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Technological advances in site-directed spin labeling of proteins

Wayne L Hubbell, Carlos J López, Christian Altenbach and Zhongyu Yang

Molecular flexibility over a wide time range is of central importance to the function of many proteins, both soluble and membrane. Revealing the modes of flexibility, their amplitudes, and time scales under physiological conditions is the challenge for spectroscopic methods, one of which is site-directed spin labeling EPR (SDSL-EPR). Here we provide an overview of some recent technological advances in SDSL-EPR related to investigation of structure, structural heterogeneity, and dynamics of proteins. These include new classes of spin labels, advances in measurement of long range distances and distance distributions, methods for identifying backbone and conformational fluctuations, and new strategies for determining the kinetics of protein motion.

Addresses
Jules Stein Eye Institute and Department of Chemistry and Biochemistry, University of California, Los Angeles, CA 90095, United States

Corresponding author: Hubbell, Wayne L (HubbellW@jsei.ucla.edu)

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Introduction

A golden age of protein physical science was ushered in by the advent of crystallographic methods which revealed how weak non-covalent interactions cooperated to stabilize the elaborate secondary, tertiary, and quaternary structures of proteins. But the information proved to be incomplete for elucidating molecular mechanisms underlying function which often rely on conformational flexibility, which is not directly revealed in the confines of the crystalline lattice. A second golden age is in progress and began when spectroscopic methods were developed to permit atomic scale resolution of protein structure and dynamics in solution. The most dramatic advances have come from solution NMR methods which provide residue specific information on protein dynamics. The technology continues to develop, but currently has relatively poor sensitivity, practical limitations for a general extension to membrane proteins in their native lipid environments, a limitation to short range inter-nuclear distance measurements, and is challenged for measuring dynamics on the μs time scale that may be important for function. These limitations provide a unique opportunity for site-directed spin labeling EPR (SDSL-EPR) to complement NMR, because they are the particular strengths of the EPR method, which is unconstrained by the size or complexity of the system under study.

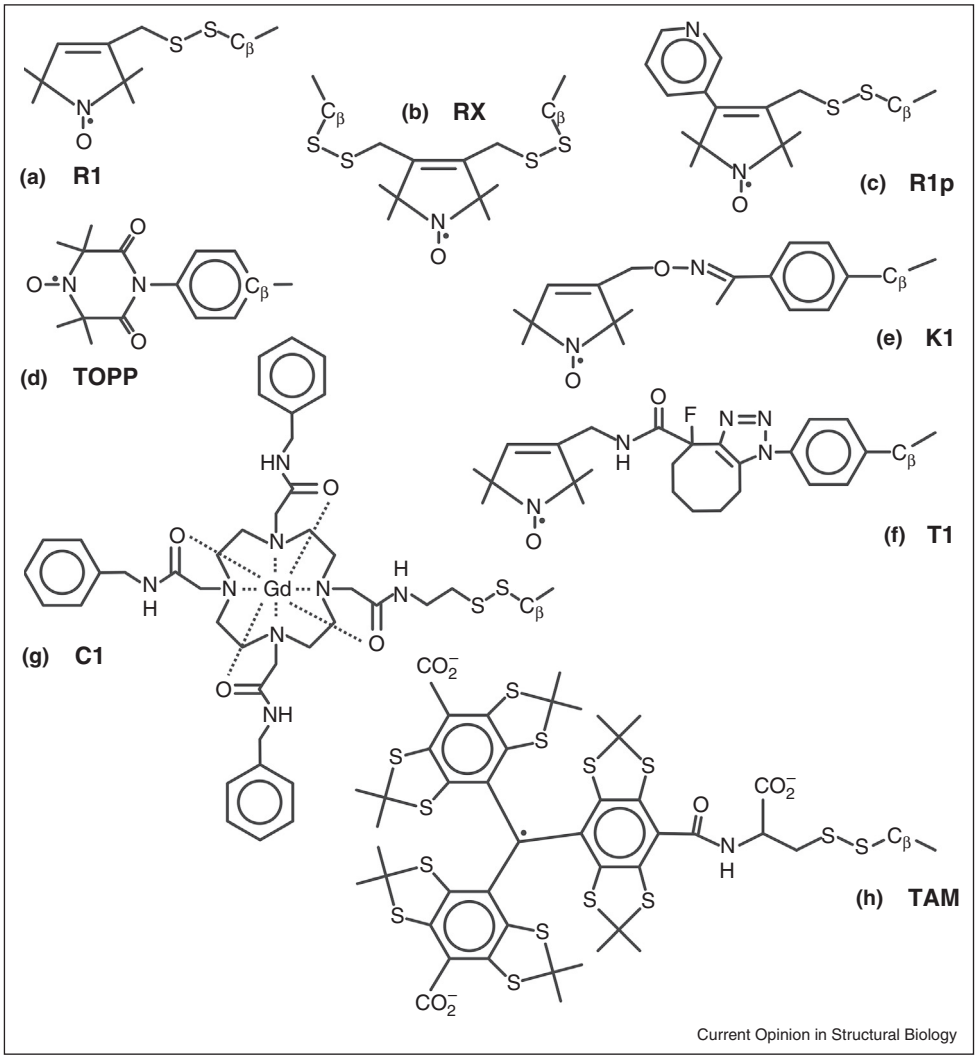
Since the last review on SDSL in this journal [1], substantial progress has been made in site-directed mutagenesis, the chemistry and physics of the spin labels, methodology and instrumentation, and in computational methods. Excellent recent reviews documenting applications of SDSL to proteins have appeared [2–5], and the present contribution will be restricted to technological advances in the above areas for exploring the structure and dynamics of proteins with an emphasis on the last two years.

