Electronic Spectroscopy and Deuteration Kinetics of Tyrosine and Tryptophan Residues: An Application to the Study of Erabutoxin *b*†

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ABSTRACT: The fluorescence increase on the deuterium oxide addition to the solvent medium was studied in various tryptophan- (or indole-) and/or tyrosine-containing model compounds. It was shown how the rates of the deuteration at the indole NH group of tryptophan and at the OH group of tyrosine could be followed independently of each other. The method was applied to a study of erabutoxin *b* molecule, a neurotoxic protein from a sea snake, to analyze the microenvironments of its single tryptophan and tyrosine residues. It was shown that the "functionally invariant" single tryptophan residue was exposed to the solvent in the surface of the molecule and that the "structurally conserved" single tyrosine residue was buried in the molecule. The rate of deuteration of the tyrosine residue (80 s^{-1} at pH 6.3 and 33 °C) was 1/20 of that of an exposed tyrosine. It was also found that the amino group of Lys-27 quenched the fluorescence of Trp-29 but its deuteration had no effect on the fluorescence.

This paper deals with the deuteration rates of tryptophan and tyrosine residues in a protein molecule. The deuteration rate of a tryptophan residue can be determined by an ultraviolet absorption measurement (Nakanishi et al., 1978). A slight blue shift of the 290-nm band (L_b) on deuteration of the indole NH group is the basis of the measurement. A coexisting tyrosine residue does not affect the measurement, because its absorption (280 nm) is located sufficiently away from the wavelength used (292 nm). The deuteration rate of a tyrosine residue can also be determined by ultraviolet absorption, provided that the sample protein has no tryptophan residue (Nakanishi & Tsuboi, 1978a). The present paper describes a method to examine the OH → OD kinetics of a tyrosine residue in a protein molecule *with* tryptophan residues. The method consists of a measurement of fluorescence (~340 nm) intensity after the addition of deuterium oxide to a protein solution in a stopped-flow device.

After the method was tested on a number of compounds with tyrosine and tryptophan residues, it was applied to a study of erabutoxin *b*, a neurotoxic protein from the venom of the sea snake *Laticauda semifasciata* (Tamiya & Arai, 1966). It is a protein which binds to the nicotinic acetylcholine receptor at the neuromuscular junction to block the stimulus transmission across the synapse (Tamiya & Arai, 1966). The protein molecule has one tyrosine (Tyr-25) and one tryptophan (Trp-29) residue (Sato & Tamiya, 1971; Endo et al., 1971; Maeda & Tamiya, 1977). There are about 120 sea snake and cobra venom components, including 60 neurotoxins, which are known to have homologous amino acid sequences. Tyrosine residues (Tyr-25) are found at corresponding positions of almost all of these venom components except those of two neurotoxins (Maeda & Tamiya, 1978) and of eight nonneurotoxic components. Phenylalanine residues occupy the positions in these exceptional cases. The Tyr-25 residue, or at least its benzene ring, is, therefore, considered to be "structurally conserved" among these homologous venom components. On the other hand, Trp-29 seems to be "functionally invariant", because it is found in all of the so far known neurotoxins but in none of the other homologous nonneurotoxic components. The microenvironments of these residues are of special interest in relation to their roles in the molecule.

Experimental Procedures

Materials. L-Tryptophan, tryptamine, indole, indoleacetic acid, indolebutyric acid, glycyl-L-tryptophan, 5-methylindole, 2-methylindole, acetyl-L-tryptophan, indole-3-ethanol, and formyl-L-tryptophan were obtained from Wako Pure Chemical Industries Ltd. *tert*-Butyloxycarbonyl-L-tryptophan (Boc-Trp)[1] and luteinizing hormone–releasing hormone (LH–RH) were purchased from Protein Research Foundation, Osaka, Japan. L-Tryptophyl-L-tyrosine was prepared by the Sagami Central Research Laboratory, Kanagawa, Japan, and was placed at our disposal by the courtesy of Professor A. Wada, University of Tokyo. *tert*-Butyloxycarbonyl-L-tryptophyl-L-tyrosine was prepared from L-tryptophyl-L-tyrosine (Nagasawa et al., 1973; Itoh et al., 1975). Deuterium oxide (99.75 atom %) was purchased from CEA-CEN-SACLAY.

