Journal of Biomolecular NMR, **25:** 235–242, 2003.
KLUWER/ESCOM

A sensitive and robust method for obtaining intermolecular NOEs between side chains in large protein complexes

John D. Gross**, Vladimir M. Gelev** & Gerhard Wagner*
Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 240 Longwood Ave. Boston, MA 02115, U.S.A.

Received 30 September 2002; Accepted 21 November 2002

Key words: biosynthetic precursors, ^{13}C-methyl, intermolecular NOEs, isotope labeling of proteins, protein complex

Abstract

A method for measuring intermolecular NOEs in protein complexes based on asymmetric sample deuteration is described. ^{13}C/^{1}H-I,L,V-methyl, U-^{2}H labeled protein is produced using the biosynthetic precursors [γ-^{13}C]-α-ketobutyrate and [γ,γ′-$^{13}C_2$]-α-ketoisovalerate. The labeled protein is mixed with its unlabeled binding partner and a 3D ^{13}C-HMQC-NOESY is recorded, yielding unambiguous intermolecular aromatic/methyl NOEs. A simple synthesis of the biosynthetic precursors via reaction of diethyl oxalate with alkyl Grignard compounds is reported. The method is demonstrated for a 35 kDa heterodimeric protein complex dissolved in a CHAPS micelle. This approach will facilitate the solution structure determination of protein/protein, protein/ligand or protein/nucleic acid complexes.

Introduction

Protein interactions regulate many biological processes, and NMR spectroscopy has become an important tool for studying protein complexes. Structural characterization of such complexes by NMR requires measurement of intermolecular distances, typically through the r^{-6} dependence of the Nuclear Overhauser effect (NOE). Considerable attention has been devoted to development of pulse sequences which enable discrimination between intra- and intermolecular NOEs (Arrowsmith et al., 1990; Ikura and Bax, 1992; reviewed in Otting and Wuthrich, 1990; Walters et al., 2001). Methods that achieve such spectral editing by the inclusion of additional spin-echo delays in NOESY experiments suffer from poor sensitivity when applied to large proteins due to efficient transverse relaxation. Substituting non-exchangeable protons with ^{2}H significantly attenuates transverse relaxation (LeMaster and Richards, 1990), but limits the number of observable proton signals. Fortunately ^{1}H spins can be re-introduced into CH_3 (Rosen et al., 1996, Smith et al., 1996; Gardner and Kay, 1997) and aromatic (Medek et al., 2000) sites of perdeuterated proteins *via* labeled amino acids or various biosynthetic precursors, and such partially protonated protein samples have been employed in global fold determination (Medek et al., 2000; Gardner et al., 1997) and ligand screening (Hajduk et al., 2000). Recently, Hajduk et al. used the γ-^{13}C/^{1}H compounds **3** and **4** as part of the *E. Coli* growth medium to produce perdeuterated proteins in which the methyl groups of Ile (δ_1), Leu and Val side chains (ILV-methyl) were exclusively labeled (Hajduk et al., 2000). Such samples had the additional advantage that ^{13}C-^{13}C J couplings were absent from the spectra, resulting in enhanced sensitivity and resolution relative to uniformly ^{13}C-enriched protein samples.

Here we present a robust method for measuring intermolecular NOEs, utilizing the above labeling scheme and based on the common occurrence of hydrophobic side chain contacts at the interface of macromolecular complexes. The precursors α-

*To whom correspondence should be addressed. E-mail: wagner@wagner.med.harvard.edu
**These authors contributed equally.

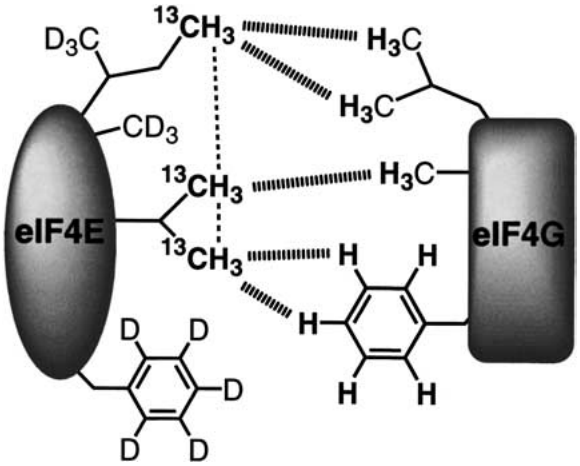

Figure 1. Asymmetric isotope labeling scheme applied in the NMR analysis of the eIF4E/eIF4G protein complex. One protein is $^{12}C/^{2}H$ – labeled except for the methyl groups of Ile (δ1), Leu and Val side chains, which are and $^{13}C/^{1}H$ – labeled. The other protein in the complex is unlabeled.

