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Assignment of Ile, Leu, and Val Methyl Correlations in Supra-Molecular Systems: An Application to Aspartate Transcarbamoylase

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Abstract: A number of complementary approaches for the assignment of Ile, Leu, and Val methyl groups in Methyl-TROSY spectra of supra-molecular protein complexes are presented and compared. This includes the transfer of assignments from smaller fragments to the complex using a "divide-and-conquer" approach, assignment transfer via exchange spectroscopy, or, alternatively, generating assignments of the complex through the measurement of pseudocontact shifts, facilitated by the introduction of paramagnetic probes. The methodology is applied to the assignment of the regulatory chains in the 300 kDa enzyme aspartate transcarbamoylase, ATCase. The "divide-and-conquer" method that has proven to be very powerful in applications to other systems produced assignments for approximately 60% of the observed methyl groups in TROSY maps of ATCase. By contrast, the combination of all approaches led to assignments for 86% of the methyls, providing a large number of probes of structure and dynamics. The derived assignments were used to interpret chemical shift changes of ATCase upon titration with the nucleotide ATP. Large shift changes in the N-terminal tails of the regulatory chain provide the first evidence for structural perturbations in a region that is known to play a critical role on the effect of nucleotide binding on distal catalytic sites of this allosteric enzyme.

Introduction

The development of methyl-TROSY NMR spectroscopy[1] and its application to highly deuterated $^{13}CH_3$-methyl labeled proteins has extended quantitative NMR studies to protein complexes with molecular weights as large as 1 MDa. Applications have been reported that involve the 120 kDa CheA−CheW complex,[2] the 200 kDa SecA translocase,[3] the 240 kDa Arp2/3 complex,[4] the 300 kDa Aspartate transcarbamoylase, ATCase,[5,6] a 300 kDa fragment of AAA-ATPase p97,[7] the 300 kDa ClpP protease at temperatures as low as 0.5 °C,[8] the 490 kDa TET2 aminopeptidase,[9] and the 670 kDa 20S core particle (CP) proteasome[10] both without and with the 11S activator complex (1.0 MDa). In all cases, spectra with high sensitivity and resolution were generated. Recording high quality data sets is a requisite first step in any NMR study; however, in most applications it is necessary to obtain chemical shift assignments that then allow NMR spectra to be used as site-specific reporters of structure, of dynamics and of interactions with target molecules. For proteins with molecular weights on the order of 50 kDa or less, site-specific backbone assignments are generated using triple resonance NMR methodology in which correlations connecting ^{1}HN, ^{15}N, and ^{13}C chemical shifts are obtained.[11,12] Assignment of side-chain signals subsequently follows by recording spectra connecting side-chain chemical shifts with those of the backbone that are already assigned.[11,12] For complexes with molecular weights larger than approximately 100 kDa many of the triple-resonance experiments become insensitive so that this approach no longer works. This is the case for ATCase, ClpP protease and the proteasome, proteins investigated in our laboratory. It is certainly the situation for other supra-molecular systems for which high quality methyl-TROSY spectra can be readily obtained. It is clear, therefore, that a different strategy is

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necessary for the assignment of methyl groups in high molecular weight proteins.

One approach is to first assign smaller molecular fragments using "traditional" methods and then transfer the assignment information onto larger constructs, primarily by comparing peak locations in 2D $^{13}C-^{1}H$ HMQC spectra and NOE patterns in 3D NOESY spectra. For SecA and the 20S CP proteasome, near complete assignments of Ile, Leu, Met, Val (SecA)[3] and Ile, Leu, Val (20S CP)[10] methyl groups were achieved in this manner, with mutagenesis used both to confirm and extend the assignments. Methyl assignments for the majority of other large complexes currently under investigation are not as of yet available, and given the early stages of most applications, it is particularly timely to evaluate the efficacy of a number of different assignment approaches that, in principle, can be used.

In the present study we consider the allosteric enzyme ATCase that comprises 6 regulatory (r-chain; 15 kDa) and 6 catalytic (c-chain; 33 kDa) chains that bind nucleotide effectors and substrates, respectively[13,14] (referred to as r_6c_6), and whose many allosteric properties are well explained by the simple Monod−Wyman−Changeux model[15] in which the enzyme exists in one of two states (T or R).[14] Crystal structures of ATCase with different ligands and in different states are available, showing that the molecule consists of a dimer of catalytic trimers (c_3) that are stabilized through interactions with three dimers of r chains (r_2).[13] Previous NMR studies have established that all r-chains are equivalent, as are all c-chains, so that only a single set of peaks for each chain is observed in methyl-TROSY spectra.[6] Herein we focus on assigning Ile (δ1 only), Leu, and Val methyl groups of the r-chains of ATCase in the T state of the intact enzyme using a number of different assignment strategies. Specifically, a "divide-and-conquer" approach is considered initially. Here the methyl assignments for the 30 kDa r_2 dimer, readily obtained using conventional triple-resonance techniques, are transferred to spectra of the intact enzyme by comparing peak positions in a variety of spectra. Although this approach has been successfully applied in studies of SecA[3] and the 20S CP proteasome[10] it was less effective in the present application. A second method is evaluated, that involves mutagenesis to create a situation whereby the time-scale of exchange between r_2 in the free form and in the enzyme complex (r_6c_6) is elevated so that exchange cross-peaks are observed connecting the spectrum of the assigned r_2 state with the unassigned spectrum of r_6c_6 (300 kDa). Such an approach has been used previously, albeit not with mutagenesis, to transfer assignments from one exchanging state to another for aromatic protons,[16] backbone amides,[17,18] and methyl groups.[2] A third approach involves making use of magnetic interactions created through the introduction of an unpaired electron into the system of interest. Pseudocontact shifts (PCSs), arising from the interaction of nuclear spins with one or more unpaired electron,[19] can be readily measured by comparing spectra recorded on a molecule in its diamagnetic and paramagnetic forms. Since the PCS depends both on the length and on the orientation of the dipole vector connecting the electron and the affected nuclear spin in a molecular frame, it provides valuable information for assignment in cases where the structure of the molecule under study is known, as has been demonstrated recently in a study of a 194 residue domain by Otting and co-workers.[20] We show here that a combination of the three strategies introduced above produces assignments for all of the Ile (δ1) methyl groups and 83% of the Leu and Val methyls of ATCase for which correlations are observed in methyl-TROSY spectra. In this way, a large number of probes are available for further studies. An application is presented that makes use of the methyl assignments in interpreting ATP−ATCase titration data. Although details of how best to implement the methodology will vary in a system dependent fashion, the approaches described are general and together provide a powerful strategy for methyl group assignment in supramolecular systems.

Materials and Methods

Protein Expression and Purification. The plasmids used for the independent expression of the r- and c-chains of ATCase have been described previously.[6] Plasmids encoding the mutant chains used for the present work were prepared with a QuikChange kit (Stratagene), with sequencing to confirm that the desired mutations were produced. All unlabeled proteins were expressed in LB medium in *E.coli* BL21(DE3) cells with overexpression induced at $OD_{600} = 0.6$ by addition of 1 mM isopropylthiogalactoside. Cultures expressing all c- and r-chain constructs with two or more mutations were induced for 18−20 h at room temperature, while WT or single r-chain mutants were induced for 4 h at 37 °C. Expression of labeled r-chain was performed in M9 medium, 99.9% D_2O, supplemented with 20 μM $ZnCl_2$. Samples used for backbone assignments and methyl out-and-back experiments were grown with $^{2}H_7$,$^{13}C_6$-glucose and 2-keto-3,3-d_2-$^{13}C_4$-butyrate and 2-keto-3-methyl-d_3-3-d_1-$^{13}C_4$-butyrate.[21,22] For all other samples $^{2}H_7$-glucose was used and methyl groups were labeled by including 2-keto-3,3-d_2-4-^{13}C-butyrate (for Ile-[$\delta1^{13}CH_3$]) and 2-keto-3-methyl-d_3-3-d_1-4-^{13}C-butyrate (for Leu,Val-[$^{13}CH_3$,$^{12}CD_3$]), as described previously.[6] To express the Co(II)-substituted enzyme, the $ZnCl_2$ supplement was omitted from the M9 medium, other trace elements were not included, and 20 μM $CoCl_2$ (final concentration) was added at the point of induction.[23]

