A Proton Nuclear Magnetic Resonance and Nuclear Overhauser Effect (NOE) Study of Human Plasma Prealbumin, Including the Development and Application to Spectral Assignment of a Combined Ring Current Shift and NOE Prediction Program*

(Received for publication, August 9, 1988)

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Human prealbumin, a homotetrameric protein with an $M_r$ of $\sim 55,000$, has been characterized by proton magnetic resonance spectroscopy. The pH dependences of the chemical shifts of the 4 histidine residues/subunit are unremarkable; variable temperature studies show that the protein tertiary and quaternary structure is stable to at least 80°C. Assignment of a number of residues was aided by the development of a computer program, PARSNIP (Programme to Analyse Ring current Shifts and nuclear Overhauser effects (NOEs) In Proteins), which employs high resolution X-ray crystal structure atomic co-ordinates (Blake, C. F., and Oatley, S. J. (1977) Nature 268, 115-120, in the case of prealbumin) and several different predictive formalisms to calculate structure-dependent ring current-induced shifts of aromatic and methyl group protons. The program combines these with distance measurements to give plots of expected NOE difference profiles resulting from irradiation at a discrete frequency. The routine should prove applicable to many proteins whose structures have been determined but which are too large for the application of sequential residue assignment techniques.

Three well resolved high field shifted methyl groups resonating at 0.18, 0.28, and 0.34 ppm are assigned to Ala-120, Leu-111, and Val-122, respectively. NOEs between these signals and the aromatics, interaromatic effects, and effects involving some of the considerable number of downfield shifted $\alpha$-protons, have been used to identify substitutions on several other residues.

Prealbumin (or transthyretin) is a mammalian plasma protein whose two known functions are the transport of thyroid hormones and of retinol, through an interaction with the retinol-binding protein (Kanai et al., 1974; Peterson, 1971). The tetrameric molecule is composed of identical 127-amino acid subunits of known sequence (Kanda et al., 1974), giving a net $M_r$ of 54,980. The protein is remarkably stable. While 6 M urea is sufficient to disrupt the prealbumin:retinol-binding protein complex (Raz et al., 1970), 6 M guanidinium chloride is required to dissociate the tetramer and even higher concentrations to affect subunit tertiary structure; strong acids and bases and detergents are without effect (Branch et al., 1971, 1972). In the former paper the authors interpret the circular dichroic properties of the protein in terms of a secondary structure which is half $\beta$-sheet and half disordered, a judgement which has been confirmed by the X-ray crystal studies of Blake and Oatley (1977). The molecule displays tetrahedral symmetry. The structure of each subunit is dominated by eight $\beta$-strands arranged in two four-stranded $\beta$-sheets. Inter-subunit interactions consist of non-covalent bonds between the edge strands of the sheets and serve to stabilize a quaternary structure which is remarkable for two features. The first of these is a channel running through the molecule, whose surface is formed by the inner $\beta$-sheets and which has been shown crystallographically to be the thyroid hormone-binding site (Blake et al., 1974). The second feature is the presence of two deep surface grooves on the molecule which Blake and Oatley (1977) propose as being stereochemically complementary to B-DNA. While a propensity to bind to DNA has not been demonstrated, and although the structure of prealbumin is at considerable variance with those determined for bacterial and viral DNA-binding proteins (see e.g. Ohlendorf et al., 1982; Fabo and Lewis, 1982; McKay and Steitz, 1981; McClarin et al., 1986; Schevitz et al., 1985), there is some reason to believe that prealbumin and thyroid hormone-modulated nucleic acid-binding proteins may be evolutionarily related (Blake, 1981; Blake et al., 1983).

Excessive line widths present a severe problem in the study of large biomacromolecules ($M_r > 20,000$) in solution by NMR and further degrade resolution which is already poor on account of spectral overlap. Equally seriously, the rapidity of $T_2$-relaxation processes, to which line widths are directly related, preclude the use of a whole range of powerful two-dimensional assignment and structural elucidation techniques (Ernst et al., 1987). Regional mobility in large proteins, however, can give rise to relatively sharp signals, superimposed on a broad envelope corresponding to nuclei in more rigid locations, and such signals can often be given useful structural interpretation (Mendez et al., 1985; Gettins and Cunningham, 1986; Perham et al., 1981; Prince et al., 1981; Endo and Arata, 1985; Highsmith et al., 1979). The molecular weight of prealbumin places it in this category of proteins which are large by NMR standards, and its line widths, and our failure to observe significant cross-peaks in two-dimensional spin and NOE correlation experiments, reflect this fact. However, its spectral complexity is mitigated by its composition of subunits which are identical with respect to primary structure. It was felt that this fact, and the existence of a high resolution crystal structure of the protein, would allow us to make assignments of sufficient generality for useful analysis of

