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Incorporation of spin-labelled amino acids into proteins†

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The elucidation of structure and function of proteins and membrane proteins by EPR spectroscopy has become increasingly important in recent years as technological advances have been made in the design of spectrometers and in the chemistry of the nitroxide group. These new developments have increased the demand for tailor-made amino acids carrying a spin label on the one hand and for reliable methods for their incorporation into proteins on the other. Here we describe methods for site-specific spin labelling of proteins. It is shown that a combination of recombinant synthesis of proteins with chemically produced peptides (expressed protein ligation) allows the preparation of site-specifically spin-labelled proteins.

KEYWORDS: EPR spectroscopy; site-directed spin labelling; chemical synthesis of proteins; protein ligation

INTRODUCTION

Unnatural amino acids are more and more employed in biophysics, biochemistry and medicinal chemistry[1] as they allow the design of novel proteins,[2] the introduction of biophysical reporter groups[3,4] or the modification of active centres.[5] An important group of such unnatural amino acids carries a reporter suitable for use in magnetic resonance techniques. Site- or fragment-specific isotope labelling, for example, enables the structural and functional analysis of proteins by solution or solid-state NMR spectroscopy.[6,7] The introduction of a paramagnetic label in the side chain allows determination of its static and dynamic properties by EPR spectroscopy.[8,9] There are three main approaches to modify peptides or proteins with spin labels. A very well established method is that of site-directed spin labelling (SDSL), which utilizes the thiol group of a cysteine residue for the incorporation of paramagnetic methanethiosulfonates (Mts) yielding the spin-label side chain R1.[10] This approach requires that the target protein possesses only cysteine residues at the desired labelling sites, which could either be naturally occurring residues or have to be introduced by recombinant methodologies. At the same time, all additional cysteines must be eliminated from the protein. Recently, a method was developed that allowed site-specific incorporation of unnatural amino acids by employing an amber suppressor tRNA chemically aminoacylated with the desired amino acid.[3,11,12] Although this elegant method might prove generally applicable in future (using unique transfer RNA (tRNA)/aminoacyl-tRNA synthetase pairs[13]), currently only very few laboratories are equipped to apply the scheme successfully. The third approach goes back to the development of solid-phase peptide synthesis (SPPS), or Merrifield synthesis.[14] Modern improvements in peptide chemistry[15] allow the synthesis of proteins containing as many as 166 amino acids.[16,17] Furthermore, combining SPPS with recombinant techniques provides the tool to introduce unnatural amino acids at sites of choice in large proteins and even membrane proteins.[18]

In the present paper, the chemistry of the incorporation of spin-labelled amino acids into peptides and proteins is critically evaluated. In a second part, it is demonstrated that the spin label can be introduced by using a combination of recombinant techniques and chemical peptide synthesis.

SPIN-LABELLED AMINO ACIDS

There are principally two different strategies for the introduction of spin labels that can be used in peptide and protein chemistry. The first group includes functionalized spin-labelled building blocks that can be introduced after the synthesis of a peptide has been completed or directly into the native protein utilizing functional groups of naturally occurring amino acids like the sulfhydryl group of cysteines. The second group relies on special amino

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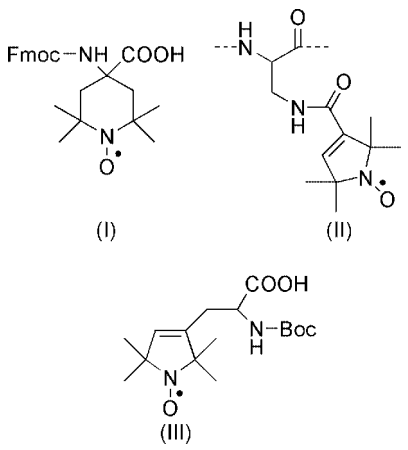
acids like 4-amino-1-oxyl-2,2,6,6-tetramethyl-piperidine-4-carboxylic acid (TOAC) I or pyrroline nitroxide-containing amino acids II and III, respectively (Scheme 1), which are directly incorporated into the peptide during chemical synthesis.