Purification of isolated r-chains, c-chains and the reconstituted ATCase enzyme was performed as reported before,[6] with a number of important modifications. To obtain NMR samples of isolated r-chain dimer, a Superdex75 gel-filtration step (50 mM TrisHCl, 200 mM KCl, 10 mM beta-mercaptoethanol (BME) pH = 8.5) was applied after Ni^{2+} affinity purification. The r-chain containing fractions were pooled and exchanged into buffer for NMR by dialysis or concentration-dilution cycles. Due to concerns about decreased stability, samples of the Co(II)-substituted protein or of the point mutants used for confirmation of assignments were prepared by lysing cell pellets with labeled r-chains, immediately adding excess of c-chain and then proceeding to Ni^{2+} affinity chromatography. Proteins containing mutant c-chains were purified by anion exchange (Q-sepharose) chromatography starting in 50 mM TrisHCl, pH = 8.0 and eluting with a 0−0.5 M KCl gradient (over 10 column volumes), with a further Superdex200 purification step (50 mM TrisHCl, 200 mM KCl, 10 mM BME, pH = 8). The

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extinction coefficient of the Co(II)-substituted enzyme was determined by the Bradford assay using the Zn-enzyme as a calibration standard. Since ε_{260} values for two independently prepared Co-enzyme samples (in 6 M guanidine hydrochloride) were within 7% of the Zn-enzyme, the same $\varepsilon_{260} = 25\,460\ M^{-1}\ cm^{-1}$ value was used for both metal bound forms of ATCase.

Measuring Elution Profiles of Mutant Enzyme. The affinity of mutant r- and c-chains along with a very qualitative measure of the "dissociation rates" of the r-dimers/c-trimers comprising ATCase were quantified using a Superdex 200 HR10/30 gel-filtration column (Pharmacia; ca. 24 mL volume) in 50 mM TrisHCl, 200 mM KCl, 10 mM BME, pH = 8, with a flow rate of 0.5 mL/min at room temperature. Samples of r- and c-chain mutants were exchanged into the same buffer as used for gel-filtration and concentrated to 0.1−0.2 mM (c-chains) and 0.2−0.5 mM (r-chains). Enzyme mixtures were prepared by mixing volumes of c-chain, r-chain and buffer so as to obtain 0.5 mL containing 0.1 mM r-chain and 0.02 mM c-chain, incubated for 15−30 min at room temperature and injected onto the gel-filtration column.

NMR Spectroscopy. Backbone assignments of r-chain dimers (30 kDa) were performed on a 1.2 mM U−^{2}H,^{13}C,^{15}N, Ile-[$\delta1^{13}CH_3$], Leu,Val-[$^{13}CH_3$,$^{12}CD_3$] r-chain sample in 50 mM NaH_2PO_4, 20 mM BME, 0.2 mM NaN_3, pH = 6.5, 10% D_2O, with all data recorded at 25 °C on a 600 MHz Varian Unity spectrometer equipped with a cryogenically cooled probe-head. Note that the stability of r-chain samples is not as high as for the full enzyme, necessitating the use of slightly different buffers than those for r_6c_6 (see below). This did not, however, interfere with the transfer of methyl assignments from r_2 to r_6c_6. ^{1}HN, ^{15}N, $^{13}C^{\alpha}$, $^{13}C^{\beta}$ and ^{13}CO resonances of the r-chain dimer were assigned by a combination of TROSY-based[24] HNCA, HN(CO)CA, HN(CA)CB, HN(CO-CA)CB, HNCO, and HN(CA)CO experiments,[11,25,26] with subsequent assignment of Ile, Leu, and Val methyl groups achieved by recording 3D out-and-back experiments−HMCM[CG]CBCA, Ile,Leu-HMCM(CGCBCA)CO, and Val-HMCM(CBCA)CO.[21,22] A second sample−2.6 mM U−^{2}H, Ile-[$\delta1^{13}CH_3$], Leu,Val-[$^{13}CH_3$,$^{12}CD_3$] r-chain in 99.9% D_2O buffer, 50 mM HEPES sodium salt, 50 mM KCl, 20 mM BME, pH* = 7.5−was produced and used to record a 3D ^{13}C/^{13}C-separated NOESY[11] data set, 250 ms mixing time, at 25 °C.

All ATCase NMR samples comprised natural abundance, protonated c-chains and labeled r-chains and were dissolved (by repeated concentration and dilution cycles in a Millipore Ultrafree-15 concentrator) in 99.9% D_2O buffer (50 mM HEPES sodium salt, 50 mM KCl, 20 mM BME, pH*=7.5). Chemical shifts were referenced to added DSS (0.2 mM) that was included in each sample. All 3D spectra were recorded on a 600 MHz spectrometer equipped with a cryogenically cooled probe-head. A 0.9 mM U−^{2}H,^{13}C,^{15}N, Ile-[$\delta1^{13}CH_3$], Leu,Val-[$^{13}CH_3$,$^{12}CD_3$] enzyme sample (monomer concentration, that is, containing 0.9 mM r- and c-chains) was used for methyl out-and-back experiments at 37 °C, including HMCMCG/CB (additionally at 45 °C), HMCM(CG/CB)CB/CA and Ile,Leu-HMCM(CGCB)CA.[10] 3D ^{13}C/^{13}C-separated NOESY spectra[11] (250 ms mixing) were recorded at 37 and 45 °C using an Ile-[$\delta1^{13}CH_3$], Leu,Val-[$^{13}CH_3$,$^{12}CD_3$] enzyme sample (0.9 mM monomer concentration).

EXSY spectra monitoring the slow exchange between free and enzyme-bound r-chain dimers made use of samples containing 1 mM Ile-[$\delta1^{13}CH_3$] or Leu,Val-[$^{13}CH_3$,$^{12}CD_3$] r-chain and 0.8−0.9 mM unlabeled c-chain (50 mM Tris-HCl, 200 mM KCl, 10 mM BME, 99.9% D_2O, pH* = 8.0; again, the decreased stability of mutant r- and c-chains necessitated sample buffer conditions slightly different from those used for WT holoenzyme) and were recorded at 37 °C on an 800 MHz spectrometer equipped with a room-temperature probe-head. A 2D ^{13}C−^{1}H experiment that follows the transfer of longitudinal order during mixing times of 2 ms (effectively no mixing), 100 and 200 ms was used, as described previously.[8,27]

Measurement of pseudocontact shifts were performed with an Ile-[$\delta1^{13}CH_3$] labeled Co-enzyme sample, 0.8 mM in r- and c-chains and an Ile-[$\delta1^{13}CH_3$], Leu,Val-[$^{13}CH_3$,$^{12}CD_3$] labeled Co-enzyme sample, 1.1 mM in r- and c-chains. HMQC spectra were recorded at 37 °C, 500 and 800 MHz (room temperature probe-heads) and compared to reference spectra of the Zn-enzyme obtained under identical conditions.

Calculation of Pseudocontact Shifts. PCS (δ^{PC}) values that derive from a single proximal paramagnetic metal ion are most conveniently calculated from the relation[19]

$$\delta^{PC} = \frac{1}{4\pi r^3}[(\chi_{ZZ} - \bar{\chi})v_1 + (\chi_{XX} - \chi_{YY})v_2 + \chi_{XY}v_3 + \chi_{XZ}v_4 + \chi_{YZ}v_5], \bar{\chi} = \frac{1}{3}\sum_i \chi_{ii} \quad (1)$$

where χ_{ij} is the ijth element of the magnetic susceptibility tensor and

$$v_1 = \frac{2z^2 - x^2 - y^2}{2r^2}, v_2 = \frac{x^2 - y^2}{2r^2}, v_3 = \frac{2xy}{r^2}, v_4 = \frac{2xz}{r^2}, v_5 = \frac{2yz}{r^2} \quad (2)$$

with $\vec{r} = (x,y,z)$ the vector connecting the metal ion and the methyl ^{13}C or ^{1}H spin in question. Note that this expression is valid in an arbitrary molecular coordinate frame; we have chosen the frame of coordinates in the PDB file 1TUG[28] in what follows. Methyl protons with standard geometry were added to the X-ray structure and their coordinates averaged into a single pseudoatom for computations. PCS values of protons only were used in all analyses reported here. In the case where PCS values are due to a single paramagnet, eqs 1 and 2 can be written in matrix form as

$$\vec{\delta} = \underline{V}\vec{\chi} \quad (3)$$

where $\vec{\delta}$ is a column vector of n elements, comprised of the n measured PCS values, with δ_k ($1 \le k \le n$) the δ^{PC} value for nucleus k (^{13}C or ^{1}H), $\underline{V}$ is a $n \times 5$ matrix where columns 1−5 are given by $(4\pi r^3)^{-1}\cdot(v_1,...,v_5)$ for each nucleus and $\vec{\chi} = (\chi_{ZZ} - \bar{\chi}, \chi_{XX} - \chi_{YY}, \chi_{XY}, \chi_{XZ}, \chi_{YZ})^+$ where the superscript + is the transpose operator.

