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An asymmetric deuterium labeling strategy to identify interprotomer and intraprotomer NOEs in oligomeric proteins

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Abstract

A major difficulty in determining the structure of an oligomeric protein by NMR is the problem of distinguishing inter- from intraprotomer NOEs. In order to address this issue in studies of the 27 kD compact trimeric domain of the MHC class II-associated invariant chain, we compared the ^{13}C NOESY-HSQC spectrum of a uniformly ^{13}C-labeled trimer with the spectrum of the same trimer labeled with ^{13}C in only one protomer, and with deuterium in the other two protomers. The spectrum of the unmixed trimer included both inter- and intraprotomer NOEs while the spectrum of the mixed trimer included only intraprotomer peaks. NOEs clearly absent from the spectrum of the mixed trimer could be confidently assigned to interprotomer interactions. Asymmetrically labeled trimers were isolated by refolding a ^{13}C-labeled shorter form of the protein with a ^{2}H-labeled longer form, chromatographically purifying trimers with only one short chain, and then processing with trypsin to yield only protomers with the desired N- and C-termini. In contrast to earlier studies, in which statistical mixtures of differently labeled protomers were analyzed, our procedure generated only a well-defined 1:2 oligomer, and no other mixed oligomers were present. This increased the maximum possible concentration of NMR-active protomers and thus the sensitivity of the experiments. Related methods should be applicable to many oligomeric proteins, particularly those with slow protomer exchange rates.

Abbreviations: invariant chain (Ii), major histocompatibility complex (MHC)

Introduction

Determination of the structures of symmetric oligomeric proteins by NMR remains challenging. The symmetry of a homooligomer, which permits electron density averaging and signal-to-noise improvement in X-ray crystallography, introduces an ambiguity into each nuclear Overhauser enhancement such that one cannot determine *a priori* which of a number stereochemically identical atoms participate in the interaction. In a symmetric *n*-mer, each NOE may indicate proximity of a proton *A* to a proton *B* either in the same protomer, or in any of the other $(n-1)$ protomers, or with *B* protons in more than one protomer.

Conventional distance geometry algorithms cannot compute molecular structures using ambiguous distance restraints such as would derive from NOEs in a symmetric oligomer. A simulated annealing approach to the problem has been proposed, in which the familiar pseudo-energy term due to violation of NOE distance restraints is replaced by an energy penalty present when the sum of expected NOE coupling strengths between *A* and *B* protons in all protomers of the simulated model (each proportional to $1/r_{AB}^6$) is less than the total coupling strength reflected by the $A-B$ distance bound entered into the distance restraints list (Nilges, 1993; 1995). In the absence of further data explicitly defining interfacial contacts, however, it is of limited value for determining the solution structures of larger or higher order oligomers.

Asymmetric isotopic labeling of oligomers facilitates a variety of NMR experiments which select

for specifically inter- or intraprotomer NOEs in a spectrum. One set of such experiments uses isotope filters or half-filters to separate NOESY spectra into subspectra containing inter- and intraprotomer NOEs (Bax and Weiss, 1987; Fesik et al., 1987; Griffey and Redfield, 1985; Ikura and Bax, 1992; Otting et al., 1986; Otting and Wüthrich, 1989; Senn et al., 1987; Wider et al., 1990). This class of experiments may be used to select for intraprotomer NOEs in cases where an isotope is present in one protomer only, or for interprotomer NOEs in cases where different protomers are labeled with ^{13}C and with ^{15}N. Because they require lengthy pulse sequences the primary drawback of these techniques is their low sensitivity, making them unsuitable for larger or less soluble proteins. Selective or uniform deuteration (Sattler and Fesik, 1996) has also been used to probe protein interfaces by NMR, including recently by using a mixed (^{2}H,^{15}N)/unlabeled dimer in H_2O to obtain interprotomer NOEs in a routinely sensitive ^{15}N-NOESY-HSQC experiment (Walters et al., 1997). In this experiment, intraprotomer H^N–H^C peaks are effectively 'whited-out' by deuterium labeling of nonlabile sites in the ^{15}N-labeled protomer, and the only crosspeaks in the amide-aliphatic region of the spectrum arise from H^N–H^C NOEs across the dimer interface. Because interprotomer NOEs are selected, an equilibrium mixture of labeled and unlabeled protomers may be used. A difficulty with this experiment is that interprotomer H^N-H^C distances will in many cases be quite large and give rise to weak NOEs which may be particularly hard to detect in bigger proteins, even when long NOESY mixing times are used. A general problem with experiments designed to detect interfacial interactions in oligomers with more than two protomers is that any resulting NOEs will still be ambiguous, because each could relate any pair of protons as long as they are not in the same protomer.

