The Additional Methionine Residue at the N-Terminus of Bacterially Expressed Human Interleukin-2 Affects the Interaction between the N- and C-Termini

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ABSTRACT: To gain insight into the origin of the difference in isoelectric point (p*I*) values for wild-type human interleukin-2 (IL-2) and IL-2 with an additional methionine residue at the N-terminus (Met-IL-2), conformational properties of the two molecular forms of IL-2 were compared by utilizing ^{1}H NMR spectroscopy. Although overall conformations were conserved in the two forms, the presence of the additional methionine residue at the N-terminus induced chemical shift changes for residues Ala1 to Lys8 as well as for Thr133, which is located at the C-terminus. These observations indicate that the effect of the additional methionine residue is confined to the N- and C-terminal regions and unveil the existence of an interaction between the N- and C-terminal regions. The chemical shift change observed for Thr133 can be interpreted in terms of a change in pK_a of the C-terminal carboxyl group, which interacts differently with the N-terminal amino group in the two forms of IL-2. It seems to be reasonable to conclude that the difference in p*I* values for the two forms of IL-2 is the consequence of the different interactions between the C- and N-terminal residues.

Interleukin-2 (IL-2)[1] is a cytokine which regulates the proliferation and differentiation of T-lymphocytes and is produced by T-lymphocytes upon antigenic or mitogenic stimulation (*1*−*3*). We previously described the purification and characterization of recombinant human IL-2 produced in *Escherichia coli* harboring the human IL-2 gene (*4*). As with other recombinant proteins expressed in *Escherichia coli*, the purified preparation of human IL-2 thus obtained was a mixture of wild-type IL-2, starting with an alanine residue at the N-terminus, and IL-2 with an additional methionine residue at the N-terminus (Met-IL-2). It was found that the two forms of the protein have different isoelectric points (p*I*) and can thus be separated by using Mono P column chromatography (*5*).

Observation of the different p*I* values for wild-type human IL-2 and Met-IL-2 prompted us to investigate the conformational differences in the two forms of IL-2 molecules. The three-dimensional structure of human IL-2 has been elucidated by X-ray crystallography, and a helix-bundle motif was revealed (*6*). The conformation of human IL-2 in solution has also been studied (*7*, *8*). Here we report a comparison of NMR spectroscopic data for the two forms of IL-2 to reveal their conformational differences. We utilized the assignment data reported by Mott et al. (*7*) and made a partial assignment which helped reveal the conformational difference between the two forms of IL-2 molecules. Although overall conformations were conserved in the two forms, the presence of an additional methionine residue at the N-terminus induced a chemical shift change for the NH resonance of Thr133, which is located at the C-terminus, as well as for residues Ala1 to Lys8 in the N-terminal region. We will show that there is a subtle but significant conformational change associated with different modes of interaction between the N- and C-termini, leading to the change in electrostatic properties as evidenced by the different p*I* values for the two forms of IL-2. Implications and possible problems associated with the N-terminal methionylation of bacterially expressed proteins will also be discussed.

MATERIALS AND METHODS

Preparation of NMR Samples. Wild-type IL-2 and Met-IL-2 were expressed and purified as described previously (*5*). With a view of facilitating the signal assignments, the same solution condition was utilized as reported by Mott et al. (*7*). Lyophilized powder of IL-2 or Met-IL-2 was dissolved in 10 mM sodium acetate-d_3 buffer in 90% H_2O/10% D_2O at pH 4.4. Protein concentration was 2.0 mM in both cases.

NMR Measurements. All NMR measurements were performed at a ^{1}H frequency of 600.13 MHz on a Bruker

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[1] Abbreviations: IL-2, interleukin-2; Met-IL-2, IL-2 with an additional methionine at the N-terminus; p*I*, isoelectric point; NMR, nuclear magnetic resonance; DQF-COSY, double quantum filtered correlation spectroscopy; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser enhancement spectroscopy; NOE, nuclear Overhauser enhancement; [C125A]IL-2, human interleukin-2 in which Cys125 is replaced with alanine; IL-1β, interleukin-1β; GH, growth hormone.

