Characterization of the Aromatic Proton Magnetic Resonance Spectrum of Crambin†

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ABSTRACT: The hydrophobic protein crambin (M_r 4715) has an aromatic content of one phenylalanyl residue (site 13) and two tyrosyl residues (sites 29 and 44). The aromatic residues have been studied by ^{1}H NMR spectroscopy at 300 and 600 MHz for crambin dissolved in deuterated glacial acetic acid and in aqueous organic media. In particular, a 3:1 acetone/water mixture affords a solvent system of low viscosity, which yields very narrow line protein spectra. The aromatic proton spectrum is unusual in that signals are doubled. Spectral simulation of modified and unmodified crambin aromatic spin systems can be accomplished only by assuming that the protein is a mixture of two species. Dynamic ^{1}H-{^{1}H} Overhauser experiments centered on the aromatic doublets of Tyr^{29} indicate that the two species do not interconvert within 2 s. NMR spectra of crambin samples obtained by repeated crystallization in 17:3 acetone/water indicate that the process enriches the mixture with one component that does not convert to the other, even upon heating of the sample to 351 K. The combined evidence strongly favors the view that the doubled spectrum results from a compositional heterogeneity, most likely a mixture of *two* homologues, rather than from an equilibrium between interconvertible forms. This is important in view of the fact that its crystallographic structure, solved to 1.5-Å resolution [Hendrickson, W. A., & Teeter, M. M. (1981) *Nature* (*London*) *290*, 107–113], is based on samples containing the two species. The ^{1}H NMR evidence is consistent with a model where the Tyr^{29} side chain is very mobile while both the Phe^{13} and Tyr^{44} rings, although flipping fast on the NMR time scale, are more constrained in their motion. Crambin was reacted with tetranitromethane and also iodinated. It is shown that, under reagent-limiting conditions, Tyr^{29} nitrates and Tyr^{44} iodinates, the latter yielding the diiodo derivative. However, excess reagent causes Tyr^{44} to nitrate and Tyr^{29} to iodinate. This confirms that the reactivity of phenolic groups is controlled by their environments and suggests that probably the iodination of Tyr^{44} is facilitated by an electrostatic effect from Asp^{43}. Hence, response to nitration or iodination cannot be taken as a single criterion to determine exposure of phenol groups in proteins. Chemical modification of Tyr^{44} causes shifts of the Ile^{33} CH_3^{δ} resonance, asserting proximity of these two residues. Upon acquiring a negative charge provided by the modifying group, crambin gains in its water solubility; furthermore, the nitrated species assists in solubilizing unmodified, native crambin in pure aqueous media. This device opens the possibility of comparing physical properties of crambin with those of other exclusively water-soluble proteins. In particular, crambin NMR spectra can now be related to those of thionin homologues from *Graminae* that are highly hydrophilic. Thus, comparison with the α_1-purothionin homologue from wheat leads to tentative identification of the crambin Ile^{34} δ-methyl triplet in the proton spectrum.

The water-insoluble protein crambin, whose X-ray structure has been solved to 1.5 Å (Hendrickson & Teeter, 1981) and is presently under refinement, constitutes a promising model for the study of hydrophobic polypeptides. It is available in large quantities from the seeds of *Crambe abyssinica*, crystallizes readily, and is quite stable. The aromatic content of crambin is low: one phenylalanine (site 13) and two tyrosines (sites 29 and 44). In preliminary ^{1}H NMR studies of crambin dissolved in organic solvents (Llinás et al., 1980; De Marco et al., 1981), its aromatic spectrum was identified. We assigned the aromatic signals of the protein dissolved in glacial acetic acid to specific residues with the help of the NOE[1] in combination with the X-ray structure. Tyr^{44}, giving rise to broadened resonances in the native state, was shown to be in dipolar contact with Ile^{33}, which exhibits high field shifted methyl signals, presumably caused by the Tyr^{44} aromatic ring current. In contrast, the spectrum of Tyr^{29} is similar to that of an unconstrained tyrosyl residue in short oligopeptides. No analysis was attempted of the Phe^{13} resonances owing to their broad, unresolved appearance in the solvents used.

The importance of aromatic residues as reporters of structure, stability, and function of proteins has been amply demonstrated (Campbell et al., 1975; Snyder et al., 1975; Wagner et al., 1976). Therefore, it is of interest to characterize their role and behavior in globular proteins. A clue to these questions can be obtained through their affinity for chemical reagents since the protein structure determines which residues are amenable to modifications as well as their reactivity kinetics. This property follows from conformational features of the protein: the reactivity of a functional group depends on factors such as steric protection, site polarity, and electrostatic interactions. In this respect, the X-ray structure of crambin (Hendrickson & Teeter, 1981) places Tyr^{29} and Tyr^{44} in quite different surroundings: Tyr^{29} belongs to the so-called "left wall", which defines a hydrophobic surface, whereas Tyr^{44} lies in a shallow pocket, near the carboxyl group of Asp^{43}. In order to probe the environments of these residues, two chemical modifications of crambin tyrosines, iodination and nitration, were studied in this work. Furthermore, other low-viscosity

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[1] Abbreviations: DMF, [$^2H_6$]dimethylformamide; D_2O, ^2H_2O; HPLC, high-performance liquid chromatography; NOE, nuclear Overhauser effect; pH*, glass electrode pH reading uncorrected for solvent or isotope effects; ppm, parts per million; rf, radio frequency; SECSY, spin echo correlated spectroscopy; 3-ITyr, 3-iodo-L-tyrosine; 3-NO_2Tyr, 3-nitro-L-tyrosine; Me_4Si, tetramethylsilane; TNM, tetranitromethane; TSP, sodium 3-(trimethylsilyl)[2,2,3,3-$^2H_4$]propionate; Tris, tris(hydroxymethyl)aminomethane.

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solvents were explored in order to narrow resonances and facilitate analysis of the aromatic spectrum.

Materials and Methods

All the reagents were reagent grade. The deuterated solvents were purchased from Merck Sharp & Dohme of Canada, Ltd. Deuterated acetic acid was distilled before use. Tetranitromethane, dithiothreitol, and triiodoacetic acid originated from Sigma Chemical Co. Crambin was isolated and purified according to a procedure similar to that reported by Van Etten et al. (1965). The wheat homologue α_1-purothionin belonged to the same batch as already described (Lecomte et al., 1982b).

Chemical Modification of Cystine. The disulfide bridges of crambin were reduced according to the procedure of Cleland (1964). After carboxymethylation (Crestfield et al., 1963), the solvent was removed under reduced pressure, and the reacted material was taken up in a minimal amount of water. Purification was performed by gel filtration on Sephadex G-10 (1.5 × 40 cm column, 0.5 M NaCl, 0.5 M NH_4HCO_3, pH 8).

Nitration of Tyrosine. The nitration of crambin was carried out with tetranitromethane under mild conditions to ensure specificity and a reasonable rate of reaction (Riordan & Vallee, 1972). The protein was dissolved in a 50% ethanol/water mixture (pH* 8, buffered with 0.005 M Tris). The trinitromethane and salts were removed by gel filtration on Sephadex G-25 (1.5 × 40 cm column, water elution); the absence of polymerization products was checked by gel filtration on Sephadex G-50 (1.5 × 40 cm column, water elution). The fractions containing the nitrated protein were combined, the solvent was removed under reduced pressure, and the material was dried under vacuum at 313 K. The product forms yellow crystals and is slightly soluble in water at high pH. Identification was performed with UV and NMR spectroscopies.