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¹ The abbreviation used is: NOE, nuclear Overhauser effect.
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FIG. 1. a, 360-MHz $^1$H NMR spectrum of 10 mg of human prealbumin at a pH meter reading of 5.5 and temperature of 23 °C, resolution-enhanced by convolution of a 1,000-scan free induction decay with a trapezoidal resolution enhancement function involving its first 300 points. Signals ascribable to histidine H2 and H4 protons are grouped as A–C and D–F, respectively. The asterisk identifies an impurity. b, as for a except at a pH meter reading of 7.4. c, as for b except run at 60 °C. The histidine H2 protons have slowly exchanged with $^1$H2O during the course of a temperature dependence study and therefore do not contribute. d, freshly made-up human prealbumin at 80 °C showing histidine H2, which do not overlap with other non-exchangeable signals. e, 360-MHz resolution-enhanced $^1$H spectrum of 1 mg of rabbit prealbumin at 80 °C; the spectrum is the result of 25,000 accumulations.

ligand binding to be possible. Indeed, the rapidly increasing body of high resolution x-ray data on biomacromolecules suggests that NMR assignment techniques for large proteins based on solid state structural information could prove very generally applicable. In spite of large line widths, “one-dimensional” NOEs may be readily observed in prealbumin under conditions where the effects of spin diffusion appear to be minimal (Kalk and Berendsen, 1976). We have accordingly adopted an assignment approach which uses x-ray-derived coordinates to combine various ring current chemical shift calculation routines (reviewed by Perkin, 1982 and discussed recently by Weiss and Hoch, 1986) with interatomic distance measurements to predict NOE difference patterns resulting from irradiations at different frequencies.

MATERIALS AND METHODS

Human prealbumin was purchased from Calbiochem. Rabbit prealbumin was a gift from Professor Johann Sundelin of the University of Uppsala (Sweden). Both materials were used as received with no further purification.

Protein samples were dissolved in 0.4 ml of Aldrich $^1$H2O which was 0.1 and 0.05 M with respect to sodium chloride and sodium phosphate, respectively, and adjusted with dilute NaOH or $^1$HCl to register a pH meter reading of 7.4, uncorrected for the deuterium isotope effect. Measurements of pH in the course of titration experiments were carried out inside 5-mm NMR tubes using a 3-mm diameter Russel glass electrode (Auchtermuchty, Scotland) and Radiometer PHM84 meter. After two lyophilizations from 99.96% isotopic purity $^1$H2O, each sample was finally made up in 0.4 ml of 99.996% $^1$H2O.

Most NMR spectra were acquired on a Bruker AM360 spectrometer, operating in the Fourier transform mode, and interfaced to an ASPECT 3000 computer, using a 3600-Hz sweep width and 8 K digitization. Normal spectra were acquired with decoupler pre-irradiation of the $^1$HOH signal, $\pi/2$ observe pulses, and a total recycle time of 3.0 s. A few spectra were obtained on a JEOL GX-500 at SK & F Laboratories, Philadelphia, PA.

NOE difference spectra were acquired in the “interleaved mode, with a DANTE pulse sequence instead of presaturation being used to suppress residual solvent (Bodenhausen et al., 1976; Morris and Freeman, 1978; Haasnoot, 1983). A 10-dB attenuator in the transmitter line increased the $\pi/2$ pulse from its normal value of about 8 to 25 μs, the carrier frequency was placed coincident with the $^1$HOH signal, and a train of about 20 1.5-μs pulses separated by 300 μs, followed by a nonselective $\pi/2$ observe pulse, effected almost complete removal of the solvent signal. The number of scans per spectrum varied from 500 to 3000 depending on the concentration of the sample and signal to noise requirements of the particular experiment. A non-volatile impurity signal at 0.15 ppm relative to trimethylsilylpropionate sulfonate was used as an internal chemical shift reference.

