Proton Nuclear Magnetic Resonance Study of Human Plasma α-2-Macroglobulin

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ABSTRACT: A proton nuclear magnetic resonance (NMR) study is reported of human α-2-macroglobulin (α-2-M). It was observed that α-2-M, which consists of four identical subunits and has a molecular weight of 720 000, gives several sharp resonances. After cleavage of the "bait" region peptide with trypsin and subsequent removal of the peptide under a high salt condition, most of the sharp resonances disappeared, indicating that the sharp resonances observed in the native α-2-M originate from the amino acid residues in the bait region. Resonances due to the aromatic protons of the Tyr residue, which exists in the bait region, have been assigned on the basis of chemical shift. It was observed that the C3– and C5–H proton resonances for the Tyr residue are especially narrow, indicating that the side chain of the Tyr residue in the bait region is in a highly mobile state. Photochemically induced dynamic nuclear polarization experiments clearly show that the Tyr residue is actually exposed to the solvent. It was possible to identify resonances due to several His residues that are exposed to solvent. Other resonances, which probably originate from Arg residues in the bait region, were also observable in the conventional NMR spectra. On the basis of the present NMR data, we conclude that the bait region of the native α-2-M is highly flexible and exposed to solvent. On treatment of α-2-M with methylamine, no significant change has been detected in the NMR spectra observed in both the conventional and CIDNP mode. This result shows that inactivation of α-2-M with methylamine does not induce any major change in the conformation of the bait region. On the basis of the present results along with those reported previously we suggest that the conformation change induced by methylamine results in sequestration of the bait region, which still keeps its flexibility but becomes inaccessible to the proteinases due to steric reason. We also suggest that the inherent flexibility observed in the present work is responsible for the susceptibility of the bait region to the proteolytic attack.

α-2-Macroglobulin, which will hereafter be abbreviated as α-2-M,[1] is an endoproteinase inhibitor that exists in human serum. It is composed of four identical polypeptide chains with molecular weight of 180 000 that are disulfide bonded in pairs (Harpel, 1973; Hall & Roberts, 1978; Swenson & Howard, 1979; Barrett, 1981). α-2-M binds and inhibits a broad spectrum of endoproteinases through a unique mechanism called the "trapping" hypothesis, which was proposed by Barrett et al. (Barrett & Starkey, 1973) and subsequently elaborated by many other workers (Harpel, 1973; Salvenson & Barrett, 1980). One important feature of this mechanism is that the trapped proteinase molecules still retain their activity to hydrolyze low molecular weight substrates (Barrett & Starkey, 1973). Proteinases are known to cleave peptide bonds in the "bait" region of the α-2-M molecule (Salvenson & Barrett, 1980). It was suggested that a reactive "thiol ester" bond that is presumed to exist in the α-2-M molecule is then spontaneously cleaved and this results in a conformational change of α-2-M, leading to trapping of the proteinase molecules that happened to be around (Howard et al., 1980).

It is known that on treatment with methylamine the reactive thiol ester bond is cleaved, resulting in a complete loss of proteinase inhibitor activity (Gonias et al., 1982). Polyacrylamide gel electrophoresis (Barrett et al., 1979), electron microscopy (Tapon-Bretaudiere et al., 1985; Nishigai et al., 1985), and circular dichroism (Gonias et al., 1982) have been used to demonstrate that a significant change of overall conformation is induced on the methylamine treatment.

The primary structure of α-2-M with 1451 amino acid residues has been determined by Sottrup-Jensen et al. (1983). The bait region comprises about 25 amino acid residues that exist between positions 675 and 700. The amino acid sequence of the bait region is so arranged that it can serve as substrate for endoproteinases of different specificities (Sottrup-Jensen et al., 1981b; Mortensen et al., 1981; Hall et al., 1981). It is presumed that a thiol ester bond is formed between Cys-948 and Gln-951 (Swenson & Howard, 1980; Sottrup-Jensen et al., 1981a). There is no direct evidence about the presence of the thiol ester bond in α-2-M molecule. However, a large amount of indirect evidence has been accumulated up to this point, leaving little doubt about the presence of the thiol ester bond (Howard, 1983).

