Studies on the Solution Conformation of Human Thioredoxin Using Heteronuclear \( ^{15}\text{N}-^1\text{H} \) Nuclear Magnetic Resonance Spectroscopy†

Julie D. Forman-Kay,†,‡ Angela M. Gronenborn,*,† Lewis E. Kay,† Paul T. Wingfield,‖,‖ and G. Marius Clore*,†

Laboratory of Chemical Physics, Building 2, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892, Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06511, and Glaxo Institute for Molecular Biology SA, 46 Route des Acacias, CH-1211 Geneva, Switzerland

Received August 2, 1989; Revised Manuscript Received September 28, 1989

ABSTRACT: The solution conformation of uniformly labeled \( ^{15}\text{N} \) human thioredoxin has been studied by two-dimensional heteronuclear \( ^{15}\text{N}-^1\text{H} \) nuclear magnetic resonance spectroscopy. Assignments of the \( ^{15}\text{N} \) resonances of the protein are obtained in a sequential manner using heteronuclear multiple quantum coherence (HMQC), relayed HMQC-correlated (COSY), and relayed HMQC–nuclear Overhauser (NOESY) spectroscopy. Values of the \( J_{\text{HN}} \) splittings for 87 of the 105 residues of thioredoxin are extracted from a variant of the HMQC–COSY experiment, known as HMQC–J, and analyzed to give accurate \( J_{\text{HN}} \) coupling constants. In addition, long-range \( J_{\text{H(i),N(i+1)}} \) scalar connectivities are identified by heteronuclear multiple bond correlation (HMBC) spectroscopy. The presence of these three-bond scalar connectivities in predominantly \( \alpha \)-helical regions correlates well with the secondary structure determined previously from a qualitative analysis of homonuclear nuclear Overhauser data [Forman-Kay, J. D., Clore, G. M., Driscoll, P. C., Wingfield, P. T., Richards, F. M., & Gronenborn, A. M. (1989) Biochemistry 28, 7088–7097], suggesting that this technique may provide additional information for secondary structure determination a priori. The accuracy with which \( J_{\text{HN}} \) coupling constants can be obtained from the HMQC–J experiment permits a more precise delineation of the beginnings and ends of secondary structural elements of human thioredoxin and of irregularities in these elements.

The application of nuclear magnetic resonance (NMR) spectroscopy to the determination of three-dimensional structures of proteins in solution has advanced rapidly in the past few years [see Wüthrich (1986) and Clore and Gronenborn (1987, 1989) for reviews]. A formidable limitation, however, still exists with respect to the size of molecules to which the methodology can be applied. One avenue for resolving ambiguities in assignment arising from severe spectral overlap associated with molecules larger than \( \sim 10 \) kDa was opened by the development of heteronuclear \( ^{15}\text{N}-^1\text{H} \) experiments. In addition to the powerful three-dimensional heteronuclear experiments reported recently (Marion et al., 1989a, b; Zuiderweg & Fesik, 1989), two-dimensional heteronuclear relayed multiple quantum and multiple bond correlation experiments can also be used to deal with problems caused by overlapping resonances and confirm assignments (Gronenborn et al., 1989a, b; Clore et al., 1988). \( ^{15}\text{N}-^1\text{H} \) HMQC, relayed HMQC–NOESY, HMQC–COSY, and HMBC experiments have been recorded on human thioredoxin.

†This work was supported by the Intramural AIDS Targeted Antiviral Program of the Office of the Director, NIH (A.M.G. and G.M.C.). J.D.F. acknowledges a graduate fellowship from the Molecular Biophysics and Biochemistry Department of Yale University and support from NIH Grant GM-22778 (to F. M. Richards, Yale University).

*National Institutes of Health.

†Yale University.

¶Glaxo Institute for Molecular Biology SA.

1 Present address: Protein Expression Laboratory, Building 6B, National Institutes of Health, Bethesda, MD 20892.

‡Abbreviations: NMR, nuclear magnetic resonance; HMQC, heteronuclear multiple quantum correlation spectroscopy; NOESY, two-dimensional nuclear Overhauser effect spectroscopy; COSY, two-dimensional correlated spectroscopy; HMQC–COSY, relayed heteronuclear multiple quantum coherence correlated spectroscopy; HMQC–NOESY, relayed heteronuclear multiple quantum coherence nuclear Overhauser spectroscopy; HMBC, heteronuclear multiple bond correlation spectroscopy; NOE, nuclear Overhauser effect; E. coli, Escherichia coli; DTT, dithiothreitol.
to obtain accurate \(^{2}J_{\text{HN}}\) coupling constants, to confirm the previous proton resonance assignments and secondary structure determination derived from purely homonuclear methods (Forman-Kay et al., 1989), and to remove ambiguities in the assignment of the long-range NOE peaks. The excellent correlation of the NMR parameters with the secondary structure, augmented by accurate coupling constant data, allows a finer determination of the beginnings and ends of the \(\alpha\)-helices and \(\beta\)-strands in human thioredoxin and yields specific information regarding irregularities in these secondary structural elements.