For the NMR monitoring, the nitration reaction was also carried out in ethanol/water. To a solution of crambin (16 mg, 3.2 μmol) in 0.5 mL of 3:2 [$^2H_6$]ethanol/D_2O (pH* 8.1, buffered with 0.08 M Tris) contained in an NMR tube was added all at once 30 μL of a mixture of TNM (20 μL, 168 μmol) and [$^2H_6$]ethanol (10 μL, 95% in D_2O). ^1H NMR observation was started immediately after the addition of TNM; the spectra were recorded at regular intervals. The temperature was maintained at 298 K.

pH Titration of the Nitrated Tyrosine. The pH titration of mononitrated crambin was monitored by UV–visible spectroscopy. To a solution of mononitrated crambin in water (A_{295} = 0.252, 60 μM, pH 3.34 reached by HCl addition) contained in a 1-cm quartz cell was added portionwise with a 10-μL syringe a solution of NaOH (1 or 0.1 M). After each addition, the solution was stirred for 2 min, and its UV–visible spectrum was recorded (240 nm < λ < 600 nm). The pH was read before and after the spectrum was taken; the value did not vary significantly over that time. The pH ranged between 3.34 and 10.65. After the titration was completed, the pH was adjusted to 7.0. The spectrum obtained was essentially the same as that observed upon raising the pH in the course of the titration.

The pK_a of the nitrated residue was calculated by nonlinear least-squares fitting to

$$A_{430} = A_1 + \frac{K_a^n(A_2 - A_1)}{K_a^n + [H^+]^n} \quad (1)$$

where A_1 and A_2 are the absorbances of the phenol and phenolate forms, respectively, and A_{430} is the absorbance at 430 nm; the parameter n takes into account cooperativity, if any.

Iodination of Tyrosine. Crambin was iodinated as reported by Covelli & Wolff (1966). The protein was dissolved in an ethanol/water mixture (50%, buffered with Tris at 0.005 M, pH* 8.5) and reacted with a solution of I_2. Quenching was achieved by addition of $Na_2S_2O_3$. The solvent was removed under reduced pressure, and the reacted material was dissolved in water and purified by gel filtration on Sephadex G-25 (1.5 × 40 cm column, water elution).

NMR monitoring of the reaction was carried out in 3:2 ethanol/water. To a solution of crambin (16 mg, 3.2 μmol) in 0.6 mL of 3:2 [$^2H_6$]ethanol/D_2O (pH* 8.1, buffered with Tris at 0.08 M) contained in an NMR tube was added 10 μL of a solution of iodine/excess iodide (12.6 μmol of I_3^-). ^1H NMR observation was started immediately after the addition. After 2 h, another 10-μL portion was added. The temperature was maintained at 298 K.

Instrumentation. UV–visible spectra were recorded on a Varian Cary 219 spectrophotometer in the double-beam mode. Proton NMR spectra were recorded at 300 MHz with a Bruker WM 300 spectrometer and at 600 MHz with the NMR Facility for Biomedical Studies at Carnegie-Mellon University. Both correlation (Dadok & Sprecher, 1974; Gupta et al., 1974) and Fourier techniques were used with homo- or heteronuclear lock, respectively.

NMR Methods. The line width of the protein signals was artifically reduced by resorting to resolution-enhancement routines: three-parameter optimal filter (Ernst, 1966) or Gaussian multiplication (Ernst, 1966; Ferrige & Lindon, 1978) for the correlation and the Fourier experiments, respectively.

The two-dimensional SECSY spectrum was acquired according to the pulse proposed by Aue et al. (1976). The resulting spectrum displays the conventional 1-D trace in the middle across the length and the cross-peaks perpendicularly, along a $\Delta\delta/2$ scale. The digital resolution in both dimensions was 5 Hz.

The proton Overhauser effects observable in the Tyr-II system were measured with the 600-MHz spectrometer in the correlation mode. The perturbing wave (B_2) power was adjusted so that the irradiation of one transition would cause a negligible perturbation of the neighbor line, separated by 8 Hz. The following sequence was applied. The B_2 frequency was set on-resonance for 2 s and then moved to a blank position outside of the spectrum. The sweep was started 5 ms later from a frequency as close as possible to the first transition to be observed. After the 1.71-s acquisition, a delay of 8 s was allowed for relaxation. The reference spectrum was obtained alternately, with the B_2 frequency off-resonance during the 2 s before acquisition. The results are presented as a difference spectrum (off- minus on-resonance) so that a negative NOE appears as a positive signal.

Simulation of the various spectra was performed with the program 'PANIC' of the ASPECT 2000 data package (Bruker Instruments). The same digital filtering was applied to both experimental and simulated spectra.

Chemical shifts are reported in parts per million. The reference for nonaqueous solvent is internal Me_4Si whereas dioxane was used in aqueous media, under the assumption it resonates at 3.766 ppm from TSP (De Marco, 1977). The temperature of the proton probe was determined with methanol or ethylene glycol samples (Van Geet, 1970; Neuman & Jonas, 1968).

Results

The spectrum of the aromatic region of crambin in deuterated glacial acetic acid is shown in Figure 1. The identification of the spin systems has been reported previously

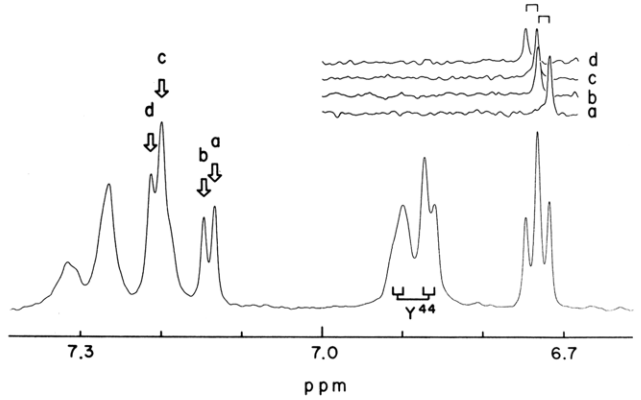

FIGURE 1: ^1H NMR spectra of crambin at 600 MHz: aromatic resonances. The vertical arrows indicate Overhauser irradiation frequencies centered on Tyr^{29} H2,6 transitions: (a–d) corresponding NOE difference spectra. The brackets denote the two Tyr^{29} H3,5 doublets. Each NOE spectrum, recorded in the correlation mode, represents 400 scans. Solvent was $[^2H_4]$acetic acid; $T \sim 295$ K. Protein concentration was 8 mM.