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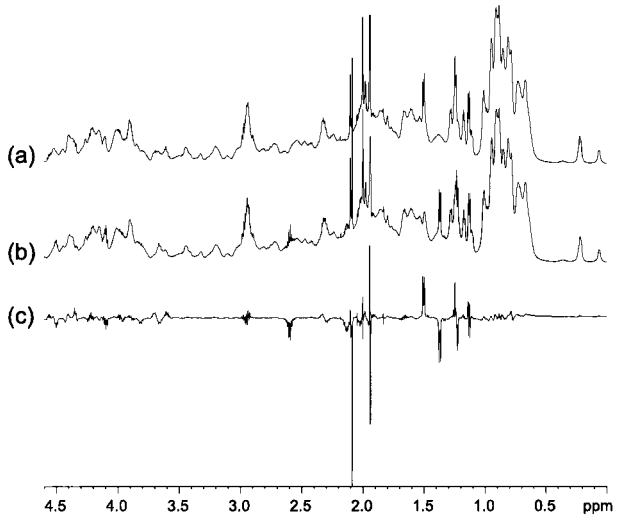

FIGURE 1: Comparison of ^{1}H one-dimensional spectra for wild-type IL-2 (a) and Met-IL-2 (b), and the difference spectrum (c) between spectra a and b. Spectra a and b were measured on 2.0 mM protein dissolved in 10 mM sodium acetate-d_3 buffer (pH 4.4) in 90% H_2O/10% D_2O at 30 °C. Spectral regions from 0.0 to 4.6 ppm are depicted. Only those signals whose chemical shifts were different between wild-type IL-2 and Met-IL-2 appear in the difference spectrum. Positive and negative peaks in the difference spectrum correspond to signals from wild-type IL-2 and those from Met-IL-2, respectively.

AVANCE600 spectrometer at 30 or 40 °C. For water suppression, WATERGATE pulse sequence (*9*) was used for two-dimensional TOCSY and NOESY experiments while presaturation was used for one-dimensional spectra and two-dimensional DQF-COSY experiments. All two-dimensional spectra were acquired with a spectral width of 7788.16 Hz and 1024(t1,real) × 2048(t2,complex) data points. Mixing times of 70 ms and 150 ms were used for TOCSY and NOESY experiments, respectively. NMR data were processed using Felix software (Molecular Simulations Inc., San Diego, CA).

Molecular Modeling. Molecular modeling was carried out using Insight II software (Molecular Simulations Inc., San Diego, CA). The coordinates of [C125A]IL-2 (*6)* were obtained from the Protein Data Bank (ID code 3INK). In the crystal structure of [C125A]IL-2, no electron densities were obtained for Ala1−Ser5. The N-terminal segment comprising Ala1−Ser5 has been added to this structure with the standard α-helical conformation. The ψ angle of Ser4 was then modified so as to place the N-terminus in close vicinity to Thr133. The resulting structure was subjected to conjugate gradient minimization using the CVFF force field with weak restraints on the backbone coordinates for Ser6−Thr133. The ϕ and ψ angles of residues Ala1−Ser5 in the final model structure were found to be in the most favored region in the Ramachandran plot using the program PROCHECK (*10*).

RESULTS

Comparison of 1D Spectra. Although it has been reported that wild-type IL-2 is susceptible to aggregation and a long measuring time cannot be used (*7*), we found no such problem with our sample. As depicted in Figure 1, the ^{1}H one-dimensional spectrum of wild-type IL-2 consisted of sharp resonances and did not show any indication of aggregation. After a total of 10 days of experiments, no difference was observed in the ^{1}H one-dimensional spectra. We believe that the susceptibility to aggregation depends on the expression and/or purification process.