ketoisovalerate (**3**) and α-ketobutyrate (**4**) are used to generate a $^{13}C/^{1}H$ ILV-methyl labeled, perdeuterated protein, that is subsequently mixed with its unlabeled binding partner. A ^{13}C HMQC-NOESY experiment allows the identification of intermolecular NOEs between ILV-methyl protons on one protein, and aromatic and aliphatic protons on the other, in a sensitive and unambiguous manner (Figure 1). We demonstrate this technique on a 35 kDa complex of the eukaryotic translation initiation factors eIF4E and eIF4G (393–490), dissolved in a CHAPS micelle.

Experimental section

Synthesis of the precursors

^{13}C-methyl iodide was obtained from Isotec (Miamisburg, OH) at $30 per gram. All solvents and reagents were used as purchased, unless noted otherwise. Anhydrous diethyl ether was degassed and passed through an active alumina column.

1,3-$^{13}C_2$-2-bromopropane (1a). (Procedure adapted from Organic Syntheses, vol. II, p. 179.) Twenty g (140 mmol) ^{13}C-methyl iodide in 25 ml of anhydrous Et_2O was added dropwise to 4 g Mg turnings (dried at 100 °C for several hours) in 50 ml Et_2O, under inert atmosphere. Initially, a few milliliters of the halide were added without stirring until the evolution of bubbles. (A few crystals of iodine may be necessary to initiate the reaction.) The remaining halide was added with stirring at a rate required to maintain gentle refluxing of the solvent, using an ice bath to prevent excessive heating. After the addition was complete, the reaction was allowed to proceed at room temperature for additional 30 min. The solution was chilled on ice and methyl formate (4.22 g, 70 mmol) in 40 ml Et_2O was added dropwise, over 5–10 min. Stirring was continued for 1 h at room temperature and the gray slurry was poured into 75 g ice, containing 12 ml conc. HCl (1.05 equiv.), with vigorous stirring. 20 g NaCl and 2 g $Na_2S_2O_3$ were added and the mixture was transferred to a narrow neck (14/20) 250 ml flask, equipped with a reflux condenser and a short distilling head. The solvent was removed by slow distillation and the second fraction (12 ml, b.p. 79–99 °C) was collected. 50 ml of 48% HBr were added to this fraction (Braverman and Duar, 1990), the solution was heated at 50 °C for 30 min and distilled again. Twenty ml of condensate (b.p. 38–100 °C) containing two layers was collected on ice. The organic layer was washed with cold 5% $NaHCO_3$/brine solution and dried over Na_2SO_4. Yield: 9.0 g = 73% (containing an additional 0.30 equiv. CH_3Br, as estimated by NMR). ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.37 − 4.24 (m, 1H), 1.83 and 1.58 (two dd, $J = 1.5$, 5.0 Hz, $J_{^{13}C\text{-}H} = 127.9$ Hz, 6H), ^{13}C NMR (125 MHz, $CDCl_3$) δ 28.4.

To minimize loss of the volatile alkyl bromides **1a** and **1b**, 20 ml of diethyl ether was added at this step, and the solution was dried over CaH_2. Quantitation of the dry compound to be used in the next step was performed following reaction with magnesium, based on the Mg residue. Note: Some of the ^{12}C-methanol displaced from the methyl formate carries over into the subsequent steps and is present in the final product **3**, in the form of unlabeled sodium pyruvate (15% by NMR). This impurity does not interfere significantly with the protein labeling efficiency, but could be avoided by using a higher formate ester, e.g., hexyl or heptyl, which form non-volatile by-products.