Prealbumin co-ordinates were obtained from the Protein Data

Fig. 2. Plots of chemical shift versus pH of the resolved histidine H2 (a, signals A, B, and C) and H4 (b, signals D, E, and F) resonances. The curves correspond to the following apparent pKₐ values: A, 6.94; B, 6.95; C, 5.92; D, 7.02; E, 7.0 and F, 6.43. Because peaks C and F consist of overlapping signals, possibly with different pKₐ values, their analysis is probably not very reliable.
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Fig. 3. a, 500-MHz $^1$H NMR spectrum of 2 mg of human prealbumin at 80 °C at pH 7.4. 8 K digitization and a 12-ppm spectral width were used in the acquisition of 2000 free induction decays. The spectrum is presented with no resolution enhancement and a 2-Hz line broadening. Some signals have been numbered in correspondence with the system used in the presentation of NOE results in Fig. 4.

b, a partial spectrum of human prealbumin simulated on the basis of the Haigh-Mallion model, and incorporating full aromatic side chain and polypeptide chain averaging. Peaks are lettered to correspond with Table I.

RESULTS AND DISCUSSION

Proton NMR spectra of 10 mg of human prealbumin at 23 °C and pH meter readings of 5.5 and 7.4 are shown in Fig. 1, a and b, respectively. The H2 and H4 protons of the 4 histidine residues (His-31, -56, -88, and -90) give rise to signals A, B, and C (apparently two overlapping singlets) and D, E, and F, respectively. The pH dependence of these signals can be monitored, and the results of such a titration are depicted in Fig. 2a for the H2 protons and Fig. 2b for the H4 protons. The derived pK$_a$ values are reported in the figure legend but are unremarkable as they do not differ greatly from those of a random coil histidyl residue. However, the upfield shift perturbations in both the protonated and unprotonated states of signals C and F (both consisting of two overlapping peaks) suggest that they may be ascribable to His-88 and His-90, which are more buried by the tertiary structure of the protein. Warming the solution from room temperature through 60 °C (Fig. 1c) to 80 °C (Fig. 1d) causes a steady line narrowing as the molecular tumbling and internal motional rates increase. This is particularly evident in the aromatic and high field-

### Table I

| Peak designation | Calculated shift | Assignment |
|------------------|-----------------|------------|
| a                | 7.8             | W-79 H7    |
| b                | 7.7             | F-44 H4    |
| c                | 7.6             | W-41 H7    |
| d                | 7.5             | F-44 H3,5  |
| e                | 7.4             | H-88 H4    |
| f                | 7.3             | W-79 H4    |
| g                | 7.2             | F-87 H3,5  |
| h                | 7.1             | Y-105 H2,6 |
| i                | 7.0             | H-90 H4    |
| j                | 6.9             | F-96 H4    |
| k                | 6.8             | W-79 H5    |
| l                | 6.7             | W-79 H6    |
| m                | 6.6             | W-41 H6    |
| n                | 6.5             | H-56 H4    |
| o                | 6.4             | F-95 H2,6  |
| p                | 6.3             | F-95 H3,5  |
| q                | 0.6             | V-122 CH$_3$ |
| r                | 0.5             | L-111 CH$_3$ |
| s                | 0.2             | A-120 CH$_3$ |

Chemical shifts of selected peaks calculated according to other models

| Peak designation | Calculated shift | Assignment |
|------------------|-----------------|------------|
| 7.7$^b$          | W-79 H7         |
| 7.6$^b$          | W-41 H7         |
| 6.1$^b$          | Y-69 H3,5       |
| 5.7$^b$          | Y-78 H2,6       |
| 0.6$^b$          | I-107 $\delta_1$ |
| 0.5$^b$          | L-111 $\delta_1$ |
| 7.8$^c$          | A-120 CH$_3$    |
| 7.7$^c$          | W-79 H7         |
| 7.7$^c$          | W-41 H7         |
| 6.2$^c$          | F-87 H2,6       |
| 6.1$^c$          | F-87 H4         |
| 5.9$^c$          | Y-78 H3,5       |
| 5.4$^c$          | Y-69 H3,5       |
| 4.2$^c$          | Y-78 H2,6       |
| 0.6$^c$          | A-25            |
| 0.5$^c$          | A-91            |
| 0.2$^c$          | L-111 $\delta_1$ |
| -0.1$^c$         | V-122 $\gamma_1$ |

$^a$ Refer to Fig. 3e.

$^b$ Johnson-Bovey, fully averaged.