**EXPERIMENTAL PROCEDURES**

Human thioredoxin was purified from a strain of *E. coli* containing a temperature-sensitive repressor and a plasmid bearing the thioredoxin gene under the control of the \(\lambda\) P\(_{r}\) promoter and phage Mu ner gene ribosome binding site, as described in Wollman et al. (1988). The bacteria were grown in minimal medium using \(^{13}\)NH\(_{4}\)Cl as the sole nitrogen source to achieve essentially complete \(^{15}\)N labeling of the protein. Two species of thioredoxin differing in the presence or absence of the N-terminal methionine were produced due to inefficient posttranslational processing, with the ratio 30% N-Met/70% N-Val as determined by N-terminal amino acid sequencing. For NMR samples, 2 mM protein was reduced with excess dithiothreitol (DTT), dialyzed against 150 mM phosphate buffer, pH 5.5, containing 0.2 mM DTT, lyophilized, redissolved in argon-purged 99.996% D\(_{2}\)O or 90% H\(_{2}\)O/10% D\(_{2}\)O, sealed in tubes with airtight rubber septa, and blanketed with argon for 30 min.

All experiments were recorded on a Bruker AM600 spectrometer at 40 °C and were processed on an ASPECT 3000 computer. Quadrature in \(F_1\) was obtained by using the time proportional phase incrementation method (Marion & Wüthrich, 1983). The \(\text{H}^1\) detected heteronuclear multiple quantum correlation (HMOC) experiment (Bax et al., 1983; Bendell et al., 1983) and the relayed HMOC-correlated (HMOC-COSY) and HMOC-nuclear Overhauser effect (HMOC-NOESY) experiments (Gronenborn et al., 1989a) were recorded in H\(_{2}\)O, collecting 256 or 512 \(t_1\) increments of 2K data points. To measure \(^{1}J_{\text{HN}}\) coupling constants, a variant of the HMOC-COSY experiment, known as HMOC-J (Kay & Bax, 1990), was recorded using the pulse scheme

\[\text{HMOC-J:} \quad 90^\circ_x \times \tau - t_1/2 - 180^\circ_x \times t_1/2 - \tau - 90^\circ_y \times \text{Acq} \]

with phase cycling \(\alpha = 2(\pi) (x) (y) (z) \times (z) \times (y) \times (z); \beta = x, -x, \delta = 8(\pi); \delta = x, -x, -x\); and Acq = \(2(x, -x, -x) (z) \times (x, x, -x) \times (z)\). The delay \(\tau\) was set to 4.5 ms, slightly less than \(1/(2J_{\text{HN}})\). This experiment was also recorded in H\(_{2}\)O with 1248 \(t_1\) increments of 2K data points to give an acquisition time of \(t_1\) of 250 ms.

Zero-filling in \(F_1\) gave a final digital resolution of 0.5 Hz/pixel on the \(15\)N dimension. The pulse sequence and phase cycling for the HMOC-J experiment (Kay & Bax, 1990) are identical with that of the HMOC-COSY experiment (Gronenborn et al., 1989a) and differ from the normal HMOC experiment by the addition of a \(90^\circ_y\) pulse immediately before acquisition. This suppresses the dispersive contributions to the line shape in \(F_2\). For all experiments in H\(_{2}\)O, water suppression was achieved with coherent presaturation of the heteronuclear multiple bond correlation (HMBC) experiment (Bax & Summers, 1986; Clore et al., 1988) was carried out in D\(_{2}\)O with 256 \(t_1\) increments of 1K data points. The HMBC spectrum was recorded in mixed mode, with pure phase absorption in the \(15\)N \(F_1\) dimension and absolute value in the \(1\)H \(F_2\) dimension (Bax & Marion, 1988).

