Detection of α-Helical Coiled-Coil Dimer Formation by Spin-Labeled Synthetic Peptides: A Model Parallel Coiled-Coil Peptide and the Antiparallel Coiled Coil Formed by a Replica of the ProP C-Terminus†

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ABSTRACT: Electron paramagnetic resonance spectroscopy was used to determine relative peptide orientation within homodimeric, α-helical coiled-coil structures. Introduction of cysteine (Cys) residues into peptides/proteins for spin labeling allows detection of their oligomerization from exchange broadening or dipolar interactions between residues within 25 Å of each other. Two synthetic peptides containing Cys substitutions were used: a 35-residue model peptide and the 30-residue ProP peptide. The model peptide is known to form a stable, parallel homodimeric coiled coil, which is partially destabilized by Cys substitutions at heptad a and d positions (peptides C30a and C33d). The ProP peptide, a 30-residue synthetic peptide, corresponds to residues 468−497 of osmoregulatory transporter ProP from *Escherichia coli.* It forms a relatively unstable, homodimeric coiled coil that is predicted to be antiparallel in orientation. Cys was introduced in heptad g positions of the ProP peptide, near the N-terminus (K473C, creating peptide C473g) or closer to the center of the sequence (E480C, creating peptide C480g). In contrast to the destabilizing effect of Cys substitution at the core heptad a or d positions of model peptides C30a and C33d, circular dichroism spectroscopy showed that Cys substitutions at the heptad g positions of the ProP peptide had little or no effect on coiled-coil stability. Thermal denaturation analysis showed that spin labeling increased the stability of the coiled coil for all peptides. Strong exchange broadening was detected for both C30a and C33d, in agreement with a parallel structure. EPR spectra of C480g had a large hyperfine splitting of about 90 G, indicative of strong dipole−dipole interactions and a distance between spin-labeled residues of less than 9 Å. Spin−spin interactions were much weaker for C473g. These results supported the hypothesis that the ProP peptide primarily formed an antiparallel coiled coil, since formation of a parallel dimer should result in similar spin−spin interactions for the spin-labeled Cys at both sites.

The study of α-helical coiled-coil structures is providing powerful insights into protein−protein interactions and the basis for new technologies (*1*). Electron paramagnetic resonance (EPR)[1] spectroscopy of spin-labeled proteins is a powerful technique for detecting coiled-coil formation by peptides/proteins. Cysteine (Cys) substitution at selected sites or cysteine scanning through the sequence allows specific labeling of those sites using Cys-specific spin labels (*2*). Oligomerization of peptides containing spin-labeled Cys residues can be detected from motional restriction of the spin label or exchange broadening if the spin-labeled sites are in close proximity or from dipolar interactions through space between residues within 25 Å of each other. Here we report application of this technique to detect formation of and determine the orientation of homodimeric α-helical coiled coils.

Coiled coils consist of two, three, or four amphipathic α-helices which wrap about each other in a left-handed superhelix. The typical sequence of a coiled coil consists of a seven-residue (heptad) repeat denoted by a b c d e f g, where the hydrophobic core residues are in positions a and d and, in general, polar and charged residues occupy positions b, c, e, f, and g. Exchange broadening was detected for a 38-residue peptide corresponding to the HIV gp41 heptad repeat, which forms a trimeric coiled coil when it was substituted with Cys in the heptad c or f positions and spin labeled (*3*). Immobilization and exchange broadening occurred for the trimeric coiled coil formed by the N-terminus of HA2 containing Cys in a and c positions, while immobilization but no broadening occurred when the Cys was in b positions

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[1] Abbreviations: MTS-SL, methanethiosulfonate spin label; Gdn·HCl, guanidine hydrochloride; EPR, electron paramagnetic resonance spectroscopy; CD, circular dichroism spectroscopy.