2-^{13}C-1-bromoethane (1b). (Procedure adapted from Organic Syntheses, vol. I, p. 188.) Methylmagnesium iodide was prepared from 20 g (140 mmol) ^{13}C-methyl iodide in a two-neck 500 ml flask equipped with a heavy stir bar and immersed in an ice bath. The Grignard reagent was diluted to 1 M with anhydrous diethyl ether (140 ml) and a cold finger filled with dry ice/acetone and connected to a drying tube was attached to the flask. Twelve g paraformaldehyde (equivalent to 400 mmol formaldehyde), placed in

a two-neck 250 ml flask and dried for several days *in vacuo* over P_2O_5, was heated in an oil bath to 180–200 °C. The produced formaldehyde gas was directed with a gentle stream of dry nitrogen into the flask containing the methylmagnesium iodide, using a wide glass tube (≥1 cm in diameter, to prevent clogging due to re-polymerization) immersed in the stirred reaction mixture. After all paraformaldehyde had decomposed (∼ 2 h) the resulting thick suspension was poured into 50 g ice with vigorous stirring, and acidified with 45 ml of 30% H_2SO_4. The mixture was distilled as described above for bromopropane, collecting 8 ml (b.p. 81–99 °C) of crude 2-^{13}C-ethanol as the second fraction. This fraction was distilled from 48% HBr, 20 ml of the first fraction (b.p. 30–100 °C) was collected on ice and washed and dried. All handling was performed on ice to minimize evaporation. Yield 10.8 g = 97%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.42 (qd, J = 7.3, 2.7 Hz, 2H), 1.80 and 1.54 (two t, J = 7.3 Hz, $J_{13C\text{-}H}$ = 127.6 Hz, 3H), ^{13}C NMR (125 MHz, $CDCl_3$) δ 19.5.

Ethyl-[γ,γ′-$^{13}C_2$]-α-ketoisovalerate (2a) and ethyl-[γ-^{13}C]-α-ketobutyrate (2b). Alkylmagnesium bromides were prepared from the corresponding alkyl bromides **1a** and **1b**, as described above, and transferred *via* cannula to a dropping funnel. The amount of reagent was calculated based on the residual unreacted magnesium.

One molar equivalent (typically, 12 g, 82 mmol) of diethyl oxalate was diluted to 0.8 M in diethyl ether (100 ml) in a two-neck flask equipped with a thermometer and a heavy stir bar. The solution was cooled to −78 °C on a dry ice/acetone bath, under inert atmosphere. 82 ml of 1 M Grignard reagent (82 mmol, 1 equiv.) was added under vigorous stirring, at such a rate that the reaction temperature did not exceed −70 °C. After the addition was complete (approx. 1 h), the pale green slurry was stirred for an additional 1 h and transferred without warming up (Singh et al., 1989) to a beaker containing 40 g ice, 50 ml diethyl ether and 6 ml conc. HCl (84 mmol, 1.05 equiv.). If necessary, the pH of the aqueous layer was adjusted to 6–7. The organic layer was washed with 50 ml H_2O, 2 × 50 ml brine, and dried over Na_2SO_4. The solvent was removed on a rotary evaporator and the oily residue (10 ml) was transferred to a 100 ml flask. The product was distilled under reduced pressure (starting at room temperature and atmospheric pressure to avoid bumping) to yield the esters **2a** and **2b** as pale green oils that contained significant amounts of diethyl ether.

Ethyl-[γ,γ′-$^{13}C_2$]-α-ketoisovalerate (2a). Collected b.p. 22–27 °C, (350 mTorr). Yield 57% (containing up to 30% ethyl pyruvate). ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.26 (q, J = 7.0 Hz, 2H), 3.24–3.16 (m, 1H), 1.31 (t, J = 7.0 Hz, 3H), 1.22 and 0.97 (two dd, J = 2.0, 5.0 Hz, $J_{13C\text{-}H}$ = 128.4 Hz, 6H), ^{13}C NMR (125 MHz, $CDCl_3$) δ 17.2.

Ethyl-[γ-^{13}C]-α-ketobutyrate (2b). Collected b.p. 28–30 °C (350 mTorr). Yield 5.9 g (36 mmol product, estimated by NMR), 44%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.26 (q, J = 7.0 Hz, 2H), 2.85–2.77 (m, 2H), 1.31 (t, J = 7.0 Hz, 3H), 1.20 and 0.94 (two t, J = 4.5 Hz, $J_{13C\text{-}H}$ = 129.9 Hz, 3H), ^{13}C NMR (125 MHz, $CDCl_3$) δ 7.0. Note: Fizet (1982) and Singh et al. (1989) both report yields greater than 80% for the above substitution reaction and suggest that the exact stoichiometry of the reagents strongly affects the yield. Increasing the reaction scale would allow more precise handling of the volatile alkyl bromides and may significantly improve the yields. Stricter control of the temperature (e.g., not to exceed −75 °C) may also be beneficial.