$^c$ Pople Dipolar, fully averaged.
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FIG. 4.
shifted methyl group regions. The absence of any significant shifts over this temperature range confirm the high stability of the protein's tertiary and quaternary structure suggested by the other physical techniques cited in the Introduction. The high temperature spectra are remarkable for the number of resonances in the 4.8–6.0 ppm region, many of which are due to downfield-shifted α-protons from prealbumin's extensive β-sheet regions (Dalgarno et al., 1983). A number of signals due to methyl groups which are shifted upfield, presumably by ring current interactions with nearby aromatic rings, resonate between 0.6 and 0.1 ppm. Fig. 1e shows the spectrum of the homologous rabbit prealbumin (Sundelin et al., 1985), which displays many broad similarities to that of the human protein. However, the differences in detail between the spectra of the two macromolecules suggest that even the few conservative amino acid changes revealed by a comparison of their sequences are sufficient to alter the relative orientations of many protons relative to other chemical shift-per-turbng groups.

Finally, a proton spectrum acquired at 500 MHz and 80 °C is shown in Fig. 3a and displays the improved dispersion characteristics expected at higher fields. It also resolves several peptide NH signals between ~8.4 and 10 ppm, which exchange very slowly with solvent deuterium and are intractably broad at 360 MHz, as well as a broad feature, presumably due to nonexchangeable aromatic protons, at ~6.5 ppm, which is poorly discernible at the lower field.

Even at 80 °C the protein line widths are too great for J-coupling to be resolved except in the cases of a few methyl and methylene signals. These probably have their origins in the 9 N-terminal and 4 C-terminal residues of the protein which are not observed in the crystal on account of motional disordering. Short transverse relaxation times even preclude the observation of useful cross-peaks in COSY and NOESY experiments at 500 MHz, at which field sensitivity is a less stringent limiting factor than it is at 360 MHz. Slow molecular tumbling also manifests itself in inefficient spin diffusion and rapid NOE buildup rates, which greatly complicate attempts to establish J connectivities by classical homonuclear decoupling techniques. On the other hand conventional one-dimensional NOE experiments can be performed effectively under conditions of short irradiation times (100–300 ms) during which the spread of saturation to nuclei by relatively non-specific spin-diffusive mechanisms is negligible. Thus it is possible to explore the environment of an irradiated nucleus between radii of ~2 and 5 Å.

It soon became obvious that attempting assignments by inspection of the protein structure to predict chemical shift perturbations intuitively, even combined with interproton distance measurements, was too complex a task to offer any change of success. A more analytical approach was therefore adopted, resulting in a program which we have designated PARSNIP (Program to Analyze Ring current Shifts and NOEs in Proteins), which will be described briefly.

From the x-ray coordinates, it is possible to calculate ring current-induced chemical shift perturbations using one of several predictive models (Pople, 1956; Johnson and Bovey, 1958; Haigh and Mallion, 1972) and observing the principles and cautions reviewed by Perkin (1982). These perturbations may be combined with random coil shifts for aromatic and methyl group protons (Bundi and Wuthrich, 1979) to simulate a structure-dependent partial protein spectrum. Protons constituting the various peaks can be identified by cursor picking, or comprehensive printed output is available.

NOEs resulting from irradiation of any peak can be predicted and difference spectra simulated by defining spheres of a given radius about all the constituent protons and denoting NOEs by intensity changes in the predicted spectrum. The user-defined search radius can be altered to reproduce the effects of different irradiation times. The program can also calculate the difference spectra which would result from irradiation of a single proton, rather than from a composite peak. We presently display spectra in a stick mode, and NOEs are given a single arbitrary intensity, but adaptation of the program to incorporate and display finite line widths and calculate absolute NOE magnitudes from considerations of relaxation pathways would not prove complicated. Hard copy output of simulated difference spectra is available and can be readily compared with experimental data sets. The program will also output contributions to the calculated shift of any proton from individual aromatic residues. In addition, the presentation mode could obviously be extended to simulate NOESY output if desired.

Calculated shifts can be overridden by the user if they are felt to be unreasonable or if a particular peak has been definitely assigned. The program also has options for averaging the shifts of nuclei which may be in rapid exchange between structurally dissimilar environments such as the ortho (2,6) and meta (3,5) protons of tyrosyl and phenylalanyl rings. Prealbumin additionally displays two crystallographically distinct polypeptide chain conformations (arbitrarily designated A and B) in the tetrameric holoprotein, and the program can simulate the effects of rapid averaging between these conformers. Estimation of the activation barriers a priori from solid state coordinates for either of these averaging processes is impractical, and therefore spectral simulations were performed using all four permutations of all aromatic rings and both polypeptide conformations averaged or unaveraged. It is believed that the high temperatures (60–80 °C) at which the NOE experiments were performed would probably impose fast exchange averaging on the ring shifts.