Ideal for determining the structure of a large oligomer would be a high-sensitivity experiment for detecting interfacial contacts among aliphatic and aromatic sidechain protons, and a way to obtain a set of unambiguously assigned NOEs defining the protomer structure and protomer–protomer contacts in order to simplify geometric or annealing calculations. We applied deuterium labeling, and a technique for generating and purifying oligomers with specific combinations of labeled protomers, to obtain lists of known intra- and interprotomer distances in a 27 kD trimeric domain of the MHC class II-associated invariant chain. Our methods proved quite effective and should be applicable, with minor modifications, to NMR studies of a wide range of oligomeric proteins.

Materials and methods

The hexahistidine tagged lumenal domain of invariant chain, Ii h94-216, was produced by overexpression in *E. coli*, as described previously (Park et al., 1995). Labeled proteins were expressed by growing cells at 30 °C on M9 minimal medium, with induction at an OD_{600} of 0.1 and harvesting after 15–24 hours of induction. For ^{15}N-labeling 1 g/L ^{15}N ammonium chloride was the only nitrogen source in the medium; for ^{13}C-labeling, 1.2 g/L of ^{13}C-glucose was the only carbon source. For ^{2}H-labeling, D_2O was used instead of H_2O and the only carbon source was uniformly deuterated glycerol, also at 1.2 g/L. Growth was approximately twice as slow as in H_2O-based M9, but the protein yield was comparable (5–10 mg/L).

Proteins were purified by nickel affinity and ion exchange chromatography, according to a published protocol (Park et al., 1995). Aprotinin, iodoacetamide, leupeptin, pepstatin, and phenylmethylsufonylfluoride were used at standard concentrations to guard against proteolysis in initial stages of the purification, and 1.0 μg/mL aprotinin was added to Ii 118–192 after trypsinolysis. Tryptic digestion of Ii h94–216 to yield Ii 118–192 was performed using 50 μM trypsin and quenched with aprotinin. Mixed trimers were generated by unfolding ^{2}H and ^{13}C labeled Ii proteins (at roughly 0.5 mM) together in 5 M urea, incubating for 15 minutes, and then refolding by tenfold dilution into 20 mM HEPES, pH 8.3. Samples were then concentrated and rediluted to reduce the amount of urea, and purified by ion exchange chromatography with a Pharmacia MonoQ column on an FPLC system. Elution peaks were collected by hand and assayed by 15% polyacrylamide SDS gel electrophoresis and by 11% polyacrylamide nondenaturing gel electrophoresis. Trimers containing both Ii h94–216 and Ii 118–192 chains were trypsinized and repurified and assayed in the same manner.

NMR spectroscopy was performed on a Varian Unity Inova 750 spectrometer. 1–3 mM protein samples were prepared in phosphate buffered saline, pH 6.7. All spectra were recorded at 25 °C in Shigemi NMR tubes with sample volumes of 230–250 μL. 100 ms mixing time ^{15}N-NOESY-HSQC spectra were acquired with WATERGATE water sup-

pression (Sklenár et al., 1993) and an optimized pulse sequence (Talluri and Wagner, 1996), with 14 ppm sweep width and 136 increments in the indirect ^{1}H dimension, and 40 ppm sweep width and 40 increments in the ^{15}N dimension. 50 ms mixing time ^{13}C-NOESY-HSQC spectra were acquired with presaturation and with a 14 ppm sweep width and 128 increments in the indirect ^{1}H dimension. Two spectra of each sample were obtained, one in the aromatic ^{13}C chemical shift range (108–143 ppm, 64 increments), and one in the aliphatic range (8–73 ppm, 108 increments), with a phase-modulated pulse train for carbonyl inversion during t_2 (McCoy and Mueller, 1992). Data were processed and transformed in Felix (Biosym, Inc.), and displayed and analyzed using XEASY (ETH, Zürich). Relative peak heights in the spectra of mixed and unmixed Ii 118–192 trimers were judged by visual comparison of each pair of peaks.