**RESULTS AND DISCUSSION**

Assignment of the \(^{15}\)N resonances in combination with a variety of heteronuclear experiments can confirm and add structurally relevant data about a protein to that already obtained from homonuclear results. Although essentially complete \(^{1}\)H assignments of human thioredoxin could be obtained by using purely homonuclear methods because of the relatively large chemical shift dispersion (Forman-Kay et al., 1989), information from \(^{15}\)N-\(^{1}\)H spectra was extracted to augment the available data. The relayed HMOC-NOESY (Figures 1A and 2A) and HMOC-COSY (Figure 1B) spectra contain essentially the same information as the NH region of their homonuclear counterparts, except that the NH \(^{1}\)H chemical shift axis is replaced by the \(^{15}\)N chemical shift axis. \(^{15}\)N sequential assignment proceeds in an analogous manner to the \(^{1}\)H sequential assignment. Direct and relayed scalar connectivities from the \(^{15}\)N atom to the intraresidue NH and C\(_{\alpha}\)H atoms are obtained from the HMOC and HMOC-COSY spectra, respectively. Sequential through-space NOE connectivities involving the NH protons are relayed to the corresponding \(^{15}\)N atom and identified in the HMOC-NOESY spectrum. Since the \(^{15}\)N assignment could be done in a manner independent of the NH assignments, it offered a useful check of the previously determined \(^{1}\)H chemical shifts (Forman-Kay et al., 1989). In addition, the \(^{15}\)N assignment aided the resolution of ambiguities in the assignment of long-range NOEs necessary for a complete tertiary structure determination, since chemical shift degeneracy or near degeneracy of particular NH proton resonances is generally not present for the corresponding \(^{15}\)N resonances and vice versa. Table I lists the \(^{15}\)N chemical shifts for resonances from both forms of human thioredoxin, the minor species containing the N-terminal methionine and the major species lacking this residue, leaving an N-terminal valine. Resonances with distinguishable NH or C\(_{\alpha}\)H \(^{1}\)H chemical shifts for the two forms are denoted with an asterisk on the amino acid name of the resonance arising from the major N-Val species.

Apparent \(^{2}J_{\text{HN}}\) splittings were measured directly from \(F_1\) cross sections of the HMOC-J spectrum (Figure 2B). These measured values do not represent the actual couplings since the line shape of the multiplets is not purely absorptive and because the two peaks of the multiplet overlap. The dispersive component of the line shape leads to overestimation of the \(^{2}J_{\text{HN}}\) value for large couplings, and the multiplet overlap causes underestimation and even disappearance of the splittings for small couplings. Figure 3A displays the apparent \(^{2}J_{\text{HN}}\) splittings as a function of the true \(^{2}J_{\text{HN}}\) coupling constants and illustrates the effect of dispersive line-shape components and finite line widths on the apparent \(^{2}J_{\text{HN}}\) splittings. The curves were generated by extracting the maxima from line shapes simulated by the Fourier transformation of a function describing the \(t_1\) evolution of magnetization for the HMOC-J sequence, modified by the application of a Gaussian apodization function. The time domain response for a multiplet centered at zero frequency is given by the expression \(\cos(2\pi J_{\text{HN}}t_1 + \phi) \exp[-\gamma^2/2T_{\text{MQ}}^2]\), where \(T_{\text{MQ}}\) refers to the multiple quantum relaxation time and \(r\) is the delay time that allows for efficient creation of heteronuclear multiple quantum coherence [in this case \(r\) was set to 4.5 ms, slightly less than \(1/(2J_{\text{HN}})\)]. The multiple quantum line width was assumed to be 10 Hz, and four different line-broadening parameters of the Gaussian apodization function were used, as indicated in Figure 3A.
FIGURE 1: $^{15}$N ($F_1$ axis)–aliphatic $^1$H ($F_2$ axis) region of the 150-ms relayed $^{15}$N–$^1$H HMQC–NOESY spectrum in H$_2$O (A), the $^{15}$N ($F_1$ axis)–$^1$C_H $^1$H ($F_2$ axis) region of the relayed $^{15}$N–$^1$H HMQC–COSY spectrum in H$_2$O (B), and the $^{15}$N ($F_1$ axis)–$^1$C_H $^1$H ($F_2$ axis) region of the HMBC spectrum in D$_2$O (C) of $^{15}$N-labeled reduced recombinant human thioredoxin (30% N-Met/70% N-Val) at 40 °C and pH 5.5. In the HMQC–NOESY spectrum (A) selected C,H(i)–$^{15}$N(i+1) connectivities are indicated. In the HMBC spectrum (C), selected C,H(i)–$^{15}$N(i+1) connectivities are also indicated. Peaks with distinguishable chemical shifts between the two forms and arising from the major N-terminal Val species are denoted with an asterisk.