Chemical shifts of NMR signals are sensitive probes for the environments where each spin is located in the three-dimensional structure of protein molecules. If there is a difference in the solution conformations of the two forms of IL-2 molecules, some spins should show chemical shift differences. We first compared the ^{1}H one-dimensional spectrum of wild-type IL-2 and that of Met-IL-2. As shown in Figure 1, the two forms of IL-2 molecules gave almost the same spectra except for small but significant differences. As shown in the difference spectrum in Figure 1c, effects of the additional methionine residue at the N-terminus were observed for only a limited number of residues. Only resonances whose chemical shifts were affected by the presence of the additional methionine residue at the N-terminus appear in the difference spectrum. Typically a pair of signals, one positive and the other negative, can be observed in the difference spectrum, representing signals from IL-2 and Met-IL-2, respectively. In the αH region (3.5∼4.6 ppm), only a limited number (6∼7) of peaks were observed in the difference spectrum, indicating that only a few residues underwent chemical shift changes upon addition of the extra methionine at the N-terminus. This means that the conformational change induced by the addition of the extra N-terminal methionine residue is confined to only limited parts of the IL-2 molecule. Sharp singlet resonances observed around 2.0 ppm can be assigned to methyl resonances of methionine residues. By comparing Figure 1a and Figure 1b, it can be seen that Met-IL-2 contains an additional singlet at 2.09 ppm. A prominent negative peak was observed in the difference spectrum depicted in Figure 1c. In addition to the singlet at 2.09 ppm, negative peaks were observed at 2.13, 2.60, and 4.10 ppm in the difference spectrum. Positive peaks corresponding to these negative peaks were not observed in the difference spectrum, indicating that these signals come from the N-terminal methionine residue which is only present in Met-IL2. On the basis of the chemical shifts, the signals at 2.13, 2.60, and 4.10 ppm can be assigned to βCH_2, γCH_2, and αCH of the N-terminal methionine of Met-IL-2. A pair of positive and negative peaks were observed at 1.50 and 1.37 ppm, respectively. Methyl resonances of alanine and threonine residues are expected to be observed in this region. The observed chemical shift change of more than 0.1 ppm indicates that the residue giving this signal is significantly affected by the addition of the methionine residue at the N-terminus of Met-IL-2. By considering the fact that wild-type IL-2 contains an alanine residue at the N-terminus while Met-IL-2 has an additional methionine preceding Ala1, the pair of doublets observed in the difference spectrum at 1.50 ppm (positive) and 1.37 ppm (negative) can be considered to be signals from the methyl group of Ala1. Further inspection of the difference spectrum of Figure 1c revealed the presence of a pair of positive and negative signals around 2.0, 2.35, and 3.6/3.8 ppm. These chemical shifts are those expected for the γCH_2, βCH_2, and δCH_2 resonances of a proline residue. Since the residue following Ala1 of IL-2 is Pro2, these signals can be assigned to Pro2. Among the residues whose chemical shifts were affected by the addition of the N-terminal methionine,

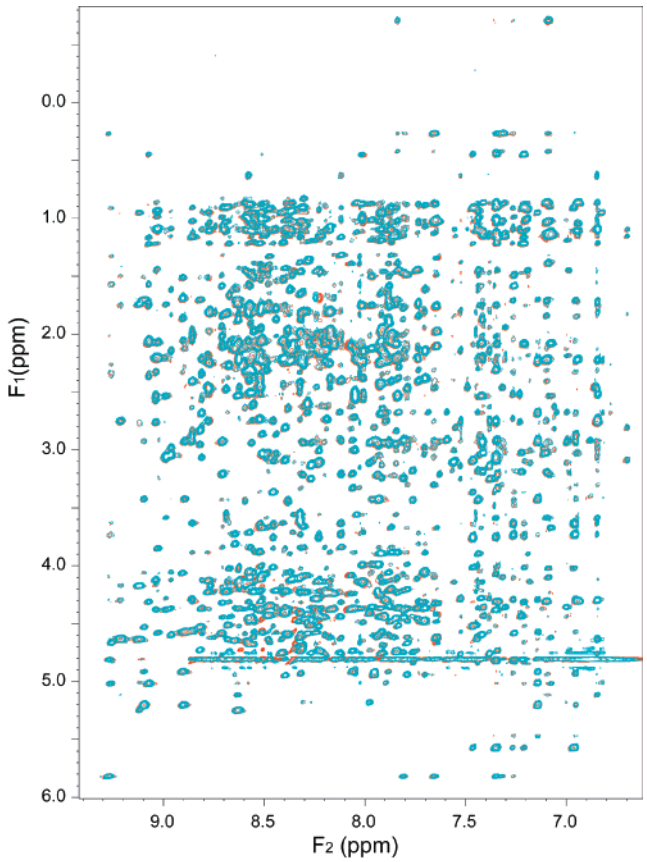

FIGURE 2: Superposition of the two-dimensional NOESY spectra of wild-type IL-2 (cyan) and Met-IL-2 (red). Each spectrum was measured on 2.0 mM protein dissolved in 10 mM sodium acetate-d_3 buffer (pH 4.4) in 90% H_2O/10% D_2O at 30 °C with a mixing time of 150 ms. The spectral region corresponding to amide protons (F_2) to aliphatic protons (F_1) is displayed.