Results and discussion

The invariant chain (Ii) is a trimeric transmembrane protein involved in antigen processing by the MHC class II antigen presentation pathway (Cresswell, 1994). In the major p31 form of the protein, residues 1–31 are cytoplasmic and contain a compartmental targeting sequence, residues 32–56 are predicted to be transmembrane, and residues 57–216 interact with MHC class II molecules in the lumen of the endoplasmic reticulum (Bakke and Dobberstein, 1990; Lotteau et al., 1990; Marks and Cresswell, 1986). Expression and purification of three hexahistidine-tagged versions of the lumenal domain have been previously described (Park et al., 1995). The Ii h94–216 molecule, which contains the histidine tag and residues 94–216 of the intact protein can be cleaved with trypsin to generate Ii 118–192, a compact 27 kD trimeric domain suitable for NMR structural studies. Standard techniques (Cavanagh et al., 1996; Wüthrich, 1986) were used to obtain a virtually complete assignment of ^{1}H, ^{13}C, and ^{15}N atoms in Ii 118–192. We used a twofold approach to distinguish inter- from intraprotomer NOEs. The first method followed the strategy first applied to obtain interprotomer H^N–H^C NOEs in PUT3 (Walters et al., 1997). Fully deuterated ^{15}N-labeled Ii 118–192 and unlabeled Ii 118–192 were purified separately, mixed in 5 M urea and refolded by dilution to generate mixed trimers containing both (^{2}H, ^{15}N)-labeled protomers and unlabeled protomers. With this sample, we acquired a 100 ms mixing time ^{15}N-NOESY-HSQC spectrum and obtained 23 clear but weak interprotomer H^N–H^C NOEs, mainly involving amide protons of residues 170–180. Due to chemical shift degeneracy of several of the methyl protons, only some of the aliphatic and aromatic protons involved could be assigned easily. The information from the experiment was insufficient for our structure determination, so we developed a second method to supplement our data with more information about the trimer interface.

Our new approach was to compare the ^{13}C-NOESY-HSQC spectrum in D_2O of a fully ^{13}C-labeled trimer with that of a single ^{13}C-labeled protomer in a trimer with two completely deuterated protomers. In the first spectrum both inter- and intraprotomer NOEs should be apparent, whereas in the second only intraprotomer NOEs will be present, assuming that the deuterium labeling is complete and that the protomers exchange between trimers on a timescale much slower than the duration of the NMR experiment. This strategy was chosen because of its sensitivity advantage over isotope-filtered applications to distributions of mixed trimers and its immunity to artifacts arising from incomplete filtering, and because of its ability (if successful) to produce reliable intraprotomer NOE data without the need for data subtraction and consequent signal-to-noise penalty. The advantage in sensitivity is due to the fact that at any given total protein concentration, fully one third of the protomers contribute to intraprotomer NOEs in a NOESY-HSQC, whereas for instance in a ^{13}C-edited/^{13}C-filtered 3D experiment on a mixed population of ^{13}C and ^{12}C-containing protomers, only 25% of protons contribute to the directly-measured interprotomer NOEs, and there is significant further signal loss due to relaxation during the longer pulse sequence. Intraprotomer NOE data can be indirectly obtained by subtracting a 3D ^{13}C-edited/^{13}C-filtered NOESY spectrum from a ^{13}C-edited/^{13}C-edited NOESY on a binomial distribution of mixed trimers, but this isotope-filtering approach would also suffer from lower sensitivity than our method as well as relative proneness to artifacts with potentially dire consequences for protein structure determination.

As with the production of (^{2}H, ^{15}N)-labeled Ii 118–192 for our first experiment, complete deuteration was achieved by growing Ii-expressing *E. coli* on an D_2O-based M9 medium containing fully deuterated glycerol as the only carbon source. The efficiency of ^{2}H-labeling was clear from a 1D spectrum of labeled Ii 118–192 (Figure 1A) and from subsequent experiments.