[γ,γ′-$^{13}C_2$]-α-ketoisovalerate (3). 5.0 g crude ester **2a** was dissolved in 10 ml H_2O. A total of 4.7 ml of 30% NaOH (1 equiv.) was added very slowly with stirring, in a water bath. The pH was checked frequently to ensure it did not exceed ∼ 7.5. The mixture was stirred for an additional 30 min, filtered, extracted with 2 × 20 ml diethyl ether and evaporated to dryness (50 °C). The product was dissolved in 60 ml boiling MeOH, filtered, the volume reduced to 20 ml and the solution cooled to room temperature. 20 ml of diethyl ether were added and the product was stored at −20 °C for several hours, collected by filtration and dried *in vacuo* to yield 1.9 g of white powder. Concentrating the mother liquor to 10 ml and adding 20 ml diethyl ether yielded additional 1.5 g. Final yield: 3.4 g (20 mmol, containing 0.15 equiv. unlabeled sodium pyruvate, 80% from ester, 29% overall yield). ^{1}H-NMR (500 MHz, D_2O) δ 2.95–2.86 (m, 1H), 1.14 and 0.88 (two dd, J = 2.0, 5.0 Hz, $J_{13C\text{-}H}$ = 128.5 Hz, 6H), ^{13}C NMR (125 MHz, D_2O) δ 16.5. LRMS $^{13}C_2C_3H_8O_3Na$: m/z = 163 (ESI+, M^+Na), 117 (ESI−, M^-Na), 257 (ESI−, $2M^-Na$).

[γ-^{13}C]-α-ketobutyrate (4). 5.9 g crude ester **2b** was added to 120 ml 1 M HCl and the mixture was heated to 70 °C for 2 h, cooled to room temperature, saturated with NaCl, and extracted with 10 × 20 ml of

diethyl ether. The organic layer was concentrated to 20 ml and mixed with 50 ml H_2O. Three % NaOH was added dropwise, while stirring the mixture on an ice bath, until the pH reached 6. The mixture was extracted with 3 × 50 ml diethyl ether, evaporated to dryness on a rotary evaporator (50 °C) and dried *in vacuo* to yield 3.6 g of white solid (29 mmol, 80% from ester, 20% overall yield). ^{1}H NMR (500 MHz, D_2O) δ 2.68–2.61 (m, 2H), 1.09 and 0.83 (two t, J = 7.5 Hz, $J_{13C\text{-}H}$ = 128.4 Hz, 3H), ^{13}C NMR (125 MHz, D_2O) δ 6.8. LRMS $^{13}CC_3H_5O_3Na$: m/z = 148 (ESI+,M^+Na), 227 (ESI−, $2M^-$Na).

Protein expression

Critical to success of this method is the production of protein in 100% D_2O. The *S. Cerevisiae* eIF4E gene (CDC33) cloned into a pGEM.2 vector (Promega) and transformed into the *E. coli* strain BL21(DE3) could not be expressed reproducibly in 100% D_2O though expression in H_2O at levels of 10 mg per liter culture was possible. We attributed the loss of expression in 100% D_2O to plasmid instability. As noted by others, plasmid loss may occur even for mildly toxic genes incorporated into vectors encoding ampicillin resistance (Studier et al., 1990). Presumably this effect is exacerbated by the diminished growth rates observed in D_2O containing media. Recloning the yeast eIF4E gene into pET-30a plasmid (Novagen) containing kanamycin resistance allowed the routine production of 10 mg of deuterated protein per liter of culture.

^{13}C/^{1}H-I,L,V-methyl; U-^{2}H labeled proteins were generated using the following protocol: The gene encoding yeast eIF4G residues 393-490 or yeast eIF4E was cloned into pGEX-2T (Pharmacia) or pET-30a (Novagen) vector, respectively. The appropriate vector was transformed into BL21(DE3) followed by inoculation of LB broth medium until an O.D. of 0.5. This was diluted 100-fold into a 10 ml M9 culture media containing H_2O and grown until an O.D. of 0.5. The cultures were centrifuged and resuspended in 1LM9 medium (100% D_2O, containing ^{2}H-Glucose and $^{15}NH_4Cl$ as the sole carbon and nitrogen sources, respectively). The media contained either 50 μg/ml kanamycin sulfate (eIF4E) or carbenicillin (eIF4G). Thirty minutes prior to induction, 100 mg of precursor **3** and 50 mg of precursor **4** and were added (Hajduk et al., 2000). Cells were induced with 1 mM IPTG at O.D. 0.4 and grown overnight at 20 °C (eIF4E) or for 8 hours at 37 °C (eIF4G). Both eIF4E and eIF4G were purified as described previously (Hershey et al., 1999).