In the present work, ^{1}H NMR was employed for the analyses of structure–function relationships of human serum α-2-M. A technique of spin diffusion, which has been shown to be quite useful in obtaining information concerning the flexible part of large protein molecules (Endo & Arata, 1985; Muto et al., 1985), will be extensively used. On the basis of the results of NMR measurements in the conventional and photochemically induced dynamic nuclear polarization (Kaptein et al., 1978a,b) modes, we discuss the structure of the bait region of the α-2-M molecule in the intact and methylamine-treated α-2-M.

Materials and Methods

Materials. α-2-M. Human α-2-M was purified from the fresh blood donated by a young Japanese male according to the method of Kurecki et al. (1979). After it was confirmed that the protein purified from pooled blood gave the same NMR spectrum as the one from fresh blood, the protein was periodically purified from pooled blood given by a local hospital. The purification procedure involved a metal chelate chromatography as a key step. We prepared Zn^{2+} chelate columns according to Kurecki et al. (1979). The purity of the protein was better than 99% when checked by polyacrylamide

[1] Abbreviations: α-2-M, α-2-macroglobulin; CIDNP, chemically induced dynamic nuclear polarization; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; PMSF, phenylmethanesulfonyl fluoride.

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gel electrophoresis in the presence of sodium dodecyl sulfate (Laemmli, 1970). The activity of the purified α-2-M was determined from the stoichiometry of inhibited trypsin per mole of the inhibitor.

Proteinases. Bovine pancreatic trypsin was purchased from Sigma Chemical Co. (St. Louis, MO). The specific activity of trypsin was determined by the method of Chase and Shaw (1967).

Chemicals. Phenylmethanesulfonyl fluoride (PMSF) was purchased from Bethesda Research Laboratories (Cambridge, MA).

Sample Preparations for ^1H NMR Measurements. α-2-M solutions containing about 10 mg of protein were concentrated on a Toyo UK-200 ultrafilter membrane (Toyo filterpaper, Tokyo) to 0.3 mL, and the buffer was replaced with deuterated phosphate buffer (0.4 M NaCl, 10 mM sodium phosphate). The pH titration of α-2-M solution was made by adding 0.3 M DCl to a D_2O solution of α-2-M at pH 7.6. All pH values reported in this paper were uncorrected meter readings of D_2O solutions with an electrode standardized for H_2O buffers.

Reaction with Trypsin. Lyophilized trypsin was added to α-2-M/D_2O solution (pH 7.2) and incubated for 10 min at room temperature.

Methylamine Treatment. An equivolume of 100 mM methylamine was added to a concentrated α-2-M solution, and pH was adjusted to 8.5; then, the solution was incubated for 18 h at 4 °C. It was confirmed by HPLC on a G4000SW column that the reaction of α-2-M with methylamine had proceeded completely (Nishigai et al., 1985). The solution was reconcentrated and replaced with D_2O as described above.

Removal of the Bait Region Peptide. The bait region peptide was removed from trypsin-reacted α-2-M as follows. After treatment with 2.2 mol equiv of trypsin, α-2-M solution was diluted with 0.6 M NaCl/phosphate buffer, incubated for 10 min at room temperature, and then concentrated on Toyo UK-200 ultrafiltration membrane. The solvent was repeatedly replaced with deuterated phosphate buffer. During the procedure, the peptide became dissociated from α-2-M and passed through the membrane, leaving the α-2-M solution free of the peptide. The ultrafiltrate was collected, and the bait region peptide was purified by reversed-phase HPLC on a Toyo ODS-120T column. The peptide collected was subjected to amino acid composition and ^1H NMR analyses.

Inactivation of Trypsin with PMSF. When the ^1H NMR spectrum of trypsin was measured, trypsin was treated with PMSF to protect the protein from autolysis. PMSF (50 mM) in dimethylformamide was added to trypsin solution in pH 7.2 phosphate buffer in a volume ratio of 1:50, and incubated for 15 min at 37 °C. The protein was then freeze-dried and dissolved in D_2O for NMR measurement.