Erabutoxin *b* was isolated and purified from the venom of *L. semifasciata* as described previously (Tamiya & Arai, 1966; Tamiya & Abe, 1972). [27-Acetyllysine]erabutoxin *b* was prepared and isolated as described previously (Hori & Tamiya, 1976).

Methods. The hydrogen ion concentration of the solution was measured with a Hitachi-Horiba F7SS pH meter. The corrections for ^2H_2O and ^2H_2O–^1H_2O mixture solvents to reach proper pH values were not made in the present work. The static fluorescence spectra were recorded by a Hitachi MPF-4 fluorometer with a 10-mm cell.

The time dependence of the fluorescence intensity was examined with a Union Giken stopped-flow spectrophotometer, RA-401. This apparatus was equipped with a rapid-mixing device of a dead time of 0.5 ms, a deuterium discharge lamp (200 W), and an ultraviolet monochromator. After rapid mixture with ^2H_2O, the sample solution was led into a 2-mm cell, and the time-dependent fluorescence change was followed by the stopped-flow method. The fluorescence was excited by a monochromatic light and observed through a Hoya UV

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[1] Abbreviations used: Boc-Trp, *tert*-butyloxycarbonyl-L-tryptophan; Boc-Trp-Tyr, *tert*-butyloxycarbonyl-L-tryptophyl-L-tyrosine; LH–RH, luteinizing hormone–releasing hormone.

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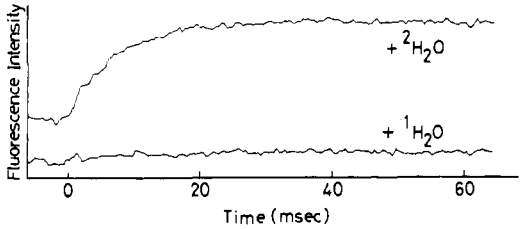


FIGURE 1: Time course of the fluorescence increase observed when tryptamine dissolved in ^1H_2O (0.14 mM; pH 6.6; 24 °C) is mixed with ^2H_2O (1:1 v/v; final concentration of tryptamine is 0.07 mM). Lower portion: a recorded curve in our control experiment in which the fluorescence was observed when the same tryptamine solution as above was mixed with ^1H_2O, instead of ^2H_2O. The fluorescence was excited at 280 nm. The total fluorescence with wavelengths longer than 300 nm was observed.

30 filter, which allowed the light with wavelengths longer than 300 nm to come into the detector. In some cases another filter, Hoya UV 33, which allowed the light with wavelengths longer than 330 nm to come through, was also used. This is for a partial discrimination of the fluorescence of tryptophan and of tyrosine. The fluorescence intensity vs. time curve was obtained by a Union Giken data processor, RA-450, a monitorscope, and an XY recorder.

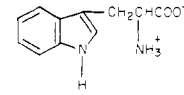
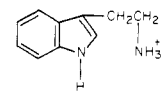
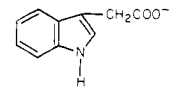
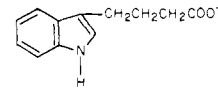
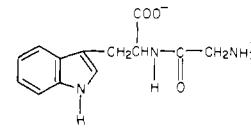
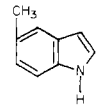
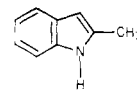
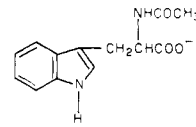
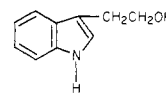
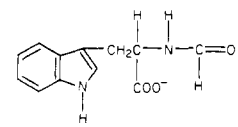
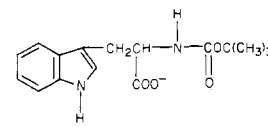
For a stopped-flow ultraviolet absorption study, the same rapid-mixing device, the same light source, and the same monochromator as those for the stopped-flow fluorescence experiments were used. Only the location of the detector (photomultiplier) was changed, and the 2-mm cell was replaced by a 10-mm cell. The absorbance vs. time curve was obtained again by the Union Giken data processor, RA-450, with the monitorscope and the XY recorder.