The situation is more complex in the application considered here where each of the r-chains of the enzyme binds a Co^{2+} ion in an identical fashion. As discussed in some detail in SI, values of δ^{PC} for a given ^{13}C or ^{1}H spin are sums of contributions from Co^{2+} ions from each of the r-chains in an r_2 dimer, but contributions from the other 4 ions that are bound to other r_2 units in ATCase can be neglected (>40 Å removed[28]). In what follows, therefore, we consider only an r_2 dimer in the calculations and distinguish each of the r-chains by A and B. We can write the PCS of a methyl spin in r-chain A, $\vec{\delta}_A$, as

$$\vec{\delta}_A = \underline{V}_{A,Co_A}\vec{\chi}_{Co_A} + \underline{V}_{A,Co_B}\vec{\chi}_{Co_B} \quad (4.1)$$

that includes contributions from Co^{2+} ions in each of chains A and B, with a similar equation for the PCS of the corresponding methyl spin from chain B, $\vec{\delta}_B$,

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$$\vec{\delta}_B = \underline{V}_{B,\mathrm{Co}_B}\vec{\chi}_{\mathrm{Co}_B} + \underline{V}_{B,\mathrm{Co}_A}\vec{\chi}_{\mathrm{Co}_A} \quad (4.2)$$

The equivalence of each of the r-chains of the dimer (see Supporting Information) implies that $\vec{\delta}_A = \vec{\delta}_B$ (which is observed experimentally) so that

$$\underline{V}_{A,\mathrm{Co}_A}\vec{\chi}_{\mathrm{Co}_A} + \underline{V}_{A,\mathrm{Co}_B}\vec{\chi}_{\mathrm{Co}_B} = \underline{V}_{B,\mathrm{Co}_B}\vec{\chi}_{\mathrm{Co}_B} + \underline{V}_{B,\mathrm{Co}_A}\vec{\chi}_{\mathrm{Co}_A}$$
$$\underline{V}_{A,\mathrm{Co}_A}\vec{\chi}_{\mathrm{Co}_A} = \underline{V}_{B,\mathrm{Co}_B}\vec{\chi}_{\mathrm{Co}_B} \quad (5.1)$$

and hence,

$$\underline{V}_{A,\mathrm{Co}_B}\vec{\chi}_{\mathrm{Co}_B} = \underline{V}_{B,\mathrm{Co}_A}\vec{\chi}_{\mathrm{Co}_A} \quad (5.2)$$

It follows therefore that

$$\vec{\delta}_A = (\underline{V}_{A,\mathrm{Co}_A} + \underline{V}_{B,\mathrm{Co}_A})\vec{\chi}_{\mathrm{Co}_A} \quad (6.1)$$

from which a susceptibility tensor for Co^{2+} in site A is calculated according to

$$(\underline{V}_{A,\mathrm{Co}_A} + \underline{V}_{B,\mathrm{Co}_A})^{-1}\vec{\delta}_A = \vec{\chi}_{\mathrm{Co}_A} \quad (6.2)$$

using in-house written Matlab (Mathworks, Inc.) scripts available upon request. A corresponding relation for $\vec{\chi}_{\mathrm{Co}_B}$ can be obtained by interchanging A and B in eq 6.2. Finally, in practice we calculate

$$\vec{\delta}_{\mathrm{avg,calc}} = \frac{1}{2}(\vec{\delta}_{A,\mathrm{calc}} + \vec{\delta}_{B,\mathrm{calc}}) = \frac{1}{2}\{(\underline{V}_{A,\mathrm{Co}_A} + \underline{V}_{B,\mathrm{Co}_A})\vec{\chi}_{\mathrm{Co}_A} + (\underline{V}_{B,\mathrm{Co}_B} + \underline{V}_{A,\mathrm{Co}_B})\vec{\chi}_{\mathrm{Co}_B}\} \quad (7)$$

that is then compared with measured PCS values to aid in the assignment process. The $\vec{\chi}$ vectors $10^4\cdot(5.4, -2.6, 0.6, -4.2, -5.6)$ ppm Å^3 and $10^4\cdot(4.7, 5.5, 0.4, -7.2, -3.1)$ ppm Å^3 were calculated for metal sites in what are referred to as chains B and D of the 1TUG[28] PDB file, respectively, and are expressed in the coordinate frame of this PDB file.

Results and Discussion

Assignments Using a "Divide and Conquer Strategy". A useful strategy for obtaining assignments of Ile, Leu, and Val methyl groups in the 300 kDa *E. coli* ATCase dodecamer (r_6c_6) is to focus initially on the individual r or c chains since these can be expressed in isolation as 30 kDa dimers (r_2) or 100 kDa trimers (c_3) that are significantly smaller than the full enzyme.[29,30] The hope is that once assignments of these smaller particles are obtained they can be transferred in a facile manner to the larger complex, as was done previously in studies of the 20S CP proteasome[10] and for SecA.[3] A sample of ^{15}N, ^{13}C, ^{2}H, Ile-[$\delta1^{13}CH_3$], Leu, Val-[$^{13}CH_3$,$^{12}CD_3$] r_2 was prepared and from a set of TROSY-based[24] triple resonance experiments[11,12] near complete backbone assignments were generated. Subsequently, HMCM[CG]CBCA, Ile,Leu-HMCM(CGCBCA)CO and Val-HMCM(CBCA)CO spectra[21,22] were recorded, connecting methyl resonances to the backbone so that the ^{13}C−^{1}H HMQC map of r_2 could be assigned, Figure 1a. Correlations from all 12 of the Ile residues were assigned in this manner, as were 47 cross-peaks from the 15 Leu and 12 Val residues. Three very broad resonances in the Leu/Val region of the spectrum could not be assigned due to the absence of either through-bond or through-space NOE connectivities, while the remaining 4 expected correlations were simply missing in 2D ^{13}C−^{1}H spectra.

Figure 1b shows an overlay of methyl-TROSY spectra of r_2 at 25 °C and of the r-chain within the intact enzyme at 37 °C (under otherwise identical conditions; spectra of r_2 were recorded at a lower temperature due to decreased stability). Eleven Ile cross-peaks and 52 Leu/Val correlations were present in the spectrum of r_6c_6. The absence of 1 Ile peak and 2 correlations from Leu/Val results from the proximity of these residues to protonated c-chains so that methyl correlations from them are completely broadened, as has been observed in other studies of supra-molecular systems.[2,10] On the basis of the spectral overlay of Figure 1b a number of assignments could be transferred immediately corresponding to 3 (27% of the observed peaks) Ile and 18 (35%) Leu/Val cross-peaks (for a total of 21 methyl groups out of the 63 that give rise to correlations in methyl-TROSY data sets, 33%).

