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Structure and dynamics of an imidazoline nitroxide side chain with strongly hindered internal motion in proteins

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ABSTRACT

A disulfide-linked imidazoline nitroxide side chain (V1) has a similar and highly constrained internal motion at diverse topological sites in a protein, unlike that for the disulfide-linked pyrroline nitroxide side chain (R1) widely used in site directed spin labeling EPR. Crystal structures of V1 at two positions in a helix of T4 Lysozyme and quantum mechanical calculations suggest the source of the constraints as intra-side chain interactions of the disulfide sulfur atoms with both the protein backbone and the 3-nitrogen in the imidazoline ring. These interactions apparently limit the conformation of the side chain to one of only three possible rotamers, two of which are observed in the crystal structure. An inter-spin distance measurement in frozen solution using double electron–electron resonance (DEER) gives a value essentially identical to that determined from the crystal structure of the protein containing two copies of V1, indicating that lattice forces do not dictate the rotamers observed. Collectively, the results suggest the possibility of predetermining a unique rotamer of V1 in helical structures. In general, the reduced rotameric space of V1 compared to R1 should simplify interpretation of inter-spin distance information in terms of protein structure, while the highly constrained internal motion is expected to extend the dynamic range for characterizing large amplitude nanosecond backbone fluctuations.

1. Introduction

Site-directed spin labeling (SDSL) is a powerful technique to study protein conformational equilibria [1–5] and dynamics [6–9] under physiological conditions. In the most common implementation, a nitroxide side chain is introduced at a selected site in a protein by mutating the residue of interest to a cysteine followed by modification with a sulfhydryl specific nitroxide reagent [10,11]. Recently, it has been shown that a nitroxide may be introduced without the use of cysteine *via* a genetically encoded unnatural amino acid [12,13]. In any case, the EPR spectrum of a nitroxide attached to a protein reflects overall Brownian rotational diffusion of the protein and internal motion of the nitroxide side chain, the latter of which can be augmented by backbone dynamics on the ns time scale [3,6]. The internal motion may be modulated by tertiary interactions of the side chain within the protein, providing information on local structure [14–18].

To isolate the contributions from internal and ns backbone motions, it is necessary to remove the influence of overall Brownian diffusion. For proteins larger than 50 kDa, the contribution from Brownian diffusion is negligible at X-band microwave frequencies; for small globular proteins, the contribution can be effectively eliminated by recording the spectra in a viscous solution [2,14], by attaching the protein to a solid support [2], or by using high-frequency EPR [19–21].

The internal motion depends on the structure of the nitroxide side chain. The nitroxide side chain designated R1 (Fig. 1A) has been widely used in EPR studies and numerous crystal structures have been determined for the side chain in T4 Lysozyme [13–16], the β protein GB1 [22] and in the membrane proteins BtuB [23] and the leucine transporter LeuT [24]. The structures for R1 and quantum mechanical calculations reveal a consistent set of preferred rotamers which are apparently stabilized by interaction of the disulfide with backbone atoms [25]. To a first approximation, this interaction restricts the internal motion to torsional oscillations of the X_4 and X_5 dihedrals (see Fig. 1A for definition of the dihedral angles) [6,11]. Because of the hindered internal motion, the anisotropies in the magnetic Zeeman and hyperfine interaction are not completely averaged, and R1 retains significant order

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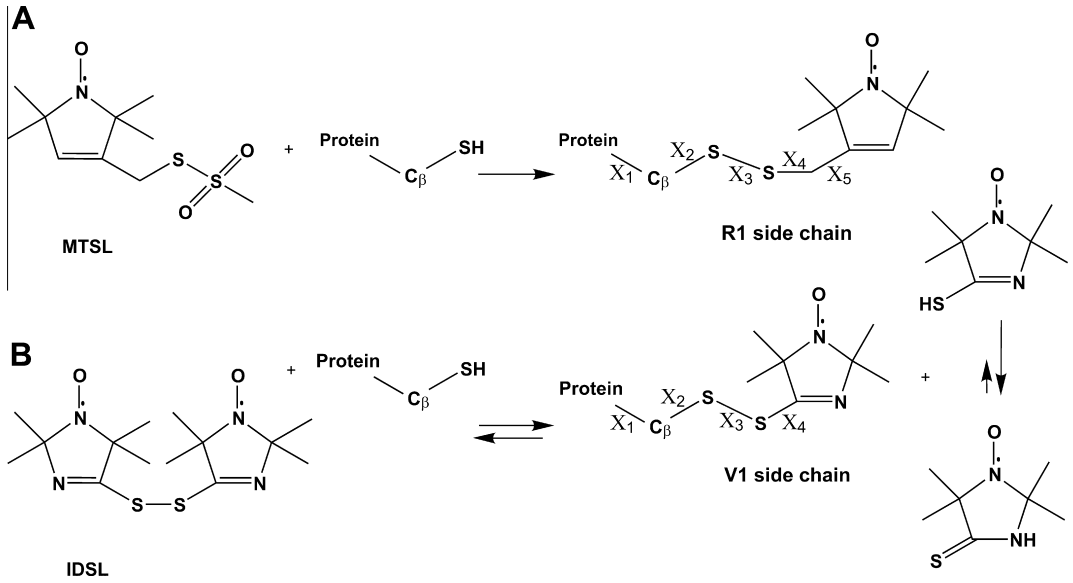