Fig. 3b accordingly shows a partial spectrum calculated on the basis of the Haigh-Mallion model under the full averaging regime. Only aromatic, methyl group, and lysine terminal methylenes are portrayed. Evaluating the accordance of each calculation with observation is necessarily somewhat subjective but was guided by certain definite criteria. The reliability of the three predictive routines is thought to decrease in the order Haigh-Mallion, Johnson-Bovey, Pople Dipolar (Perkin, 1982). All three models and averaging schemes predict the highest field-shifted peak to be the methyl group of Ala-120; the Haigh-Mallion chain-averaged prediction of the chemical shift of this group (0.2 ppm) is also quantitatively consistent with the experimentally observed shift (0.18 ppm) of peak 76 (see the control spectrum in Fig. 4). This model also ascribes the two next highest field-shifted methyls to Leu-111 â (0.5 ppm) and Val-122 γ (0.6 ppm) which possibly correspond to the experimental peaks 75 (0.28 ppm) and 74 (0.34 ppm). The other predictive models also assign high field shifts to these signals, although the Pople technique also predicts a fre-

**Fig. 4.** Control spectrum and NOE difference spectra resulting from 200-ms irradiations at the frequencies denoted by vertical arrows. All NOEs resulting from irradiation of any peak can be predicted and difference spectra simulated by defining spheres of a given radius about all the constituent protons and denoting NOEs by intensity changes in the predicted spectrum. The user-defined search radius can be altered to reproduce the effects of different irradiation times. The program can also calculate the difference spectra which would result from irradiation of a single proton, rather than from a composite peak. We presently display spectra in a stick mode, and NOEs are given a single arbitrary intensity, but adaptation of the program to incorporate and display finite line widths and calculate absolute NOE magnitudes from considerations of relaxation pathways would not prove complicated. Hard copy output of simulated difference spectra is available and can be readily compared with experimental data sets. The program will also output contributions to the calculated shift of any proton from individual aromatic residues. In addition, the presentation mode could obviously be extended to simulate NOESY output if desired.
Fig. 5. Simulated NOE difference spectra resulting from irradiation of the aromatic and individual high field-shifted methyl groups. Irradiated peaks are identified by the single bold vertical lines in each calculated difference spectrum. NOEs, of a single arbitrary intensity, are generated between nuclei within 4.5 Å of each other. The control was calculated according to the Haigh-Mallion predictive routine and by assuming full conformational averaging. Analogous printouts or screen displays are readily obtained for other models and search radii, and the contributions of individual nuclei to the effect of the irradiation of a composite peak can be easily interrogated.

Quantitative differences, and differences with respect to chemical shift rank order, do exist between the predictions of the different models, but these will not be discussed in detail. However, the calculations are encouragingly similar considering the size and complexity of the system. Comparison of experiment with predictions cannot distinguish between slow and rapid exchange between chain conformations. The Haigh-Mallion prediction under full conformational averaging conditions was eventually judged to yield the most satisfactory correspondence with experiment, and this model was chosen...
FIG. 6. Stereo plots of selected 10-Å radius regions of prealbumin, conveying some of the geometric features on which NMR assignments have been based. Each region is centered on the following residue: a, Trp-41; b, Ala-120; c, Tyr-78; and d, Tyr-69.
Close approaches between aromatic and α-protons in prealbumin

Both peptide chain conformations were searched and any approach less than 3 Å in either was registered, and distances to both Phe and Tyr ortho and meta protons were measured. The mean interproton separation, r, was then calculated over both conformation and ring orientations using the formula

$$r = \left( \frac{1}{\sigma} \right)^{1/6}$$

which takes account of the geometric dependence of NOE magnitudes (Noggle and Schirmer, 1971). F, Phe; G, Gly; K, Lys; S, Ser; W, Trp; Y, Tyr.