^1H NMR Measurement. ^1H NMR spectra were recorded on a Bruker WM-400 spectrometer operating at 400 MHz in the Fourier transform mode. The 8K data points were acquired with a spectral width of ±2500 Hz. A large flip angle of 80–90° was employed for the measurements, which is optimum for most signals at a sacrifice of the fidelity for some of the signals with longer T_1. All chemical shifts are given (ppm) from external DSS (5% in D_2O). NMR measurements were made at 25 °C. Photochemically induced dynamic nuclear polarization difference spectra of α-2-M and 0.2 mM 3-*N*-(carboxymethyl)lumiflavin were obtained as described by Kaptein et al. (1978a).

Results and Discussion

An example of ^1H NMR spectra of the intact α-2-M is reproduced in Figure 1. It should be noted that there are several resonances that are unusually narrow in line width for a protein with a molecular weight of 720000. The chemical shift values indicate that at least Tyr, His, Arg, and Ala residues are responsible for the observed resonances.

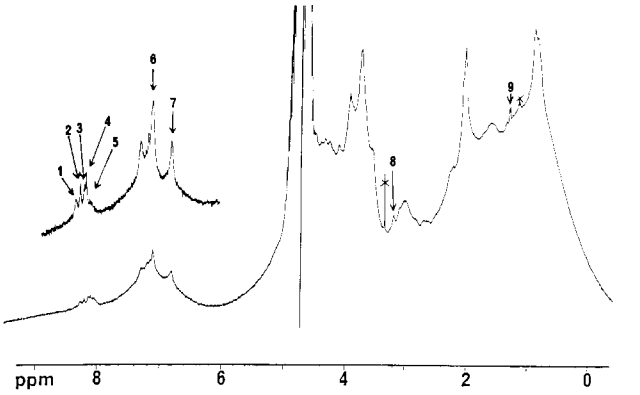

FIGURE 1: 400-MHz ^1H NMR spectra of human α-2-M (10 mg in 0.3 mL of 400 mM NaCl/D_2O, 10 mM phosphate, pH 6.7). The HDO peak was presaturated in gated decoupling mode for 0.5 s at a radiofrequency level of $\gamma(H_2)/2\pi = 70$ Hz, when the inset spectrum was recorded. The free-induction decay was recorded with 8K data points and a spectral width of ±2500 Hz. A total of 3000 transients was accumulated. Chemical shifts are (ppm) from external DSS (5% in D_2O). The probe temperature was 25 °C. The peaks marked × are due to low molecular weight contaminants.

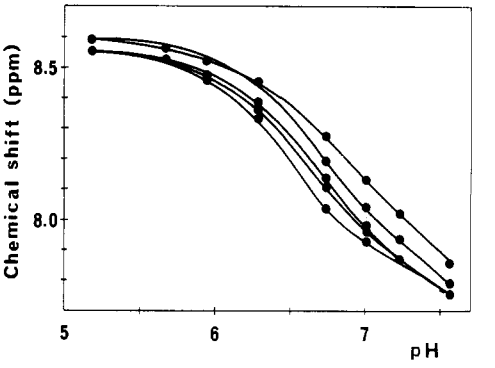

FIGURE 2: pH dependence of the chemical shifts of the C2–H proton of His residues observed for human α-2-M. Spectral conditions employed to collect the titration data are as described in Figure 1.

The pH dependence of the chemical shifts of resonances observed between 7.7 and 8.7 ppm is plotted in Figure 2. This result indicates that resonances 1–5 in Figure 1 are due to the C2–H protons of His residues. Above pH 9, resonances 6 and 7 were observed to shift downfield. This result clearly shows that resonances 6 and 7 originate from a Tyr residue. It is likely that resonances 8 and 9 are due to Arg and Ala residues from their chemical shift data and, in the case of resonance 9, from spin coupling constant data (Bundi & Wüthrich, 1979).