To correct the experimentally measured $J_{HNa}$ values for the errors demonstrated in Figure 3A, the following strategy was employed. The HMQC–$J$ spectrum was processed with a Gaussian apodization function in $F_1$ using four different negative line-broadening parameters (−4, −6, −8, and −10 Hz) and a curve with a maximum at the end of the $t_1$ time domain data to optimize resolution. Processing the data with at least two different negative line-broadening parameters is required to extract $J_{HNa}$ and line-width values from each multiplet. In practice, more accurate $J_{HNa}$ values can be obtained by using estimated splittings based on three or more line-broadening parameters. Splittings measured from experimental spectra processed with the different parameters, along with initial guesses of the true $J_{HNa}$ values and multiple quantum line widths, were entered into a least-squares minimization program. Corrected values of $J_{HNa}$ were obtained by varying the input values of $J_{HNa}$ and the multiple quantum line widths using Powell’s nonlinear optimization algorithm (Powell, 1965) to minimize the differences between the measured values of $J_{HNa}$ for the different values of the line-broadening parameter and the corresponding calculated values of the apparent $J_{HNa}$ splittings obtained from the line-shape simulated as described above. The corrected values of the $J_{HNa}$ coupling constants are presented in Table I.

The significantly narrower multiple quantum line widths in the $F_1$ dimension (Bax et al., 1989), coupled with the analysis described above which corrects for multiple quantum line widths and dispersive contributions, result in $J_{HNa}$ values whose accuracy far exceeds that of the apparent coupling constants measured directly from homonuclear COSY-type experiments without correction for the broad homonuclear NH line widths. This latter technique results in substantial overestimation in the values of the coupling constants unless similar methods for correcting the $J_{HNa}$ values to that used here are employed. A fundamental limitation, however, of the homonuclear COSY experiment is that the minimum separation of the anti phase components of the NH–C,H COSY cross-peaks is approximately half the NH line width (Neuhaus et al., 1985). Typical homonuclear NH line widths for proteins the size of thioredoxin are between 10 and 20 Hz, making it impossible to measure many couplings in α-helical regions, which normally lie between 3 and 6 Hz. The lower limit for
Solution Conformation of Human Thioredoxin

Biochemistry, Vol. 29, No. 6, 1990

1569

105-

10

I

G87

10

G91

d'

li5L0

9

8

OF80

mK82

10

H

PPW

muRe

2: 15N FI axis)-NH/aromatic 'H (F2 axis) region of the

150-111s relayed 15N-lH HMQC-NOESY spectrum (A) and the

HMOC-J spectrum (B) of 15N-labeled reduced recombinant human

thioredoxin (30% N-Met/70% N-Val) at 40 °C and pH 5.5. In the

HMOC-NOESY spectrum (A) selected 15N(i)-NH(i+1) connect-

ivities from residue Ser-7 to residue Gln-12 and from Phe-41* to

Lys-48* are indicated. Peaks with distinguishable chemical shifts

between the two forms and arising from the major N-terminal Val

species are denoted with an asterisk.

measurable couplings in the HMQC-J experiment is well

below that of the COSY experiments due to the intrinsically

narrower multiple quantum line widths. The smallest apparent

splittings measured in the HMQC-J spectrum of human

thioredoxin were 2.0 Hz with corrected 3JHNa values of 3.8

and 4.0 Hz for Asp-102* and Lys-94, respectively.

To estimate the precision of the corrected values of the 3JHNa

coupling constants and to ensure that the values derived from

the least-squares fitting procedure were not in local minima,
different initial guesses for the line width and 3JHNa values

were used as input. Values of 3JHNa varying within ±0.5 Hz

for large couplings and within ±1.0 Hz for small couplings

were obtained for different initial guesses. Large couplings

seem to be more robust to experimental errors in measuring

the 3JHNa splittings since there is less of a dependence on the

multiple quantum line width, as indicated in Figure 3B.

Within the 10-14-Hz regime, where the multiple quantum line

widths of human thioredoxin at 40 °C lie, this dependence is

only significant for 3JHNa values less than 6 Hz. The accuracy

of these small 3JHNa values, nevertheless, is still extremely good.

In addition, only four 3JHNa splittings in the HMQC-J

spectrum of human thioredoxin were too small to be measured,

namely, those for Thr-9, Gln-12, Thr-30, and Asn-39.

The 3JHNa values determined by using the HMQC-J ex-

periment and subsequent numerical analysis correlate well with

the secondary structure determined from a qualitative inter-

pretation of homonuclear NOESY patterns, NH exchange
data, and the COSY-derived couplings. The structure of

human thioredoxin elucidated from the previous homonuclear

experiments and supported by these accurate 3JHNa couplings

is a mixed five-stranded β-sheet and four α-helices (Form-

man-Kay et al., 1989). The couplings measured are related to

the φ torsion angle of the peptide backbone by a Kar-

plus-like relationship (Karplus, 1963; Pardi et al., 1984).

