of the covalent labeling took place. The knowledge of the primary amino acid structure of hDBP enabled us to choose reagents suitable to cleave the labeled protein at specific sites as well as to predict the molecular weights of the peptide fragments obtainable from such treatments. The expected site-specific cleavage patterns (by BNPS-skatole and CNBr) of hDBP, as well as the expected lengths and molecular weights of the peptide fragments, are shown graphically in Figure 3.

Digestion of the labeled protein with BNPS-skatole, a tryptophan-specific reagent (Fontana, 1972), produced two Coomassie Blue stained peptide fragments, 16.5 and 35.5 kDa (lane 1, Figure 4A), in an expected manner. Importantly, the corresponding fluorogram (lane 1, Figure 4B) contained a single radiolabeled band corresponding to the smaller fragment

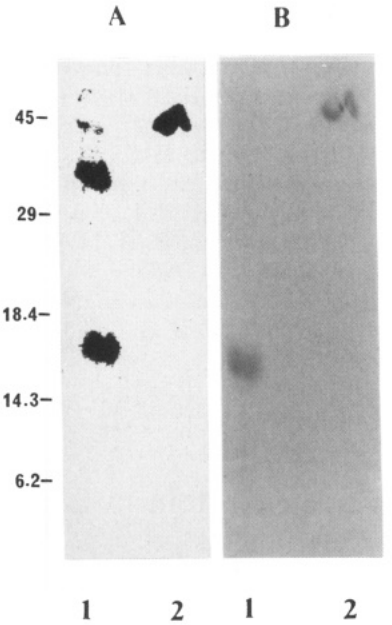

FIGURE 4: SDS–PAGE and autoradiographic analysis of the BNPS-skatole digest of a sample of hDBP photoaffinity labeled with ^{3}H-25-ANE: (A) the gel stained with Coomassie Blue; (B) autoradiogram. The lanes represent (1) the BNPS-skatole digest and (2) photoaffinity labeled hDBP. Positions of the standard protein molecular weight markers are noted on the left.

(16.5 kDa). According to the predicted cleavage pattern of hDBP (Figure 3), this labeled peptide represented the portion of the hDBP molecule that spans the amino terminus to the residue 145.

On the other hand, cleavage of the protein with CNBr, a methionine-specific reagent (Allen, 1981), was expected to produce four peptides with predicted molecular masses of 4.7, 11.8, 17, and 17.7 kDa (Figure 3). When the labeled protein was digested with CNBr, and the digest was analyzed on a 15–24% denaturing SDS gel along with standard protein molecular weight markers, three Coomassie blue stained bands were obtained (lane 2, Figure 5A). Although peptides 4.7 kDa and 11.8 kDa clearly separated in this gel, the peptides 17 kDa and 17.7 kDa overlapped, not unexpectedly, to a relatively broad peptide band (lane 2, Figure 5A). Most importantly, the majority of the covalently attached radioactivity migrated to the peptide band 11.5 kDa (lane 2, Figure 5B). According to Figure 3, this CNBr fragment represented the residue 1–107 part of the peptide. This finding is consistent with the results obtained from the BNPS-skatole experiment, in which a residue 1–145 peptide was found to be labeled by ^{3}H-25-ANE. Therefore, our results strongly suggest that the 11.5-kDa peptide, which encompasses the N-terminus through residue 107 of the protein, contained the putative 25-OH-D_3-binding site.

It is well recognized that DBP is the third member of the albumin and α-fetoprotein gene family, and considerable amino acid and nucleotide homologies exist among these proteins (Yang et al., 1985b). All of them are rich in aspartic acid, glutamine, and cysteine (as disulfide bonds) residues. Moreover, the positions of the cysteine residues are highly conserved among these proteins. Some important differences, however, do exist. The presence of a tryptophan residue at position 145 in hDBP is noteworthy. DBP also contains two additional cysteines at positions 13 and 59. The disulfide linkage between these cysteine residues provides an extra folding in the first internal domain of this highly folded and symmetrical molecule. Interestingly, our results strongly