In spite of the large number of very successful applications, several serious shortcomings of the method of cysteine exchange mutagenesis and SH-specific spin labelling have to be considered, which may include distortions of the protein conformation or unspecific labelling in the presence of more than one SH group. Furthermore, the extended length of spin labels like the commonly used 1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl-sulfonate (MTSSL) as compared to naturally occurring amino acid side chains and the conformational flexibility (Fig. 1) does not unequivocally allow relating of the data obtained directly to the properties of the side chain in its native state. For example, the label could fold in an environment different from that of the original side chain, thereby experiencing conditions that can falsely be attributed to the native state.

However, these disadvantages can be overcome by the recently developed techniques for the incorporation of unnatural amino acids into proteins and by taking advantage of tailor-made amino acids. New, *N*-Boc–protected paramagnetic amino acids with various side chains (e. g. differences in polarity, orientation and conformationally constrained structures) obtained by O'Donnell synthesis have been reported very recently.[20–22] The synthesis of a paramagnetic amino acid containing the simplest and smallest 3-methylene-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-1-yloxyl radical moiety in the side chain (III in Scheme 1) is reported in the next section. Spin-label amino acids with reduced residual side chain motion provide defined orientations with respect to the backbone. This will simplify discrimination between protein backbone dynamics and its modulation upon conformational changes and residual side chain dynamics. Furthermore, the reduced residual motion and the defined orientation of the nitroxide side chain with respect to the backbone will provide reliable data of inter-spin distances and relative orientations of the nitroxide side chains (molecular axes orientations). Using sets of doubly spin-labelled engineered proteins, the determination of



Scheme 1. Chemical structures of nitroxide-containing amino acids.

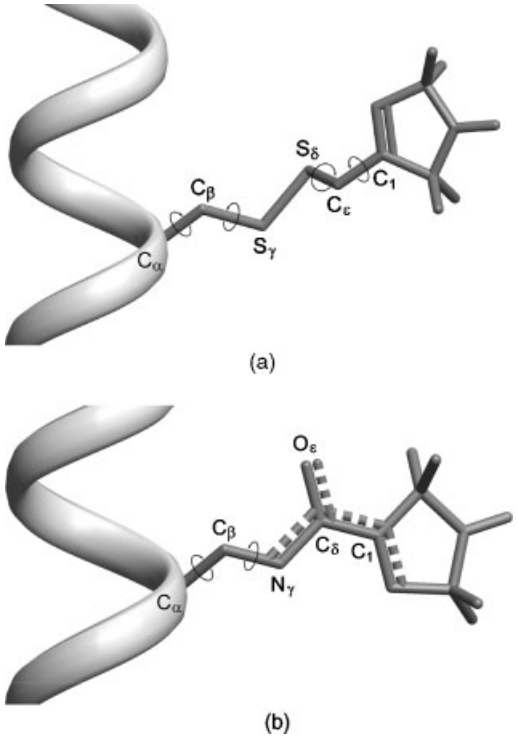
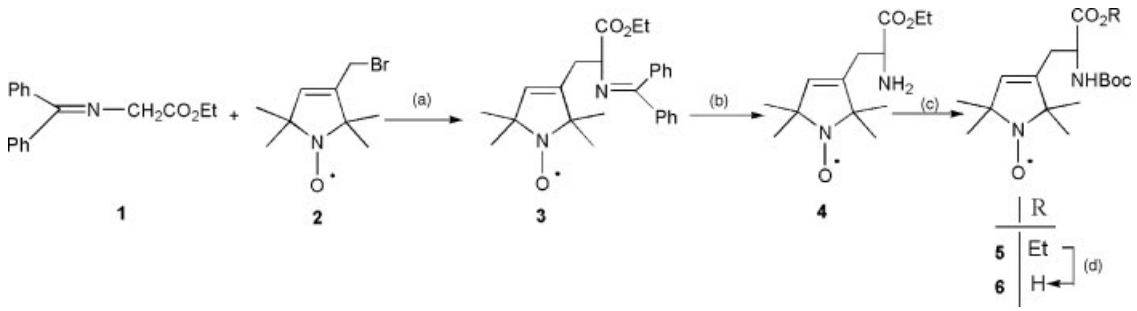


Figure 1. Conformational space of two spin-labelled amino acids (MTSSL (a) and II (b)). The double bond character of the peptide bond in conjugation with the double bond in the five-membered ring is indicated by dots.

structural details and conformational changes with atomic resolution will be achievable.