In order to confirm that signals 6 and 7 are actually due to four protons of a single Tyr residue, we measured the area under respective peaks as well as the total area for the aromatic envelope using a Numonix 1250 planimeter. The combined area obtained for the two peaks is 1.2 ± 0.2% of that for the total area. The area under peak 6 was multiplied by $^2/_3$, because the C4–H proton of a His residue is superimposed on it. The expected contribution of four protons of a Tyr residue as calculated from the amino acid composition of α-2-M is 0.6%. The agreement between experimental and calculated results is acceptable for the following reasons. First, spectral conditions used in the present study are by no means ideal for reproducing the right intensities for all the signals. Since α-2-M is not a stable protein, we had to finish data collection

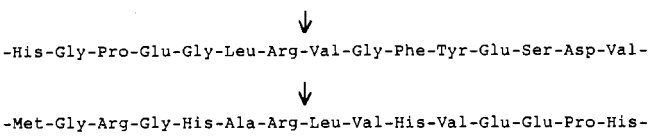

FIGURE 3: Amino acid sequence of the bait region of human α-2-M (Mortensen et al., 1981). Two peptide bonds cleaved by trypsin are shown with arrows.

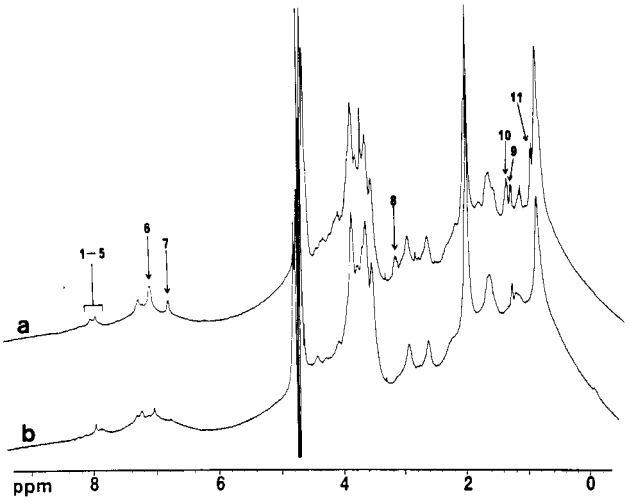

FIGURE 4: 400-MHz ^{1}H NMR spectra of α-2-M reacted with trypsin. The HDO peak was presaturated in gated decoupling mode for 0.5 s at a radiofrequency level of $\gamma(H_2)/2\pi$ = 70 Hz. Spectra were recorded at pH 7.2 before (a) and after (b) the removal of the bait region peptide. Trypsin was added at a molar ratio of 2.2:1.0 (trypsin/α-2-M). The reaction with trypsin and the removal of peptide were done as described in text. Totals of 3000 and 800 transients were accumulated for spectra (a) and (b), respectively. A line broadening of 1 Hz was applied prior to Fourier transformation. Other spectral conditions are as described in Figure 1.

as soon as possible after the sample preparation. A large flip angle of 80–90° was employed along with a short repetition time for the measurements, which appears to be optimum for most signals at a sacrifice of the fidelity of intensity for some of the signals with longer T_1. T_1 for the C2–H and C4–H protons of His residues of peptides and proteins are usually longer than other aromatic protons such as 2,6-H of Tyr residues. Under the conditions used in the present experiments, a significantly reduced intensity is expected for protons such as those of C2–H and C4–H of His residues. Second, the area measurement cannot be very accurate because of a severe overlap of resonance lines and an inherent uncertainty in defining the base line, especially in the case of the aromatic envelope.

Signals 6–9 are more or less superimposed on other signals. This can be seen in Figure 4, where signal 6 is still observable, even though Tyr has been removed in the sample used for measuring spectrum b.

The portion of the spectrum given as an inset in Figure 1 was observed under a condition where *spin diffusion* prevails by a strong irradiation of the protons of the peptide backbone. This of course results in differential loss of intensities of each signal depending upon the flexibility of the environments for each amino acid residue (See Endo & Arata, 1985).