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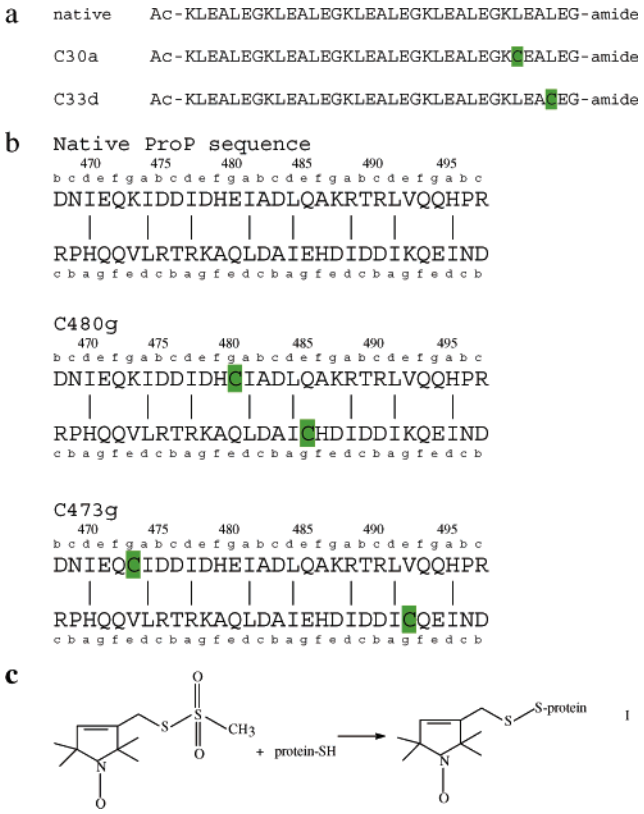

FIGURE 1: (a) Sequences of model peptides, native, C30a, and C33d, with Cys substitutions highlighted and (b) sequences of the ProP C-terminal replica of residues 468−497, native, C473g, and C480g, all shown in a proposed antiparallel alignment. Heptad a through g positions are indicated. Lines indicate a−d and d−a interactions across the hydrophobic core. Cys substitutions are highlighted. (c) Structure of MTS-SL in a disulfide linkage to cysteine.

(*4*). Motional restriction, exchange broadening, and dipolar broadening were detected for the heterotetrameric SNARE complex containing two spin-labeled peptides, labeled at a, d, and other positions (*5*−*7*). Similarly, exchange and dipolar broadening were observed for a 75-residue peptide replica of the trimerization domain from the heat shock transcription factor containing spin-labeled Cys in an e position (*8*). All of the above contained peptides in parallel orientation.

Despite all of these studies on parallel trimeric or tetrameric coiled coils, peptides which form parallel or antiparallel dimers have not yet been studied by this technique. Further studies with model peptides containing spin-labeled Cys at different positions are necessary in order to identify heptad positions at which Cys can be introduced and spin-labeled without significantly affecting coiled-coil stability. Such sites must also be close enough within the coiled-coil structure to allow detection of coiled-coil formation by EPR spectroscopy. Replacement of alanine (Ala) with Cys in a peptide forming a monomeric α-helix was found to cause some loss of helical structure, but spin labeling of the Cys was helix supporting, similar to the effects of non-β-branched aliphatic amino acids such as Ala, methionine (Met), and leucine (Leu) (*9*). Similarly, the parallel, dimeric coiled-coil structure of a 35-residue model peptide (Figure 1a) was partially destabilized by substitution of Cys for Leu at a and d positions (*10*).

In the present study we have used EPR and circular dichroism (CD) spectroscopy to investigate the effect of Cys substitution and spin labeling on the stability of the helical coiled coils formed by this model peptide and by the ProP peptide, a replica of a naturally occurring structure that is believed to form an antiparallel, homodimeric coiled-coil structure. Cys was introduced at heptad a position 30 or heptad d position 33 in the model peptide, creating peptides denoted C30a and C33d (Figure 1a). These peptides were spin-labeled, and their propensity for coiled-coil formation was determined in both unlabeled and labeled states.

