Journal of Biomolecular NMR **30:** 205–210, 2004.

NMR resonance assignment of selectively labeled proteins by the use of paramagnetic ligands

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Received 22 March 2004; Accepted 7 July 2004

Key words: drug design, NMR spectroscopy, protein structures, selective isotopic labeling, TROSY

Abstract

Selective isotopic labeling of larger proteins greatly simplifies protein NMR spectra and reduces signal overlap, but selectively labeled proteins cannot be easily assigned since the sequential assignment method is not applicable. Here we describe a strategy for resonance assignment in selectively labeled proteins. Our approach involves a spin-labeled analog of a ligand of which the three-dimensional structure in complex with the target protein is known. Other methods for introduction of the spin label are possible. The paramagnetic center causes faster relaxation of all neighboring nuclei in a distance-dependent manner. Measurement of this effect allows to deduce distances between isotopically labeled residues and the paramagnetic center which can be used for resonance assignment. The method is demonstrated for the catalytic domain of Abl kinase in complex with the inhibitor, STI571.

Introduction

Protein NMR spectroscopy is a valuable technique to investigate protein structure and dynamics, and to characterize protein interactions with ligands (Zerbe, 2003). Before the advent of TROSY techniques, only smaller proteins of up to 25 kDa were amenable for NMR studies. While TROSY significantly extended this size limit with respect to correlation time (Wuethrich and Wider, 2002; Pervushin, 2003), NMR studies of large uniformly labeled proteins are still hampered by signal overlap caused by the many resonances. A possible remedy to eliminate overlap is selective isotope labeling of the protein, for example isotopic labeling of one particular amino acid type. This is well established for E.coli expression (Goto and Kay, 2000; Lian and Middleton, 2001), and we have recently introduced a protocol for amino-acid-type specific labeling of proteins expressed in Baculovirus-infected insect cells (Strauss et al., 2003). Selective isotope labeling solves the signal overlap problem, but at the cost that the few remaining signals cannot be easily assigned since the sequential assignment method is no longer applicable. Yet resonance assignment is the basis of all detailed NMR investigations. In order to alleviate this dilemma, we here propose a novel strategy for resonance assignment of residues near the active site, which are typically the most important ones. It involves the application of a spin-labeled ligand or another paramagnetic center and the subsequent measurement of distances between the paramagnetic center and selected protein sites. Spin labels have previously been used to aid structure calculation based on known resonance assignment (Battiste and Wagner, 2000). This manuscript describes the reverse approach by aiding resonance assignment based on known structure.

Protein tyrosine kinases have emerged as pharmaceutical targets of great importance (Fabbro et al., 2002a). Inhibition of the tyrosine kinase activity of the Bcr-Abl oncoprotein by the anti-leukemia drug, imatinib mesylate (STI571; Glivec®) has become a paradigm for the concept of therapeutic modulation of kinase activity by selective kinase inhibitors (Capdeville et al., 2002; Fabbro et al., 2002b). Several crystal structures of protein tyrosine kinases complexed to potent inhibitors are known (Hubbard and

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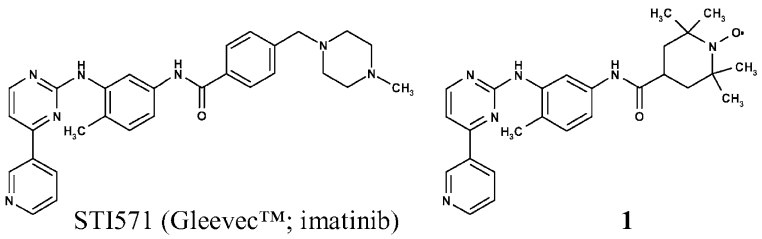

Scheme 1. Chemical structures of STI571 and spin-labeled analog **1.**

Till, 2000), but essentially no NMR investigations have shed light on the solution structure and dynamics of these pharmaceutically relevant proteins. In an effort to do so, we have prepared several samples of the 32 kDa Abl kinase domain complexed with STI571, each isotopically labeled with a different amino acid type (Strauss et al., 2003). In order to interpret structural and dynamic data on an atomic level, resonances have to be assigned to their respective position in the protein sequence. Interesting residues near the ligand binding site have previously been assigned by comparing chemical shift perturbations of structurally similar ligands (Medek et al., 2000; Pellecchia et al., 2002). Differential conformational changes, however, can severely obscure the perturbations caused by direct contact, so that this method is not very reliable. Measurement of protein-ligand NOEs can also lead to resonance assignment, but this requires a deuterated protein in addition to selective isotopic labeling (Pellecchia et al., 2002). A pair of two adjacent amino acid residues can be isotopically labeled by ^{13}CO and ^{15}N, respectively, thus allowing the assignment of the ^{15}N-labeled component (Weigelt et al., 2002). However, this yields only one backbone assignment per sample preparation, and requires that both amino acid types in the pair do not dilute the isotopic label by scrambling. Very recently, Pintacuda et al. (2004) described an elegant strategy for NMR resonance assignment in paramagnetic proteins. Unfortunately, this approach is only applicable to metalloproteins. If a metal binding site has to be deliberately introduced as for example by the ATCUN motif (Donaldson et al., 2001), the assigned resonances do not correspond to the interesting residues near the active site.