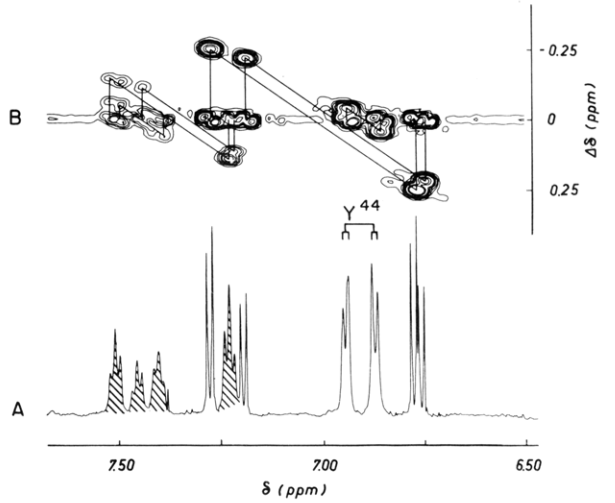

FIGURE 2: ^1H NMR spectrum of crambin at 600 MHz: aromatic region. (A) 1-D spectrum, digital resolution, 0.12 Hz, 1024 scans; (B) SECSY spectrum, contour plot of the region shown in (A), digital resolution, 5 Hz in both dimensions. Connectivities are indicated for the three aromatic spin systems: Phe^{13} (hatched resonances at ~7.45 and 7.2 ppm), Tyr^{29} (~6.77, ~7.20, and 7.30 ppm), and Tyr^{44} (~6.9 ppm). Solvent was 15:5:1 $[^2H_6]$acetone/D_2O/$[^2H_6]$acetic acid; $T \sim 295$ K. Protein concentration was 7 mM.

(Llinás et al., 1980; De Marco et al., 1981): one of the tyrosines (Tyr^{44}) gives rise to a broad, "two-doublet" AA′BB′ system centered around 6.88 ppm. The second tyrosine (Tyr^{29}) appears as four doublets of intensity 1:1:1:1 arranged in two pairs resonating close to the random-coil chemical shifts of ~6.73 and ~7.17 ppm. Phe^{13} exhibits three broad signals below 7.17 ppm, whose multiplicity is not resolved in this solvent.

The interpretation of the spectral pattern observed for Tyr^{29} is not straightforward. Indeed, it is not clear whether rotation of the rings around the C^{β}–C^{γ} bond is slow in the NMR time scale, in which case one can observe one signal per proton, or whether the protein exists in two different conformations in equilibrium (Llinás et al., 1980). Furthermore, since crambin is chemically heterogeneous (Hendrickson & Teeter, 1981; Teeter et al., 1981), it is also possible that the spectrum results from two different compounds, in which case the "doubled" set of Tyr^{29} resonances would arise from nonconvertible forms.

NOE experiments were implemented in order to check for interconversion and rotation of the Tyr^{29} ring. In four independent experiments, the individual transitions within each downfield doublet of Tyr^{29} were selectively irradiated for 2 s and the responses of the Tyr^{29} upfield aromatic transitions recorded as difference spectra (inset of Figure 1). It can be seen that the excitation of one downfield transition induces an intensity decrease of only one upfield resonance. This phenomenon can be explained by reference to the standard energy-level diagram of two spins coupled by scalar spin–spin interactions (Noggle & Schirmer, 1971). The decrease of the intensity of only one directly coupled transition indicates that, over the wait period implemented to build up the NOE, a meta proton does not transform into the other meta proton giving rise to the second doublet pair. In other words, flipping of a presumably "immobilized" ring or interconversion between the two forms does not occur within 2 s.

Despite crambin's low molecular weight (4715), its spectra in acetic acid at room temperature exhibit relatively poor resolution. The viscosity of the sample is partly responsible for the large line widths to the extent of interfering with a detailed study of the Phe^{13} spin system. Dilution of the preparation did not bring about any improvement in resolution. In order to obtain a high-resolution spectrum of the aromatic region, a sample of crambin was dissolved in a 3:2 mixture of $[^2H_6]$acetone and D_2O. This solvent system was chosen for the following reasons. (1) Acetone/water mixtures are used in the extraction of the protein (Van Etten et al., 1965). They do not cause irreversible denaturation and, therefore, are not likely to damage the protein as glacial acid does under prolonged exposure or high temperature. (2) The viscosity of the solution is low, especially at large levels of acetone. (3) Deuterated acetone and D_2O are available with a degree of purity higher than that of most organic solvents.

The aromatic spectrum in 3:2 acetone/water after H exchange against solvent deuterons is presented in Figure 2. A trace of acetic acid was added to shift and narrow signals (De Marco et al., 1981) and to minimize overlap. As is readily observed, the various multiplets are well resolved; moreover, the spread of chemical shifts and line widths characterizing the signals throughout the entire spectrum (not shown) ensure that the protein is not unfolded. Although the overall aspect of the aromatic region is similar to that exhibited in acetic acid, the identification of the spin esystems was repeated in this solvent: at 600 MHz it was possible to decouple selectively all the signals including those of Phe^{13}, which shows five multiplets and therefore raises the question of a locked ring as Tyr^{44} does. The 1-D results were confirmed by the 2-D SECSY technique; the spin–spin connectivities are indicated in Figure 2. The simple cross-peak pattern does not support the interpretation of either four distinct coupled spins for tyrosine-29 or five distinct coupled spins for Phe^{13}. This reinforces the hypothesis of coexistence of two protein forms, each with freely flipping rings.

Inspection of the spectrum of Figure 2 suggests that the two sets of multiplets of Tyr^{29} and of Phe^{13} have different integrated intensities. To ascertain that a variable line width is not responsible for the appearance, the aromatic resonances were simulated. Reference and calculated spectra are shown in Figure 3A,B. It was difficult to reproduce the experimental spectrum by attributing to each signal an intensity of one proton: a satisfactory match was achieved only when two complete aromatic spectra were added in a 3:2 ratio (Figure 3B–D). These spectra each contain the following: (1) Phe^{13}, characterized by an AA′BB′C spin system of broadened resonances, consistent with a rapidly flipping phenylalanyl ring somewhat constrained in its motion by a restricted environment; (2) Tyr^{29}, characterized by an AA′XX′ spin system of

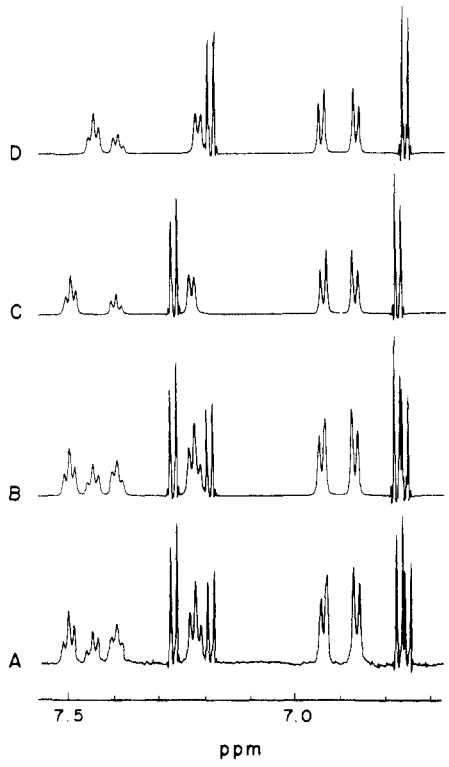

FIGURE 3: ^{1}H NMR spectra of crambin at 600 MHz: analysis of the aromatic resonances. (A) Experimental; (C) computer simulation of crambin I; (D) computer simulation of crambin II. (B) simulates (A) [it is a weighed sum of (C) and (D)]. Solvent was 15:5:1 [$^2H_6$]acetone/D_2O/[$^2H_6$]acetic acid; $T \sim 295$ K. Protein concentration was 7 mM. (Parameters for the simulation are available as supplementary material; see paragraph at end of paper regarding supplementary material.)

sharp resonances, indicative of unhindered motion, consistent with a freely flipping tyrosyl ring with chemical shifts close to the random-coil values; (3) Tyr^{44}, characterized by an AA'BB' spin system of broadened resonances, consistent with a rapidly flipping tyrosyl ring, somewhat constrained in its motion by a restricted environment.