Further assignments were obtained from an analysis of a 3D ^{13}C/^{13}C-separated NOESY data set[11] recorded on a sample of ^{15}N, ^{12}C, ^{2}H, Ile-[$\delta1^{13}CH_3$], Leu, Val-[$^{13}CH_3$,$^{12}CD_3$] ATCase. This analysis was done in concert with distance information from the X-ray structure of the enzyme,[28] as described in our previous study of the proteasome.[10] By means of example, Figure 1c shows a number of strips isolated from the 3D NOESY data set showing correlations that link Ile 134, Leu 76, Leu 99 and Ile 103. The lack of intraresidue methyl correlations in the case of Leu and Val ([$^{13}CH_3$,$^{12}CD_3$]-labeling) complicates assignment of each pair of prochiral methyls to a given residue. Such information is most reliably obtained by recording 3D through-bond methyl out-and-back experiments[10] performed on a U−^{15}N, ^{13}C, ^{2}H, Ile-[$\delta1^{13}CH_3$], Leu, Val-[$^{13}CH_3$,$^{12}CD_3$] ATCase sample. Particularly useful for ATCase were those experiments transferring magnetization from the methyl carbon to the adjacent ^{13}C spin[10] ($^{13}C^{\gamma}$ or $^{13}C^{\beta}$ for Ile/Leu or Val, respectively; HMCMCG/CB, Figure 1d) or from the methyl carbon to the carbon nucleus two bonds removed[10] ($^{13}C^{\beta}$ or $^{13}C^{\alpha}$ for Ile/Leu or Val; HMCM(CG/CB)CB/CA, Figure 1e). In addition to connecting pairs of prochiral methyls in this way, Figures 1d,e, the experiment is also useful in providing additional assignments since strip-plots of connectivities between methyl ^{13}C, ^{1}H spins and side-chain aliphatic carbons can be compared between spectra of r_2 and r_6c_6 and a high degree of similarity provides a very reasonable basis for transfer of assignments from spectra of the r-dimer to the complex. Analysis of NOESY and the through-bond methyl correlation data increased the number of assignments to 6 Ile (54%) and 32 Leu/Val (62%) correlations, corresponding to 60% of the observed Ile/Leu/Val methyl cross-peaks in ^{13}C,^{1}H methyl-TROSY spectra. This is somewhat less than the success rate noted in studies of the 'half-proteasome', a 360 kDa complex that was used to assist in the assignment of methyl groups for the full 20S CP complex,[10] that reflects the poorer spectral quality for the ATCase system. A second complicating effect is that for ATCase the transfer of assignments from r_2 to r_6c_6 is made more difficult by rather significant changes in many of the peak positions in spectra of these two particles that makes this approach less transparent in this case than for SecA,[3] for example. Clearly, alternative, complementary strategies are needed to increase the fraction of methyl correlations that can be assigned.

Assignments Based on Exchange Spectroscopy (EXSY). In cases where the exchange between a pair of interacting

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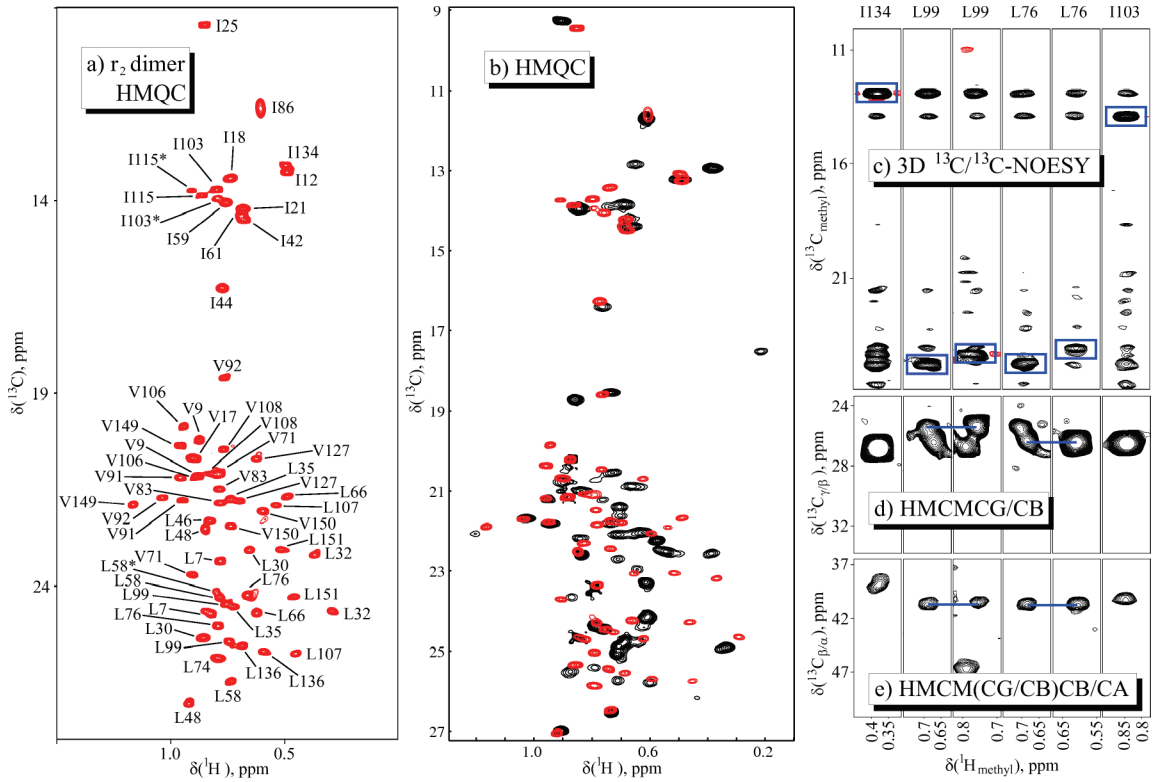

Figure 1. Assignment of Ile, Leu, and Val methyl groups of the r-chain of ATCase obtained by through-bond and through-space 3D NMR spectroscopy. (a) Methyl-TROSY spectrum of the isolated r_2-dimer, 25 °C, 600 MHz, showing the assignments of methyl correlations obtained using 'standard' 3D triple resonance experiments.[12] Resonances marked with * are minor peaks due to alternate conformations at that site. (b) Overlay of Methyl-TROSY spectra of the Ile/Leu/Val labeled r-chain in ATCase, black, 37 °C, 800 MHz and the free r-chain dimer, red, 25 °C, 600 MHz. (c) Strip-plots isolated from the 3D $^{13}C/^{13}C$-separated NOESY spectrum of ATCase, 37 °C, 600 MHz, at $^{1}H_{methyl}$ and $^{13}C_{methyl}$ positions of residues that form a cluster at the interface between the N- and C-terminal domains of the r-chain (labels at top). NOESY diagonal peaks are indicated by blue boxes. (d) Strips from the 3D HMCMCG/CB spectrum, 37 °C, 600 MHz, of the same residues as in (c). Peaks from isopropyl δ-methyls of a given Leu residue are linked by the identical C^{γ} correlations, connected by horizontal blue lines. (e) Same as (d) with strips isolated from the 3D HMCM(CG/CB)CB/CA spectrum and blue lines connecting identical C^{β} correlations.

molecules is slow on the NMR chemical shift time-scale, a separate set of peaks is observed for each of the partners in the free and bound states.[11] If both states are similarly populated, with rates of exchange on the order of $\sim 1-50\ s^{-1}$, exchange correlation peaks will be observed in an EXSY-type experiment[8,27,31] that connects corresponding nuclei from each of the two interconverting states. In this manner the assignments from one spectrum (corresponding to one of the states) can be transferred to the second spectrum (unassigned molecule). Elegant studies using this approach have been described previously, focusing on an interaction between a domain of the c-AMP-dependent protein kinase and a binding partner,[17] diamagnetic and paramagnetic forms of cytochrome c,[16] complexes involving subunits of DNA polymerase III,[20] and an exchanging complex between the chemotaxis proteins CheW and CheA.[2] For these systems exchange rates were serendipitously in the appropriate regime for the appearance of exchange peaks in EXSY spectra. The situation is not so fortuitous for ATCase. In this case one would predict that the very tight binding between r- and c-chains ($\sim$10 nM[32]) would lead to extremely slow off-rates for dissociation of r_2 from the complex. For example, assuming that 5% of the enzyme dissociates in 40 h based on a previous study[33] at 25 °C, and simple bimolecular association kinetics, the off-rate is estimated to be $4 \cdot 10^{-7}\ s^{-1}$, too slow for the observation of exchange peaks in EXSY spectra. Hence, altering solution conditions and/or introducing mutations to the subunits of ATCase is necessary to enhance the exchange process.