Our approach takes advantage of the fact that the structures of pharmaceutically interesting proteins are often known before NMR resonance assignment is undertaken, and it results in assignment of residues near the active site, which are typically the most interesting residues. The approach involves a spin-labeled analog of a ligand for which the three-dimensional structure of the complex is known. For Abl, we chose the STI571 analog **1** (Scheme 1), designed on the basis of the available crystal structures of Abl complexed to STI571 and to an STI571 variant (Schindler et al., 2000; Nagar et al., 2002; Manley et al., 2002), and prepared by propylphosphonic anhydride-mediated coupling of 4-carboxy TEMPO with 4-methyl-*N*-3-[4-(3-pyridinyl)-2-pyrimidinyl]-1,3-benzenediamine. Compound 1 was purified by recrystallization from EtOH to give beige-colored crystals, mp 217–218 °C.* Compound **1** inhibits the kinase activity of Bcr-Abl with a similar potency as that of STI571 (IC_{50} 473±23 nM for **1**, versus 191±6 nM for STI571). Based on the similar potencies and on our knowledge about the structure-activity relationship of STI571 variants, it can be assumed that both binding modes are identical. Paramagnetic moieties such as spin labels or paramagnetic metals drastically increase T1 and T2 relaxation of neighboring spins. This paramagnetic relaxation enhancement depends on the inverse sixth power of the distance between nuclear spin and paramagnetic center and can therefore be used as a sensitive measure of distances up to 2 nm (Dwek, 1973; Kosen, 1989; Jahnke, 2002).

Figure 1a and b show ^{15}N,^{1}H-HSQC spectra of ^{15}N-Val / ^{15}N-Phe-labeled kinase domain of Abl complexed to **1**. The valine residues had been previously identified (Strauss et al., 2003) and are circled. Figure 1a displays a spectrum of the complex with ligand **1** in its radical and paramagnetic form. Figure 1b shows a spectrum of the same sample after reduction of the spin label with ascorbic acid to form the diamagnetic N-hydroxylamine. Conformational exchange processes within the kinase domain are expected to be identical for the paramagnetic and the

*1H NMR (400 MHz, DMSO-d6 + aq. ascorbic acid) d 9.82 (s, 1H), 9.25 (s, 1H), 8.91 (s, 1H), 8.70 (d, 1H), 8.50 (d, 1H), 8.45 (dd, 1H), 7.51 (dd, 1H), 7.42 (d, 1H), 7.26 (dd, 1H), 7.15 (s, 1H), 7.12 (d, 1H), 2.74 (dt, 1H), 2.19 (s, 3H), 1.59 (m, 4H), 1.08 (s, 12H). Anal. Calcd. For C26H31N6O2: C, 67.95; H, 6.80; N, 18.29; O, 6.96. Found: C, 68.17; H, 6.83; N, 18.16; O, 7.36.

diamagnetic form, so that weaker intensities of selected resonances in the upper spectrum directly reflect spatial proximity to the paramagnetic center. In contrast, dynamic processes are generally different in unliganded and liganded protein, and it is therefore not advisable to compare intensities of the spin-label sample with apo protein. Paramagnetic relaxation enhancement can be determined by comparison of peak intensities in the paramagnetic (Figure 1a) and diamagnetic (Figure 1b) samples. Arrows point to the three valine resonances with highest paramagnetic relaxation enhancements. For comparison, Figure 1c shows a ^{15}N,^{1}H-HSQC spectra of ^{15}N-Val-labeled Abl kinase domain complexed to STI571: The valine resonances of the complex with **1** can be readily mapped to the complex with STI571.