Nitroxide side chains

The common implementation of SDSL employs the disulfide-linked nitroxide side chain, designated R1, introduced via cysteine substitution mutagenesis (Figure 1a). In general, interpretation of EPR data from R1 in terms of protein structure and dynamics requires knowledge of the rotamers and internal dynamics of the side chain itself, and this information is most complete for the simple case of R1 at non-interacting solvent exposed sites in helices and loops. For R1 at such sites, crystallographic data from T4 lysozyme (summarized in [6]) and other proteins [7–8], including a membrane protein [9], and density functional calculations [10] document a ubiquitous intra-residue interaction of the disulfide with backbone atoms that defines a limited rotamer library [6] and constrains the nitroxide to an anisotropic motion, as suggested by an early model [11]. As discussed below, such sites are the key to mapping backbone dynamics in helices and loops. The conformation and dynamics of R1 in β structures are less well understood, but the first members of a rotamer library have been identified in recent crystal structures of R1 in β sheets [12,13•,14•].

One of the most popular applications of SDSL-EPR is inter-spin distance determination using pulsed dipolar (PD) spectroscopy in doubly labeled systems. To interpret inter-nitroxide distances in terms of protein structure, the spatial distribution of the nitroxide at an arbitrary site must be considered, and this is modulated by interactions with the environment. To account for the spatial distribution of R1 in experimental distance distributions, different strategies have been considered, including a rotamer library biased by interaction with the protein [15••] and MD simulations and Monte Carlo conformational searches [16••]. Comparison of these methods

Figure 1



Structures of paramagnetic protein labels represented as side chains. **(a)** The R1 side chain. **(b)** The cross-linking side chain RX. The cross-link can be formed between *i* and $i \pm 3$ or $i \pm 4$ cysteine residues within a regular helix, between *i* and $i \pm 2$ residues in a β strand, or between any elements with properly spaced cysteine residues. **(c)** The R1p side chain. **(d)** The TOPP residue introduced by peptide synthesis. Although there may be rotation about individual bonds, the nitroxide is fixed in the same spatial location, because all bonds connecting the nitroxide with the protein are collinear. **(e)** The ketoxime-linked side chain K1 generated by reaction of a p-acetyl-phenylalanine unnatural amino acid with a hydroxylamine nitroxide reagent. **(f)** The triazole-linked side chain T1 generated by the reaction of a p-azido-phenylalanine unnatural amino acid with a strained cyclooctyne nitroxide reagent using Cu-free Click chemistry. **(g)** A disulfide-linked Gd^{3+} chelate side chain. **(h)** A disulfide-linked TAM spin label.

came to the conclusion that the rotamer library is the method of choice, and for the sites studied, the agreement with experiment was best when crystal structure rotamers were selected from the complete rotamer library [16••]. Simpler approaches sample a large conformational space, either with no assumptions about rotamer probabilities [17] or with the option to weight the population according to the crystallographically favored rotamers [18].

Another means for dealing with the rotamer distribution of R1 is to eliminate it. With this in mind, new side chains with constrained geometry are being explored. For the cross-linked RX side chain (Figure 1b) [19–22] inter-spin distance distributions were narrow compared to R1 [20•], and for R1p (Figure 1c) proton relaxation enhancement suggested a single rotamer populated in solution [23]. Crystal structures of RX (PDB entry 3L2X) and the 4-phenyl analog of R1p (PDB entry 1ZUR) in T4 lysozyme show resolved electron densities for the entire side chain, unlike those for R1 which are resolved only to the disulfide due to internal disorder. The side chain TOPP (Figure 1d) [24] provides a fixed position of the spin in a protein, but so far is introduced by total synthesis, restricting its practical use to peptides or small proteins.