Results

Effects of Deuteration on the Fluorescence of Tryptophan and Related Compounds with the Indole Rings. Our first question to be answered is "what kind of deuteration rate is reflected in the stopped-flow fluorescence photometry of a tryptophan residue"?

Tryptophan and other compounds with the indole rings gave fluorescence with a peak at ~350 nm when excited at 280 nm in aqueous solutions at room temperature. In ^2H_2O, the fluorescence intensity of L-tryptophan was markedly greater than that in ^1H_2O (Stryer, 1966). The intensity difference is partially ascribable to the solvent–isotope effect, which reaches its maximal value within 1 ms (Nakanishi & Tsuboi, 1978b). The remaining part of the difference is attributable to the quenching efficiency change of the α-NH_3^+ group of the compounds on deuteration, which proceeds more slowly, and this rate is considered to be equal to that of the $NH_3^+ \rightarrow ND_3^+$ deuteration (Nakanishi & Tsuboi, 1978b). In the present study, we examined the deuteration effects on the fluorescence intensity of various indole compounds. The results are summarized in Table I. All of the 13 compounds listed here show an appreciable fluorescence increase on going from the ^1H_2O solution to the ^1H_2O + ^2H_2O (1:1) solution. A slow, time-dependent fluorescence increase, however, was detected only with compounds with free α-amino groups, namely, with tryptophan and tryptamine (see Figure 1). Thus, it is clear that 1-NH deuteration of the indole ring does not cause any *time-dependent* fluorescence change [note that the 1-NH deuteration does cause a *time-dependent* absorption change (Nakanishi et al., 1978)]. In addition, the deuterations of various substituents, so far examined, of the indole ring have apparently nothing to do with the slow fluorescence increase, except the deuteration of position 3-C-C-NH_3^+.

Table I: Effects of Deuteration on the Fluorescence Intensity

compd	structure	static difference[a] (%)	kinetic difference[b]
tryptophan		37	+
tryptamine		20	+
indole		17	–
indoleacetic acid		17	–
indolebutyric acid		23	–
glycyl-tryptophan		9	–
5-methyl-indole		31	–
2-methyl-indole		36	–
acetyl-tryptophan		16	–
indole-3-ethanol		21	–
formyl-tryptophan		11	–
Boc-Trp		9	–

[a] Difference in the fluorescence intensity of a compound dissolved in ^1H_2O (2.7×10^{-6} M) and that in 1:1 mixture of ^1H_2O and ^2H_2O. The difference is given by percent increase on going from ^1H_2O to ^1H_2O + ^2H_2O. [b] The time-dependent increase in the fluorescence intensity found after a rapid mixing of the compound in ^1H_2O with the same volume of ^2H_2O. (+) Detected; (–) no increase found.

Effects of Deuteration on the Fluorescence Intensity of Tryptophyltyrosine with Its N-Terminal Blocked. Figure 2 shows the time dependence of the fluorescence intensity of Boc-Trp-Tyr on its deuteration. This intensity change was not

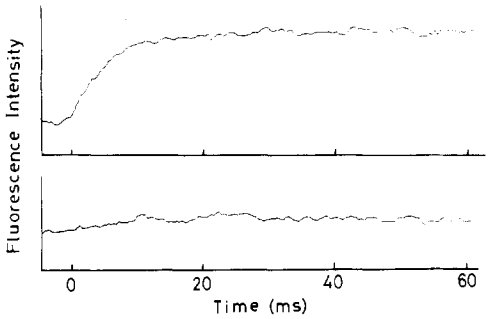


FIGURE 2: Time course of the fluorescence change observed when Boc-Trp-Tyr dissolved in ^1H_2O (0.12 mM; pH 6.3; 12 °C) is mixed with ^2H_2O (1:1 v/v; final concentration of Boc-Trp-Tyr is 0.06 mM). The fluorescence was excited at 280 nm (for upper curve) and at 295 nm (for lower curve). Each time, the total fluorescence with wavelengths longer than 330 nm was observed.