Figure 2a displays a model of the structure of the kinase domain of Abl complexed to **1**, based on the Abl-STI571 crystal structure. Figure 2b plots the calculated distances of the 18 valine amide protons of Abl to the paramagnetic center of **1**. Figure 2c plots the sixth power of this distance, normalized to the most remote valine residue (Val 338). This is done since paramagnetic relaxation enhancements are proportional to the inverse sixth power of distance. Figure 2d shows the observed paramagnetic relaxation enhancements on a scale from 0 (signal completely quenched) to 1 (signal intensity unchanged). The paramagnetic relaxation enhancement was defined as I(ox)/I(red), where I(ox) is signal intensity with paramagnetic 1 and I(red) is signal intensity with diamagnetic (reduced) **1**. Small amounts (about 10%) of diamagnetic 1 impurities were present in the paramagnetic sample, and this was appropriately corrected for. Ideally, Figure 2c and 2d would perfectly correlate so that the highest paramagnetic relaxation enhancement corresponds to the smallest distance, and all valine resonances could be assigned by this method.

Figures 2b–d show indeed a good correlation: There are three valine amide protons (Val299, Val289, Val379) within 1 nm of the paramagnetic center, and there are three resonances which are quenched by more than 85% so that $I_{ox}/I_{red} < 0.15$. These three resonances must correspond to Val299, Val289, and Val379, respectively. Similarly, there are six valine amide protons more than 2 nm away from the paramagnetic center, and six resonances which are less than 10% affected by the spin label. Again, these resonances must come from the corresponding amide protons. By these very simple ^{15}N-HSQC experiments, which require no ^{13}C or ^{2}H labeling, we were

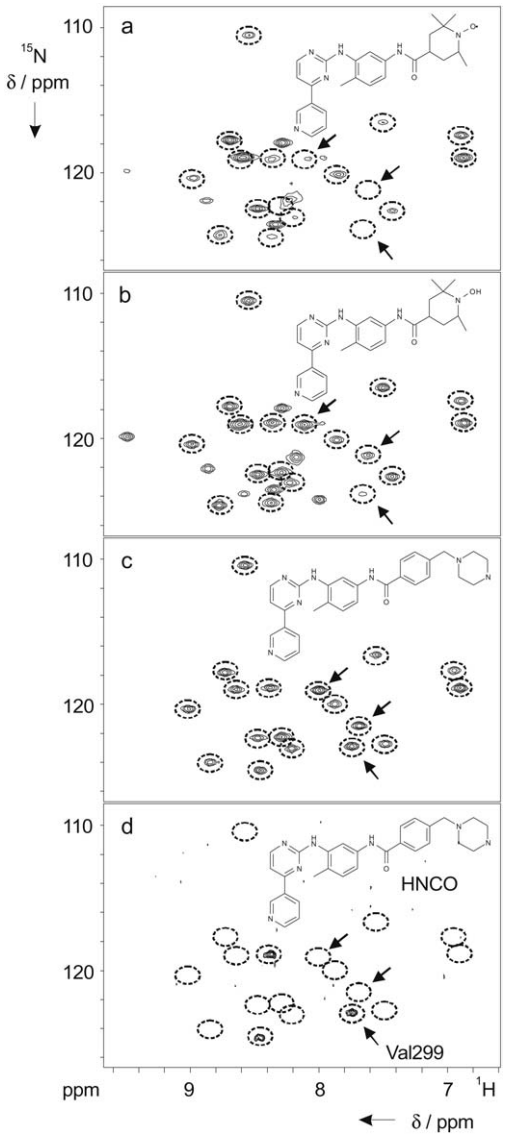

Figure 1. (a) ^{15}N-^{1}H HSQC spectrum of ^{15}N-Phe/^{15}N-Val-labeled Abl kinase domain complexed with **1**, and (b) complexed with **1** after reduction by ascorbic acid. (c) ^{15}N-^{1}H HSQC spectrum of ^{15}N-Val-labeled Abl kinase domain complexed with STI571. (d) 2D HNCO spectrum of ^{13}CO-Leu, ^{15}N-Val-labeled Abl kinase domain. Sample conditions are 200 μM complex, 20 mM Bis-Tris, 100 mM NaCl, 2 mM EDTA, pH 6.5. For a paramagnetic spin label, it is mandatory that no reducing agents such as DTT are present. Reduced signal intensity in the upper panel indicates distances < 2 nm from the paramagnetic center. All valine resonances are circled, and the three resonances experiencing greatest paramagnetic relaxation enhancement, which are thus assigned to Val289, Val299 and Val 379, are marked by arrows. Val299, independently assigned by the 2D HNCO experiment using the ^{13}CO-Leu, ^{15}N-Val labeled sample, is annotated in (d).