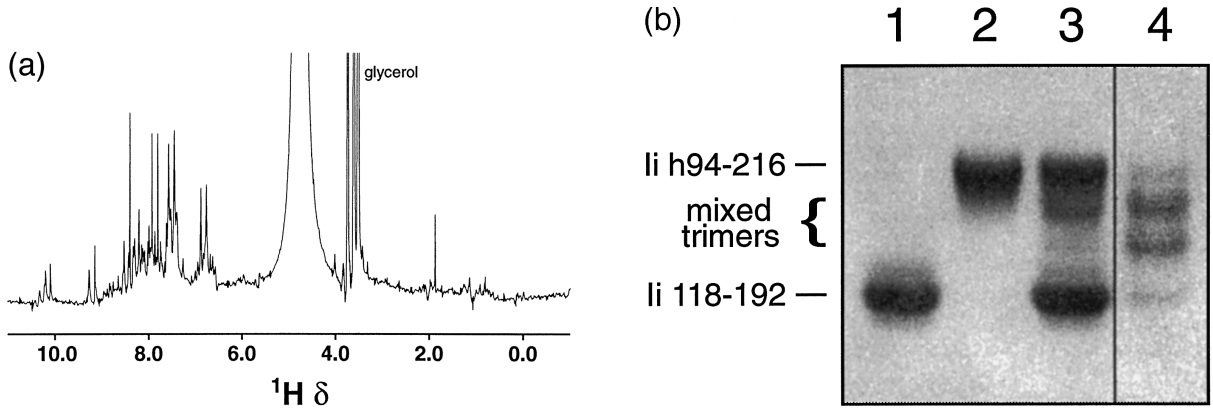

Figure 1. (A) 1D proton NMR spectrum of fully deuterated Ii 118–192 in H_2O. The spectrum was acquired on Varian Unity Inova 750 spectrometer, at 25 °C and in phosphate-buffered saline, pH 6.7. (B) Nondenaturing 11% polyacrylamide gel showing bands corresponding to: (1) Ii 118–192 alone, (2) Ii h94–216 alone, (3) Ii 118–192 mixed with Ii h94–216 and incubated for 3.5 days at room temperature, (4) Ii 118–192 mixed with Ii h94–216 and heat denatured to produce mixed trimers.

A solution of trimers all labeled with ^{13}C in one protomer and ^{2}H in the other two protomers is not at equilibrium and over time will tend to mix into a solution where populations of (^{13}C Ii 118–192)$_3$, (^{13}C Ii 118–192)$_2$(^{2}H Ii 118–192), (^{13}C Ii 118–192)(^{2}H Ii 118–192)$_2$, and (^{2}H Ii 118–192)$_3$ are in a 1:6:12:8 ratio. In order for all NOEs in a ^{13}C-NOESY-HSQC spectrum of ^{13}C- and ^{2}H-labeled mixed trimers to be interpretable as intraprotomer contacts, it is crucial that no significant protomer exchange towards this equilibrium take place during the course of a long experiment. To probe this issue before significant expenditure of resources, the rate of exchange of protomers in Ii was estimated by mixing trimers of Ii h94–216 with trimers of Ii 118–192, incubating at room temperature for various lengths of time, and monitoring the resulting trimeric species by nondenaturing gel electrophoresis (Figure 1B). The Ii h94–216 trimer has a significantly lower mobility on nondenaturing gels than Ii 118–192, and trimers containing mixtures of Ii h92–216 and Ii 118–192 have intermediate mobility. Mixed trimers were purposely produced by boiling a 1:1 mixture of Ii h94–216 and Ii 118–192 for one minute. Although there was some loss of protein due to precipitation during the heat-shock, the four resulting species formed in roughly the 1:3:3:1 ratio expected from mixed trimers with comparable reassociation rates or stabilities (Figure 1B, lane 4). Apart from residues in the 118–192 segment, most of Ii h94–216 is disordered (Jasanoff et al., 1995), so it is not surprising to find evidence consistent with mixed and unmixed trimers of Ii h92–216 and Ii 118–192 having similar association properties. Importantly, after ten days of room temperature incubation there was no apparent change in the distribution of species in the heat-shocked sample and no formation of mixed trimers was visible in an unboiled mixture of Ii h92–216 and Ii 118–192. This indicated that the half-life for trimer mixing is on the order of weeks at least, and that no significant trimer mixing would be expected in the course of a 5–10 day NMR experiment.