In order to determine whether the two forms are interconvertible, a temperature study was carried out in acetone/water, solvent that provides the resolution necessary for intensity comparison. Those signals that at room temperature exhibit peculiar resonance frequencies move monotonically toward their random-coil chemical shift as the sample is heated. Nevertheless, even at the highest temperature reached in the experrment (351 K), the spectrum retains numerous features displayed by the native protein. This resistance toward thermal denaturation was also observed in other organic solvents such as acetic acid and DMF (De Marco et al., 1981). The spectral pattern of Tyr^{29} ($\sim$6.90 and $\sim$7.25 ppm) is not significantly influenced by heating the sample (Figure 4). Moreover, the proportion of the two forms as represented by doubled Tyr^{29} spectra remains constant over the explored temperature range. Harsh treatments such as incubation at pH* >11, use of trifluoroacetic acid as a solvent, or reduction of the disulfide bridges are required in order to simplify the Tyr^{29} pattern to two doublets and the Phe^{13} pattern to three signals. When the evolution of the spectrum could be followed, it appeared that both forms yield concomitantly a denatured protein; in no instance did we observe a deviation from the initial ratio of the two species. In contrast with the behavior of Tyr^{29}, the spectrum of Tyr^{44} (6.95 ppm) is altered upon raising the temperature: the AA' and BB' multiplets move toward each other yielding a singlet pattern at 351 K, the highest temperature investigated (Figure 4A). The same behavior was observed in the other media, which allowed the temperature to be raised above the coalescence point, showing that an AA'BB' system is recovered, with diverging halves (Lecomte & Llinâs, 1984). It is concluded that at room temperature the ring H2,6 and H3,5 resonances are reversed from their usual random-coil ordering at low and high fields, respectively. That the ratio of the two protein forms is not changed by bringing the temperature to 351 K argues against interconversion between two species.

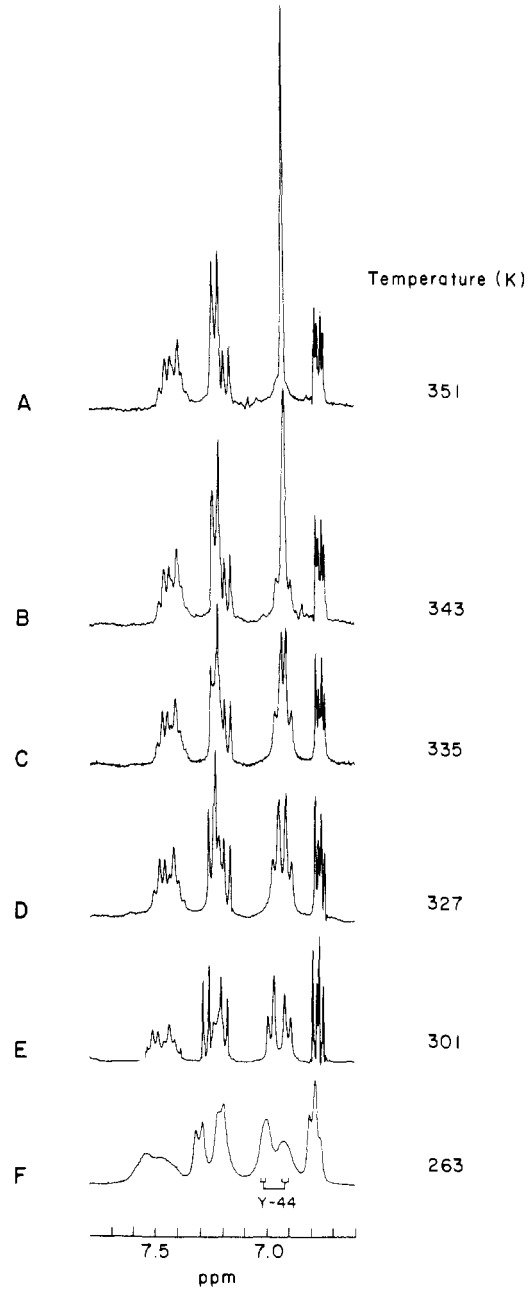

FIGURE 4: ^{1}H NMR spectra of crambin at 300 MHz: aromatic resonances. Solvent was 3:2 [$^2H_6$]acetone/D_2O. Recorded at (A) 351, (B) 343, (C) 335, (D) 327, (E) 301, and (F) 263 K. Protein concentration was 5 mM.

Interestingly, it is possible to alter the ratio of the two components by successive recrystallizations in 17:3 acetone/water. The ratio of the two forms, as monitored by the relative integrated intensities of the H3,5 doublets of Tyr^{29}, drops from approximately 5:4 to 2:1, after four recrystallizations (a fifth recrystallization did not significantly alter the relative amount of the components). Such an enrichment demonstrates that

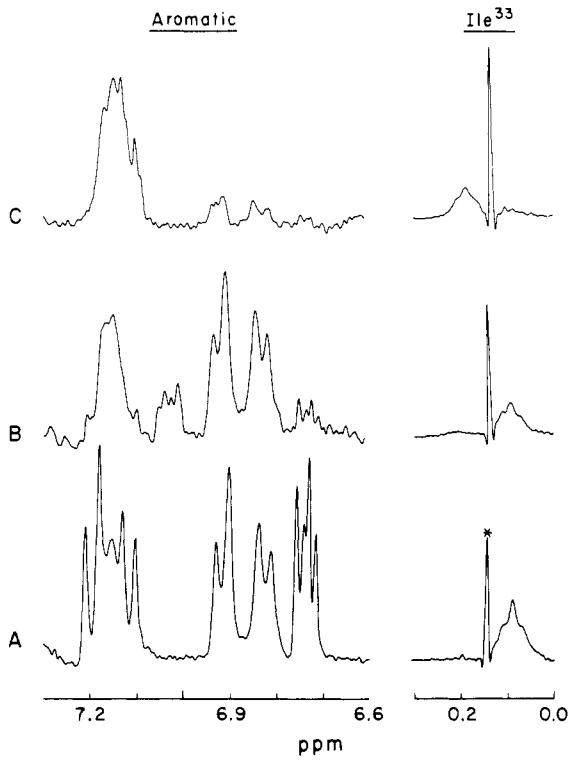

FIGURE 5: Nitration of crambin as monitored by ^1H NMR at 300 MHz: tyrosyl aromatic and Ile^{33} δ-methyl resonances. (A) Reference spectrum; (B) spectrum recorded 10 min after addition of 168 μmol of TNM; (C) spectrum recorded 1 h after addition of 168 μmol of TNM. The strong singlet absorption at ~0.15 ppm stems from an impurity. Solvent was 3:2 [$^2H_6$]ethanol/D_2O; protein concentration was 6.4 mM in 80 mM Tris buffer (pH* 8.1); T = 298 K.