the signals from Ala1 and Pro2 can thus be assigned based on analysis of the one-dimensional ^{1}H spectra of IL-2 and Met-IL-2.

Comparison of NOESY Spectra. Figure 2 shows the superposition of the NOESY spectrum of wild-type IL-2 (cyan) and that of Met-IL-2 (red). Except for a few residues, chemical shifts as well as intensities of NOE signals were conserved for the two forms of human IL-2 molecules. Since the intensity of the NOE signal is proportional to the inverse sixth power of the distance between protons which give the NOE peak, conservation of the NOE intensities means that proton−proton distances were not changed. These observations suggest that only limited, if any, conformational change is induced upon the addition of the N-terminal methionine residue.

Assignments of Resonances Which Showed Chemical Shift Changes. To further define the conformational difference between wild-type IL-2 and Met-IL-2, signal assignments are required. Assignments for backbone resonances of wild-type human IL-2 have already been reported (*7*). By comparing the chemical shifts of the NH-αH cross-peaks reported by Mott et al. and those obtained here, unambiguous assignments were obtained for well-resolved cross-peaks. To avoid erroneous assignments for the crowded part of the fingerprint region (NH-αH), we resorted to the standard sequential assignment procedure (*11*) and obtained assignments of NH-αH cross-peaks for about 75% of the whole protein.

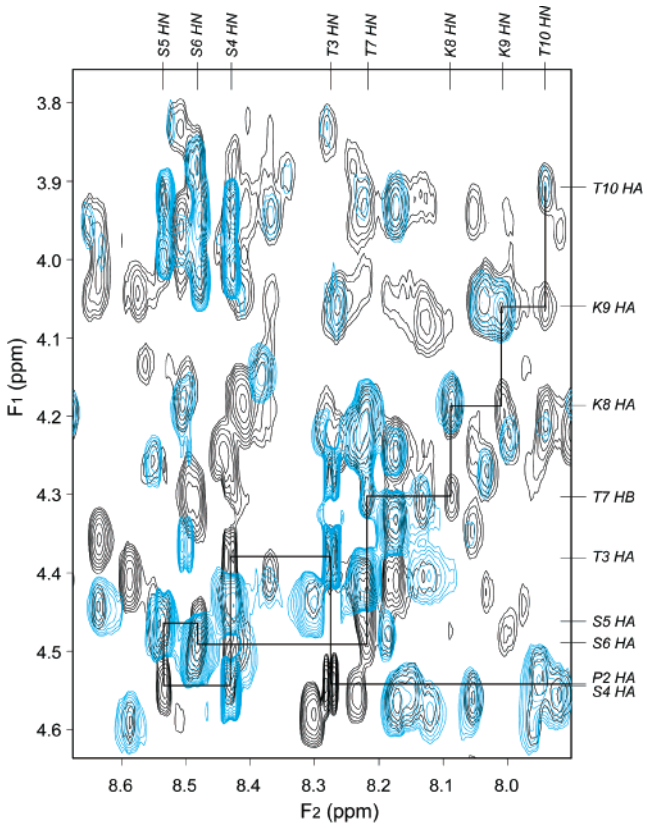

FIGURE 3: Sequential assignments for residues Thr10 to Pro2 of Met-IL-2. The fingerprint regions of the NOESY (black) and the TOCSY (cyan) spectra of Met-IL-2 are superimposed. Chemical shifts for amide and α-protons are indicated by labels outside the spectra. These labels represent the residue type as one letter code and the residue number. For example, the labels *T10 HN* and *K9 HA* indicate the chemical shifts for the amide proton of Thr10 and the α-proton of Lys9, respectively.