It is known that 1.5−2 M urea causes dissociation of c-chain trimers into folded monomers,[34] and as a first step the addition of urea was tested for enhanced dissociation of the intact enzyme. Addition of up to 2 M urea did not lead to exchange cross-peaks in EXSY spectra recorded on samples containing free (r_2) and enzyme-bound (r_6c_6) WT r-chains at 25 or 37 °C. Several mutations have been described in the literature that cause the enzyme to dissociate more readily but again exchange cross-peaks between free and bound r-chains were not observed for these systems. Based on these negative results with previously characterized mutants a more focused protein engineering strategy was taken. It is extremely difficult to predict *a priori* the number and locations of mutations that will be needed to achieve weakened binding and enhanced exchange. Further, construction and characterization of mutants one-by-one is too time-consuming, in particular if labeled samples for NMR are prepared. Instead, it seems prudent to take an approach whereby a set of mutants is generated and characterized by a rapid and

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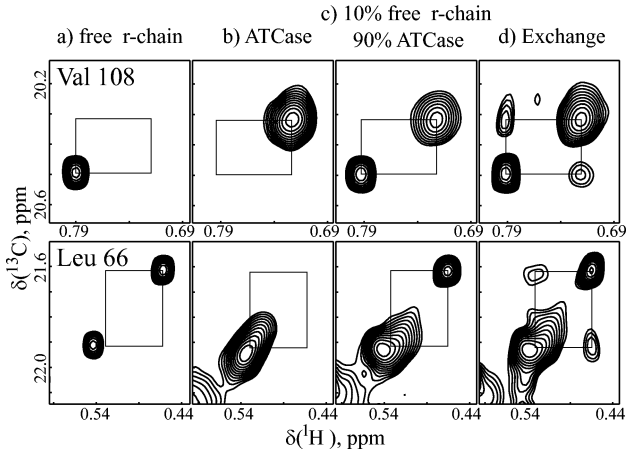

Figure 2. Assignment of r-chain methyl correlations in ATCase via magnetization transfer between free r_2 dimer and intact enzyme. Top row: Val 108 methyl region of Methyl-TROSY spectra of (a) free r_2 dimer, (b) ATCase, (c) 10% free r_2, 90% ATCase. The same spectral region isolated from a magnetization exchange spectrum recorded with a mixing time of 100 ms, 37 °C is shown in (d). Only r-chains are ^{13}C labeled, as described in Materials and Methods. Bottom row: same as top but for Leu 66.

inexpensive method with the aim of finding those changes that lead to complete dissociation of enzyme into r_2 and c_3. If no such mutants are found, a second iteration is performed, the levels of dissociation characterized in this set of proteins, and the process repeated until dissociation is complete. Subsequently, some of the abolished interactions can be reintroduced to tune affinities and rates to levels which are likely to yield exchange cross-peaks in NMR spectra. Two cycles of such a procedure were required for ATCase, as described in detail in Supporting Information.

The protein engineering approach we used identified the E119A r-chain and the E109A/E117A/D129A/S131A/N132A c-chain (referred to in what follows as 5 Ala c-chain) as a good pair for NMR studies. Notably, the HMQC spectrum of the E119A r-chain dimer nearly superimposes on the spectrum of the WT r_2 protein so that all of the assignments are immediately transferable. When excess of unlabeled 5Ala c-chain is added a different set of peaks appears, with much broader line-widths indicative of the formation of intact ATCase, Figure 2a,b. Based on our analysis of spectra of WT protein, the majority of correlations from r_2 whose assignments could be directly transferred to r_6c_6 derived from residues in the N-terminal nucleotide-binding domain, far from the r-chain/c-chain interfaces. This is also true in the mutant enzyme prepared from E119A r-chain and 5Ala c-chain where 3 out of the 10 observed correlations for Ile (30%) and 17 out of 47 for Leu/Val (36%) could be immediately assigned and indeed have the same resonance positions as the corresponding cross-peaks in WT ATCase.

To detect exchange between r-chains in the 'free' (r_2) and 'bound' (r_6c_6) states, samples with r:c chain molar ratios between 1:0.8 and 1:0.9 were prepared so that approximately 80−90% of the r-chains reside in the bound state. This ratio ensures that approximately equal intensity signals are observed for peaks derived from each state, despite the large sensitivity losses that are associated with the 300 kDa r_6c_6 complex, Figure 2c. Exchange cross-peaks were observed in spectra recorded with magnetization exchange delays of either 100 or 200 ms (at both 30, 37 °C), Figure 2d. Exchange correlations were detected for 3 Ile (30%) and 18 Leu/Val (38%) methyl groups. It is, of course, not possible to observe exchange peaks in those cases when chemical shifts in r_2/r_6c_6 are degenerate in one or two dimensions (although in the latter case the assignment is clear), when intensities of peaks from r_6c_6 are very low, or in regions of severe overlap. Combining assignments based on direct overlay of two-dimensional $^{13}C-^{1}H$ correlation spectra and observation of exchange cross-peaks via EXSY, a total of 5 Ile (50% of observed peaks) and 33 Leu/Val signals (70% of observed peaks) were assigned in the spectrum of the mutant enzyme. These are similar numbers to what is obtained for the WT enzyme from analysis of 3D spectra, but includes six additional signals derived from Ile18, Leu30, Leu35, Leu66 and Leu151 (2 peaks) that were not assigned previously. Of these six cross-peaks assigned by exchange, three (Leu35, Leu66 and one of the Leu151 correlations) are shifted to such an extent between spectra of mutant and WT ATCase that transfer to the WT enzyme is impossible. Assignments for the remaining correlations can be transferred, however, and the second peak from Leu151 assigned on the basis of the HMCMCG/CB spectrum. Adding these assignments to those previously obtained from 3D NOESY and "out-and-back" experiments extends the assigned peaks in the spectrum of WT ATCase to 7 Ile (63%), 35 Leu/Val (67%).

Although it is not surprising that mutations, in some cases, introduce significant changes to spectra that make transfer of assignments to WT ATCase difficult, the aspartate transcarbamoylase system is particularly unfavorable in this regard. ATCase exists in an equilibrium between R and T conformations and it is well-known that mutations, especially at the interface between r- and c-chains (as was done here), can alter the equilibrium,[33] leading to shifts in peak positions. One strategy might be to work with the mutant system from the start (i.e., assign it and not WT) if the biochemical properties of the mutant and WT are similar. That 50% of the Ile and 70% of the Leu/Val correlations could be assigned in the mutant using a strategy which includes EXSY speaks to the power of this approach, although establishing conditions for exchange is certainly a limiting feature.

Assignments from Pseudocontact Shifts. In principle PCSs (δ^{PC}) are a powerful way of extending Ile/Leu/Val methyl assignments in high molecular weight proteins. They are relatively easy to measure by simply comparing spectra of the molecule in its diamagnetic and paramagnetic forms, $\delta^{PC} = \delta$(paramagnetic) $- \delta$(diamagnetic), and they contain valuable structural information in the form of both the length and the orientation of the interdipole vector connecting the electron and nucleus in a molecular frame (see eqs 1,2 of Materials and Methods). In protein NMR applications PCS values have been used to define locations of metal ions in proteins with known structures,[35] and conversely, as distance restraints from a paramagnetic center for structure determination.[36−38] Naturally, matches between PCSs predicted from a known protein structure with experimentally determined PCS values for unassigned resonances provide assignments for the peaks in question, as has been demonstrated recently by Otting and co-workers as an approach for the assignment of methyl resonances in a 194 residue protein.[20] In this case, PCSs were calculated based on a paramagnetic susceptibility tensor derived from a significant

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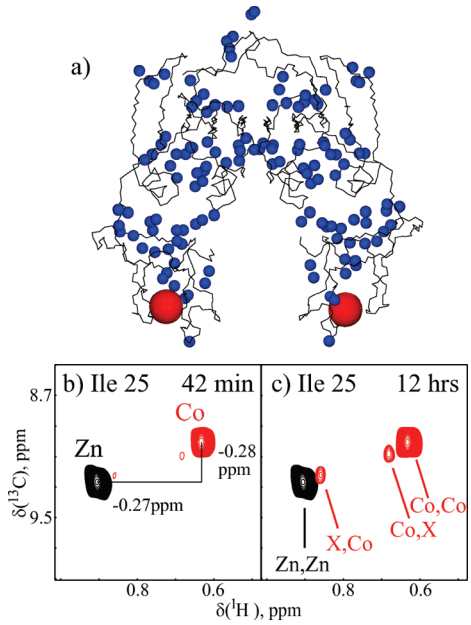