### Table II

| Peaks in proximity | Mean approach |
|--------------------|---------------|
| F-33 H,2,6-F-33 Ha | 3.0           |
| W-41 H-4-K-33 Ha  | 2.4           |
| W-41 H-4-W-41 Ha  | 2.6           |
| W-41 H-5-Y-69 Ha  | 2.6           |
| F-44 H-4-F-64 Ha  | 2.7           |
| F-64 H,2,6-F-64 Ha| 2.2           |
| Y-78 H,2-Y-78 Ha  | 2.9           |
| Y-78 H,3-A-38 Ha  | 3.0           |
| W-79 H-4-W-79 Ha  | 2.3           |
| W-79 H-7-S-112 Ha | 2.7           |
| W-79 H-6-S-112 Ha | 3.1           |
| Y-114 H,2,6-G-22 Ha| 2.8           |

### Table III

Assignments in human prealbumin

| Peak number | Chemical shift (ppm) | Constituents |
|-------------|---------------------|--------------|
| 1, 2        | 7.60                | W-41 M4; F-33 H,3,5 and H2,6 |
| 3           | 7.46                | W-79 H7      |
| 6           | 7.22                | W-41 H6; F-64 H,6; F-87 H,3,5; Y-105 H,3,5 |
| 7           | 7.12                | Y-105 H,2,6; W-79 H6     |
| 9, 10       | 6.93                | F-87 H,2,6; Y-114 H,2,6  |
| 11          | 6.72                | F-64 H,3,5    |
| 12          | 6.43                | Y-78 H,3,5    |
| 16          | 5.63                | Y-78 H,2,6    |
| 20          | 5.23                | F-33 H6; S-112 Ha |
| 24          | 4.86                | F-64 Ha       |
| 31          | 4.15                | G-22 H       |
| 33          | 4.03                | A-120 Ha     |
| 66          | 1.06                | T-75 CH3     |
| 74          | 0.34                | V-122 CH3    |
| 75          | 0.28                | L-111 CH3    |
| 76          | 0.18                | A-120 CH3    |

* The designations correspond to the control spectrum in Fig. 4.

As discussed previously, the identification of the highest field ring current-shifted methyl signal at 0.18 ppm with Ala-120 is consistent with all predictions. Irradiation in this spectral region results in distinct NOEs to aromatic signals 6 and 10. All the aromatic protons of Phe-87, which is disposed to exert a strong shielding influence on Ala-120, are on average within 4 Å of the latter’s methyl protons. Chemical shift predictions suggest that signals 6 and 10 ought to be observed to Phe-87 H,3,5 and H2,6, respectively. Tyr-114 H,2,6 are also close to Ala-120’s methyl group, and could contribute to signal 6. Fig. 6b portrays these geometric relationships in stereo. Ala-120 Hα probably contributes to signal 33 at 4.03 ppm. NOEs to aromatic signals 7 and 12 are also observed from irradiations at the frequencies of the other two isolated high field methyl groups at 0.28 and 0.34 ppm, suggested by the Haigh-Mallion calculation to be due to Leu-111 δ and Val-122 γ. The latter is within 4 Å of Tyr-105 H,3,5 and also close to its 2,6 protons, predicted at 7.2 and 7.4 ppm, respectively. These nuclei could contribute to peaks 7 (7.12 ppm) and 6 (7.22 ppm). Irradiation of peak 75 results in a NOE to peak 66, a composite peak at a chemical shift (1.1 ppm) consistent with calculations for Thr-75 CH3 (1.2 ppm), which is less than 4 Å from Leu-111 δ, CH3.

The short T values manifested by prealbumin make the establishment of aromatic scalar coupling connectivities by one- and two-dimensional techniques difficult. In this respect it has been assumed that the strongest NOEs will be observed between protons in an ortho configuration relative to each other on the same ring. The strongest effect resulting from irradiation of peak 13 is to a peak 16, which at 5.65 ppm resonates in the frequency band appropriate to β-sheet α-protons. However, all the shift calculation routines ascribe the highest field-shifted aromatic signal to Tyr-78 H,2,6 (at 5.5 ppm according to the Haigh-Mallion formalism) by virtue of their strong shielding by Trp-79 (conveyed by a stereo projection in Fig. 6c). Peak 13 may thus be assigned to Tyr-78 H,3,5, and indeed both sets of protons on this residue are expected to receive NOEs from the peaks corresponding to the benzylic ring of Trp-79. Signal 20, assigned as containing Phe-33 Hα, also receives NOEs from signals 3 and 7. Table II indicates that Trp-79 H7 and H6 are close to Ser-112 Hα, close to the ortho and meta protons of Phe-33 whose predicted chemical shifts (7.3 and 7.4 ppm, respectively) are also not significantly different from those of signals 1 and 2. Thus, that 1 and 2 contain contributions from some combination of Trp-41 H4 and H7 and Phe-33 H2,6 and H3,5 is consistent with chemical shift and geometric criteria. The spatial relationships which suggest these assignments are shown in a stereo presentation in Fig. 6.
and ascribing those two indole protons, respectively, to signals 3 and 7, which are connected by NOEs, and 20 to the \( \alpha \)-proton of the tryptophan residue, is consistent with chemical shift criteria. The NOEs observed between signals 3 and 7 and 13 and 16 are thus in accord with these assignments. A predicted NOE is also observed to the methyl signal 68, probably Val-28, \( \gamma \), or Ala-25.