It is known that trypsin cleaves two peptide bonds in the bait region, releasing a peptide segment that comprises 15 amino acid residues (Mortensen et al., 1981). See Figure 3. Figure 4 shows ^{1}H NMR spectra of α-2-M reacted with trypsin. Given in parts a and b are observations before and after removal of the "bait" region peptide, respectively. In the present experiment, the released peptide was removed by ultrafiltration. As Figure 5 shows, the free trypsin does not

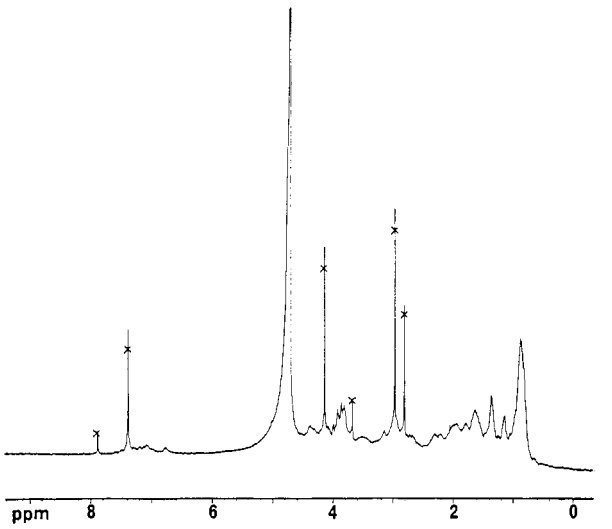

FIGURE 5: 400-MHz ^{1}H NMR spectrum of trypsin. Trypsin was inactivated with PMSF before measurement as described in text. The spectrum was recorded at pH 7.2 in the absence of a presaturation pulse. A total of 2000 transients was accumulated. Other spectral conditions were as described in Figure 1. The peaks marked × are due to PMSF and low molecular weight contaminants.

give any sharp resonances such as those observed in Figure 1.[2]

On treatment with trypsin, resonances 6 and 7 were observed to become narrow in line width. Resonance 7 was split into a clear doublet upon trypsin treatment. Differences of the chemical shifts of the His resonances observed in Figures 1 and 4a are not quite simple. This may be due to a small difference of pH at which spectra were measured. It was observed that some of the His resonances observed in the intact α-2-M disappear in the spectrum of the trypsin–α-2-M complex. See Figure 4b. However, identification of the His residue that exists in the bait region was not possible due to severe overlap of the resonances. It should also be noted that two new resonances become observable in the trypsin–α-2-M complex. See Figure 4a. Chemical shift data show that these peaks originate from the CH_3 protons of Ala and Val residues (Bundi & Wüthrich, 1979).

The ^{1}H NMR spectrum of the trypsin–α-2-M complex observed after removal of the bait region peptide by ultrafiltration is clearly devoid of most of the sharp resonances mentioned above. The peptide recovered from the ultrafiltrate had an amino acid composition corresponding to a bait region sequence of Val-Gly-Phe-Tyr-Glu-Ser-Asp-Val-Met-Gly-Arg. It is known that trypsin cleavage gives a 15-residue peptide that has Gly-His-Ala-Arg segment at the C terminal of the 11-residue peptide given above (Mortensen et al., 1981). Presumably the four-residue segment has been cleaved off during the process of separation of the 15-residue peptide. These results clearly indicate that most of the amino acid residues giving sharp resonances in Figures 1 and 4 are actually those of the bait region.

The aliphatic proton of some of the amino acid residues give extremely complicated signals even in simple peptides and are barely detectable in the case of large proteins. Gly, Ser, Pro, and Asp residues undoubtedly fall in this category. This would mean that even if they belong to the bait region, which is flexible, there is little hope that we can detect any of these

[2] The peaks, which have unusually narrow line widths for proteins, are primarily due to PMSF and dimethylformamide, which was used as solvent for PMSF. When the spectra of the α-2-M/trypsin complex were measured, no PMSF had been added in the sample.