Figure 1(a) presents all flexible bonds within the R1 side chains bound to a cysteine in comparison with those of the unnatural amino acid II. For the R1 side chain, molecular dynamics (MD) simulations have been performed (Beier and Steinhoff, unpublished results). For rotation around the Cα–Cβ bond, MD simulations reveal a potential according to the familiar three-peaked behaviour showing the *gauche*/*anti* states of pure sp^3 hybridizations. Both –C–S– potentials show almost ideally staggered conformations. The torsion angle distributions of the –S–S– bond reveal two characteristic states around 90 and 270°. In these cases, the lone-pair electrons in the $3p_\pi$ atomic orbitals of both sulfur atoms are orthogonal to each other and their repulsion is minimized. Rotation around the last bond, –Cε–C1–, shows the highest flexibility. Particularly, apart from the *trans* conformation between the –Sδ–Cε– and the double bond, all orientations are possible. If the entire R1 side chain is located in a more restrictive environment, the rotation about –Cε–C1– is dominant. Since this bond is nearly parallel to the nitroxide bond, it leads to an anisotropic reorientational motion of the spin label approximately around the molecular *x*-axis.

A low flexibility is expected for the side chain of the unnatural amino acids II and III (Fig. 1(b)). The partial double bond character of the N–C bond keeps the four atoms HNCO in a plane and also restricts the reorientation about the single bond connecting the ring to this plane. For side chain III, the



Scheme 2. The reaction scheme for the synthesis of the paramagnetic Boc-protected amino acids **5** and **6**. (a) 10% aq. NaOH, CH_2Cl_2, Bu_4NHSO_4; (b) 5% aq. H_2SO_4, EtOH; (c) Boc_2O, THF; (d) 10% aq. NaOH, EtOH.

sterical restrictions due to the interaction of the ring with the backbone provide a spin-label probe with even less mobility.

CHEMICAL SYNTHESIS OF SPIN-LABELLED AMINO ACIDS

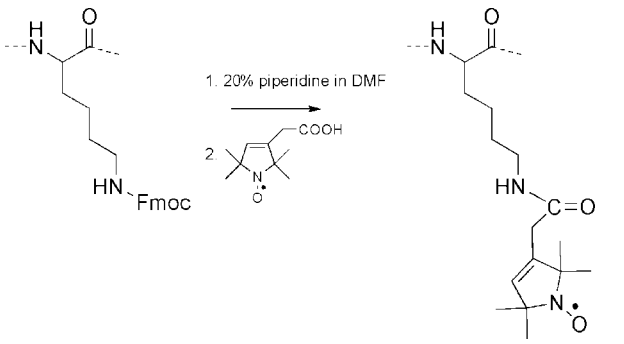
The chemical synthesis of the novel spin-labelled amino acid **5** (Scheme 2) was carried out by alkylating ethyl *N*-diphenylmethylene glycine **1** with paramagnetic allylic bromide[23] **2** under phase-transfer conditions[24] in aqueous $NaOH/CH_2Cl_2$ in the presence of Bu_4NHSO_4. This procedure afforded the monoalkylated product **3**, which could be readily hydrolysed under acidic conditions to the corresponding amine[25] **4**, without affecting the *N*-oxyl radical moiety. The treatment of DL-amino acid esters with *t*-butoxycarbonyl anhydride in THF gave the corresponding protected *N*-Boc amino acid ethyl esters **5**, which can be hydrolysed[20] to amino acid **6** ready for use in SPPS (Scheme 2).