Values obtained for human thioredoxin are generally less than

6 Hz for α-helical regions and greater than 7 Hz for β-sheet

regions. This contrasts with the data from the COSY ex-

periment, where apparent couplings could only be categorized

into ranges of less than 9 Hz for α-helical regions and greater

than 9 Hz for regions of β-sheet (Forman-Kay et al., 1989).

Figure 4 illustrates the correlation between the 3JHNa coupling

constants derived from the HMQC-J spectrum (Figure 4A)

and the secondary structure of human thioredoxin (Figure 4C).

The secondary structure of human thioredoxin which was
determined on the basis of homonuclear NOEs and COSY-
derived splittings can also now be reevaluated by using in-
formation inherent in the accurate 3JHNa splittings obtained

FIGURE 2: 15N (F1 axis)–NH/aromatic 1H (F2 axis) region of the

150-ms relayed 15N–H HMQC-NOESY spectrum (A) and the

HMOC-J spectrum (B) of 15N-labeled reduced recombinant human

thioredoxin (30% N-Met/70% N-Val) at 40 °C and pH 5.5. In the

HMOC-NOESY spectrum (A) selected 15N(i)-NH(i+1) connect-

ivities from residue Ser-7 to residue Gln-12 and from Phe-41* to

Lys-48* are indicated. Peaks with distinguishable chemical shifts

between the two forms and arising from the major N-terminal Val

species are denoted with an asterisk.

FIGURE 3: (A) Apparent 3JHNa splittings extracted from line shapes

simulated by Fourier transformation of the t1 time domain response,

plotted as a function of the true 3JHNa value for different negative

line-broadening values (LB) of the Gaussian apodization function of

-10 Hz (---), -8 Hz (-), -6 Hz (-), and -4 Hz (---), assuming

a multiple quantum line width of 10 Hz. Note that the Gaussian curve

used has a maximum at the end of the time domain (i.e., GB = 1).

To guide the eye, a straight line given by 3JHNa(apparent) =

3JHNa(true) is also drawn. (B) Apparent 3JHNa splittings as a function

of multiple quantum line width, for values of the true 3JHNa coupling

of 4 Hz (-), 6 Hz (--), 8 Hz (--), and 10 Hz (---), assuming LB

= -6 Hz and GB = 1.

Published on 01 December 1990. DOI: 10.1021/bi00064a008
Table I: \(^{15}\)N Assignments and \(^{1}J_{\text{HN}}\) Coupling Constants for the N-Met and N-Val* Forms of Human Thioredoxin*