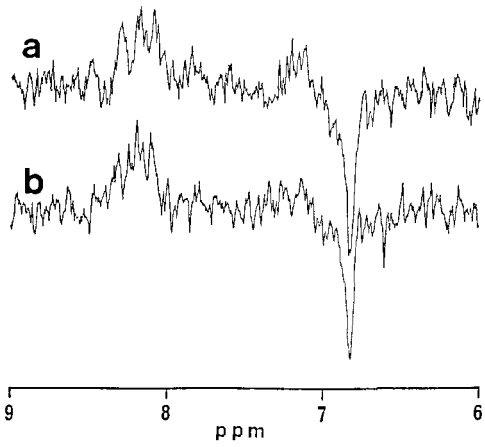

FIGURE 6: Aromatic region of photochemically induced dynamic nuclear polarization difference spectra of α-2-M. Difference spectra (light minus dark) of α-2-M before (a) and after (b) methylamine treatment were obtained in phosphate/D_2O buffer, pH 6.7. CIDNP spectra were obtained as described in text. A total of 100 transients was accumulated.

signals. In general, it is easier to detect at least some parts of signals due to Leu, Val, and Met. One can see these signals in Figure 1, although spectral resolution is by no means satisfactory for most of them. The methyl protons of Leu and Val gave signals around 1 ppm. Unfortunately, α-2-M gave a large signal at around 2 ppm originating from the methyl protons of sugar chains. Therefore, signals due to the methyl protons of Met residues cannot be observed separately. As seen in Figure 4, this region of the spectra shows a substantial change upon cleavage of the peptide from the bait region. See also spectra given for the hinge region of IgG by Endo and Arata (1985).

Figure 6 shows the aromatic region of a CIDNP difference spectra of α-2-M. Spectra a and b were observed before and after the methylamine treatment, respectively. The Tyr residue, which gives peaks 6 and 7 in Figure 1, was observed to give a strong emission (negative) peak. This result clearly indicates that the Tyr residue is accessible to photoexcited flavin molecules (Kaptein et al., 1978a,b). In addition, some enhanced absorption (positive) peaks were observed in the region of the C2– and C4–H protons of His residues, indicating that exposed His residues exist in the α-2-M molecule.

Very little change was observed in the photochemically induced dynamic nuclear polarization difference spectra for the methylamine–α-2-M complex. Similar observations were made on conventional NMR spectra of the methylamine–α-2-M complex. See Figure 7. These results strongly suggest that the conformation of the bait region does not change significantly upon hydrolysis of the thiol ester bond. This means that the bait region in the methylamine-treated α-2-M still retains a similar degree of flexibility as in the case of the native protein. It is known that on treatment with methylamine the overall conformation of α-2-M is significantly affected (Gonias et al., 1982). We also observed that the bait region becomes resistant to the proteolytic attack after methylamine treatment (Arakawa & Ikai, to be published). On the basis of the present results along with those reported previously, we suggest that the conformation change induced by methylamine results in sequestration of the bait region, which still keeps its flexibility but becomes inaccessible to the proteinases due to steric reasons. This model is consistent with the observation of the photochemically induced dynamic nuclear polarization measurements that the Tyr residue in the bait region is accessible to the flavin molecule even after the methylamine treatment.

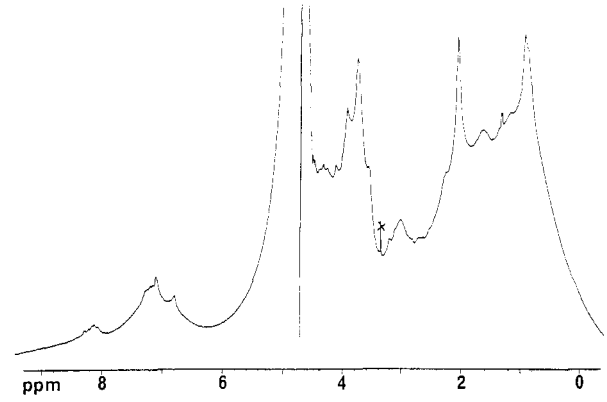

FIGURE 7: 400-MHz ^1H NMR spectrum of methylamine-treated α-2-M. α-2-M was reacted with 50 mM methylamine for 18 h at 4 °C (pH 8.5). The spectrum was recorded at pH 6.7. Other spectral conditions are as described in Figure 1.