NMR spectroscopy

Backbone assignments of eIF4E and eIF4G(393–490) were obtained at 750 MHz using TROSY triple resonance sequences (Salzmann et al., 1998, 1999), modified with C_β decoupling during HNCA/HN(CO)CA (Matsuo et al., 1996a) and shaped refocusing pulses during HN(CA)CO (Matsuo et al., 1996b). Assignments of the ^{13}C, ^{1}H - labeled ILV methyl groups in uniform ^{15}N,^{13}C,^{2}H - enriched eIF4E and eIF4G complexes were obtained by performing H(CCO)NH (Grzesiek et al., 1993) and (H)C(CO)NH (Gardner et al., 1996) on a 500 MHz spectrometer optimized for implementation with a cryoprobe: The CACO transfer was implemented with doubly selective R-SNOB pulses (Matsuo et al., 1996b; Kupce et al., 1995) and TOCSY mixing was performed with an rf inhomogeneity compensated, numerically optimized adiabatic mixing sequence (Bennett et al., in preparation). Aromatic proton assignments of eIF4G in complex with perdeuterated eIF4E were obtained using aromatic edited 2D Clean-TOCSY (Cavanagh and Rance, 1992), aromatic edited 2D NOESY and ^{15}N NOESY-HSQC, recorded at 750 MHz. Aromatic editing in the 2D NOESY and TOCSY experiments was required to eliminate excitation of CHAPS signals in the aliphatic region and was achieved by replacing the last pulse of the sequence with an aromatic selective E-SNOB (Kupce et al., 1995) pulse of width 1.5 ms. Aromatic assignments of eIF4E were obtained by performing ^{15}N NOESY HSQC on the complex of ^{15}N-labeled eIF4E and U-^{2}H-labeled eIF4G. The eIF4E aromatic assignments were corroborated by the initial structures of the complex obtained with the restraints derived from ^{13}C HMQC-NOESY spectra recorded on ^{13}C/^{1}H-ILV, U-^{2}H-eIF4E in complex with unlabeled eIF4G. The indirect proton dimension of the ^{13}C HMQC-NOESY was recorded in semi-constant-time mode (Grzesiek and Bax, 1993; Logan et al., 1993).

Results and discussion

The precursors **3** and **4** are not commercially available and, in our hands, the previously reported synthesis (Hajduk et al., 2000) was difficult to implement. We followed an alternative synthetic route (Figure 2) based on the reaction of diethyl oxalate with alkyl Grignard compounds at low temperatures that gave the mono-substituted ethyl α-ketoesters **2a** and **2b**

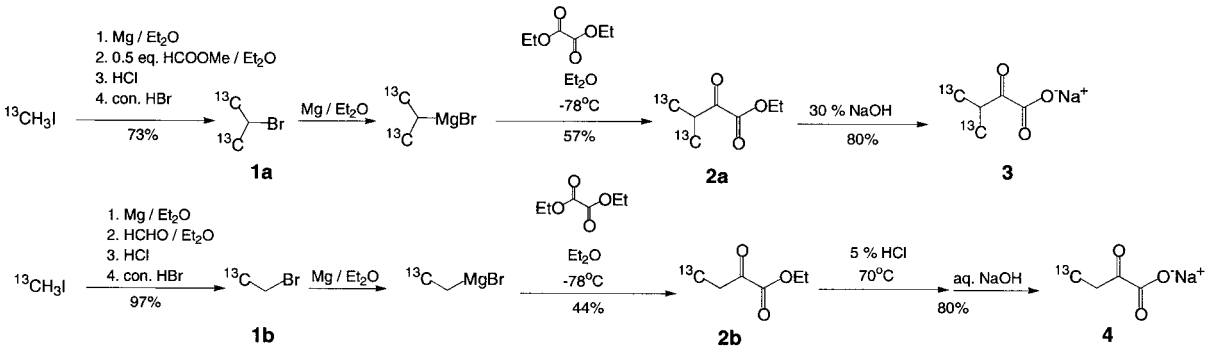

Figure 2. Synthesis of the biosynthetic I,L,V precursors **3** and **4**.