Figure 3. Measurement of PCSs in Co-ATCase as a tool for the assignment of Ile, Leu and Val methyl groups. (a) X-ray derived structure of the r_2 dimer (in the context of the intact enzyme) showing the positions of metal ions (red spheres, one per r-chain). Co^{2+} ions bound to both of the metal sites contribute to the ^1H and ^{13}C chemical shifts of each r-chain methyl group (blue balls). (b) Overlay of selected regions of methyl-TROSY correlation maps of Zn-ATCase (black) and Co-ATCase (red), 37 °C, 800 MHz recorded in 42 min. (c) Same as (b) but with spectral acquisition times increased to 12 h so that low intensity peaks can be observed. These peaks derive from ATCase bound with only a single Co^{2+} ion (referred to in the text as X_1Co_1−ATCase). The peak labeled "X,Co" derives from ATCase where the metal site proximal to Ile25 (of the r-chain) is occupied by a diamagnetic ion, while the metal-binding site farther from Ile25 contains Co^{2+}. "Co,X" denotes the correlation that results when the diamagnetic and Co^{2+} ions in "XCo" are swapped.

amount of high quality ^{15}N data. The utility of this methodology for a much larger system such as the 300 kDa ATCase where *a priori* information about the susceptibility tensor is not available, remains to be established, however.

An advantage of ATCase is that the structural Zn^{2+} ions in the C-terminal domains of the regulatory chains, Figure 3a, can be substituted with Co^{2+} by simply including the latter ion in a minimal expression medium in the absence of other transition metal salts.[23] The UV−vis spectrum of the resulting protein is identical to that of Co-substituted ATCase (Co-ATCase) prepared by stripping Zn^{2+} ions and adding extraneous Co^{2+} (ref 39). Co-ATCase shows the same kinetic properties as "regular" Zn-ATCase,[39] implying that the structure of the protein is not altered by metal substitution. Of course, PCSs can only be calculated if the positions of the corresponding peaks in the diamagnetic and paramagnetic forms of the protein are known. Otting and co-workers used EXSY of a metal-binding protein with a 1:1 mixture of diamagnetic and paramagnetic lanthanide ions to transfer the known assignments of the diamagnetic protein to the paramagnetic species.[20] This approach requires that metal exchange is in the correct regime (~0.5 to several tens/sec). As an alternative, they also developed an algorithm that solves the combinatorial problem of pairing and assigning cross-peaks from diamagnetic and paramagnetic species so long as the magnetic susceptibility tensor and the structure of the protein are available.[20] In the case of ATCase, metal exchange is not a possibility since the metal site is entirely buried in the enzyme. However, for sites distal from the metal (>12 Å; note that sites within 12 Å are broadened extensively) the Co−^1H and Co−^{13}C vectors are nearly coincident for a given methyl group so that the experimental ^1H and ^{13}C pseudocontact shifts are expected to be nearly the same (in ppm). An overlay of small regions of Methyl-TROSY spectra of Zn- and Co-ATCase is shown in Figure 3b, focusing on Ile25, establishing that the shifts in each of the ^{13}C and ^1H dimensions are essentially equivalent. Moreover, all of the experimentally measured shifts are in the same direction, reflecting the fact that the paramagnet is positioned at one end of the r-chain (Figure 3a) with angles between the metal-nucleus bond vectors and the Z-principal axis of the magnetic susceptibility tensor between 70°-115°. The fact that all of the shifted Ile/Leu/Val peaks in the methyl-TROSY spectrum of Co-ATCase could be connected to the corresponding peaks from the Zn-ATCase enzyme by tracing along the line $\delta(^{13}C)=\delta(^1H)$ resulted in a total of 10 measured PCSs for Ile and 42 for Leu/Val (note that there are fewer observed correlations for the paramagnetic form of the enzyme).

The X-ray structure of ATCase reveals that pairs of r-chains interact, Figure 3a, so that PCSs for many methyl groups will be a composite of contributions from metal ions in both of the chains that comprise the dimer (r_2), as described in Materials and Methods and in more detail in SI. By contrast, metal ions from the remaining 4 r-chains within the enzyme are at distances of over 40 Å from methyl groups of the r_2-dimer in question and are not expected to contribute appreciably.[28,40] That two cobalt ions affect the resonance positions of methyl groups of each r-chain in ATCase is apparent in high sensitivity methyl-TROSY spectra (recorded for 12 h), Figure 3c: a set of lower intensity (ca. 5% peak height) peaks was detected for several residues at chemical shifts intermediate between those corresponding to Zn-ATCase and Co-ATCase. The presence of these correlations must reflect the fact that only ~95% of the metal sites in the r-chain are occupied by Co^{2+}, with a diamagnetic ion incorporated from the growth medium bound to the other 5% (a "vacancy" with no metal is unlikely as it would destabilize the C-terminal domain). This predicts a 0.25%: 4.75%: 4.75%: 90.25% distribution of doubly diamagnetic (i.e., 0 Co^{2+} per r_2, referred to in what follows as X_2-ATCase), singly paramagnetic (i.e., 1 Co^{2+} per r_2, X_1Co_1-ATCase) and doubly paramagnetic (i.e., 2 Co^{2+} per r_2, Co_2-ATCase) molecules, which is what is observed. It is also noted that the PCS due to each of the Co^{2+}-ions is additive, implying a negligible interaction between electron spins of the two paramagnets in the double Co^{2+} species. This then allows the few PCSs that could be quantified from X_1Co_1-ATCase to be used together with those from Co_2-ATCase in the analysis described below.

PCSs of many of the already assigned Ile/Leu/Val methyl groups were readily obtained and some of these were used to calculate the 5 unknown parameters of the susceptibility tensor based on the known X-ray structure (see eq 6.2 of Materials and Methods); in particular, correlations from Ile12, 25, 44, 103, and 134 (Co_2-ATCase) and from Ile25, 103, and 134 (X_1Co_1−ATCase) were used. Once the tensor is known it can be used to predict PCSs of unassigned residues, which are then compared to experimental values to aid in the assignment process. Initially, we focused on assignments for Ile 18, 21,

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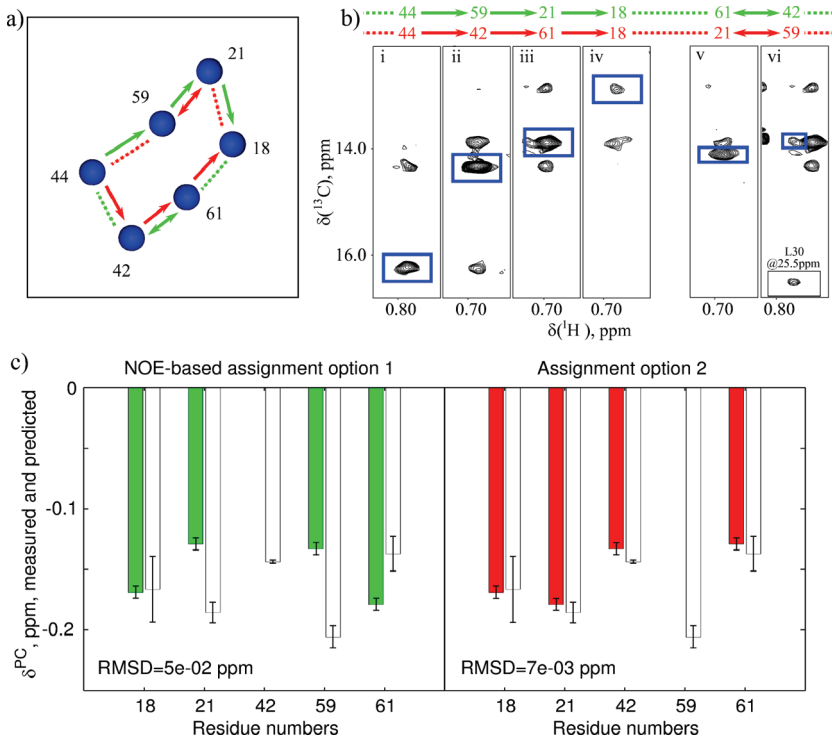