We also suggest that the inherent flexibility observed in the present work is responsible for the susceptibility of the bait region to the proteolytic attack. The bait region of α-2-M can be readily split by a variety of endoproteinases with different specificities. The sequence of the bait region has been shown to satisfy such requirements (Roberts & Hall, 1983). The conformation of the bait region must also be very important for its availability to proteolysis. The present conclusion is that the bait region is exposed to solvent and is highly flexible. This is certainly a property that would make this part of the molecule susceptible to a quick and efficient attack by many kinds of endoproteinases. However, at the present stage, it is difficult to know how the bait region, which is highly flexible and exposed to solvent, retains the conformation of α-2-M with the intact thiol ester. Splitting of the bait region triggers hydrolysis of the thiol ester bond, inducing a conformational change in the protein and eventually resulting in trapping of the proteinase. If the bait region is too free from the main body of the α-2-M molecule, splitting of the peptide bonds in the bait region would not be able to induce any significant degree of change in the molecule. As Figure 1 shows, the effect of spin diffusion is transmitted differentially to each of the signals originating from the bait region. This presumably reflects different degrees of flexibility at different sites of the amino acid residues in the bait region. It has been demonstrated by Campbell et al. (1975) that observation of the Fourier-transformed spin–echo spectrum makes it easier to separate signals with narrow line widths from the severely overlapped broad background. It is hoped that combination of the NMR method described in this work with the spin–echo method will lead to the understanding of the mechanism with which the cleavage of the bait region triggers the trapping of the proteinases.

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A Comparative Carbon-13, Nitrogen-15, and Phosphorus-31 Nuclear Magnetic Resonance Study on the Flavodoxins from *Clostridium MP*, *Megasphaera elsdenii*, and *Azotobacter vinelandii*†

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ABSTRACT: The flavodoxins from *Megasphaera elsdenii*, *Clostridium MP*, and *Azotobacter vinelandii* were studied by ^{13}C, ^{15}N, and ^{31}P NMR techniques by using various selectivity enriched oxidized riboflavin 5′-phosphate (FMN) derivatives. It is shown that the π electron distribution in protein-bound flavin differs from that of free flavin and depends also on the apoflavoprotein used. In the oxidized state *Clostridium MP* and *M. elsdenii* flavodoxins are very similar with respect to specific hydrogen bond interaction between FMN and the apoprotein and the electronic structure of flavin. *A. vinelandii* flavodoxin differs from these flavodoxins in both respects, but it also differs from *Desulfovibrio vulgaris* flavodoxin. The similarities between *A. vinelandii* and *D. vulgaris* flavodoxins are greater than the similarities with the other two flavodoxins. The differences in the π electron distribution in the FMN of reduced flavodoxins from *A. vinelandii* and *D. vulgaris* are even greater, but the hydrogen bond patterns between the reduced flavins and the apoflavodoxins are very similar. In the reduced state all flavodoxins studied contain an ionized prosthetic group and the isoalloxazine ring is in a planar conformation. The results are compared with existing three-dimensional data and discussed with respect to the various possible mesomeric structures in protein-bound FMN. The results are also discussed in light of the proposed hypothesis that specific hydrogen bonding to the protein-bound flavin determines the specific biological activity of a particular flavoprotein.

Flavodoxins are a group of relatively small flavoproteins (14 000–23 000 Da) consisting of one polypeptide chain and containing a single molecule of noncovalently bound riboflavin 5′-phosphate (FMN)[1] (Mayhew & Ludwig, 1975).

The flavin coenzyme can exist in three redox states, i.e., oxidized, one electron reduced or semiquinone, and two

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[1] Abbreviations: FMN, $FMNH_2$, and $FMNH^-$, riboflavin 5′-phosphate in the oxidized, two-electron-reduced neutral, and anionic state, respectively; TARF and $TARFH_2$, tetraacetylriboflavin in the oxidized and two-electron-reduced state, respectively; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; Me_4Si, tetramethylsilane; OYE, "old yellow enzyme"; *C.MP*, *Clostridium MP*; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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