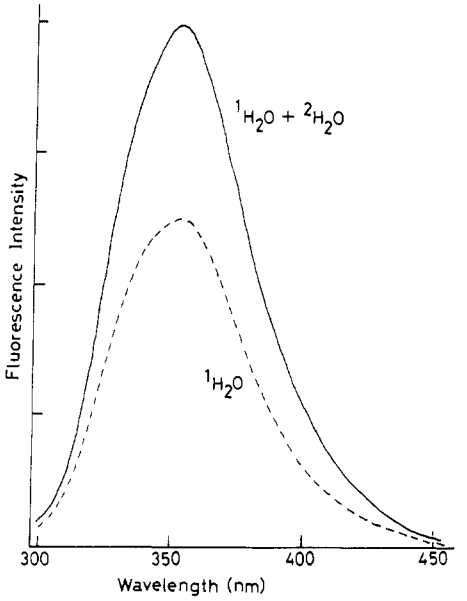


FIGURE 3: Fluorescence emission spectra of LH–RH in ^1H_2O (---) and in ^2H_2O + ^1H_2O (1:1 v/v) (—).

observed when the compound was excited with 295-nm light; it was observed only when excited with 280-nm light. The intensity change was detectable not only through a Hoya UV 30 filter but also through an UV 33 filter, suggesting the participation of the tryptophan residue in the fluorescence change. It is known that the 295-nm light can excite the tryptophan residue only, while the 280-nm light can excite both tryptophan and tryosine residues. The electronic energy of the excited tyrosine is known to be often transferred to the tryptophan in the same molecule (Eisinger, 1969). The efficiency of this energy transfer in Boc-Trp-Tyr is estimated to be 84%. The fluorescence is thus emitted mostly from tryptophan and only weakly from tyrosine. The energy transfer efficiency is probably greater if the tyrosine residue is deuterated on its phenolic oxygen. The time-dependent fluorescence increase thus reflects the deuteration time course of the tyrosine residue.

Note that Boc-Trp shows no time-dependent fluorescence increase on its deuteration. Also, the time-dependent fluorescence change on deuteration of a tyrosine derivative without a tryptophan residue was already studied in detail (Nakanishi & Tsuboi, 1979).

Deuteration Kinetics of the Tyrosine Residue in a Decapeptide. Luteinizing hormone-releasing hormone (LH–RH) is a decapeptide with each one of the tyrosine (Tyr-3) and

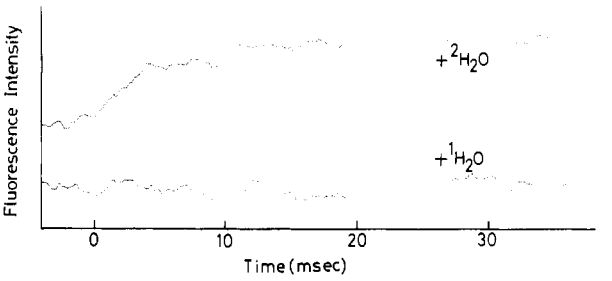


FIGURE 4: Time course of the fluorescence change observed when LH–RH dissolved in ^1H_2O (0.088 mM; pH 6.5; 11 °C) is mixed with ^2H_2O (1:1 v/v; final concentration of LH–RH is 0.044 mM). Lower portion: a recorded curve in our control experiment in which the fluorescence was observed when the same LH–RH solution as above was mixed with ^1H_2O, instead of ^2H_2O. The fluorescence was excited at 280 nm. The total fluorescence with wavelengths longer than 330 nm was observed.