Once we had ascertained that a population of asymmetrically-labeled Ii trimers would be stable for sufficient periods of time, we isolated trimers with the desired composition of ^{13}C- and ^{2}H-labeled protomers. We produced and purified separately ^{2}H-labeled Ii h94–216 and ^{13}C-labeled Ii 118–192, mixed and denatured them in 5 M urea, refolded, and passed them over an ion exchange column. Previous work had shown that different combinations of long (h94–216) and short (118–192) Ii protomers produce trimers which elute from an anion exhanger at different salt concentrations (Park, 1995). Here the ratio of long to short chains in a trimer corresponded to the ratio of ^{2}H to ^{13}C labeling, enabling purification of the specifically desired mixed trimers. Refolded mixed trimers eluted in five major peaks from the anion exchange column (Figure 2A). The peaks were analyzed by nondenaturing PAGE, which indicated the number of distinct trimeric species in each peak, and by SDS-PAGE (Figure 2B), which indicated the molecular weights of protomers in each peak and their rough ratios. Peaks 2–4 contained significant amounts of the desired trimers: peaks 2 and 3 consisted entirely of trimers with one ^{13}C-labeled protomer and two ^{2}H-labeled protomers, and peak 4 was a mixture

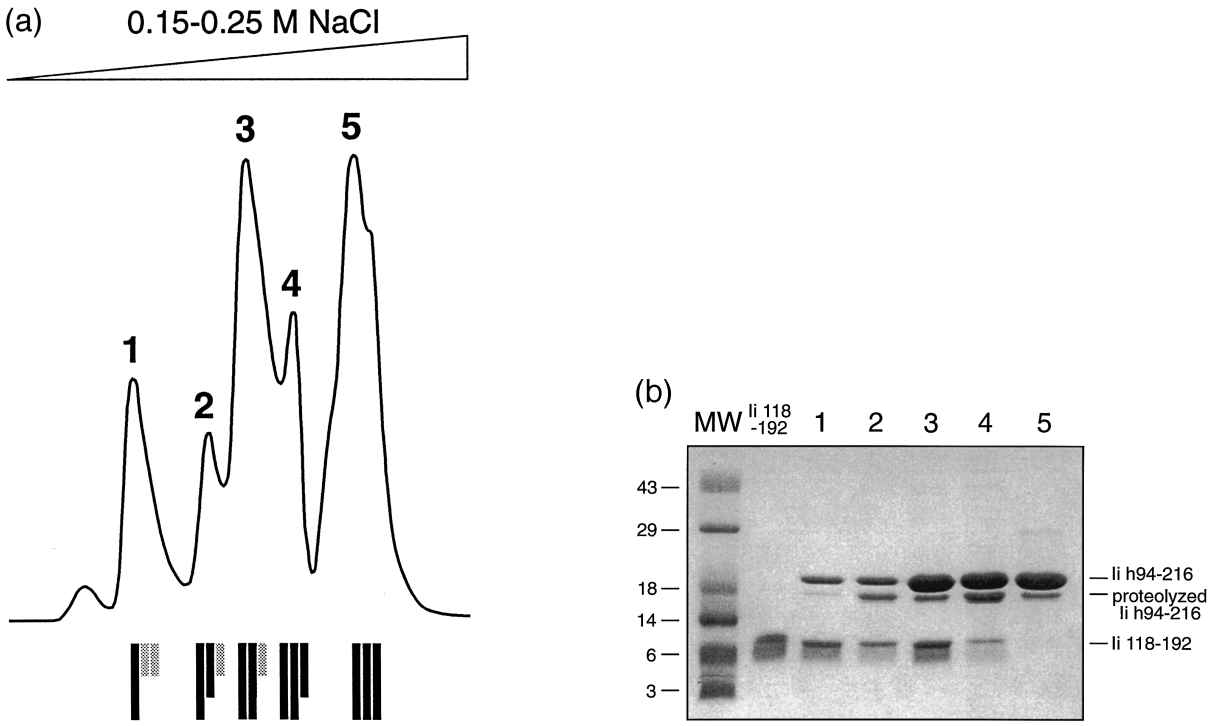

Figure 2. (A) UV absorbance trace showing the elution profile of mixed Ii trimers from a Pharmacia MonoQ anion exchange column. Peaks 1–5 are labeled and their principal components are denoted below by 'trimers' of short, medium, and long lines (see text and panel B); ^{2}H labeled polypeptides are represented in black, ^{13}C in grey. (B) A 0.1% SDS, 15% polyacrylamide gel showing composition of elution peaks labeled 1–5 in (A). Three Ii species of different length were present in the trimers: ^{2}H Ii h94–216 (16 kD), proteolyzed ^{2}H Ii h94–216 (14 kD), and ^{13}C Ii 118–192 (9 kD). Peaks 2–4 were used in the mixed trimer NMR sample. 2 and 3 consisted entirely of trimers with one ^{13}C-labeled protomer and two ^{2}H-labeled protomers, and peak 4 was a mixture of uniformly ^{2}H-labeled trimers and trimers with one ^{13}C-labeled protomer. Protein standards (left lane) are labeled with their molecular weights in kD, and the second lane contains Ii 118–192 alone as a further reference.