<table>
<thead>
<tr>
<th>residue</th>
<th>(^{15})N (ppm)</th>
<th>(^{1}J_{\text{HN}}) (Hz)</th>
<th>residue</th>
<th>(^{15})N (ppm)</th>
<th>(^{1}J_{\text{HN}}) (Hz)</th>
<th>residue</th>
<th>(^{15})N (ppm)</th>
<th>(^{1}J_{\text{HN}}) (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>b</td>
<td>b</td>
<td>E68</td>
<td>123.8</td>
<td>5.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V2</td>
<td>b</td>
<td>b</td>
<td>E70</td>
<td>116.9</td>
<td>6.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K3*</td>
<td>128.9</td>
<td>9.7</td>
<td>V71</td>
<td>119.6</td>
<td>7.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q4*</td>
<td>126.5</td>
<td>6.9</td>
<td>K72</td>
<td>126.9</td>
<td>11.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q3 NH₂</td>
<td>112.3</td>
<td>f</td>
<td>Q6</td>
<td>114.3</td>
<td>6.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I5*</td>
<td>127.3</td>
<td>7.3</td>
<td>T74</td>
<td>112.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I5</td>
<td>127.7</td>
<td>e</td>
<td>P75</td>
<td>114.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E6</td>
<td>123.4</td>
<td>7.6</td>
<td>T76</td>
<td>119.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S7</td>
<td>111.4</td>
<td>6.3</td>
<td>F77</td>
<td>125.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K8</td>
<td>122.3</td>
<td>4.3</td>
<td>Q78</td>
<td>118.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T9</td>
<td>115.3</td>
<td>g</td>
<td>Q84 NH₂</td>
<td>105.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A10</td>
<td>123.7</td>
<td>5.4</td>
<td>F79</td>
<td>119.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F11</td>
<td>120.5</td>
<td>4.4</td>
<td>G83</td>
<td>104.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q12</td>
<td>117.3</td>
<td>g</td>
<td>K81</td>
<td>118.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q12 NH₂</td>
<td>113.4</td>
<td>f</td>
<td>K82</td>
<td>128.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E13</td>
<td>117.6</td>
<td>4.4</td>
<td>G89</td>
<td>118.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A14</td>
<td>121.7</td>
<td>5.1</td>
<td>Q84 NH₂</td>
<td>112.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L15</td>
<td>117.2</td>
<td>g</td>
<td>G85</td>
<td>125.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D16</td>
<td>119.7</td>
<td>4.9</td>
<td>V86</td>
<td>120.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A18</td>
<td>121.3</td>
<td>5.9</td>
<td>N51</td>
<td>118.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G19</td>
<td>105.8</td>
<td>5.7</td>
<td>N51 NH₂</td>
<td>112.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D20</td>
<td>128.5</td>
<td>h</td>
<td>V52*</td>
<td>120.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K21</td>
<td>119.3</td>
<td>5.9</td>
<td>V52</td>
<td>120.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L22</td>
<td>122.8</td>
<td>5.4</td>
<td>S69</td>
<td>116.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V23</td>
<td>126.0</td>
<td>10.0</td>
<td>F54*</td>
<td>125.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V24*</td>
<td>126.9</td>
<td>9.6</td>
<td>F54</td>
<td>126.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V24</td>
<td>126.8</td>
<td>h</td>
<td>L55*</td>
<td>122.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V25*</td>
<td>126.8</td>
<td>9.7</td>
<td>L55</td>
<td>122.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V25</td>
<td>126.7</td>
<td>h</td>
<td>E56*</td>
<td>121.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D26</td>
<td>123.5</td>
<td>8.8</td>
<td>E56</td>
<td>121.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F27</td>
<td>125.4</td>
<td>9.1</td>
<td>V57*</td>
<td>125.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S28</td>
<td>117.7</td>
<td>8.5</td>
<td>V57</td>
<td>125.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A29</td>
<td>121.7</td>
<td>e</td>
<td>D58*</td>
<td>128.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T30</td>
<td>116.2</td>
<td>g</td>
<td>V59</td>
<td>119.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W31</td>
<td>122.6</td>
<td>c</td>
<td>D61</td>
<td>120.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C32</td>
<td>122.6</td>
<td>e</td>
<td>D60</td>
<td>119.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G33</td>
<td>128.8</td>
<td>d</td>
<td>C62</td>
<td>120.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C35</td>
<td>131.8</td>
<td>i</td>
<td>Q63</td>
<td>122.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K36</td>
<td>120.7</td>
<td>h</td>
<td>Q63 NH₂</td>
<td>111.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M37</td>
<td>116.2</td>
<td>5.1</td>
<td>D64</td>
<td>118.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I38</td>
<td>115.8</td>
<td>8.1</td>
<td>V65</td>
<td>121.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N39</td>
<td>125.3</td>
<td>g</td>
<td>A66</td>
<td>120.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N39*</td>
<td>125.3</td>
<td>e</td>
<td>S67</td>
<td>111.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Residues which give distinguishable NH or CH₃CH proton chemical shifts for the two forms of human thioredoxin are denoted with an asterisk (*) at the amino acid name for the resonances arising from the major N-terminal valine species. \(^{15}\)N chemical shifts are reported with respect to external liquid NH₃. *Unassigned resonance. **Resonance not observable at 40 °C. **Proline residue has no NH proton and no \(^{1}J_{\text{HN}}\) coupling. Hence, the \(^{15}\)N chemical shift can only be assigned through the observation of \(^{1}H(i-1)\) - \(^{1}H(i)\) scalar connectivities. These were not observed for Pro-75. **Cross-peak too weak to measure coupling. \(^{1}J_{\text{HN}}\) group, therefore, no \(^{1}J_{\text{HN}}\) coupling to measure. **The coupling is too small to measure, probably less than 3.75 Hz. **\(^{1}J_{\text{HN}}\) coupling impossible to measure due to overlap. **Splitting pattern uninterpretable, perhaps due to multiple conformations.

by the method described above. The beginnings and ends of β-strands are well-defined by the NOEs across the sheet, but the extent of α-helices is much less obvious. Although the coupling constants of turns and loops may cover a broad range of values, they are not usually as small as expected for an ideal α-helix (4-5 Hz). Therefore, using the NH(i-1)-NH(i+1,7,2,3,4), CH(i-1)-NH(i+1,2,3,4), and CH(i)-CH(i+1) NOEs in conjunction with the \(^{1}J_{\text{HN}}\) values derived from the HMOC-J spectrum, the four α-helices of human thioredoxin were reexamined to obtain a better definition of their starting and ending points. As a result, the previous placement of secondary structure elements was found to be essentially correct except for α₂, which seems to begin at Gln-63, rather than at Cys-62 as thought earlier, due to the very high \(^{1}J_{\text{HN}}\) value of 9.9 Hz for this residue, which is inconsistent with helical structure.