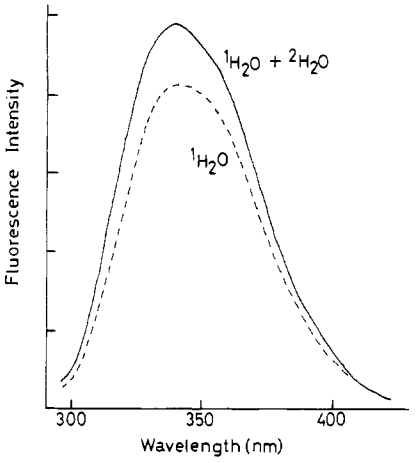


FIGURE 5: Fluorescence emission spectra of erabutoxin *b* in ^1H_2O (---) and in ^2H_2O + ^1H_2O (1:1) (—).

tryptophan (Trp-5) residues (Marche et al., 1976; Shinitzky & Fridkin, 1976). The fluorescence spectrum of this decapeptide is shown in Figure 3. As shown here, the quantum yield is greater in the deuterated form. The stopped-flow experiment on medium change from ^1H_2O to ^1H_2O + ^2H_2O (1:1 v/v) showed an appreciable time-dependent fluorescence increase when excited with 280-nm light (see Figure 4). If it is excited at 295 nm, on the other hand, no such a time-dependent fluorescence increase was observed. As the tryptophan residue is located at the middle of the peptide chain and has no free amino group, the fluorescence increase is ascribed to the deuteration of the hydroxy oxygen of the tyrosine residue. The increase rate is estimated to be 140 s^{-1} at pH 6.5 and 11 °C. This value is close to that of free tyrosine (Nakanishi & Tsuboi, 1978a), indicating that the tyrosine residue in the LH–RH molecule is well exposed to the solvent.

Deuteration Kinetics of Tyr-25 in Erabutoxin b. Erabutoxin *b* has 62 amino acid residues involving single Tyr (position 25) and single Trp (position 29). Its emission spectrum (shown in Figure 5) has a peak at 340 nm (and has no peak in 305–310-nm region) when excited at 280 nm, suggesting that a considerable amount of the electronic energy is transferred from Tyr-25 to Trp-29. Such a Tyr → Trp energy transfer in this protein was once suggested by Montenay-Garestier et al. (1978), and the efficiency for the excitation energy transfer was estimated by them to be 40%. The emission spectrum is essentially the same as that of erabutoxin *a* (Seto et al., 1970), which is a molecule with an asparagine residue at the 26th position and otherwise the same as erabutoxin *b*. When a ^1H_2O solution of erabutoxin *b* is rapidly mixed with ^2H_2O, a

FIGURE 6: Time course of fluorescence change observed when erabutoxin *b* is dissolved in ^1H_2O (1:1 v/v; final concentration of erabutoxin *b* is OD_{280} = 0.475). The fluorescence was excited at 280 nm. The total fluorescence with wavelengths longer than 300 nm was observed.

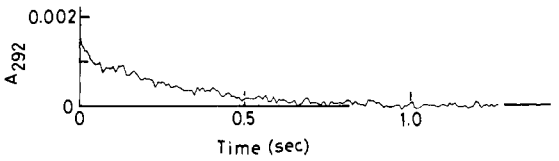


FIGURE 7: Time course of the absorbance change at 292 nm observed when erabutoxin *b* in ^1H_2O (OD_{280} = 1.5; pH 6.4; 34 °C) is mixed with ^2H_2O (1:1 v/v; final concentration of erabutoxin *b* is 0.75 OD_{280}).

time-dependent fluorescence increase is found with 280-nm excitation (see Figure 6). This is not observed, however, when the fluorescence is excited at 295 nm. The rate of the fluorescence increase, which is taken as the rate of tyrosine deuteration, is found to be 80 s^{-1} at pH 6.3 and 33 °C.