CHEMICAL SYNTHESIS AND SEMI-SYNTHESIS OF PROTEINS CONTAINING SPIN LABELS

The chemical synthesis and semisynthesis of proteins relies on the ability to produce the constituent peptides, typically by SPPS, either based on the Boc or Fmoc protection strategy. Chemoselective ligation methods, like native chemical ligation (NCL), are then used to link two ore more of the resulting peptides in a head-to-tail fashion or to attach them to recombinantly produced proteins.[26–28] The synthesis and purification of peptides consisting of up to 60 amino acids is now routinely done and even the incorporation of a wide variety of biophysical probes (fluorophores, isotope labels, sterically hindered amino acids) can be achieved.[29,30] This often requires identification of synthesis conditions that are chemically compatible with the desired biophysical probe as well as optimization of the coupling condition by varying activation agents, solvents and coupling times to avoid inefficient coupling reactions.

However, the site-directed incorporation of spin-labelled amino acids in a given protein by chemical means still presents a challenge. This is mostly due to the delicate nature of the nitroxide moiety that is readily protonated under acid conditions, such as TFA treatments during Boc-synthesis or cleavage from the solid support during Fmoc synthesis, and can then decompose. This problem can be overcome by employing Fmoc-based synthesis strategies either on the solid phase in combination with HF-cleavages or in solution as described by Marchetto *et al*. and Monaco *et al*., respectively.[31,32] In both cases, Fmoc-protected TOAC (I) was used to achieve successful incorporation of the spin-labelled amino acids into short peptides. The solution-phase approach yielded the pure, EPR-active TOAC-containing peptide without any further treatment, whereas the HF-cleavage requires reconstitution of the *N*-oxide under mildly basic conditions to revert the protonation of the nitroxide followed by oxidation. Another approach to achieve site-specific incorporation of a spin label into a peptide synthesized by Boc-chemistry was presented by McNulty *et al*.[19] They used N^{α}-Boc, N^{β}-Fmoc-L-2,3-diaminopropionic acid [Boc-Dap(Fmoc)-OH] as a scaffold to couple a spin label to the side chain of this amino acid (Scheme 1, II) just prior to HF-cleavage. This avoids exposure of the spin label to repeated cycles of TFA treatment, which can result in irreversible destruction of the nitroxide. Reconstitution of full spin activity was again achieved by treatment with a weak base.

We have tried to achieve direct incorporation of a pyrroline-based nitroxide amino acid into peptides by Boc-chemistry relying on the fact that pyrroline nitroxides are less sensitive towards reducing, oxidative and acidic condition, probably due to a lower accessibility of the nitroxides when compared with six-membered rings such as in TOAC.[33] Incorporation and deprotection of amino acid III positioned at or close to the *N*-terminus using Boc-chemistry in combination with an *in situ* neutralization protocol[34] could be successfully conducted. However, nine coupling cycles after introduction of III into the peptide chain, decomposition of the nitroxide-containing amino acid III became obvious, and after 14 cycles, no product could be identified anymore. Comparison of synthesis results obtained for similar sequences where the nitroxide amino acid was replaced by leucine or L-2,3-diaminopropionic acid (Dap) clearly indicated that the five-membered nitroxide amino acid is also prone to irreversible destruction under Boc-synthesis conditions. This irreversible destruction of the spin label can probably be avoided if the nitroxide moiety is masked during peptide elongation, e.g. as a benzyl protected hydroxylamine (Hideg *et al*., unpublished). Deprotection of the *O*-benzyl-hydroxylamine should occur upon treatment with HF and the resulting hydroxylamine can be oxidized to produce the paramagnetic nitroxide.



Scheme 3. Coupling of spin label to the ε-amino group of lysine.[19]

In order to demonstrate incorporation of a spin label into semi-synthetic proteins using peptides synthesized by Boc-chemistry, we took refuge in an approach similar to that described by McNulty *et al*.[19] and introduced an orthogonally protected lysine residue (Boc-Lys(Fmoc)-OH) into the peptide sequence (CGK(SL)GHHHHHH, the His_6 tag was introduced for purification purposes). Coupling of the spin label was achieved after complete assembly of the peptide and selective deprotection of the ε-amino group of lysine with 20% piperidine in *N*,*N*-dimethylformamide (DMF) by using 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) activation in the presence of DIEA as a base (Scheme 3).