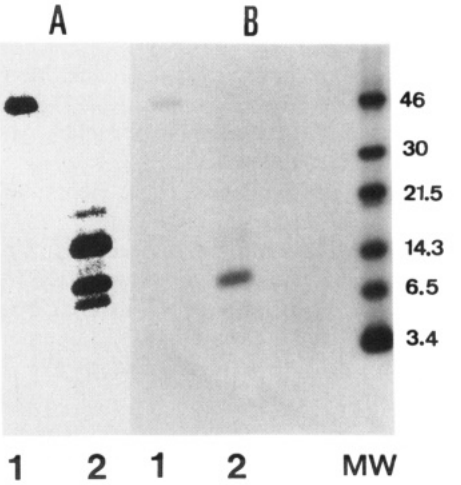

FIGURE 5: SDS–PAGE and autoradiographic analysis of the CNBr digest of a sample of hDBP photoaffinity labeled with ^{3}H-25-ANE: (A) Coomassie Blue stained gel; (B) autoradiogram. The lanes represent (1) ^{3}H-25-ANE-labeled hDBP and (2) ^{3}H-25-ANE-labeled hDBP + CNBr. The lane marked MW shows the positions of ^{14}C-labeled standard molecular weight proteins.

suggest the location of the 25-OH-D_3-binding site specifically in this part of the molecule.

We are currently engaged in the isolation of the CNBr-labeled peptide to obtain its amino acid sequence. It will provide an unambigous identity of this peptide. Obviously we will cleave this peptide further, which will provide us with a more focused identity of the 25-OH-D_3-binding site in hDBP.

In conclusion, we have developed a highly site-specific photoaffinity labeling reagent for hDBP and have identified a putative 25-OH-D_3-binding site in it.

Registry No. 25-OH-D_3, 19356-17-3; 25-ANE, 133191-08-9.

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Fluorescence Studies on the Interactions of Myelin Basic Protein in Electrolyte Solutions†

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ABSTRACT: This paper examines the influence of electrolytes on fluorescence spectral properties of the single tryptophanyl residue, Trp-115, within the 18.5-kDa species of myelin basic protein from bovine brain. Steady-state fluorescence spectra and intensities and time-correlated fluorescence lifetimes increased in the presence of increasing concentrations of mono- and divalent electrolytes (Li^+, Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^-, ClO_4^-, SO_4^{2-}, and PO_4^{3-}). In all cases, the increases closely paralleled the ionic strength of the bulk aqueous medium and resembled that observed upon immersion of the protein in solutions of urea. This behavior was therefore concluded to reflect changes in the solution conformation of myelin basic protein. Bimolecular quenching of Trp-115 by acrylamide was rapid (10^9 M^{-1} s^{-1}), approaching the diffusion limitation, and markedly dependent on the viscosity of the bulk aqueous medium. Rotational depolarization of myelin basic protein was rapid ($\phi \leq 1$ ns), occurring at rates exceeding those predicted for a rigid particle of revolution, and markedly dependent on the viscosity of the surrounding medium. Whereas the bimolecular quenching constants were unaltered in the presence of electrolytes, rotational depolarization of myelin basic protein underwent substantial slowing as indicated by the appearance of an additional decay component characterized by a correlation time of 5-10 ns. These studies indicate that Trp-115 of myelin basic protein is readily accessible to the bulk aqueous medium and is associated with a highly mobile segment of the protein. The slowing of rotational depolarization upon immersion of myelin basic protein in electrolyte solutions is consistent with an electrolyte-induced self-association of myelin basic protein molecules and indicates a relationship between the lability of solution conformation on the one hand and the capacity for self-association on the other.

Myelin basic proteins are a class of cytoplasmic species that are synthesized in Schwann cells and oligodendrocytes and are present in myelin as physically distinct molecular forms of differing molecular weights. These species arise through alternative splicing of a single primary gene transcript (de Ferra et al., 1985; Kamholz et al., 1986, 1988; Lemke, 1988; Campagnoni & Macklin, 1988), are uniformly small, appearing as 14-, 17-, 18.5-, and 21-kDa species, and highly basic, with isoelectric points estimated to be greater than 10, and are required for myelination (Lemke, 1988). They are localized to the cytoplasmic appositions, referred to as *major dense lines*, and are therefore confined to the interior of the cell. As a consequence of their highly basic properties, it might be expected that the ionic composition of the cytoplasmic environment influences myelin basic protein with respect to conformation and function.

This question is of interest in that unlike a number of enzyme species, for which functional responses can be examined under differing physical conditions, myelin basic protein displays no readily discernible functional index. It is clear that myelin basic protein is required for normal myelination within the central nervous system. While the genetics underlying expression of myelin basic protein are becoming increasingly understood (Lemke, 1988; Campagnoni & Macklin, 1988), the physical determinants governing its structure and function with respect to the wrapping and compaction of myelin remain unknown. What is known is that, as discerned from studies of intrinsic viscosity (Epand et al., 1974), low-angle X-ray scattering (Krigbaum & Hsu, 1975), and circular dichroism (Gow & Smith, 1989), myelin basic protein in solution displays a near-random-coil configuration and a concentration-dependent capacity to undergo self-association in the absence

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