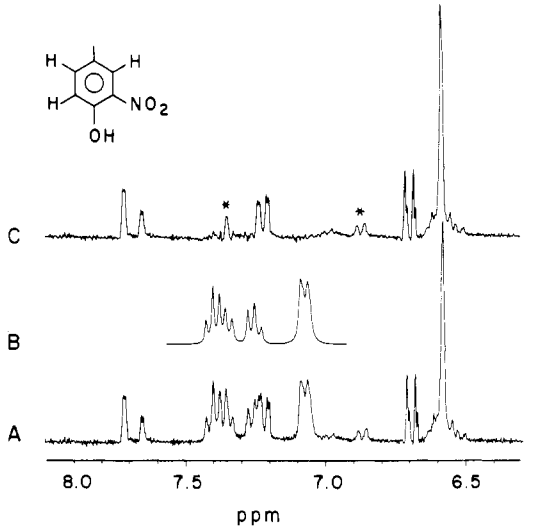

FIGURE 6: ^1H NMR spectrum of nitrated crambin at 300 MHz: aromatic region. (A) Experimental; (B) simulation of the phenylalanine spin system; (C) difference spectrum where (B) has been subtracted from (A). Spectrum C contains resonances from reacted and unreacted tyrosines only. Signals produced by incipient nitration of Tyr^{44} are indicated by an asterisk (*). Solvent was D_2O, pH* 10.9; T ~ 300 K; 4096 scans were taken. Protein concentration was ~2 mM. (Parameters for the simulation are available as supplementary material.)

the components in the crambin mixture have slightly different solubilities in acetone/water. It seems reasonable to associate this minute difference with the variable presence of Pro and Ser at position 22 in the sequence. The separation of two species, even though partial, is of interest as it suggests the possibility of developing a method for fractionating the two forms with suitable solvent systems, e.g., by reverse-phase HPLC.

In order to determine whether or not the distinction between the Tyr^{29} and Tyr^{44} phenol rings goes beyond the magnetic properties of their environments, chemical modifications based on nitration and iodination reactions were performed. Nitration of the tyrosyl ring with TNM was carried out under mild conditions. The material obtained is slightly soluble in water and could be titrated. UV–visible spectra showed the presence of an isosbestic point at 381 nm, which is in agreement with the literature value (Riordan & Vallee, 1972). The curve of A_{430} vs. pH yields a pK_a of 6.98, fitted with a simple noncooperative ionization (Hill coefficient = 1) for the modified side chain. The pK_a reported for the model compound *N*-acetyl-3-nitrotyrosine is 7.0 (Snyder et al., 1975; Riordan et al., 1967).

To determine which of the two residues reacts preferentially with TNM, progress of the reaction was monitored by NMR. A mixture of ethanol and water was used because of buffer considerations and greater similarity with an aqueous medium even though the NMR spectrum obtained is somewhat broader than that in acetone/water, the best solvent for spin-system analysis. Figure 5A presents the spectrum of native crambin in 3:2 ethanol/water between 7.3 and 6.6 ppm, where signals from both tyrosines are found, and between 0.3 and 0.0 ppm, where the Ile^{33} triplet resonates. The resonances of Tyr^{29} have all but disappeared 10 min after the addition of TNM (Figure 5B) whereas the Ile^{33} triplet broadens without shifting. Unmodified Tyr^{29} is not detected 20 min after the start of the reaction. If the mixture is allowed to incubate for another 40 min (Figure 5C), Tyr^{44} is transformed by ~90%. At the same time, the Ile^{33} triplet moves ~0.1 ppm downfield.

It is interesting that the Ile^{33} triplet shifts only upon reaction of Tyr^{44}, and then by only a small amount. The implications bear on the assignment of the aromatic residues and on the stability of the crambin structure. First, Tyr^{29} does not seem to interact with Ile^{33} whereas Tyr^{44} does; this parallels the conclusions reached previously through NOE experiments (De Marco et al., 1981) and confirms the assignment based on the crystallographic structure. Second, the nitration reaction, even when forced upon the less reactive phenol ring, does not perturb much the secondary and tertiary structures of the tyrosine sites as witnessed by the Ile^{33} upfield-shifted triplet.

The aromatic ^1H NMR spectrum of *N*-acetyl-3-nitro-L-tyrosine ethyl ester, a 3-NO_2Tyr model compound (Snyder et al., 1975), consists of three distinct signals since the H2/H6 degeneracy is removed upon substitution of the H3 ring hydrogen. The H5 and H6 resonances appear as doublets whereas the H2 signal is a singlet. Although the chemical shift of the three protons depends upon pH, their relative ordering does not: H2 resonates at lower fields than H6, which in turn resonates at lower fields than H5. When, as described above, the reaction was forced to achieve maximal effect, a high concentration of reagents and protein was used, which caused side reactions to occur as shown, e.g., in Figure 5B, where transient peaks not readily interpretable are detected. In order to isolate a mononitrated crambin for better NMR characterization, the experiment was repeated with diluted reagents and carefully controlled.

The 300-MHz spectrum of the aromatic region of nitrated crambin in D_2O, pH* 10.9, is presented in Figure 6. The spin systems of Phe^{13}, Tyr^{44}, and 3-NO_2Tyr^{29} are well resolved and were identified by selective ^1H–{^1H} spin-decoupling experiments. The Phe^{13} multiplets resonate between 7.0 and 7.5 ppm. As observed for unreacted crambin in acetone/water, Phe^{13} displays three triplets and two doublets of noninteger

intensities. Simulation of the spectrum requires two AA'BB'C spin systems, of slightly different line widths, in a ratio of 3:2; the result is shown in Figure 6B. The trace of Figure 6C is obtained when the calculated Phe spectrum (B) is subtracted from the experimental data (A). Figure 6C therefore contains signals from the two tyrosines, reacted or not. Integration yields a total intensity of about seven protons by reference to the Phe[13] signals. The highly degenerate Tyr[44] multiplet at ~6.58 ppm accounts for slightly less than four protons, which indicates that Tyr[44] underwent little modification (<10%). The ring protons of reacted Tyr[29] account for the remaining signals. When the multiplet at 7.21 ppm is irradiated, a 1.8-Hz coupling is removed from both peaks at 7.72 and 7.65 ppm, and the signals at 6.70 ppm loose their 8.8-Hz splitting. This behavior is consistent with that expected of a 3-NO_2Tyr provided allowance is made for a duplication of signals. The two spin systems were simulated and found to exist in the same ratio as determined for the Phe[13] signals. The detection of the long-range coupling between H2 and H6 is unusual for a molecule of this size.