The signal assignments revealed that residues which showed significant chemical shift differences in the two forms of the protein were confined to the N-terminal region (Ala1−Lys8) and the C-terminal Thr133 residue (chemical shift differences between wild-type IL-2 and Met-IL-2 are listed in Table S1 in Supporting Information). Sequential assignments for residues Thr10 to Pro2 of Met-IL-2 are represented in Figure 3. Since chemical shifts are sensitive probes for the environments in which each spin is located, these results indicate that the overall conformation of the two forms of IL-2 is the same and that the conformational changes are confined to a few N-terminal residues and the C-terminal Thr133. Considering that the only structural difference between wild-type IL-2 and Met-IL-2 is the presence of the additional methionine residue at the N-terminus of Met-IL-2, the chemical shift change observed for the C-terminal Thr133 is quite intriguing. Since the chemical shifts were measured on different samples, they must be interpreted with caution. This is true, in particular, for C-terminal Thr133 because the NMR measurements were performed at pH 4.4, which is close to the pK_a of the C-terminal carboxyl group. To confirm the chemical shift difference observed for Thr133, ^{1}H NMR spectra of a mixture of wild-type IL-2 and Met-IL-2 were measured under the same condition as for the individual protein sample. Figure 4 shows the TOCSY spectrum for a mixture of wild-type IL-2 and Met-IL2. The signal enclosed with a dotted circle

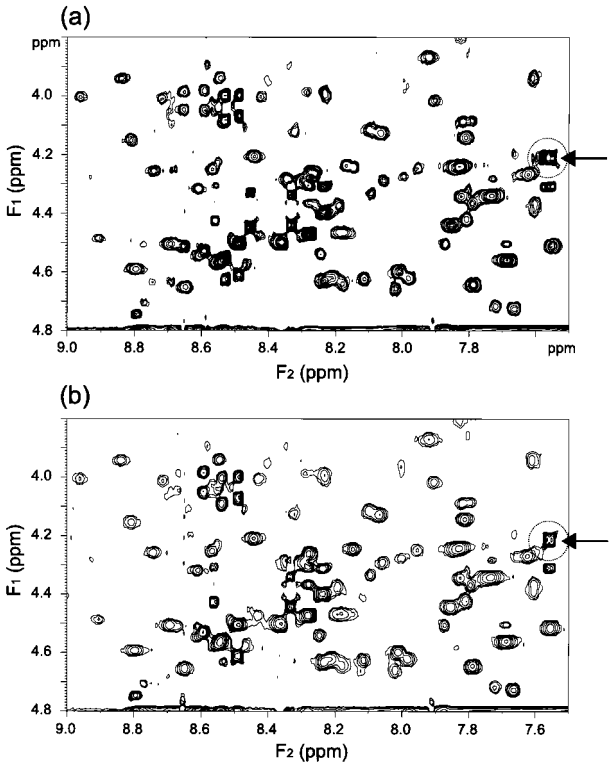

FIGURE 4: Comparison of the two-dimensional TOCSY spectrum for a mixture of wild-type IL-2 and Met-IL-2 (a) and that for Met-IL-2 alone (b). Each spectrum was measured on 2.0 mM protein dissolved in 10 mM sodium acetate-d_3 buffer (pH 4.4) in 90% H_2O/10% D_2O at 30 °C with a mixing time of 70 ms. Only fingerprint regions (NH-αH) are shown. The signal enclosed with a dotted circle represents a cross-peak beween an amide proton and an α-proton of Thr133. An arrow indicates the position of the cross-section depicted in Figure 5.