Secondary transporter ProP from *Escherichia coli* is an osmosensor and H^+-osmoprotectant symporter (*11*). ProP activity is a function of medium osmolality in both intact bacteria and, after purification, proteoliposomes (*12*). The cytoplasmic C-terminal domain of ProP was predicted to form an α-helical coiled coil since sequence analysis revealed heptad repeats characteristic of coiled-coil structures (*13*). A synthetic peptide corresponding to residues 456−500 of ProP was shown to form a dimeric coiled coil of low stability in vitro (*14*), and residues 468−497 were the minimum required for coiled-coil formation (*26*). Deletion of residues 475−500 from ProP inactivated the protein in vivo, suggesting a physiological role for this structure (*14*).

The low stability of the coiled coil formed by the ProP C-terminal domain was thought to result from the presence of Arg488 and His495 at heptad a positions. Replacement of His495 with isoleucine in the synthetic peptide stabilized the coiled coil, as expected, and caused it to form higher order oligomers. However, replacement of R488 with isoleucine further destabilized the coiled coil formed by the synthetic peptide. Interestingly, the R488I substitution dramatically increased the osmolality at which ProP became active and reduced its ability to remain in an active conformation (*14*). These results suggested that coiled-coil formation by the C-terminal domain of ProP may be required for its sustained activation (*15*). They further suggested that the orientation of the coiled coil was antiparallel, stabilized by interchain electrostatic interactions involving R488 (*14*).

A peptide corresponding to ProP residues 468−497 was synthesized with Cys substitutions to determine if coiled-coil formation by a peptide replica of the C-terminus of ProP would tolerate Cys substitution, whether it could be detected by spin labeling, and whether the structure formed was parallel or antiparallel. Cys was placed at g positions in order not to decrease the stability of the coiled coil further. Replacement of residues E480, near the center of the peptide (creating peptide C480g), or K473, closer to the N-terminal end (creating peptide C473g, Figure 1b), allowed us to determine the orientation of the resulting peptide monomers in the coiled coil. If the peptide forms an antiparallel structure, the labeling at the central 480 sites should result in stronger broadening than at the 473 sites, which will be much more distant in an antiparallel than in a parallel coiled-coil structure.

MATERIALS AND METHODS

Materials. Peptides with the sequences shown in Figure 1a,b were synthesized as described earlier (*10*, *14*). The spin label (1-oxy-2,2,5,5-tetramethylpyrroline-3-methyl)methanethiosulfonate (MTS-SL) (Figure 1c) was from Toronto Research Chemicals, Inc. (North York, Ontario).

Spin Labeling of Peptides. The peptides were dissolved in deionized water at a concentration of 1 mg/mL. The pH of the solution was adjusted to 7 by titration with 100 mM sodium bicarbonate, and nitrogen gas was slowly bubbled through the solution for 5 min. A 10-fold molar excess of MTS-SL in dimethyl sulfoxide or dimethylformamide [to a final solvent concentration of 1.4% (v/v)] was then added dropwise with stirring. The solutions of the C30a and C33d peptides and spin label were stirred at room temperature for 2 h in an atmosphere of nitrogen. The mixture was then partially lyophilized to reduce the volume by 50% before purification. The solutions of the ProP peptides and spin label were stirred in capped tubes at room temperature overnight. The spin-labeled peptides were purified on a reversed-phase C18 HPLC column, 3.9 mm i.d. × 300 mm (Waters, μBondapak), using a linear AB gradient, where A is 0.05% TFA and B is 0.05% TFA in acetonitrile at a gradient rate of 0.75% B/min with a flow rate of 0.7 mL/min. For study of unlabeled peptides, the thiol group was blocked with iodoacetamide by adding a 5-fold molar excess of iodoacetamide to the peptide solution. The reaction was carried out at 4 °C overnight. Purification of the blocked peptide was carried out as described for the spin-labeled peptide. Peptide concentrations were determined by amino acid analysis. Spectral integration and spin-label quantification for C30a and C33d showed that the mole ratio of bound spin label to peptide was 0.9:1. Mass spectrometry analysis of the spin-labeled C480g and C473g showed that the peptides were pure and that the mole ratio of bound spin label to peptide was 1:1.