Fig. 1. Introduction of nitroxide side chains *via* cysteine substitution mutagenesis. In each case, a cysteine residue is introduced at the site of interest, followed by reaction with the desired sulfhydryl specific reagent. (A) Reaction with 1-oxyl-2,2,5,5-tetramethypyrroline-3-methyl (MTSL) to generate R1. (B) Reaction with bis(2,2,5,5-tetramethyl-3-imidazoline-1-oxyl-4-il)-disulfide (IDSL) to generate V1. Numbering of dihedral angles (*X*) is shown for each side chain.

reflected in an EPR spectrum characteristic of an anisotropic motion [11]. On one hand, the internal modes of R1 bias the nitroxide motion to a region of maximum sensitivity for detecting small differences in amplitude and rate on the ns time scale, making R1 ideal for monitoring site dependent backbone motion in well-ordered proteins [9]. On the other hand, the internal modes reduce the dynamic range over which the amplitudes of backbone motions can be measured [7]. In addition, the internal motions and multiple rotamers lead to uncertainty in the spatial location of the nitroxide, complicating the interpretation of inter-spin distance measurements in terms of protein structure. To constrain the internal motion, side chains with a 4-substituent on the nitroxide ring have been investigated [7,11,26]. In each case the presence of the substituent increased the order and decreased the effective rate of the internal motion, thus extending the dynamic range for characterizing large amplitude backbone motions. The most constrained nitroxide side chain is TOAC in which the nitroxide ring is fused to the peptide backbone, but so far has only been introduced by peptide synthesis or peptide ligation methods [27–31].

In this report an imidazoline nitroxide side chain, designated V1 (Fig. 1B), is shown to have a highly ordered internal motion and restricted rotameric space relative to R1 and thus should be useful to extend the dynamic range for detection of backbone motions and to provide a more localized nitroxide for interspin distance measurements either by linewidth methods [32,33] or pulsed dipolar spectroscopy [34,35]. Crystallographic and computational studies reveal an intra-residue N–S interaction as the structural origin of the high order.

2. Materials and methods

2.1. Preparation, expression and spin labeling of mutant T4L protein

T4 Lysozyme cysteine substitution mutants employed in the study were prepared as previously reported in a cysteine-less pseudo-wild type background having C54T and C97A substitutions [12,14–16]. All mutant proteins were at least 95% pure as judged by SDS–PAGE electrophoresis. Dithiothreitol (DTT) was added to the purified protein in 50 mM MOPS, 25 mM NaCl pH 6.8 to a final concentration of 5 mM and the protein was stored at −20 °C in 20% glycerol.

The spin labeling reagent bis(2,2,5,5-tetramethyl-3-imidazoline-1-oxyl-4-il)-disulfide (IDSL), was prepared as previously described [36]. The reagent 2,2,5,5-tetramethyl-pyrroline-1-oxyl methanethiosulfonate (MTSL) was a generous gift of Prof. Kalman Hideg (University of Pecs, Hungary). Before spin labeling, the protein was loaded onto a 5 ml HiTrap desalting column (GE Healthcare) equilibrated with 50 mM MOPS, 25 mM NaCl pH 6.8 and eluted with the same to remove DTT. The eluted protein was immediately reacted with 5–10-fold excess of spin labeling reagent overnight at room temperature. The excess spin label was removed on a HiTrap desalting column. The spin labeled protein was concentrated to ~200 μM using an Amicon Ultra centrifugal filter Device (10 kDa cutoff) (Millipore, Bedford, MA) and stored at −20 °C.

2.2. EPR spectroscopy and spectral simulations

EPR measurements were performed on a Bruker Elexsys E580 fitted with a high sensitivity resonator. Protein solutions of 5 μl were contained in quartz capillaries (0.60 mm ID × 0.84 mm OD) and spectra were recorded using 20 mW incident microwave power at 298 K. Unless otherwise noted, spectra were recorded in 30% w/v sucrose to reduce the rotary diffusion rate of the protein [2].

For lineshape fitting studies, mutant T4L 72 was immobilized on cyanogen bromide activated Sepharose 4B beads as previously described [3]. The EPR spectrum for T4L 72V1 immobilized on Sepharose was fit to the MOMD model of Freed and coworkers [37]. The principle components for the **A** (hyperfine interaction) and **g** tensors used were determined from a least-squares fit to the spectrum of T4L 72V1 in frozen solution at 193 K. The values are: Azz = 34.9 Gauss, Axx = 5.9 Gauss, Ayy = 5.18 Gauss; gzz ≡ 2.0023, gxx = 2.0089, gyy = 2.0062.

Four pulse Double Electron Electron Resonance (DEER) was conducted on a Bruker Elexys 580 spectrometer operated at Q-band and fitted with an Bruker EN5107D2 resonator. Samples of 150–200 μM spin labeled T4L containing 20% glycerol (v/v) were loaded into borosilicate capillaries (1.4 mm ID × 1.7 mm OD), and then flash-frozen in liquid nitrogen; data were collected at