The 3.5 protons of Tyr-69 are predicted to resonate at a frequency intermediate between the ortho and meta hydrogens of Tyr-78. They are tentatively assigned to a signal at \( \sim 6.3 \) ppm which is only well observed at 80 °C and 500 MHz and putatively broadened by an intermediate exchange rate process. Indeed, Tyr-69’s phenol ring is situated in a region of the protein interior which is particularly crowded sterically and is conceivably hindered rotationally by the close rings of Phe-44, Phe-64, and Phe-87, and several aliphatic side chains. These configurations are conveyed in Fig. 6d.

Irradiation of signal 6 (at 7.22 ppm) results in a prominent NOE to the \( \alpha \)-proton peak 24. The shift of Phe-64 H2,6 is predicted as \( \sim 7.5 \) ppm, and these protons are on average only 2.2 Å from the \( \alpha \)-hydrogen of the same residue, which is in a conformational region of the protein characterized as a turn. The actual shift of peak 24 (4.86 ppm) is consistent with this assignment. The number of protons contributing to signal 6, however, makes the positive identification of Phe-64 H5,5, predicted to resonate at 6.7 ppm, doubtful, although the effects between signals 6 and 10 and 11 could associate these protons with either of the latter two. Phe-64 H3,5 is also reasonably close to Phe-64 H4,5, and a NOE between the latter (signal 24) and signal 11 suggests that it contains the 3.5 protons of this residue.

The peak designated 9,10 has been proposed to contain a resonance from the 2.6 protons of Phe-114, which are close to the \( \alpha \)-hydrogens of Gly-22 on a neighboring polypeptide chain in the tetramer. Irradiation of this peak produces a NOE to two signals, 26 (4.71 ppm) and 31 (4.20 ppm) in the \( \alpha \)-proton spectral region, and the latter is probably ascribable to an \( \alpha \)-proton of Gly-22.

Finally, all the assignments derived and discussed above are summarized in Table III. A number of strong NOEs are observed between aromatic signals and the envelope of highly overlapping methyl group signals between 0.5 and 1.5 ppm. While the pattern of these effects is qualitatively well predicted, specific assignments were felt to be of limited use because of the high degree of spectral degeneracy and were not attempted.

**CONCLUSIONS**

The difficulties of studying large proteins by NMR have already been alluded to in the introduction. In addressing the central and general problem of achieving residue-specific assignments in a large protein, such as prealbumin, we were encouraged by the high thermal and chemical stability of this molecule, which allowed time-consuming experiments to be performed under temperature conditions which gave remarkably well resolved spectra. Furthermore, the protein yielded a particularly specific set of NOE data and was already fairly well characterized crystallographically. The difficulties and uncertainties inherent in an "inspection" approach to estimating structure-dependent chemical shifts and NOE correlations led naturally toward a more rigorous analytical procedure.

The program we have developed to this end, and which certainly proved indispensable in the derivation of the assignments discussed, may be readily applied in principle to any protein whose atomic coordinates are known and published.

It was not felt necessary in this case to include certain refinements such as allowances for limited side chain motions, but such additions would be straightforward. The assignments derived in this paper have been used subsequently in studies of regional mobility changes induced in the protein by different natural and synthetic thyromimetics, the results of which are reported in the accompanying paper (Reid et al., 1989).

**Acknowledgments**—We would like to thank Prof. Johann Sundelin for his gift of rabbit prealbumin, our colleagues John Emmett, Andy Vinter, Ted Pepper, Luciano Mueller, and Sally Heald for their interest in this work, and Neville Haskins for sharing his skill in deciphering acronyms. We are also indebted to Colín Blake and Patricia de la Paz for helpful discussions and sharing unpublished results.

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