of uniformly ^{2}H-labeled trimers and trimers with one ^{13}C-labeled protomer. The situation had been complicated by partial degradation of Ii h94–216, which had taken place between its purification and the separation of refolded mixed trimers. As a result, three molecular species contributed to mixed trimers: ^{2}H Ii h94–216 (16 kD), proteolyzed ^{2}H Ii h94–216 (14 kD), and ^{13}C Ii 118–192 (9 kD). SDS gel electrophoresis indicated that peak 2 contained one each of the three species, and that peak 3 contained two ^{2}H Ii h94–216 and one ^{13}C Ii 118–192 protomer per trimer. Because of incomplete resolution of peaks 3 and 4 (verified by nondenaturing PAGE), peak 4 contained some (^{13}C Ii 118–192)(^{2}H Ii h94–216)$_2$ trimers, but mostly singly proteolyzed ^{2}H Ii h94–216 trimers. Other trimeric combinations of Ii protomers were not present in significant enough quantities to warrant purification and analysis. In order to maximize the final yield of (^{13}C Ii 118–192)(^{2}H Ii 118–192)$_2$, peaks 2–4 were combined, trypsinized and repurified to generate a sample 1.3 mM in Ii 118-192 trimers of which at least 70% was (^{13}C Ii 118–192)(^{2}H Ii 118–192)$_2$, and the remainder was (^{2}H Ii 118–192)$_3$, which does not contribute to ^{13}C-NOESY-HSQC spectra in D_2O.

For both mixed and unmixed Ii 118–192 samples, two 50 ms mixing time ^{13}C-NOESY-HSQCs (one in the aliphatic and one in the aromatic carbon chemical shift range) were aquired over a period of ten days on a Varian 750 MHz spectrometer. Figure 3 shows a comparison of several strips from the spectra of uniformly ^{13}C-labeled Ii 118–192 (left panel) and of (^{13}C Ii 118-192)(^{2}H Ii 118–192)$_2$ (right panel). Significant differences in relative peak heights are striking. The complete absence of some peaks present on the right in the spectrum of the mixed trimer confirms that ^{2}H-labeling was effective and that little trimer exchange took place. If any significant exchange had occurred in the mixed trimer NOESY experiment one would never find the complete absence of a peak observed in the NOESY-HSQC of the unmixed trimer sample. Based on the peak height differences between the two spectra, NOEs were sorted into three classes. Any