EPR Spectroscopy. For EPR measurement, solutions of the peptides C30a and C33d, at concentrations of 80 and 68 μM, respectively, were made in 50 mM potassium phosphate buffer containing 0.5 M KCl, pH 7.0. Guanidine hydrochloride (Gdn·HCl) denaturation studies were carried out by combining mixtures of a stock solution of peptide and a stock solution of 8 M Gdn·HCl, both in the above buffer, with buffer alone to give the appropriate Gdn·HCl concentration and a constant peptide concentration. The ProP peptides, C473g and C480g, were dissolved at a concentration of 3 mM in 50 mM potassium phosphate buffer, pH 7, containing 0.1 M KCl and then diluted 1:1 with the same buffer with and without 8 M Gdn·HCl. Samples were incubated for 1 h at room temperature before measurement of spectra at room temperature. For exchange with unlabeled peptide, a solution of the labeled peptide was combined with a solution of the unlabeled iodoacetamide-blocked peptide to give a mole ratio of labeled peptide to unlabeled peptide of 1:3. Spectra were measured with time after addition of unlabeled peptide at room temperature until no further change occurred. Spectra of peptides C473g and C480g were also measured at 77 K in a dewar filled with liquid nitrogen. However, the large degree of broadening obtained indicated that the peptides may have aggregated at the low temperature, resulting in broadening independent of orientation. Spectra were measured on a Bruker ECS106 EPR spectrometer at 10 mW power. The motional parameter was calculated as described earlier (*16*).

CD Spectroscopy. CD spectra of spin-labeled and unlabeled reduced peptides (the latter in DTT) were acquired in 50 mM potassium phosphate buffer containing 100 mM KCl at pH 7.5 and in this buffer containing 50% TFE. The concentration of C30a and C33d was 26 μM and that of C473g and C480g was about 100 μM as specified in Table 2. The ratios of the CD signals at 222−208 nm were calculated. A ratio of above 1.00 is indicative of a coiled-coil structure whereas a ratio less than 1.00 suggests a non-coiled-coil structure. The effect of thermal denaturation on the CD spectra was determined by heating from 5 to 80 °C at a rate of 45 °C/h. It was assumed that the peptides in buffer were 100% folded at 5 °C and 0% folded at 80 °C in order to calculate the fraction folded at intermediate temperatures. $T_{1/2}$, the temperature at which a 50% decrease in folding of the peptides occurred, was determined from a plot of fraction folded against temperature.

RESULTS

Model Coiled-Coil Peptides C30a and C33d. Cys-containing peptides C30a and C33d were shown earlier to form stable parallel two-stranded α-helical coiled coils (*10*). These peptides were spin-labeled with MTS-SL in order to determine if exchange broadening occurred for spin labels at these positions and to what degree the spin label affected the stability of the coiled coil. The CD spectra of spin-labeled C30a and C33d in buffer with and without 50% TFE are shown in Figure 2. The molar elipticity value at 222 nm indicates that the peptides are highly α-helical, and the fact that TFE did not increase the magnitude of this value further indicates that the peptides were fully folded in buffer. For both peptides in buffer, spin-labeled or not, the ratios of the CD signals at 222−208 nm were greater than 1, confirming that they formed a coiled coil (Table 1). For both peptides in TFE, the ratios were less than 1, indicating that the peptides formed a monomeric helical structure in this solvent. Thermal denaturation (Figure 3) showed that the unlabeled peptides formed very stable coiled coils with $T_{1/2}$ values of 54.2 °C for C30a and 51.8 °C for C33d (Table 1). Spin labeling increased the $T_{1/2}$ values to 58.1 and 60.1 °C, respectively, indicating an increase in stability of the coiled coil. The spin label stabilized the coiled coil twice as much in position d (8.3 °C increase in $T_{1/2}$) compared to position a (3.9 °C increase in $T_{1/2}$), as would be expected due to the more efficient packing at position d (*10*).