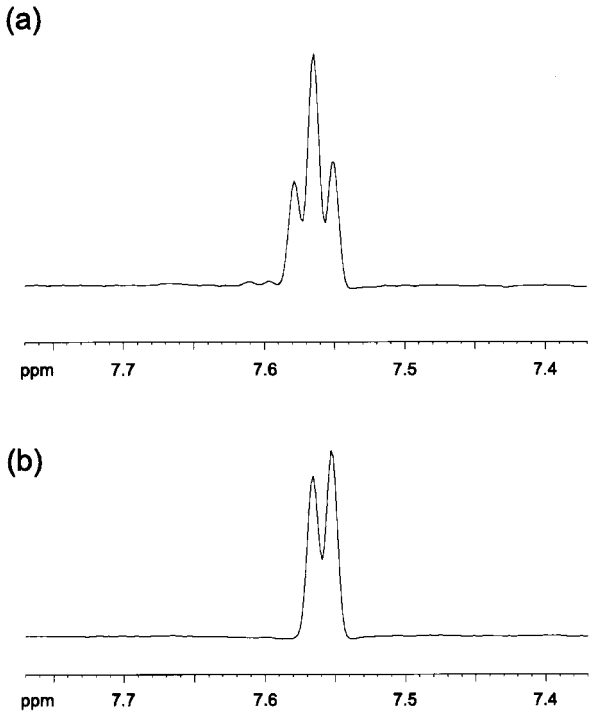

FIGURE 5: Comparison of the cross-section along the F_2-axis of the cross-peak between NH and αH resonances of Thr133 for a mixture (a) and Met-IL-2 alone (b). Cross-sections along the F_2-axis as depicted by arrows in Figure 4 are shown.

is a cross-peak between the NH and αH protons of Thr133 located at the C-terminus of IL-2. As is clearly shown in the intersection along the F2-axis (Figure 5a), this peak gave a triplet-like structure. The doublet observed for Met-IL-2 corresponded to the two upper field peaks observed for the mixture, as shown in Figure 5b. On the other hand, the doublet observed for wild-type IL-2 corresponded to the two lower field peaks of the mixture. This result demonstrates that the chemical shifts for the NH resonance of Thr133 are different for wild-type IL-2 and Met-IL-2.

DISCUSSION

The N-terminus of a recombinant protein expressed in *E. coli* starts with formyl-methionine (*12*), which is subsequently processed by peptide deformylase (*13, 14*). The methionine aminopeptidase then removes the N-terminal methionine to finally produce the N-terminal methionine-free mature protein. However, removal of the N-terminal methionine does not always take place, in particular, when the side chain of the adjacent amino acid is bulky or charged (*15, 16*). Even when the side chain of the neighboring amino acid is small and uncharged such as alanine as in the case of IL-2, the action of the methionine aminopeptidase could result in the incomplete cleavage of the N-terminal methionine, possibly due to the high level expression and the accumulation as inclusion bodies of the protein.

In the case of recombinant human interleukin-1β (IL-1β), wild-type and N-terminally methionylated IL-1β were separated by chromatofocusing (*17*). The success of this separation was attributed to a small difference in pK_a between N-terminal methionine and N-terminal alanine. We previously described the separation of wild-type IL-2 and Met-IL-2 based on the different p*I* values between the two forms of the protein (*5*). Here we investigated the origin of the difference in p*I* values. It was demonstrated that the chemical shifts of the NH resonances of Thr133 were different between wild-type IL-2 and Met-IL-2. The chemical shift change observed for Thr133 at the C-terminus induced by an additional methionine residue at the N-terminus suggests some sort of interaction between the two termini. In the crystal structure of human IL-2 with C125A mutation (*6*), electron densities for the N-terminal region were not observed beyond Ser5, but Ser6 is located in the vicinity of the C-terminus. By modeling the missing Ala1−Ser5 part onto the crystal structure of IL-2 (Figure 6), it was confirmed that the C-terminal Thr133 can be in close vicinity of the N-terminus with a reasonable conformation. Considering the fact that NMR measurements were performed at pH 4.4, which is close to the pK_a value of carboxyl groups, the difference in the chemical shift of the NH resonance of Thr133 can be interpreted in terms of the difference in the ionization state of the C-terminal carboxyl group. Since residues which showed chemical shift changes were confined to the N-terminal region, except for Thr133, it is most probable that the chemical shift change for the NH resonance of Thr133 was brought about by the different mode of interaction between the C-terminal carboxyl group and the N-terminal amino group in the two forms of IL-2. This kind of difference in the ionization state of a charged group seems to be the cause for inducing the difference in p*I* values for the two forms of IL-2. Similar conformational effects of the additional N-terminal methionine on the electrostatic proper-