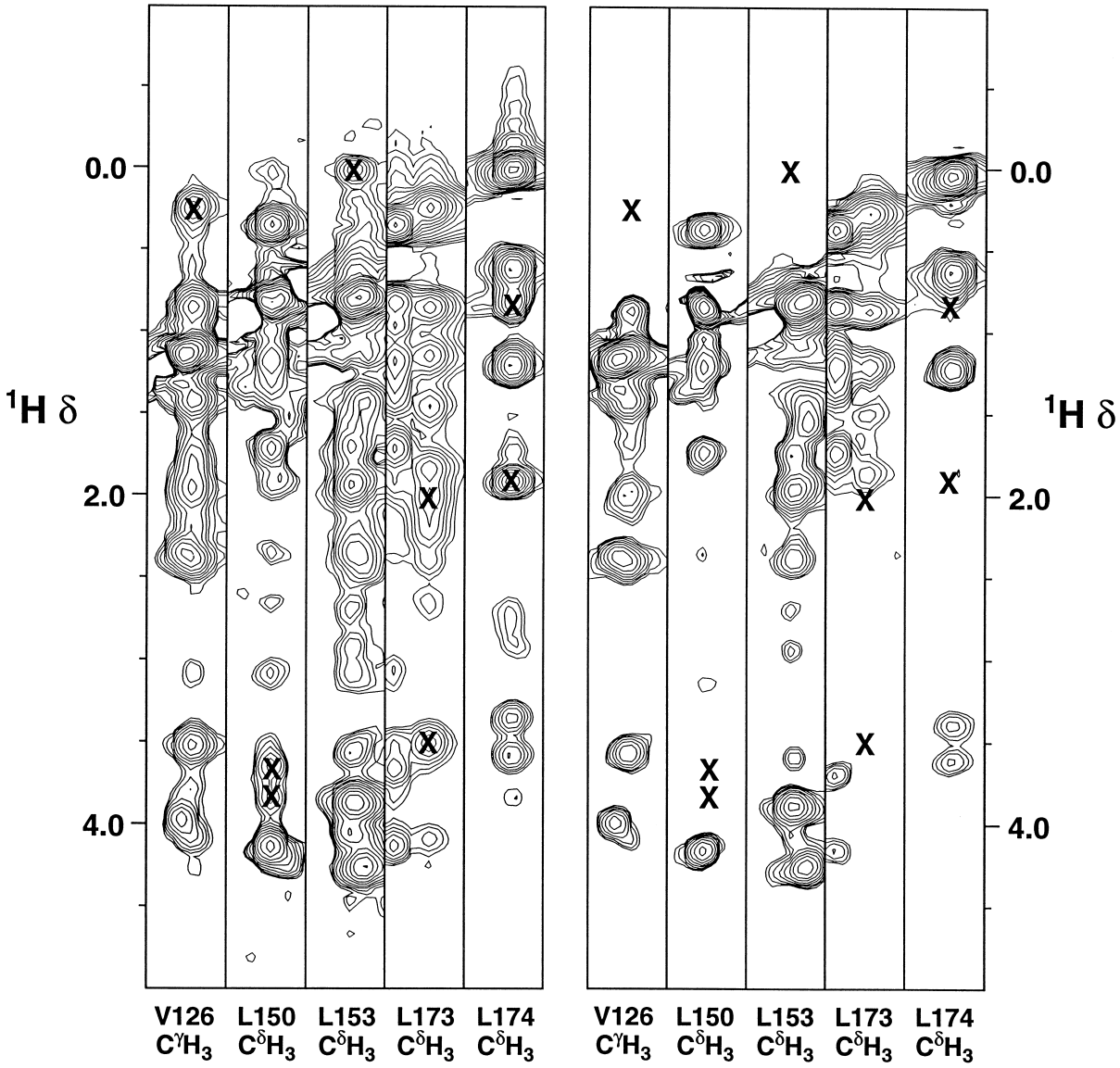

Figure 3. A comparison of several methyl strips from the D_2O ^{13}C-NOESY-HSQC spectra of (^{13}C Ii 118–192)$_3$ (left) and (^{13}C Ii 118–192)(^{2}H Ii 118–192)$_2$ (right). ^{13}C and direct-detected ^{1}H chemical shifts (perpendicular to the page and horizontal, respectively) correspond to the those of the group named below each strip. Large NOEs present in strips on the left but absent in strips on the right (marked by X's) had predominantly interprotomer character.

NOE in the spectrum of the mixed trimers indicated an intraprotomer interaction; most of these had approximately the same relative height in the two spectra and were determined to indicate 'mainly' intraprotomer interactions. Peaks which were absent in the spectrum of the mixed trimer were determined to indicate interprotomer interactions. The third class arose because the spectra of the unmixed ^{13}C-labeled trimer were taken using a higher sample concentration, and had a higher signal-to-noise ratio. Small peaks in these spectra which appeared to be absent in the spectra of the mixed trimer were not assigned as interprotomer NOEs but were left entirely ambiguous. This was particularly an issue in the aromatic-aliphatic region, where most of the NOESY peaks had low amplitude even in the spectrum of the unmixed trimer.