EPR spectra of MTS-SL bound to C30a and C33d are shown in Figure 4. The spectrum of C30a consists of two components, a sharp component due to a fraction of the spin label that is mobile and not exchange broadened and an underlying broadened component. The latter is evident from the increase in intensity above the baseline on the low-field side and increase in intensity below the baseline on the high-field side. The broadened component is the major one indicating that most of the peptide forms a coiled coil. The unbroadened spectral component may be due to unbound spin label or a small fraction of spin-labeled peptide that either is a monomer or is in a coiled coil which is unraveled at the C-terminal end, causing the spin labels to be more than 25 Å apart. A broadened component is not obvious in the spectrum of C33d. However, it is present in that spectrum also, since exchange of unlabeled peptide for labeled peptide, added in a 3:1 mole ratio, caused an increase in intensity of the EPR peaks for both peptides due to loss of the exchange broadening (Figure 5). This increase was significantly greater for C30a than C33d (compare spectra in Figure 5 for C30a after 5 and 375 min plotted normalized to the same amount

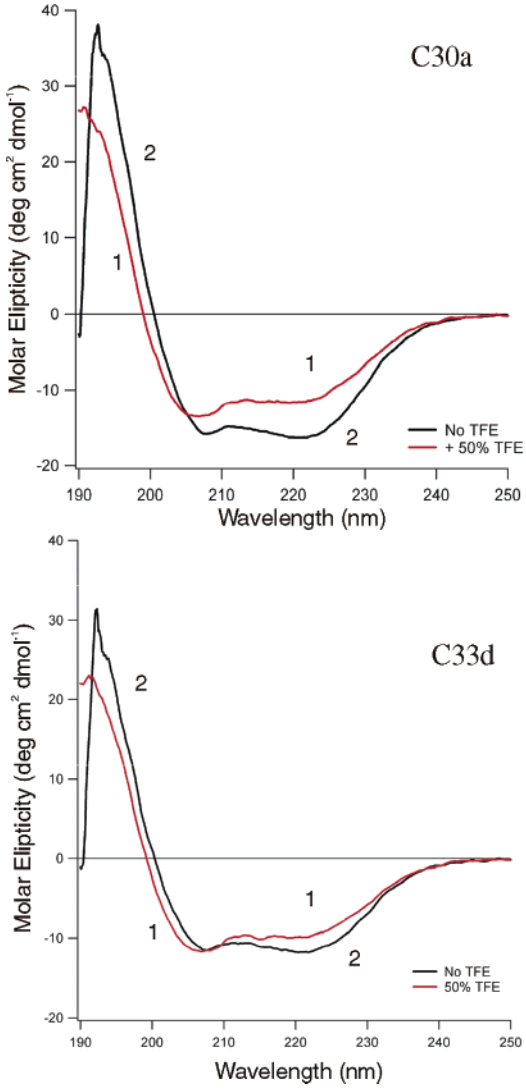

FIGURE 2: CD spectra of C30a and C33d in 50 mM potassium phosphate buffer, pH 7.5, containing 100 mM KCl, with (curve 1) and without (curve 2) 50% TFE.

of spin-labeled C30a and spectra for C33d after 1.5 and 245 min plotted normalized to the same amount of spin-labeled C33d). However, the fact that some increase in intensity occurred for C33d indicates that some broadening of the spectrum of C33d also occurred when only labeled peptide was present (Figure 4). The loss of broadening reveals the true line shape of the spectra of the spin-labeled peptides and shows that the spin label bound to C30a is less mobile than that bound to C33d. The very sharp component in the spectrum of C30a is still present in the spectrum after dilution with unlabeled peptide (apparent on the high-field side) and can now be seen clearly to be due to a small percentage of the total spin label.