In addition, an assessment of the degree to which both the α-helices and the β-strands of human thioredoxin maintain ideal geometry can be made. The β-strands β₁, β₂, β₃, and β₄ appear to be quite regular, with couplings ranging from 8 to 10 Hz. However, β₂, a strand at the edge of the sheet, deviates substantially from a completely extended chain. This is evident from both the pattern of NH(i)-NH(i+1) NOEs, which are not seen for an ideal β conformation, and the \(^{1}J_{\text{HN}}\) values of 4.7, 6.2, and 5.6 Hz for residues Gly-87, Glu-88, and Phe-89, respectively, which correspond to backbone torsion angles more consistent with an irregular than an extended structure. A number of the α-helices in human thioredoxin contain distortions from ideal geometry as well. In α₁, which contains Pro-40, residue Ile-38 has a coupling of 8.1 Hz and Phe-41 has one of 6.5, both larger than expected within a perfect α-helical stretch. The end of this helix is also distorted with couplings of 7.2, 7.5, and 8.9 Hz for residues Glu-47, Lys-48, and Tyr-49, respectively. Similar effects are apparent at the ends of α₂, which seem to exhibit conformations deviating from the ideal α-helical geometry, with Cys-69 and Glu-70 in α₁ having couplings of 8.5 and 6.9 Hz and residues Leu-104 and Val-105 in α₂ having couplings of 8.9 and 5.8 Hz. Thus, the accurate \(^{1}J_{\text{HN}}\) values obtained from the
Solution Conformation of Human Thioredoxin errors from dispersive components to the line shape and multiplet connectivities due to overlapping or unidentified CuH(i)-ISN(i+1) qualitative analysis of N-terminal Val species of reduced recombinant human thioredoxin, on the basis of the data obtained from homonuclear experi-


tures with the secondary structure of the protein. Therefore, C,H(i)-15N(i+1) cross-peaks from the HMBC experiment and corrected to remove errors from dispersive components to the line shape and multiplet overlap effects. The reasons for the absence of several values in the figure is indicated in Table I. (B) Relative intensities of the scalar C,H(i)-15N(i+1) connectivities from the HMBC experiment, plotted as a function of residue number. Missing data represent unassigned connectivities due to overlapping or unidentified 15N resonances. (C) Secondary structure of human thioredoxin determined from a qualitative analysis of 1H NOE patterns and NH exchange data, provided as a reference to illustrate the correlation of these two NMR parameters with the secondary structure of the protein.

HMBC-J spectrum permit the secondary structure of human thioredoxin to be described more precisely than can be achieved on the basis of the data obtained from homonuclear experiments alone.

A second heteronuclear 15N-1H experiment was analyzed that also correlates well with the secondary structure of the protein. The HMBC experiment yields correlations between atoms separated by two and three bonds, including those to C,H(i) from both 15N(i) and 15N(i+1). The presence of scalar C,H(i)-15N(i+1) connectivities confirms the sequential assignments. More importantly, the intensities of these connectivities, which are related to the ψ backbone torsion angle, also confirm the secondary structure determination. The magnitude of the 3JHNO coupling constant is related to the ψ angle via a Karplus-type relationship and has a maximum absolute value of 6 Hz for ψ = -60°. Couplings greater than 2 Hz arise for -120° < ψ < 0°, which overlaps the torsion-angle range found within α-helices, and couplings less than 2 Hz are seen for all other ψ values, including those found in β-strands (Bystrov, 1976). The intensity of the scalar C,H(i)-15N(i+1) cross-peak is strongly dependent on the size of the heteronuclear coupling, the transverse relaxation time, and the multiplicity of the 1H multiplet (Clore et al., 1988). Considering that the 1H T2 values for a protein the size of thioredoxin are relatively short (<50 ms), couplings smaller than about 3 Hz are not expected to yield observable correlations. Consequently, C,H(i)-15N(i+1) cross-peaks are predicted to be observed principally in α-helical regions. This makes the HMBC experiment useful for confirming sequential assignments or determining them a priori within α-helical regions where the sequential C,H(i)-NH(i+1) NOEs are weakest.

As expected from this analysis, the scalar C,H(i)-15N(i+1) cross-peaks are most apparent between residues Lys-8 and Lys-21, Pro-34 and Tyr-49, Gln-63 and Cys-69, and Lys-94 and Thr-100 of human thioredoxin, overlapping significant portions of α-helices α1, α2, α3, and α4, respectively. A summary of the relative intensities of the C,H(i)-15N(i+1) connectivities of the protein is presented in Figure 4B. The correlation between the magnitude of the HMBC peaks and secondary structure features is clearly demonstrated. Here again, irregularities within the secondary structure elements can easily be detected. For example, Phe-42, within helix α2, exhibits a very weak C,H(i)-15N(i+1) connectivity, confirming the finding, based on 3JHNO coupling constant analysis, of irregular geometry near Pro-40. Similar effects of nonideal conformations are found at the end of helix α4.