Figure 4. Measurement of PCS values resolves ambiguities in assignment of methyl NOE spectra. (a) Arrangement of Ile residues that form a hydrophobic cluster in the N-terminal region of the r-chain. Blue balls denote $C^{\delta 1}$ methyl groups of Ile residues 44, 59, 21, 18, 42, and 61 that are localized to a β-sheet in the T-state structure (1TUG) of ATCase.[28] The Ile side-chains protrude to one side of a nearly planar β-sheet with all the $\delta 1$ methyls also arranged roughly in one plane. The view shown here is perpendicular to the plane of the β-sheet. The green and red arrows denote the 2 possible sets of NOE connectivities starting with Ile 44 that is already assigned. Solid arrows connect those residues for which NOE correlations are observed in 3D NOESY spectra (see b, below), while dashed lines indicate that the connectivity between residues is broken (i.e., NOEs connecting residues are not observed). (b) Strips from a 3D $^{13}C/^{13}C$-separated NOESY spectrum of ATCase (r-chain labeled), 45 °C, 600 MHz, isolated at $^{1}H_{methyl}$ and $^{13}C_{methyl}$ positions of Ile residues shown in (a). Labels at the top denote the Ile residues that are connected via the green or red pathways (a), leading to two sets of possible methyl group assignments. The diagonal peaks in the NOESY spectrum are indicated by blue boxes. (c) Comparison of measured PCS values (δ^{PC}) for residues based on "green" (left) and "red" (right) assignment pathways vs predicted values from the X-ray structure of ATCase (pdb code 1TUG[28]) (white bars). The level of agreement is quantified by rmsd values that are an order of magnitude lower for the 'red' assignment.

42, 59, and 61 that form a hydrophobic cluster in the r-chain and that were not available from the methods discussed above. Figures 4a,b illustrate the ambiguities that were encountered via the NOE approach, preventing further assignments using this method. The paucity of NOEs leads to a 2-fold degeneracy in the ways in which the NOE connectivities (and hence the Ile methyls) can be assigned, following either the green or red pathways of Figure 4a. Focusing on the green pathway for the moment and starting from Ile 44 (which is assigned, panel i of Figure 4b) the single NOE connection observed for this residue can be assigned to Ile 59 (panels i−ii), based on the X-ray structure, Figure 4a. Continuing with the green pathway an NOE connectivity to Ile 21 is assigned (again using the X-ray structure, panels ii−iii), leading finally to Ile 18 (panels iii−iv), confirming the Ile 18 assignment that was obtained originally from magnetization exchange (see previous section). Ile 18 does not show the expected NOE correlation to residue 61 that would lead to its assignment. However, the two remaining Ile methyl correlations, from Ile 61 and 42, can be assigned by noting that Ile 42 is proximal to Leu 30 and only one of the two candidate methyl groups for these residues shows an NOE to this leucine, panel vi. Similar arguments to those discussed above can be made for the red pathway, so that there are two sets of potential assignments that must be distinguished.

Values of δ^{PC} were calculated for Ile 18, 21, 42, 59, and 61 (white bars in Figures 4c,d) and compared with measured values obtained from the two different possible assignments. The very different rmsd values between calculated and measured PCSs for the pair of "solutions" (close to a factor of 10 difference) allows one to be chosen (corresponding to the red pathway above), bringing the total number of assigned Ile residues to 11 (100% of those observed in spectra).

It would, of course, be useful to confirm these assignments by an independent method. This can be achieved through a comparison of spectra of the WT enzyme and r-chain point mutants of ATCase. The generation of point mutations is relatively inexpensive, both in cost and time, if protein yields are reasonable and if multiple mutant plasmids can be produced, cultures grown and samples purified in parallel. Here we substituted Ile with Leu (one at a time) at positions 18, 21, 42, 59, and 61 and recorded methyl-TROSY correlation maps (Figure 5). Taken together all of the mutational data indicate that the assignments we have obtained are correct (confirming the red pathway). However, on an individual basis it is clear that some of the mutations are not definitive, such as I18L, for which two candidate peaks emerge; this ambiguity is resolved in the I21L spectrum. This emphasizes that (1) even single, fairly conservative mutations in hydrophobic cores can lead to significant changes in chemical shifts so that assignments may not be forthcoming and that (2) it may, nonetheless, be possible to obtain unambiguous assignments, as in the present case, by recording a series of spectra of different mutants within the same core.

As a final step, additional Leu and Val methyl groups were assigned by the PCS approach. A new susceptibility tensor based on data from all Ile residues and from Val 106 ($\gamma 1$ and $\gamma 2$; stereoassigned from NOEs and the known X-ray structure) and Val 127 ($\gamma 1$ only) was calculated and then used to predict PCSs

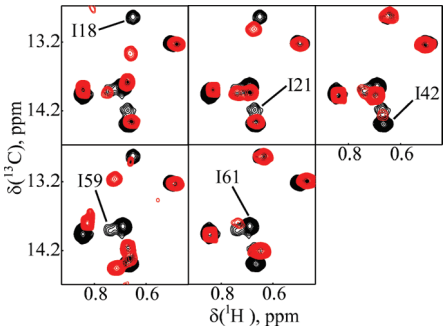

Figure 5. Verification of assignment of Ile residues (Ile 18, 21, 42, 59, and 61) obtained from PCS values using point mutagenesis. Each panel shows an overlay of methyl-TROSY spectra of WT-ATCase (black) and mutant-ATCase (an Ile to Leu r-chain point mutant) (red). The label in each panel indicates the site mutated. The correlation in the WT spectrum corresponding to the mutated site and assigned by analysis of NOE spectra and PCS data is indicated.

for each Ile/Leu/Val methyl group in the r-chain. Figure 6a shows the comparison between predicted and measured δ^{PC} values for the methyls that were used to generate the susceptibility tensor; not surprisingly the agreement is excellent. As a cross-validation, Figure 6b illustrates the correlation between predicted vs experimental δ^{PC} values for those methyl groups already assigned but not used in the tensor calculation and again the agreement is high. Importantly, 8 peaks that could not be assigned using other approaches (see above) could now be connected to the appropriate Leu/Val residues, Figure 6c. This brings the total number of assigned Leu/Val methyl groups to 43 (83% of the observed peaks in spectra) and of all Ile/Leu/Val methyls to 54 of 63 (86%), Figure 7a,b. Interestingly, the pseudocontact shift analysis also provided stereospecific assignments for methyl groups of seven residues. For the majority of Leu and Val residues, however, such assignments were not possible primarily because measured PCS values were very similar for both methyl groups. However, in some cases (Leu32, Leu151) stereospecific assignments could not be obtained due to the poor definition of methyl rotamers in the X-ray structure.

Correlation of Chemical Shift Changes and Ligand Binding. An important use of biomolecular NMR spectroscopy is in the mapping of ligand binding sites to specific regions of biomolecules.[41] This, of course, presupposes that assignments are available. We have previously titrated T-state ATCase with MgATP and determined that binding is best described by a model in which each equivalent r-chain binding site in the r_2-dimer interacts with the second site, with K_D values of 7.9 and 0.25 mM.[6] The near complete Ile/Leu/Val methyl assignments for the r-chain in the context of the 300 kDa enzyme allows the amplitudes of peak displacements during the titration ($\Delta\delta$) to be mapped onto the sequence. Figure 7c shows that the titration data is in excellent agreement with expectations based on the X-ray structure of ATCase and in particular with the position of the nucleotide binding site in the r-chain. None of methyl groups in the C-terminal Zn-binding domain (that is distal from the nucleotide binding site; blue in Figure 7c) titrate beyond 0.1 ppm for ^{13}C, with still smaller differences for ^{1}H. Residues in the N-terminal nucleotide-binding region segregate into two groups, separated by a central β-sheet. Those on the