The spin-labelled peptide was then cleaved from the solid support using liquid HF at 0 °C in the presence of 5% *p*-cresol as a scavenger and yielded a paramagnetic compound (data not shown). The applicability of the method was demonstrated by fusing CGK(SL)GHHHHHH to the Ras-binding domain of *c*-Raf1 (RBD) at its *C*-terminus (Fig. 2 and section 'Spin Labelling of RBD By Expressed Protein Ligation') by using an expressed protein ligation (EPL) scheme.

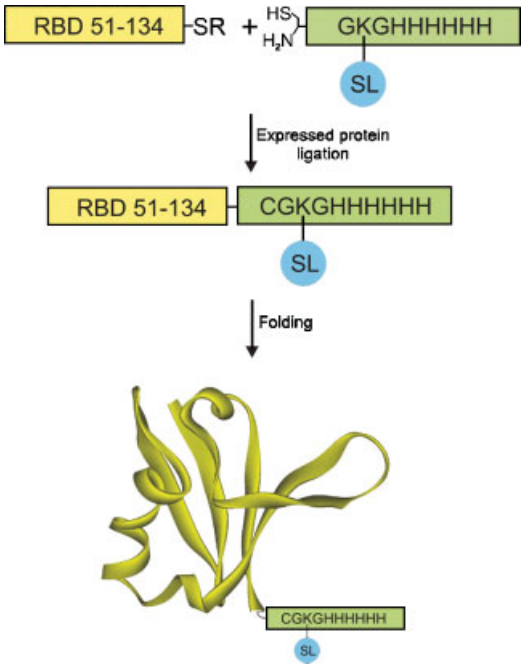
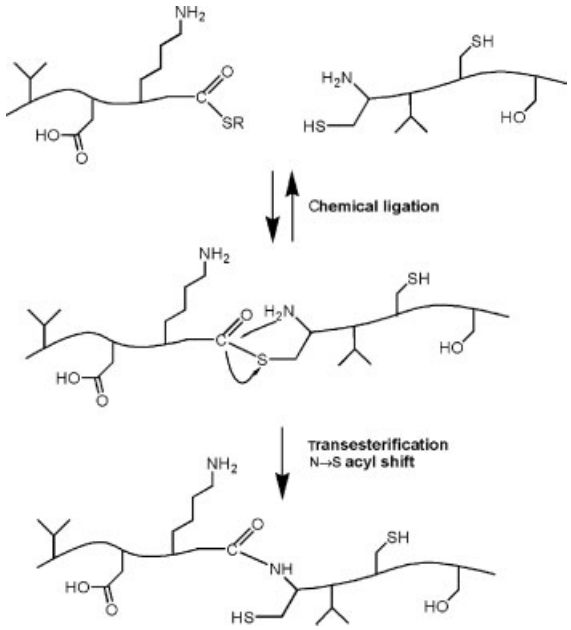


Figure 2. Strategy for the *C*-terminal spin labelling of RBD.



Scheme 4. Mechanism of native and expressed protein ligation.

EPL and the closely related NCL strategies rely on the reaction of the sulfhydryl group of an *N*-terminal cysteine with a *C*-terminal thioester (Scheme 4). After rearrangement through an S → N acyl shift, a native peptide bond is formed. The reaction can be performed even in the presence of other unprotected cysteine residues because of a first reversible reaction and a second irreversible step. The combination of recombinant techniques and peptide chemistry allows introduction of the spin labels at every position of the protein. The only requirements are *N*-terminal cysteine residues that can be generated by protein splicing, proteolysis of recombinant proteins and chemical synthesis of peptides and *C*-terminal thioesters that are accessible by protein splicing and chemical synthesis.

It should be noted that applying the EPL technique to a spin-labelled peptide is inherently problematic because of the use of a large excess of thiols to mediate the transesterification step and to keep the cysteine residues in a reduced state.[35] The reducing properties of frequently used thiols like thiophenol in NCL or sodium 2-mercaptoethanesulfonate (Mesna) in EPL can lead to reduction of the nitroxide to the corresponding hydroxylamine and therefore render the label inactive,[36] which makes it necessary to reoxidize the sample after the ligation step. However, reoxidation of the spin label might be accomplished by treatment with a weak base in the presence of atmospheric oxygen with or without added PbO_2.