Denaturation of the peptides with Gdn·HCl caused a large increase in intensity of the spectrum of both peptides, partly due to increased mobility but mainly due to loss of exchange broadening following disruption of structure (compare spectra of C30a plotted normalized to the same amount of spin-labeled C30a and spectra of C33d plotted normalized to the same amount of spin-labeled C33d, before and after addition of Gdn·HCl in Figure 6). The dependence of spectral intensity on Gdn·HCl concentration for both peptides is shown in Figure 7. The concentration of Gdn·HCl required to give a 50% increase in spectral intensity is a measure of the stability of the coiled coils. It was 2.2 M for C30a and 2.4 M for C33d. This is similar to the concentration of Gdn·HCl required to attain 50% denaturation of the reduced, unlabeled peptides as determined by CD spectroscopy and reported earlier, 1.8 M for C30a and 2.0 M for C33d (*10*).

Peptide Replica of the C-Terminal Domain of ProP. The CD spectra of the ProP peptide, its spin-labeled and non-spin-labeled derivatives with amino acid substitution K473C or E480C (peptides C473g and C480g), were similar (not shown). None of these peptides were fully folded at the concentration used (approximately 100 μM). In addition, thermal denaturation showed that the stability of the coiled coil formed by these peptides was much less than those of the model parallel coiled-coil peptide or its derivatives C30a and C33d. $T_{1/2}$ values were 26.5 and 28 °C (Table 2) for the ProP C-terminal peptides compared to a value above 50 °C for C30a and C33d. Cys substitution at either K473 and E480 had no effect on the stability of the coiled coil. In contrast, spin labeling the introduced Cys increased the stabilities of the peptides with these substitutions. Spin labeling of C480g increased the $T_{1/2}$ value by 10.5 °C compared to 4 °C for spin-labeled C473g (Table 2). Thus the spin label contributes more stability when added to the central heptad than the more peripheral heptad.

It was found earlier that the amount of dimer formed by a peptide replica of the C-terminus of ProP (residues 456−500) increased with peptide concentration, and at 1.5 mM, only a dimer was detected by sedimentation equilibrium ultracentrifugation (*14*). Therefore, we measured the EPR spectra of 1.5 mM solutions of spin-labeled peptides C473g and C480g, shown in Figure 8A, normalized to the same amount of spin label. The lower intensity of the spectrum of labeled peptide C480g indicates that a larger percentage of the spectrum is broadened but the residual narrow spectral component appears narrower than that in the spectrum of peptide C473g. The spectra of both peptides are also shown plotted out to a larger scale in Figure 8B so that the underlying broad spectral component present for C480g can be seen. This reveals a very broad component with peaks separated by almost 90 G. This component was not seen in the spectrum of C473g. Such large splitting has been observed only for proteins with two nitroxides separated by less than 15 Å (*7*, *17*, *18*) and is due to rotational dipolar relaxation. It occurs also in the spectrum of C30a in Figure 4, but the spectral component is less distinct. Rotational dipolar relaxation can be observed only for proteins with a rotational correlation time less than $r^3h/3\pi g^2\beta^2$ where r, h, g, and β are the interspin distance, the Planck constant, the electronic g factor, and the Bohr magneton, respectively (*18*). Small rotational correlation times are to be expected for small peptides such as those used in the current study. In the case of lysozyme, with 164 residues and a rotational correlation time of 6 ns, similar broadening was observed for nitroxides 7−9 Å apart (*18*, *19*). Double integration of the spectrum of C480g in Figure 8 showed that 80% of the spin label contributed to the broad component with large hyperfine splitting. The residual sharp spectral component observed for C480g, resulting from 20% of the spin label, is probably due to undimerized or denatured