Unconcern of Lys-27 with the Time-Dependent Fluorescence Increase of Trp-29 in Erabutoxin b. There is a possibility that tryptophan fluorescence is affected not only by the deuteration of tyrosine but also by that of the nearby amino group. We know that, if an ammonium ($-NH_3^+$) is located as close as 3 Å from the position 4 carbon of the indole ring, as in the case of tryptophan [whose conformation was studied by Koshio et al. (1976)] or tryptamine, a disturbing fluorescence change is introduced due to its deuteration (see above). In the erabutoxin *b* molecule, the ε-amino group of Lys-27 bears such a possibility. However, this was not the case. A stopped-flow experiment showed that [27-N^6-acetyllysine]erabutoxin *b* gave exactly the same fluorescence vs. time curve as that of the native erabutoxin *b*, when its ^1H_2O solution was rapidly mixed with ^2H_2O. It should be mentioned also that the fluorescence of [27-N^6-acetyllysine]erabutoxin *b* was appreciably weaker than that of intact erabutoxin *b*; the former was 2/3 times as strong as the latter.

Deuteration Kinetics of Trp-29 in Erabutoxin b. As has been stated in the introduction, the deuteration of Trp-29 can be followed by a stopped-flow ultraviolet absorption measurement at 292 nm. The result is shown in Figure 7. The rate constant is estimated to be 4.3 s^{-1} at pH 6.4 and 34 °C.

Discussion

In the present study, we have established a method to trace the deuteration process of tyrosine residues in a protein which has tryptophan residues as well. The method consists of a rapid mixing of a ^1H_2O solution of the protein with ^2H_2O and a measurement of the fluorescence intensity (excited at 280 nm) as a function of time. The fluorescence comes from tryptophan, but its intensity does not depend upon the extent of the deuteration of the tryptophan residue itself; it depends upon the extent of the deuteration of the tyrosine residue. This method works as long as the Tyr → Trp intramolecular energy transfer takes place and no disturbing ammonium group (NH_3^+) is located in the vicinity of the indole ring of the tryptophan residue. The method requires only a small amount of the protein sample, 0.1 mg or so.

For erabutoxin *b*, new pieces of information have been obtained as for the environments of Tyr-25 and Trp-29 in the molecule. The observed deuteration rate (80 s^{-1} at pH 6.3 and 33 °C) of Tyr-25 is only 0.05 times as high as the rate of free tyrosine [1600 s^{-1} at pH 6.3 and 33 °C (Nakanishi & Tsuboi (1978a)]. This fact suggests that Tyr-25 is buried in the molecule, so that the solvent (^2H_2O) is not readily accessible. However, the deuteration of Tyr-25 in the erabutoxin *b* molecule requires a greater activation free energy than the deuteration of free tyrosine. In other words, to bring Tyr-25 to the completely exposed state, $-RT \ln 0.05$ = 1.8 kcal/mol of free energy is needed (where R is the gas constant and T, the absolute temperature, = 306 K). This result is consistent with the fact that the tyrosine residue of erabutoxin *b* is not readily modified (Sato & Tamiya, 1970). A nuclear magnetic resonance study (Inagaki et al., 1978) on erabutoxin *b* also showed that the tyrosine residue is buried in the molecule. Trp-29, on the other hand, seems to be exposed in the surface of the molecule. Its deuteration rate (4.3 s^{-1} at pH 6.4 and 34 °C) is nearly equal to that of free tryptophan [4.8 s^{-1} at pH 6.4 and 34 °C (Nakanishi et al., 1978)]. These findings are inconsistent with what was previously found by fluorescence study (Seto et al., 1970), by chemical modification study (Seto et al., 1970), by NMR study (Inagaki et al., 1978), and by X-ray crystallography (Low et al., 1976; Tsernoglou & Petsko, 1977; Kimball et al., 1979).

Lastly, a comment is to be added on Lys-27. The present study showed that the ε-NH_3^+ of Lys-27 was located sufficiently close to the indole ring of Trp-29, so that it decreased the fluorescence of Trp-29 to some extent (directly or indirectly through, for example, Glu-38). The location and/or the orientation of the ε-NH_3^+ group with respect to the indole ring, however, must be different from those of the α-NH_3^+ group of tryptophan (or tryptamine) with respect to its indole ring. The result agrees with the structure given by the X-ray crystallographers (Low et al., 1976; Tsernoglou & Petsko, 1977; Kimball et al., 1979).