SPIN LABELLING OF RBD BY EXPRESSED PROTEIN LIGATION

Using the strategy outlined above, a semi-synthetic protein that carries a site-specifically incorporated spin label within its *C*-terminus was generated. As a test system for this approach, the RBD was used. This protein is a downstream

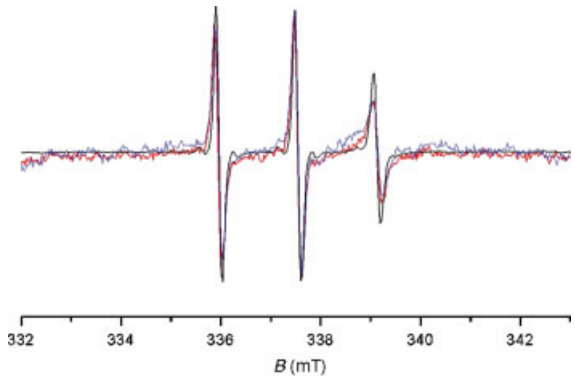


Figure 3. EPR spectra of the spin-labelled peptide (CGK(SL)GHHHHHH (black), RBD-CGK(SL)GHHHHHH (red), and RBD-CGK(SL)GHHHHHH in complex with Ras(GppNHp) (blue). Experimental conditions: The spin-labelled peptide was measured in 6 M guanidinium hydrochloride buffered with 100 mM NaOAc at pH 4.0. RBD and RBD–Ras complex have been measured in 50 mM Tris HCl, 100 mM NaCl, 5 mM $MgCl_2$ at pH 7.4. Measurements were performed with a home-built EPR spectrometer equipped with a Bruker dielectric cavity with the following settings: microwave power 0.4 mW, modulation amplitude 0.1 mT, scan time 60 s, 10 scans accumulated for RBD and RBD in complex with Ras.

effector of the Ras protein, and Ras itself functions as a molecular switch in eukaryotic signalling cascades.[37] In its guanosine triphosphate (GTP) bound form, it activates downstream signal transduction chains by binding to RBD, a protein domain of the kinase *c*-Raf1. To generate the RBD thioester, the protein was expressed as a fusion protein containing RBD-intein-CBD (chitin binding domain).

The construct was transformed to an RBD carrying a *C*-terminal thioester by treatment with 150 mM Mesna, which was purified and used for ligation with the spin-labelled peptide in the presence of 100 mM Mesna.[38] The reduced spin label was reoxidized with oxygen alone and in the presence of PbO_2. The latter protocol, although quite successful for the *C*-terminal peptide, resulted in a loss of protein probably due to adsorption by the PbO_2 powder.

The EPR spectra of the peptide, the spin-labelled RBD, as well as the corresponding RBD/Ras complex are shown in Fig. 3. As expected, the line widths increase for RBD as compared to the small peptide because of the decrease of the reorientational correlation time. Complexation with Ras(GppNHp) does not further alter the spectrum, indicating that the *C*-terminus of RBD does not interact with Ras as can be assumed from the crystal structure of RBD in complex with Rap1A, a Ras homologue.[39]

CONCLUDING REMARKS

The chemical synthesis of peptides in combination with recombinant techniques provides the means to incorporate unnatural spin-label amino acids into proteins with sufficient yields and reasonable effort. The NCL of unprotected peptides has become a viable method for the preparation of a wide variety of biologically active proteins. Furthermore, the EPL technique has enabled the production of polypeptide thioesters by recombinant means. The combination of chemically synthesized peptides with recombinant polypeptides greatly increases the versatility and applicability of chemical synthesis of labelled proteins. It has now become possible to introduce spin labels via these methods not only in the *C*- or *N*-terminal part of the protein but also in its centre, thus allowing EPR studies with a new quality of labelled proteins.

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