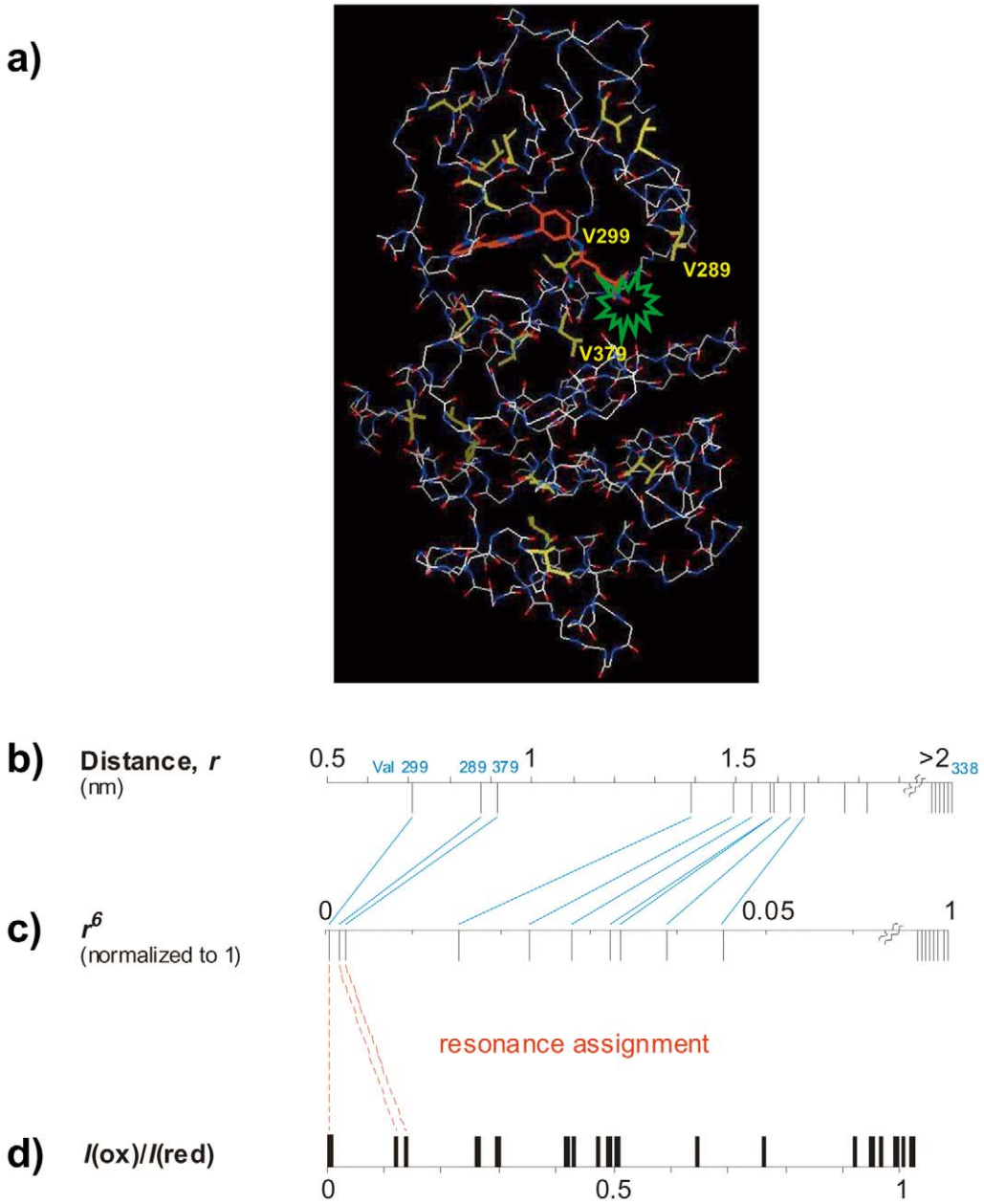

Figure 2. (a) Model of the structure of the kinase domain of Abl complexed with **1**, based upon the Abl/STI571 x-ray structure (Nagar et al., 2002, Manley et al., 2002) All valines are colored yellow. (b) Distances between the valine amide protons and the paramagnetic center, as derived from the model. (c) The same distances, raised to the sixth power, and normalized to the most distant valine residue. (d) Experimentally observed paramagnetic relaxation enhancements, I(ox)/I(red), for the valine resonances (average of at least five measurements). Correlations between Figures 2c and 2d are used for resonance assignement. Note that distances will be blurred if dynamic processes in the protein or in the ligand occur.

able to assign the three valine residues which are most interesting for structural studies of abl kinase complexes with ligands, since they are near the active site and thus directly affected by different ligands. Abl complexes selectively ^{15}N-labeled with another amino acid type would yield corresponding resonance assignments. If selectively ^{13}C-labeled protein was used instead of selectively ^{15}N-labeled protein, resonances of selected side chains could be assigned.