Crambin was also iodinated. For the controlled reaction, a stoichiometric quantity of I_2 was added at 273 K whereas for exhaustive iodination, excess reagent was allowed to react at 298 K. The products from yellowish crystals that are sparingly soluble in water at high pH. The identification was achieved by UV spectroscopy: 3-ITyr has an ultraviolet absorbance maximum at 283 (acidic) or 305 nm (basic), while 3,5-I_2Tyr peaks at 287 (acidic) or 311 nm (basic). Thyroxin, the coupling product of two diiodotyrosines, shows a maximum at 298 (acidic) or 325 nm (basic) (Edelhoch, 1962). Progress of the iodination of crambin was monitored by its absorbance at 310 nm. A pH titration of the material dissolved in water was attempted but failed because of precipitation.

The iodination was also monitored by ^{1}H NMR spectroscopy. The spectrum of the model 2-I-4-$CH_3C_6H_3OH$ (Cambie et al., 1976) consists of three peaks, a doublet at 6.87 ppm (H6, ^2J = 8 Hz), a doublet of doublets at 7.08 ppm, (H5, ^3J = 8 and 2 Hz), and a doublet at 7.52 ppm (H3, ^3J = 2 Hz), whereas that of 2,6-I_2-$CH_3C_6H_2OH$ contains only one singlet, at 7.53 ppm (Cambie et al., 1976). The spectrum of partially iodinated crambin in glacial acetic acid shows that, in contrast with the nitration experiment, Tyr[44] is substituted first. Moreover, the reaction yields the diiodo compound with a broad singlet appearing at 7.58 ppm. The time course of iodination can be followed in Figure 7. The bottom trace presents the aromatic part of the reference spectrum for 3:2 ethanol/water, pH* 8.1. Also shown is the Ile[33] triplet at 0.1 ppm. Upon a 12-min wait after the addition of triiodide, Tyr[44] has essentially disappeared, and the Ile[33] signal is split into three resonances at 0.1, 0.2, and 0.3 ppm. Spectrum D was recorded after complete modification of Tyr[44]; only the low-field Ile[33] triplet remains, indicating that the transient signal at 0.2 ppm arises from the 3-I-Tyr[44] protein. After several hours and further addition of fresh triiodide, Tyr[29] has reacted almost fully to give mono- and diiodo derivatives. The behavior of the Ile[33] triplet over the course of the substitution is instructive: once Tyr[44] has reacted, there is no further perturbation of the signal, which remains at 0.3 ppm. Therefore, the modification of Tyr[29] does not influence site 44, and the transient peak at 0.2 ppm in Figure 7B,C comes from the intermediate monoiodotyrosine-44.

Discussion

In the study of the aromatic spectrum of unreacted crambin, the variable-temperature experiments do not lend support to the hypothesis of interconversion between two conformational

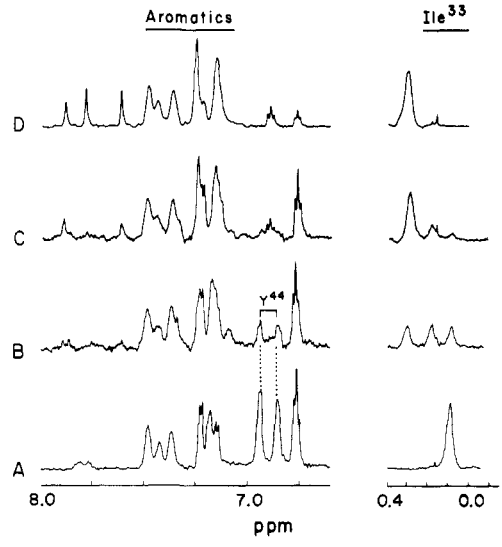

FIGURE 7: Iodination of crambin as followed by ^{1}H NMR at 600 MHz: aromatic and Ile[33] regions. (A) Reference spectrum; (B and C) spectra recorded 12 and 30 min after addition of 12 μmol of I_2; (D) spectrum recorded 4 h after the addition of another 12 μmol of I_2 to the (C) mixture. Solvent was 3:2 [$^2H_6$]ethanol/D_2O; protein concentration was 5.3 mM in 80 mM Tris buffer (pH* 8.1); T = 298 K.

forms of the same molecule in thermal equilibrium: the relative intensity of the two sets of Tyr[29] resonances does not vary upon heating or cooling. As for the possibility of an immobilized ring, it is also impaired by the same observations: upon heating the sample, the increase of backbone flexibility and partial unfolding that take place as monitored by the temperature response of the aliphatic signals should speed up the rate of ring flipping. In the bovine pancreatic trypsin inhibitor, the immobilized Tyr[35] displays four doublets of equal intensity at room temperature, but at 340 K the ring becomes freely rotating and gives rise to an AA'XX' spectrum (De Marco et al., 1977). The pattern exhibited by Tyr[29] does not show any such sign of a change in dynamics.

On the other hand, the NOE experiment sets an upper limit to the rate of Tyr[29] ring exchange between two hypothetical intramolecular environments: it does not exceed 2 Hz at room temperature. This rate is quite low and hard to reconcile with the narrow appearance of the Tyr[29] resonances, similar to those encountered for solvent-exposed unrestricted rings. Furthermore, the spectral simulations (Figure 3) yield noninteger values for the two components, and the SECSY spectrum affords no evidence of connectivity between the two sets. Therefore, the possibility of locked rings is ruled out, and the sole explanation left is the coexistence of two forms of the protein. We thus conclude that the duplication of the spectrum is a consequence of the protein being compositionally heterogeneous, with Pro/Ser and Ile/Leu vicariances at sites 22 and 25, respectively (Hendrickson & Teeter, 1981; Teeter et al., 1981). It is noteworthy that only two forms (henceforward called crambin I and crambin II) are distinguished: a random permutation of the two variable occupancies would yield four different polypeptides. Since two noninterconvertible species are present, it follows that the increased number of resonances is generated by some tertiary structure feature of the individual crambin variants. It would also appear that the three aromatic residues sense the existence of the chemical heterogeneities in a nonuniform fashion, Tyr[29] being influenced the most and Tyr[44] the least.

In the solid-state structure the two variable sites are exposed. Residue 22 terminates a turn ahead of the second α-helix,

which contains residue 25 (Hendrickson & Teeter, 1981). The substitution of an isoleucine for a leucine at an exposed site is not likely to affect either the polarity of the protein or its spatial organization. In homologous proteins, the Ile/Leu substitution has a relative frequency of 5 on a scale of 0 to 9, the average value being 3 (McIachlan, 1971). In contrast, a Pro/Ser replacement might well affect the structure since a proline imposes stronger constraints on the conformation than a serine does and the two residues have different polarizabilities. Indeed,the substitution is rarely encountered among homologous proteins, scoring 0 on a relative frequency scale (Mclachlan, 1971).

Assignments of tyrosyl resonances derived on the basis that only exposed phenol groups are susceptible to chemical modifications seem verified as of now for nitration; unfortunately, contrary examples are many in the case of iodination (Sherman & Kassel, 1968). The bovine pancreatic trypsin inhibitor illustrates the reactivity range imposed by the structure on the tyrosine residues (Sherman & Kassel, 1968; Meloun et al., 1968). The protein contains four tyrosines, at positions 10, 21, 23, and 35. Residue 23 is completely inert. Nitration affects Tyr^{10} first and then Tyr^{21}. Ring iodination takes place at sites 35, 21, and 10, but only the partially buried Tyr^{35} phenol undergoes disubstitution. This is understandable by extrapolating to proteins the polarity effect discussed by Mayberry & Hockert (1970). For a tyrosine embedded in a nonpolar polypeptide matrix, the formation of a monoiodo derivative is disfavored. However, once it occurs, the second substitution is slowed less than it would be in a high dielectric constant medium, so that exhaustive iodination more readily yields diiodo derivatives at buried than at surface residues.