(Fizet, 1982; Rambaud et al., 1988; Singh et al., 1989). 2,2-$^{13}C_2$-isopropyl bromide (**1a**) and 2-^{13}C-ethyl bromide (**1b**) were synthesized using traditional Grignard chemistry, starting with ^{13}C-methyl iodide and then converted to the corresponding alkylmagnesium bromides. Following reaction with diethyl oxalate, compound **3** was obtained by saponification of the corresponding ester in aqueous NaOH (Fizet, 1982; Kozlowski et al., 1989). Compound **4** is sensitive to basic conditions (Kozlowski et al., 1989) and was obtained from the ethyl ester by acid hydrolysis, followed by neutralization with dilute base. The overall yields were 20–30% but could be significantly improved if the reactions are scaled up (see the Experimental section). As reported here, the cost of precursors in terms of ^{13}C-methyl iodide is $\sim$ \$30 per 1L of D_2O cell culture.

ILV-labeled protein samples were obtained by adding precursors **3** and **4** to minimal growth medium, containing D_2O and ^{2}H-glucose, 30 min prior to induction (Hajduk et al., 2000). As with free eIF4E (Matsuo et al., 1997), CHAPS is employed to increase the stability of the complex against precipitation. Only four CHAPS (^{1}H,^{13}C) cross-peaks are upfield of (1.7 ppm, 30 ppm) in both dimensions; therefore, resonances from CHAPS do not significantly overlap with ILV methyl groups. Figure 3 shows a typical HSQC spectrum of an ILV-methyl labeled perdeuterated protein (eIF4G) in complex with its unlabeled partner (eIF4E). The 2D ^{1}H planes of the corresponding ^{13}C HMQC-NOESY spectrum are shown in Figure 4. Signals in the aromatic region of the directly detected proton dimension derive from intermolecular aromatic/methyl NOEs. Also observed are intramolecular NOEs between the ILV-methyl groups and intermolecular NOEs with aliphatic protons of the unlabeled binding partner (data not shown). Though

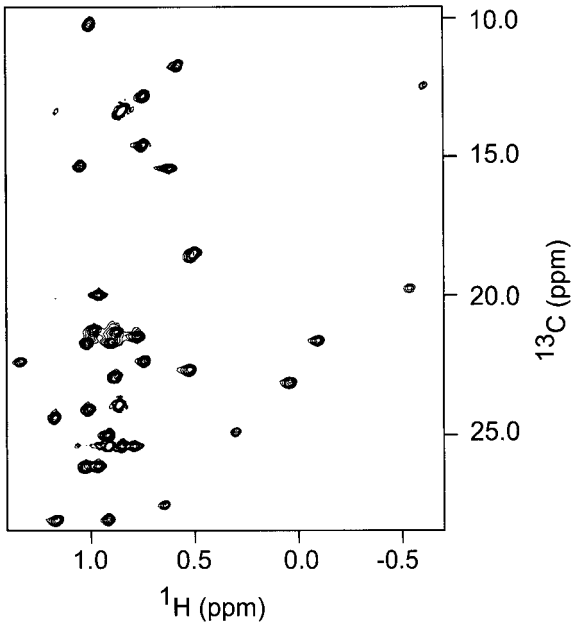

Figure 3. Gradient enhanced ^{13}C-HSQC spectrum of ^{13}C/^{1}H methyl ILV U-^{2}H eIF4G(393-490) in complex with unlabeled eIF4E, recorded at 750 MHz. Sample conditions: 0.4 mM 1:1:1 complex of m7GDP / eIF4E / eIF4G in 50 mM Na phosphate buffer (pH 6.5) in 100% D_2O containing 50 mM KCl, 1 mM DTT and 12 mM CHAPS.

analysis is still underway, 73 unambiguous intermolecular NOEs were observed from these spectra. These data, combined with 55 unambigous NH-aliphatic NOEs using a complementary assymetric deuteration scheme described previously (Walters et al., 1997) have allowed structure determination of the complex to a backbone RMSD of 0.95 Å resolution (Table 1). It should be noted that the intermolecular restraints provided by assymetric deuteration were useful in refining the eIF4G(393-490) structure in addition to defining the eIF4E/eIF4G(393-490) complex (Figure 5