As with any novel method, our method of assigning selectively labeled proteins by the use of a spin-labeled ligand should be validated independently. Unfortunately, assignment of Val289, Val299 or Val379 by conventional methods is far from being straightforward – if it was, our novel method would not be necessary. The strategy proposed by Weigelt et al. (2002), however, does offer the possibility for independent assignment. In this method, two adjacent amino acids are labeled with ^{13}CO and ^{15}N, respectively, and the ^{15}N-labeled amino acid is then identified in a 2D HNCO experiment. In the case of Val289, Val299 and Val379, such an approach is not possible for Val289 (preceded by Ala288) and Val379 (preceded by Lys388) since we have insufficient knowledge about the scrambling behavior of alanine and lysine in Baculovirus-infected insect cells. But the approach is possible for Val299, which is preceded by Leu298. From our own experiments, we know that the leucine label does not scramble (Strauss et al., 2003). Unfortunately, the Leu-Val pair is not unique in abl, since besides the interesting pair Leu298-Val299, there are also the pairs Leu370-Val371 and Leu376-Val377. Nonetheless, this was considered the best approach for independent assignment of Val299. A sample with ^{13}CO-labeled Leu and ^{15}N-labeled Val was therefore prepared by published methods (Strauss et al., 2003), and a 2D HNCO experiment was recorded and is shown in Figure 1d. As expected, one of the three valines identified by the spin label showed indeed a clear signal in the 2D HNCO spectrum. This peak at (7.73 ppm, 123.0 ppm) can therefore be assigned to Val299, and an independent assignment is thereby achieved. The other two signals experience moderate quenching by the spin label ($I_{ox}/I_{red} = 0.5$–0.64), consistent with distances to the paramagnetic center of 1.49 and 1.58 nm for Val 371 and Val377, respectively. While assignment of three important valine resonances was achieved, the required data for assignment of the phenylalanine resonances is incomplete, since out of the 11 Phe in the catalytic domain of Abl kinase, only 8 are observed at pH 6.5.

The proposed method will not only be useful for resonance assignment of selectively labeled proteins, for which sequential assignment is not possible, but also for uniformly labeled proteins. In this case, ambiguities in sequential assignment by degenerated chemical shifts can be resolved using the distance from the paramagnetic center as an additional constraint. Other ways for introduction of the paramagnetic center are conceivable: The protein itself can be covalently spin labeled e.g., at a cystein residue (Kosen, 1989), preferably if there is only a single reactive cystein near the active site. In the case of metal-binding proteins, the same approach is applicable by comparing signal intensities and pseudocontact shifts in complexes with diamagnetic and paramagnetic metal complexes (Pintacuda et al., 2004). In cases where ligand binding requires a metal ion, such as in kinases where ATP binding requires Mg^{2+}, replacement of the diamagnetic metal with a paramagnetic metal, such as Mn^{2+}, can be beneficially used for the same purpose. The proposed method is far more reliable than simple chemical shift mapping with different ligands: Comparison of ^{15}N,^{1}H spectra of Abl kinase catalytic domain with reduced spin label **1** and STI571 (Figures 1b,c) reveals that Val289, Val299, and Val379 (the valines nearest to the paramagnetic center and assigned by the spin label) experience significant chemical shift changes, but that also 7 other valine resonances experience similar chemical shift changes. Without the spin label experiments, the three nearest valines would not have been identified.

In summary, we have developed a method to assign resonances of some of the most important residues near the active site of a protein, based on knowledge of the three-dimensional structure of the complex. The correlation between distance and paramagnetic relaxation enhancement is good – but why is it not even better? Protein and ligand dynamics are likely to constitute the main factor that limit the resolution of the proposed method since they average paramagnetic relaxation enhancements. Kinases are inherently flexible, and flexibility is an essential feature for their control (Engh and Bossemeyer, 2002). However, this limitation could become an opportunity: Established NMR relaxation studies provide a powerful tool to study protein dynamics on the pico- to nanosecond time scale, but it is more difficult to characterize dynamics on the micro- to millisecond time scale, which appears to be the most relevant time scale for protein function (Palmer, 2001). Paramagnetic relaxation enhancement, in contrast, is highly sensitive for dy-

namics on this slower time scale, and it is expected that investigations using paramagnetic ligands will be extremely helpful to understand the function and control of protein kinases.

Acknowledgements

We thank Dr S. Cowan-Jacob for the coordinates of the Abl/STI571 complex and Dr J. Mestan for comparing the activities of STI571 and **1** against Bcr-Abl.

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