Of 967 interresidue ^{13}C-NOESY-HSQC assigned crosspeaks (counting symmetric peaks separately for

these purposes), 138 had definite interprotomer character, 494 had definite intraprotomer character, and the remaining 335 were ambiguous or appeared to have both inter- and intraprotomer character. Of the 361 assigned NOEs belonging to pairs of protons separated by more than four residues, 120 were interprotomer, 88 were intraprotomer, and 153 were ambiguous. This indicates that the protomers of Ii 118–192 make extensive contacts with one another, and simulated annealing trials have shown that well-defined protomer structures can only be generated when both inter- and intraprotomer constraints are included in calculations of the entire Ii 118–192 trimer structure. Because labeling was efficient and protomer exchange was slow, the principal sources of artifact in these experiments were probably the differences in the spin diffusion pathways among nuclei near the oligomer interface in the fully ^{13}C-labeled trimer and in the mixed trimer with two deuterated protomers. The fact that spin diffusion can be noticeably affected by the ^{1}H/^{2}H ratio at the oligomer interface was demonstrated by our observation of several clear spin diffusion peaks among amide protons in the H_2O ^{15}N-NOESY-HSQC spectrum of mixed (^{2}H, ^{15}N)/unlabeled Ii 118–192 trimers which were not in the same spectrum of unmixed (^{2}H, ^{15}N Ii 118–192)$_3$. With the ^{13}C-NOESY-HSQC spectra of (^{13}C Ii 118–192)$_3$ and (^{13}C Ii 118–192)(^{2}H Ii 118–192)$_2$, the effects of spin diffusion on assignment of intra- and interprotomer NOEs were minimized because of the relatively short mixing time (50 ms) and because only a dramatic decrease in peak intensity was considered grounds for assignment of an NOE with interprotomer character.

The relative numbers of inter- and intraprotomer NOEs elucidated by our experiments demonstrate the importance of this work as a step towards obtaining a structure of Ii 118–192. A similar approach will probably be equally valuable in many oligomeric structure determinations by NMR. A principal requirement for success was that oligomers with the desired distribution of labels be separable from oligomers with different distributions. We accomplished this by creating longer and shorter versions of the protein labeled with ^{2}H and ^{13}C, respectively. Mixed oligomers with different combinations of long and short protomers could be purified from one another and then trypsinized to produce trimers with only protomers of the desired length. We used ion exchange chromatography to purify mixed oligomers from one another, but in principle size exclusion chromatography would have worked as well. Using modern molecular biological and chemical techniques it would be simple to reversibly or irreversibly modify an overexpressed protein to perturb one of its physical properties, mix modified and unmodified protomers, and then purify oligomers based on the number of modified protomers they contain. Adding a short acidic tag to a protein's N- or C-terminus could enable separation of mixed oligomers by ion exchange chromatography; a bulkier modification could allow separation by gel filtration, or an epitope, ligand, or polyhistidine tag could allow separation of mixed oligomers by titration from a corresponding affinity column.

We were fortunate that the protomers of Ii 118–192 do not exchange on or faster than the timescale of the ^{13}C-NOESY-HSQC spectra we wanted to obtain. The protomers of many stable oligomers will be in faster exchange and will not be as amenable to asymmetric labeling for that reason. This can be accommodated in several ways. If an oligomer is not too large already it should be possible to slow artificially the rate of protomer exchange by adding an oligomeric extension of the same order which stabilizes the protomers against exchange. For a coiled-coil dimer or trimer this could be achieved for instance by adding a stable segment from dimeric GCN4 or its trimeric derivative GCN4-pII (Harbury et al., 1993) to one end of the protein. For a fairly soluble oligomer with fast protomer exchange, a population of oligomers with only one ^{13}C-labeled protomer could be maintained simply by adding an excess of ^{2}H-labeled protomers. In a population of trimers at equilibrium for which ^{2}H-labeled protomers were in fivefold excess over ^{13}C-labeled protomers, over two-thirds of ^{13}C-labeled protomers would be in trimers with only one ^{13}C-labeled protomer, and most of the rest would be in trimers with two ^{13}C-labeled protomers. Relative peak heights for interprotomer NOEs in a spectrum of uniformly labeled ^{13}C trimers would differ by roughly a factor of five from peak heights in the mixed sample. Technical modifications such as these could increase the class of oligomers for which our strategy of asymmetric labeling and sorting of NOEs between carbon-bound protons is feasible, and facilitate solution structure determination for further proteins.

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