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Nuclear Magnetic Resonance Determination of Metal–Proton Distances in the EF Site of Carp Parvalbumin Using the Susceptibility Contribution to the Line Broadening of Lanthanide-Shifted Resonances†

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ABSTRACT: The substitution of the paramagnetic lanthanide ion ytterbium for the calcium ion in the EF calcium binding site of carp parvalbumin results in a series of ^{1}H NMR resonances which are shifted well outside of the envelope of the ^{1}H NMR spectrum of the diamagnetic form of the protein. The line broadening of these shifted resonances has been measured as a function of ^{1}H NMR frequency between 200 and 400 MHz, and the spin–lattice relaxation rates have been measured at 270 MHz. The analysis of the relaxation rates based upon the theories of Vega & Fiat [Vega, A. J., & Fiat, D. (1976) *Mol. Phys. 31*, 347–355] and Guêron [Guêron, M. (1975) *J. Magn. Reson. 19*, 58–66] indicates that a major contribution to the line widths comes from the novel susceptibility relaxation mechanism and that the metal to proton distances can be directly calculated from this contribution.

Calcium binding proteins play an important role in the regulation of many biochemical processes (Kretsinger, 1976; Rasmussen et al., 1972). Among the most studied of these proteins are the skeletal and cardiac troponins (Potter et al., 1977) and the myosin light chains (Weeds et al., 1977) which are involved in the regulation of muscle contraction and calmodulin which is involved in the regulation of bovine brain phosphodiesterase (Lin et al., 1974), brain adenylate cyclase (Westcott et al., 1979), chicken gizzard myosin light-chain kinase (Dabrowska et al., 1978), rabbit skeletal myosin light-chain kinase (Yagi et al., 1978), and a number of other enzymes [for a recent review, see Cheung (1980)]. The elucidation of the X-ray structure of the calcium binding protein parvalbumin from carp revealed that its two calcium binding sites are each completely formed from a contiguous polypeptide sequence folded into the homologous "CD and EF hands" (Kretsinger & Nockolds, 1973). Each homologous calcium binding domain contains in turn a helix, a loop around the metal ion, and a second helix. The loop around the metal ion contains regularly spaced liganding carboxyl, carbonyl, or hydroxyl ligands. Homologous sequences to parvalbumin (Kretsinger, 1976) can be found in rabbit skeletal troponin C and myosin alkali light chains (Collins, 1974; Weeds & McLachlan, 1974), DTNB light chains (Collins, 1976), bovine calmodulin (Vanaman et al., 1977; Stevens et al., 1976), rat testis calmodulin (Dedman et al., 1978), bovine intestinal calcium binding protein (Fullmer & Wasserman, 1977), porcine intestinal calcium binding protein (Hofmann et al., 1979), and other calcium binding proteins. The number of times in a given protein the sequence repeats and the substitutions therein can be correlated with the number of metals bound to the protein and their binding strengths, respectively. These findings have lead to the proposal that homologous structures, at least at the level of the calcium binding sites, exist for all these proteins.

In a separate paper (Lee & Sykes, 1980b) we have described the strategy of a NMR methodology, the final goal of which is to compare in detail protein structures in solution This technique will enable us to test the hypothesis that all of these calcium binding proteins have homologous structures. The technique is based upon the substitution of paramagnetic lanthanide ions for the calcium ions and the subsequent analysis in structural terms of the shifts and broadenings induced in the NMR spectrum (^{1}H NMR in this specific example). Our approach is to study carp parvalbumin initially and to use the known X-ray structure of this protein to determine the unknown parameters of the NMR experiment which are required before the shifts and broadenings can be interpreted in terms of the structure of the protein. With these parameters and the knowledge of the amino acid substitutions for different proteins, we will then be able to compare the calculated and observed NMR spectra of a new protein as a probe of its structure.

In this paper we focus on the analysis of the line broadening of the lanthanide-shifted ^{1}H NMR resonances as a method for the determination of metal–proton distances in the EF site

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