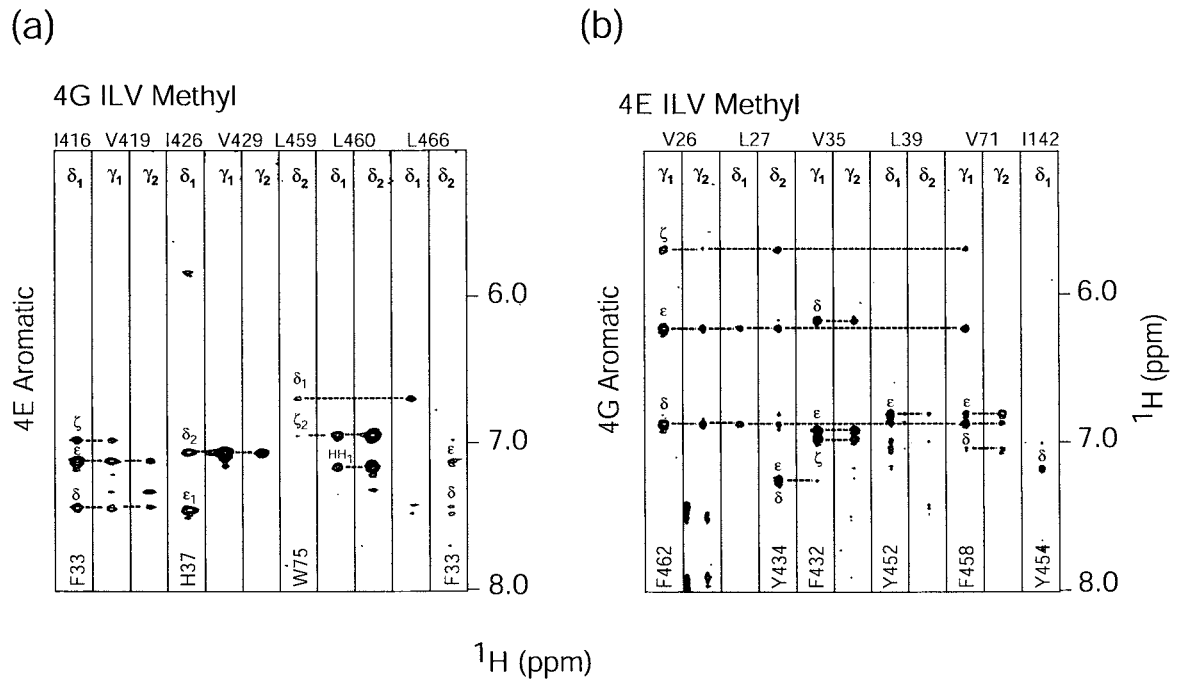

Figure 4. Aromatic region of the ^{13}C HMQC-NOESY (mixing time 150 ms) spectra of (a) 0.4 mM $^{13}C/^{1}H$ methyl ILV U-^{2}H eIF4G(393-490) in complex with unlabeled eIF4E, recorded at 500 MHz using a cryoprobe(tm); (b) 0.7 mM $^{13}C/^{1}H$ ILV U-^{2}H eIF4E in complex with unlabeled eIF4G, recorded at 750 MHz.

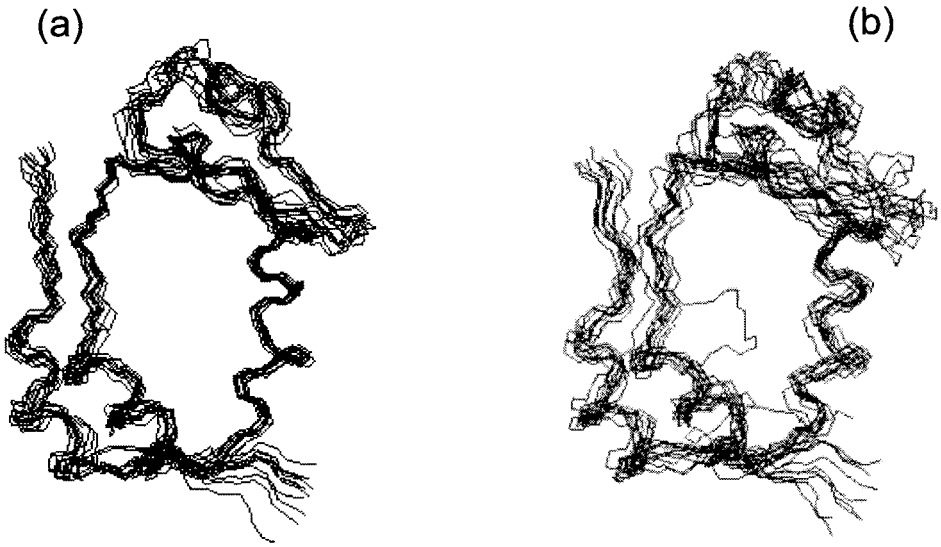

Figure 5. Superimposed structures of the complexed eIF4G calculated (a) with both NH–aliphatic and ILV-aliphatic intermolecular NOEs, (b) using NH-aliphatic intermolecular NOEs. The intramolecular restrains are the same in both calculations. NOEs were grouped into three bins (3.5, 4.5 and 5.5 Å) based on cross-peak intensities. An additional 0.5 Å was added for NOEs involving methyl groups.