CONCLUDING REMARKS

Two-dimensional heteronuclear 15N-1H NMR experiments on completely 15N labeled proteins provide significant information relevant to a solution structure determination that can confirm and complement data extracted from homonuclear 1H experiments. The HMQ, HMQC-NOESY, and HMQC-COSY experiments yield an independent check on the sequential proton resonance assignment, and the HMQC-NOESY permits the resolution of many ambiguities in long-range NOE assignments necessary to extract distance data for use in tertiary structure calculations. 3JHNO coupling constants measured from the HMQ-J spectrum are more accurate, and the number of measurable couplings is greater than could be derived from homonuclear COSY-type spectra, particularly for a protein of the size of human thioredoxin and larger ones where broad NH line widths preclude the measurement of small couplings (Kay et al., 1989). The high level of confidence in the values for these couplings has led to a more precise determination of the secondary structure of human thioredoxin in terms of both the extents of the secondary structural elements and the degree to which they maintain ideal geometry. Finally, sequential scalar C,H(i)-15N(i+1) connectivities in the HMBC experiment allow confirmation of sequential assignments, particularly since the corresponding cross-peaks are strongest in α-helical regions where the homonuclear C,H(i)-NH(i+1) NOE is weakest. Moreover, since the 3JHNO values and the intensity of the HMBC C,H(i)-15N(i+1) peaks are related to the ϕ and ψ torsion angles of the peptide backbone, respectively, these parameters are invaluable to confirm the secondary structure obtained by homonuclear experiments alone and can be analyzed in their own right to determine the secondary structure. Thus, even in cases where the 1H chemical shift dispersion is sufficient for assignment using only homonuclear methods, heteronuclear 15N-1H experiments can be extremely useful for larger proteins in providing additional data to be incorporated into a structural study of these macromolecules.

REFERENCES


Environments and Conformations of Tryptophan Side Chains of Gramicidin A in Phospholipid Bilayers Studied by Raman Spectroscopy†

Hideo Takeuchi, Yasuhisa Nemoto,‡ and Issei Harada*

Pharmaceutical Institute, Tohoku University, Aobayama, Sendai 980, Japan

Received July 12, 1989; Revised Manuscript Received October 9, 1989

ABSTRACT: Raman spectroscopy has been used to investigate the hydrophobic interaction of the indole ring with the environments, the water accessibility to the N1H site, and the conformation about the Cg-C3 bond for the four tryptophan side chains of gramicidin A incorporated into phospholipid bilayers. Most of the tryptophan side chains of the head-to-head helical dimer transmembrane channel are strongly interacting with the lipid hydrocarbon chains, and the hydrophobic interactions for the rest increase with increasing hydrocarbon chain length of the lipid. One tryptophan side chain (probably Trp-15) is accessible to water for the four tryptophan side chains of gramicidin A incorporated into phospholipid bilayers. Most of the tryptophans (Trp-11 and Trp-13) depend on the bilayer thickness. The torsional angle about the Cg-C3 bond is found to be ±90° for all the tryptophans irrespective of the membrane thickness. Binding of the sodium cation to the channel does not change the torsional angles but decreases the water accessibilities of two tryptophans (Trp-11 and Trp-13) considerably. In conjunction with a slight spectral change in the amide III region, it is suggested that the sodium binding causes a partial change in the main-chain conformation around Trp-11 and Trp-13, which results in the movements of these side chains toward the bilayer center. Two models consistent with the present Raman data are proposed for the tryptophan orientation in the dominant channel structure.

Gramicidin A is a linear pentadecapeptide composed of alternating L- and D-amino acids with the N- and C-terminal residues blocked by a formyl and an ethanolamide group, respectively: HCO-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-

NHCH2CH2OH (Sarges & Witkop, 1964). This peptide forms transmembrane channels that induce permeability to monovalent cations and water in natural and artificial lipid membranes (Hladky & Haydon, 1972; Myers & Haydon, 1972; Rosenberg & Finkelstein, 1978; Finkelstein & Andersen, 1981). The structure of the channel has been studied extensively by various physicochemical methods, and a model proposed by Urry (1971) for the backbone conformation is now generally accepted (Weinstein et al., 1980; Urry et al., 1982b, 1983; Wallace et al., 1986). According to the model, the channel is a dimer consisting of two left-handed β-helical