That unexposed residues can substitute the H5 faster than exposed ones has also been concluded for human serum albumin (Perlman & Edelhoch, 1967). In the case of insulin, triiodide tends to modify the protein at Tyr^{A19} (Massaglia et al., 1969). It is noteowrthy that the crystallographic model places the more reactive Tyr^{A19} in a shalow surface pocket while the less reactive Tyr^{A14} is fully solvent exposed (Blundell et al., 1972). Therefore, it appears that the results obtained depend upon a number of factors such as type of reagent, polarity of the solvent, local dielectric constant, pK_a of the phenol, and accessibility of the side chain.

The experiments presented above demonstrate that in crambin Tyr^{29} reacts with TNM to yield $3\text{-}NO_2Tyr^{29}$ at a faster rate than Tyr^{44} does. On the other hand, Tyr^{44} is readily disubstituted by iodine whereas Tyr^{29} displays a reduced reactivity. Both modifications seem to leave the protein intact, but they alter its solubility properties. Even when the two reactions were carried out in the same solvent, at identical temperature and pH, the reactivities of the two crambin tyrosines toward iodine and TNM were found opposite. Since the mechanism of both substitutions (Mayberry et al., 1964; Mayberry & Bertoli, 1965; Bruice & Benkovic, 1968) involves phenolate ion as the reactive tyrosine species, a difference in the acidity of the rings cannot be held responsible for the outcomes of the two reactions. Therefore, it becomes clear that the rates of modification are indeed controlled by the effect of the microenvironments around the side chains, other than pK_a differences.

In the crystal structure of crambin (Hendrickson & Teeter, 1981), Tyr^{29} is solvent exposed, so that the approach of a relatively bulky reagent and the formation of a charge-transfer complex, postulated for the nitration (Bruice et al., 1968), can take place with minimal hindrance. Tyr^{44}, located in a sterically less accessible position, might offer more resistance to the attack. With regard to the iodination, one should consider the equilibria

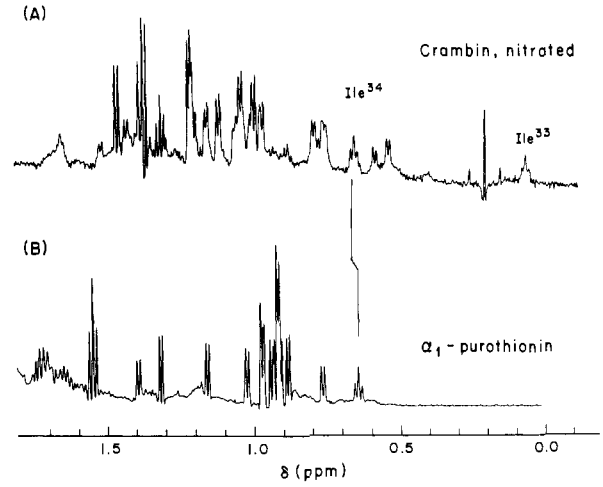

FIGURE 8: ^1H NMR spectra of crambin and α_1-purothionin at 600 MHz: methyl resonances. The crambin sample is a mixture of unmodified and nitrated crambin. The sharp singlet at ~0.2 ppm in spectrum (A) arises from an impurity. The vertical broken line connects a triplet in crambin (A) with the Ile^{34} CH_3^{δ} resonance from α_1-purothionin (B). The samples were dissolved in D_2O, pH* ~9 at a concentration of ~1 mM (A) and pH* ~7 at a concentration of 2 mM (B). The spectra, recorded at ~300 K, are resolution enhanced.

$$I_3^- \rightleftharpoons I_2 + I^-$$

$$I_2 + H_2O \rightleftharpoons H_2OI^+ + I^-$$

At pH 8.1, Asp^{43} is deprotonated, contributing one electrostatic charge to the Tyr^{44} surroundings. It is quite plausible that, owing to the reduced dielectric constant of the solvent, the carboxylate attracts strongly the reagent, K^+ and its counterion I_3^-, thus favoring the reaction. Another possibility is a general base catalysis effect. Precedence for this behavior has been observed in small systems (Bruice & Benkovic, 1966). This hypothesis also justifies why the second iodination of Tyr^{44} is relatively fast, in contrast with the predictions based on model compounds. If the mechanism is not an initial approach by I_3^- but rather by a hypoiodous species, the effect is expected to go in the same direction.

From this study it would appear that crambin is composed of two closely homologous species. This is consistent with a previous study of crambin dissolved in [$^2H_6$]acetic acid showing that to simulate the methyl spectrum at most two subspectra were required for each multiplet (Lecomte et al., 1982a). The amino acid substitutions at sites 22 and 25 appear to affect the NMR spectra of Phe^{13} and Tyr^{29} but very little that of Tyr^{44}. Mononitrated crambin retains doubled Phe^{13} and Tyr^{29} spectra; Tyr^{44}, with its single AA'BB' pattern, does not seem to sense either the chemical modification or the heterogeneities. This means that the amino acid substitution responsible for the duplication of some lines in the unreacted crambin remains equally efficient in the nitrated material and, therefore, that the chemical modification does not perturb significantly the local structures about sites 44 and 29.

In order to obtain ^1H NMR spectra of crambin in water, advantage was taken of the fact that when eluted from a Sephadex G-25 column with ethanol/water, the nitrated product does not separate well from unreacted crambin. A solution of the mixture can be prepared in water at high pH, opening the possibility to compare the spectrum of the nitrocrambin/crambin complex with those of protein homologues

from wheat and barley, which were previously studied in aqueous medium (Lecomte et al., 1982b). Figure 8 shows the methyl-region spectra of the mixed crambin multimer (A) and the α_1-purothionin from wheat (B). The highly shifted signal from the crambin Ile[33] CH_3^{δ} is readily recognized at ~0.1 ppm and finds no counterpart in the thionin spectrum as, in the *Craminae* homologues, site 33 is occupied by lysine. On the other hand, the Ile[34] CH_3^{δ} triplet of the thionin, unequovically identified as there is only one such residue in the sequence, yields a triplet at ~0.67 ppm (Lecomte et al., 1982b). The crambin spectrum contains a triplet at about the same chemical shift: it is suggested that it also arises from the Ile[34] CH_3^{δ} group present in crambin. The upfield shift of the triplet in both proteins—the random-coil shift is ~0.88 ppm (Bundi & Wüthrich, 1979)—can be attributed to the ring current generated by the aromatic side chain at site 44.