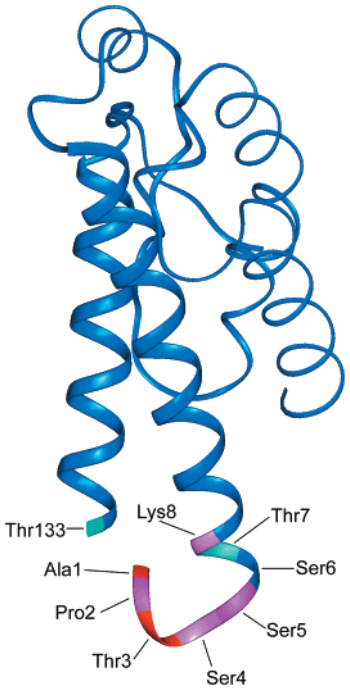

FIGURE 6: Putative model which depicts the interaction between the N- and C-termini of IL-2. The N-terminal and the C-terminal helices are depicted by thick ribbons while other parts of the model structure were represented by thin ribbons. Residues are colored from blue to red according to the size of chemical shift difference ($\Delta\delta$) between wild-type IL-2 and Met-IL-2 (blue for $\Delta\delta < 0.01$ ppm; cyan for $0.01 < \Delta\delta < 0.02$ ppm; pink for $0.02 < \Delta\delta < 0.04$ ppm; magenta for $0.04 < \Delta\delta < 0.10$ ppm; red for $\Delta\delta > 0.10$ ppm). In the crystal structure of [C125A]IL-2 (6), no electron densities were obtained for Ala1−Ser5. In this model the N-terminal segment comprising Ala1−Ser5 has been added to the crystal structure of [C125A]IL-2 starting from Ser6. From this model, it seems to be possible that C-terminal Thr133 is in close vicinity of the N-terminus.

ties of a recombinant protein have been described for α-lactalbumin (*18*). In this case, detailed structural comparisons between wild-type and N-terminally methionylated α-lactalbumin employing X-ray crystallography revealed that the differences in the electrostatic interactions of the N-terminal amino group with the side chain atoms of nearby residues (Thr38, Asp37, and Asp83) brought about a difference in the pK_a value of the N-terminal amino group for the two proteins.

The effects of the extra N-terminal methionine residue on the structure, stability, and folding of *E. coli*-expressed recombinant proteins have been the focus of recent studies. There has been compelling evidence that the additional methionine at the N-terminus of α-lactalbumin destabilizes the native-state folding (*18*−*20*). Similar destabilizing effects have been reported for recombinant hen egg-white lysozyme (*21*, *22*) and apomyoglobin (*23*). In the case of an immunoglobulin V_L domain expressed in *E. coli*, an unprocessed N-terminal methionine destabilizes the protein by approximately 3 kJ/mol (*24*). On the other hand, the presence of the extra N-terminal methionine residue does not interfere with the native-state stability in some proteins (*25*, *26*). The antigenicity of methionylated recombinant proteins could be different from that of wild-type proteins. It is known that human growth hormone (GH), a member of the long-chain cytokines, is rendered more immunogenic by the extra N-terminal methionine (*27*). Although elevated antigenicity of Met-IL-2 has not been described, it may be of interest to note that both IL-2 and GH belong to the four-helical cytokine superfamily and have a similar arrangement of four helices with the N- and C-termini close in space in both cases (*28*).

Comparison of ^{1}H NMR spectra of wild-type IL-2 and Met-IL-2 has unexpectedly revealed the presence of an interaction between the N- and C-termini. Evidence in this study demonstrated that the presence of an extra methionine at the N-terminus can change the electrostatic properties of IL-2 molecules, as exemplified by the difference in the isoelectric points, through modification of the interaction of the C-terminus with the N-terminus. Details of the effects of the additional methionine residue at the N-terminus on the structure and function of the recombinant proteins are not yet understood. In view of the fact that electrostatic as well as conformational properties of Met-IL-2 are different from those of wild-type IL-2, we think that structural comparison as described here between IL-2 and Met-IL-2 should provide insights into the role of the extra N-terminal methionine residue.

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SUPPORTING INFORMATION AVAILABLE

Chemical shift differences between IL-2 and Met-IL-2 are listed in Table S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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