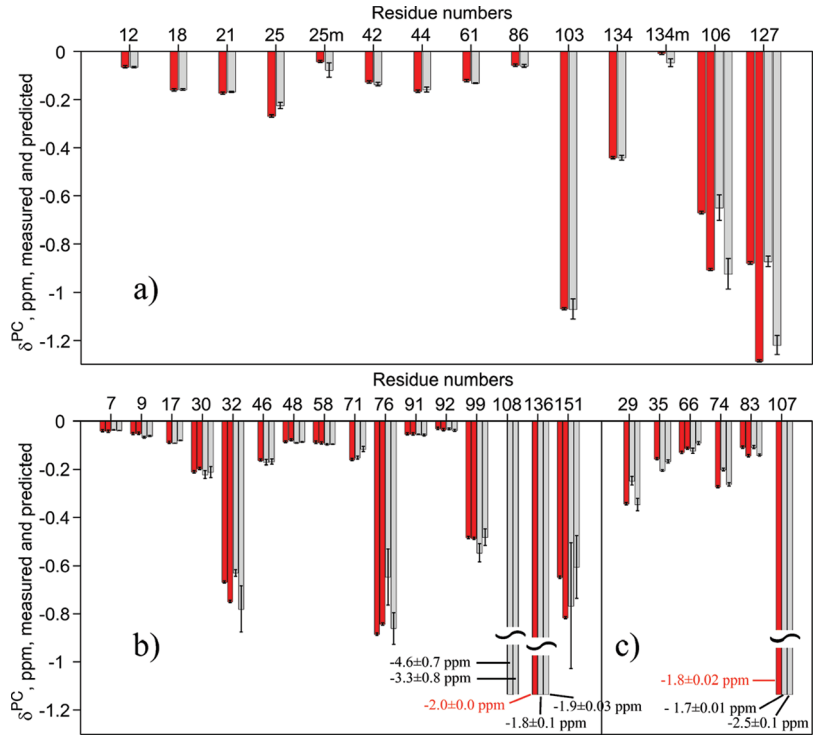

Figure 6. Assignment of Leu and Val methyl correlations by PCS analysis. Red and gray bars denote experimental and predicted PCS values (δ^{PC}), respectively. (a) Comparison of δ^{PC} values for those residues whose experimental PCS values were used to predict the magnetic susceptibility tensor (all Ile, Val106, Val127). Labels "25 m" and "134 m" indicate δ^{PC} values from the minor peaks of Ile25 and Ile134 that originate from molecules with only one of two cobalt ions present for each r_2-dimer in the context of the intact ATCase molecule. (b) Comparison of δ^{PC} values for those residues for which assignments were available, excluding those residues in (a). The residues highlighted here were not used in the calculation of the magnetic susceptibility tensor and hence the good agreement between measured and predicted δ^{PC} values serves as a cross-validation of the derived tensor values. (c) Residue numbers of methyl groups for which assignments were not available prior to PCS analysis (absent or ambiguous NOEs) and for which assignments could be generated on the basis of a comparison between measured and predicted δ^{PC} values.

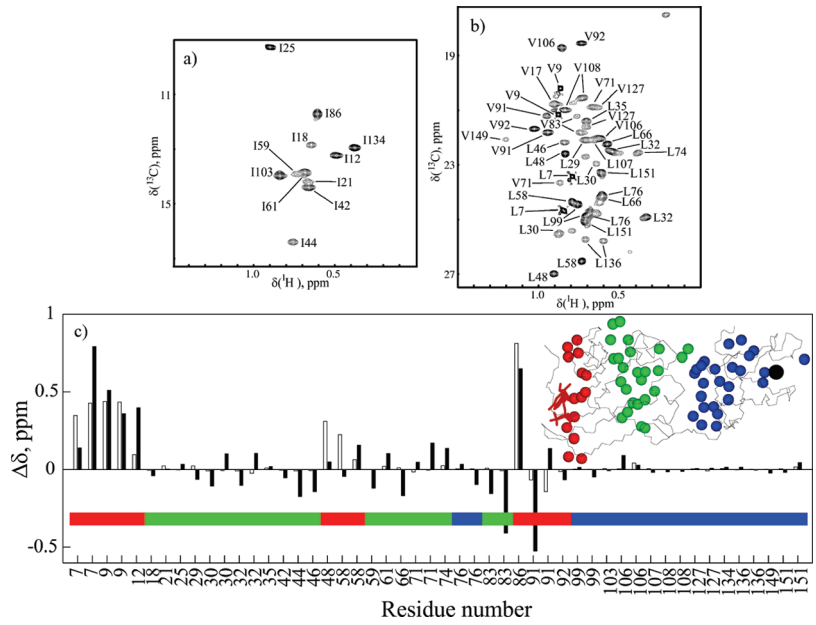

Figure 7. Methyl assignments provide insight into ATP titration data. (a,b) Methyl-TROSY spectrum of WT-ATCase (only r-chain labeled), 37 °C, 800 MHz, with chemical shift assignments for selected residues indicated, focusing on the Ile (a) and Leu/Val (b) regions. (c) Mapping of the ATP binding site. ^{1}H and ^{13}C chemical shift changes ($\Delta\delta$) upon saturation with 20 mM ATP are indicated by white and black bars, respectively. The colored horizontal bar shows the region of the r-chain from which each methyl group derives. The inset (black trace) illustrates the backbone structure of the r-chain in the T-state ATP-bound form of the enzyme,[42] with the colored balls corresponding to Ile, Leu, and Val methyl groups, the black ball denotes the Zn^{2+} ion and bound ATP is indicated by a stick representation (red).

C-terminal side show moderately sized differences in chemical shifts, up to 0.4 ppm in ^{13}C (green), while methyl groups on the N-terminal side of the β-sheet, comprising the nucleotide-binding site, experience the largest shift differences upon saturation with MgATP (red). Notably, all of the residues with sizable ^{1}H shift changes (0.1 ppm or more) reside in this cluster. The δ1 methyl group of Ile86 stacks onto the purine ring of ATP, producing a very large $\Delta\delta$ value (0.8 ppm in ^{1}H).[42] Interestingly, there is a large perturbation in the positions of cross-peaks of Leu7 and Val9 upon addition of MgATP. This implies a substantial structural change, in agreement with results of functional studies. R-chain constructs where the 10 amino-terminal residues are either removed[43] or replaced by Ala[44] have established that the N-terminal tails play an important role in the interactions between the two adjacent nucleotide sites in the r-chain dimer and in the generation of the allosteric response of enzyme kinetics to nucleotide binding. In X-ray structures the first 10 N-terminal residues of the r-chain are defined poorly[42,45] due to weak electron density and any conclusions about how they respond to nucleotide binding based on this data are tentative at best. The large upfield shifts of all methyl groups of Leu7 and Val9, obtained in this NMR study, provide the first firm evidence of changes in the structure of the N-terminal tail upon nucleotide binding.

Concluding Remarks. In the Supporting Information we elaborate in some detail on the advantages and disadvantages of each of the complementary approaches described in the text. To summarize briefly, although many of the methyl correlations in spectra of the r-chains in ATCase could be assigned by the "divide-and-conquer" approach (60%) that we and others have used successfully in the past,[3,10] a combination of the methods described here was found to significantly increase the level of assignment (86%). Such assignments provide a large number of probes for further studies of enzyme function and for quantifying the structural changes that occur upon ligand binding. In this regard, the Ile/Leu/Val assignments of the r-chain reported here establish that ATP binding leads to significant changes in shifts of functionally important N-terminal residues that play a critical role both in stabilizing the r-chains within the r_2 dimer and in the effects of nucleotide binding on distal catalytic regions of this allosteric enzyme. Obtaining site-specific chemical shifts remains a bottleneck of current studies involving supra-molecular systems. It is clear, however, that a combination of several approaches, optimized on a per-system basis, can be particularly effective in generating assignments for large numbers of methyl groups so that these moieties can then serve as powerful reporters of structure, function and dynamics in large macromolecular complexes.

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Supporting Information Available: Discussion of the approach used for isolation of suitable r- and c-chain mutants for EXSY experiments, summarized in Figure S1 and Table S1. Discussion of the contributions to measured PCS values from two symmetrical r-chains. Summary of the strengths and weaknesses of each of the assignment methods presented. A table (Table S2) summarizing the approximate experimental and laboratory times that must be dedicated to each of the approaches described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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