Both iodination and nitration bring to the protein an additional polar group. They also decrease the pK_a of the phenol by 2 units (3-ITyr), 4 units (3,5-I_2Tyr), or 3 units (3-NO_2Tyr) (Wolff & Covelli, 1966; Riordan et al., 1967). The p*I* of the protein is lowered by the two modifications, so that the reacted protein has one more charge at pH 11. We propose that bearing the extra charge is responsible for the enhanced water solubility of modified crambin. The water solubility of native crambin when in the presence of the nitro derivative suggests that solubilization is assisted by interaction(s) between the modified and unmodified protein species and that the observed spectrum is probably that of a complex. As determined from the aromatic spectrum (not shown), the ratio of nitrocrambin/crambin is ~2:3, so that the minimal putative aggregate is integrated by about five protein molecules. Considering the overall narrow appearance of the spectrum, this is somewhat puzzling and suggests that crambin retains a high degree of mobility in the multimeric state.

In summary, as isolated, purified crystalline crambin consists of a mixture of two magnetically distinguishable, noninterconvertible, species. These result from the Pro[22]/Ser[22] and Leu[25]/Ile[25] substitutions that perturb the Phe[13], Tyr[29], and, to a much lesser extent, Tyr[44] aromatic ^{1}H NMR spectra. The heterogeneities are also thought to be responsible for the observed solubility differences of the two forms in mixed organic media. Tyr[44], whose ^{1}H NMR spectrum is extremely sensitive to solvent and temperature perturbations, exhibits definite chemical reactivity differences when compared with Tyr[29], whose side chain is unhindered from freely flipping while in contact with the exterior medium. We conclude that the environmental differences about the two residues, apparent in their local magnetic shieldings, are responsible for their distinct chemical properties. We also present evidence suggesting that in solution crambin aggregates, its multimeric state retaining much of the dynamic flexibility that can be associated to a monomeric species.

Supplementary Material Available

Two tables showing ^{1}H NMR spectral characteristics of crambin aromatic resonances and nitrated crambin phenylalanine-13 resonances (2 pages). Ordering information is given on any current masthead page.

Registry No. Tyr, 60-18-4; hydrogen, 1333-74-0.

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Localization of the *N*-Ethylmaleimide Reactive Cysteine in the Beef Heart Mitochondrial ADP/ATP Carrier Protein†

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ABSTRACT: Alkylation of the ADP/ATP carrier protein in beef heart mitochondria by *N*-ethylmaleimide (NEM) results in inactivation of transport. One out of the four cysteinyl residues contained in 1 mol of carrier subunit of M_r 32000 is alkylated by NEM. The identification of the alkylated residue to Cys-56 has been achieved by chemical and enzymatic cleavages. The chemical cleavages included cleavages at the nonalkylated cysteinyl residues by cyanide at alkaline pH and at methionyl residues by cyanogen bromide. Enzymatic cleavage involved the use of trypsin and chymotrypsin; the resulting peptides were resolved by high-performance liquid chromatography. Analysis of a small size [^{14}C]NEM-labeled peptide obtained by tryptic and chymotryptic digestion of the [^{14}C]NEM-labeled carrier protein yielded the following amino acid sequence:

$$\begin{array}{c}[^{14}C]NEM\\ |\\ X_{51}\text{-}Gly_{52}\text{-}Ile_{53}\text{-}Ile_{54}\text{-}Asx_{55}\text{-}Cys_{56}\text{-}Val_{57}\text{-}Val_{58}\text{-}Arg_{59}\text{-}\end{array}$$

where X is probably a substituted lysine.

When ADP or ATP is added to isolated mitochondria at micromolar concentrations, the sensitivity of ADP/ATP transport to the inhibitory effect of *N*-ethylmaleimide (NEM)[1] increases markedly (Leblanc & Clauser, 1972; Vignais & Vignais, 1972). Concomitantly with inhibition of ADP/ATP transport, covalent binding of [^{14}C]NEM could be demonstrated (Vignais et al., 1975). It was postulated that the functioning of ADP/ATP transport could induce the unmasking of the SH group of a strategic cysteinyl residue in the carrier protein. The SH unmasking was prevented by uncoupling and conversely enhanced in respiring mitochondria developing a protonmotive force (Vignais & Vignais, 1972). The component of the protonmotive force that governs the SH unmasking was identified as the pH gradient (Michejda & Vignais, 1981). The reactivity of the membrane-bound ADP/ATP carrier to NEM is abolished by atractyloside (ATR) or carboxyatractyloside (CATR) and in contrast enhanced by bongkrekic acid (BA). The interest of these data lies in the fact that the ADP/ATP carrier protein can assume two conformations that are specifically recognized and trapped by ATR or CATR (CATR conformation) or by BA (BA conformation) and that the transition between the two conformations is facilitated by the substrates of the carrier, i.e., ADP or ATP (Block et al., 1983). One out of the four cysteinyl residues per carrier subunit (M_r 32000) reacts with NEM (Aquila et al., 1982a). The present report is a study of the localization of the reactive cysteine in the amino acid sequence of the carrier protein isolated from beef mitochondria.

Experimental Procedures

Chemicals and Enzymes. Atractyloside and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were obtained from Sigma Chemical Co. Guanidinium chloride, trifluoroacetic acid, succinic anhydride, and phenyl isothiocyanate were from Pierce; DABITC was from Fluka; acetonitrile, hydrochloric acid, and Coomassie blue R250 were from Merck; acrylamide and bis(acrylamide) were from Eastman Kodak. Na^{14}CN-(30–50 Ci/mol) and [^{14}C]-*N*-ethylmaleimide (20–40 Ci/mol) were from the Commissariate à l'Energie Atomique, Saclay, France. TPCK–trypsin was from Worthington and α-chymotrypsin from Miles Laboratories. All reagents used were of the purest grade commercially available.

Polyacrylamide Gel Electrophoresis. Electrophoretic separation of peptides was performed overnight in 20% acrylamide slab gels following the method described by Cabral & Schatz (1979) for resolution of peptides with molecular weights ranging from 20000 to 3000. The lyophilized peptide samples (20–30 μg) were dissolved first in 40 μL of 8 M urea, followed by 40 μL of a buffer consisting of 50% glycerol, 2% $NaDodSO_4$, 0.1 M sodium phosphate, pH 7.5, 7% β-mercaptoethanol, and traces of bromophenol blue.

^{14}C-Labeled peptides were detected by fluorography as described by Laskey & Mills (1975) after impregnation of the gel with an autoradiography enhancer (EN^{3}Hance) from New England Nuclear. The gels were dried and exposed for several weeks to a preflashed Fuji RX film at −70 °C with an intensifying Cronex screen.

N-Ethylmaleimide Covalent Labeling of the ADP/ATP Carrier. Fifty milligrams of beef heart mitochondria was suspended in 10 mL of an aerated sucrose buffer made of 0.25 M sucrose and 10 mM Hepes, pH 6.8, and supplemented with 100 μM ADP plus 20 μM BA. Additions of ADP and BA

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[1] Abbreviations: TPCK–trypsin, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone treated trypsin; $NaDodSO_4$, sodium dodecyl sulfate; ATR, atractyloside; CATR, carboxyatractyloside; BA, bongkrekic acid; DTNB, Ellman's reagent or 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; NEM, *N*-ethylmaleimide; PITC, phenyl isothiocyanate; DABITC, 4-(dimethylamino)azobenzene 4′-isothiocyanate; DABTH, 4-(dimethylamino)azobenzene 4′-thiohydantoin.

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