Table 1. RMSD of the calculated eIF4G(393-490)/eIF4E structures in the presence and absence of intermolecular NOEs obtained as described here and in Walters et al. (1997)

RMSD (Å)	All NOEs	Without ^{15}NH NOEs	Without ^{13}C-ILV NOEs
eIF4G(393-490)	0.64	1.24	1.14
eIF4G(393-490)/eIF4E	0.95	1.45	1.15

and Table 1). The detailed NMR structure of the complex will be reported elsewhere.

Previously, intermolecular NOEs have been measured using half-filtering methods (Otting et al., 1986). Within this framework, magnetization is selected for (editing) or against (filtering) based on a spin-echo difference module where the sign of magnetization coupled to a heteronucleus may be manipulated independently of uncoupled magnetization. These modules are separated by NOE mixing amongst proximal protons resulting in considerable lengthening of the standard ^{15}N or ^{13}C dispersed NOESY experiment (Walters et al., 2001). Signal losses in large proteins due to short transverse relaxation times result in a reduction in the number of observed NOEs. A more sensitive and robust approach for distinguishing intermolecular NOEs is to use asymmetric deuteration (Walters et al., 1997). This approach does not require any additional delays in the pulse scheme as filtration is achieved by deuteration. For example, ^{15}N-^{1}H intermolecular NOEs may be measured by mixing 100% ^{2}H, ^{15}N labeled protein with an unlabeled ligand and performing a ^{15}N NOESY-HSQC in H_2O (Walters et al., 1997) . Any observed aliphatic to NH NOEs are intermolecular. Moreover, the amide proton transverse relaxation rates of deuterated protein are attenuated, resulting in an additional sensitivity gain.

With the approach presented here, aromatic resonances are observed in the directly detected dimension, F_3. For large proteins containing many aromatic residues, one would expect spectral overlap to seriously hinder analysis, however, this effect is partially reduced for globular proteins where only a subset of the total number of aromatic residues would appear on the complex interface. If spectral overlap is a problem, the experiment can be performed on samples where specific protonated aromatic amino acids are incorporated into an otherwise deuterated background, in complex with an ILV-methyl labeled binding partner as described above. Alternatively, one may perform 4D HMQC-NOESY-TROSY with one protein ILV-^{13}C labeled as above, and the other protein ^{13}C-enriched at phenylalanine and/or tyrosine aromatic positions (Wang et al., 1999). Though the sensitivity of the latter approach might be expected to suffer due to the additional delays, this effect would be partially offset by the absence of ^{13}C-^{13}C J couplings and the use of ^{13}C aromatic TROSY (Pervushin et al., 1998; Meissner and Sorensen, 1999a, b) in F_3.

Conclusion

In sum, the ^{13}C/^{1}H ILV-methyl/U-^{2}H and ^{15}N/U-^{2}H asymmetric labeling schemes provide robust and sensitive means to obtaining intermolecular NOEs. This asymmetric deuteration technique may be extended to the analysis of protein/nucleic acid, protein/drug or homooligimeric complexes. Together with residual dipolar couplings (Prestegard et al., 2000) and site-directed spin labeling (Bertini et al., 1999; Battiste et al., 2003), the distance restraints provided by these experiments will facilitate the structure determination of large macromolecular complexes in solution. The alternative synthetic scheme for generating [γ,γ'-$^{13}C_2$]-α-ketoisovalerate (**3**) and [γ-^{13}C]-α-ketobutyrate (**4**) presented here is simple, cost-effective and amenable to large scale implementation.

Acknowledgements

J.D.G acknowledges support from an American Cancer Society postdoctoral fellowship. We gratefully acknowledge Dr S. Fesik for providing the ILV precursors used in the initial portion of this study, Drs R.S. Lokey, Tim Sprigings and J. Tallarico at the Institute for Chemistry and Cell Biology for providing lab space and helpful advice, and Dr Mallika Sastry for critically reading the manuscript. This research was supported by grants from NIH to GW (GM47467, GM38608 and CA68262) and the MIT/Harvard Center for Magnetic Resonance (RR-00995).

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