Table 1: Impact of Spin Labeling on Coiled-Coil Stability for the Model Peptide

	C30a				C33d			
	unlabeled		spin labeled		unlabeled		spin labeled	
	buffer	TFE	buffer	TFE	buffer	TFE	buffer	TFE
ratio[a] (222/208)	1.1	0.95	1.03	0.86	1.1	0.89	1.03	0.84
$T_{1/2}$ (°C)[b]	54.2 ± 0.1	nd[c]	58.1 ± 0.1	nd	51.8 ± 0.1	nd	60.0 ± 0.1	nd

[a] Ratio of CD signal at 222 nm to that at 208 nm. [b] Thermal denaturation measured by CD spectroscopy. [c] nd = not determined.

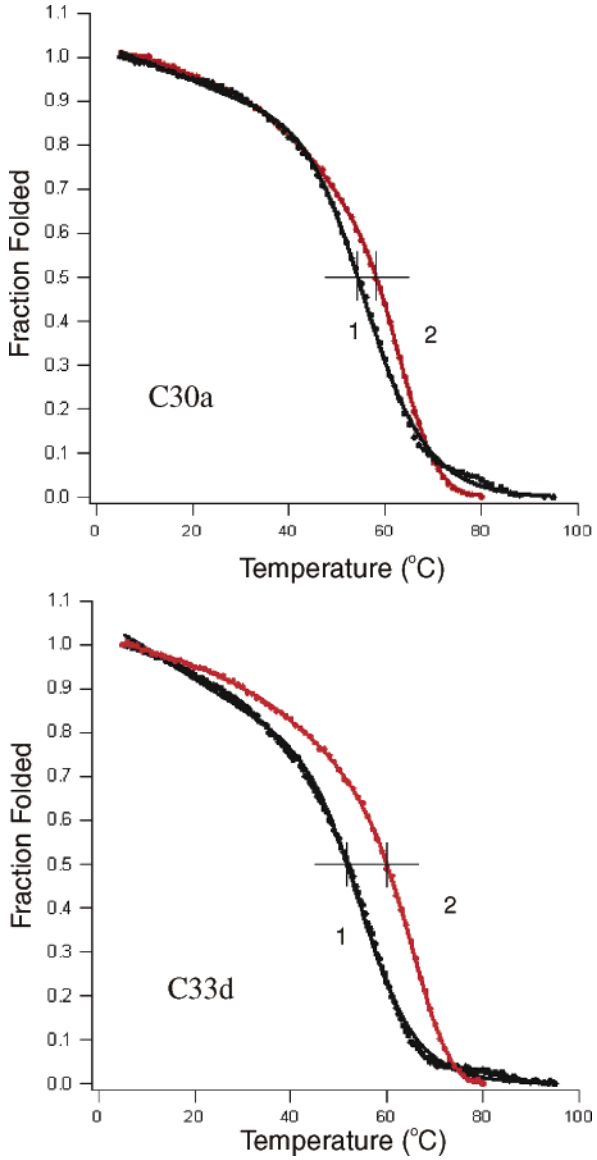

FIGURE 3: Thermal denaturation of C30a and C33d, in 50 mM potassium phosphate buffer, pH 7.5, containing 100 mM KCl, determined by CD spectroscopy as described in Materials and Methods. Data for unlabeled (black points, curve 1) and spin-labeled (red points, curve 2) peptides are shown. A smooth curve (solid line) was fit to the data by nonlinear regression, and the temperature ($T_{1/2}$) at which the peptides were 50% folded was determined.

peptide. A similar sharp component may also be in the spectrum of C473g (Figure 8) but cannot be distinguished from the predominant spectral component which is indicative of somewhat slower motion.

Dilution of the labeled peptides with unlabeled peptide in a 1:3 mole ratio resulted in a large increase in signal intensity (Table 3) and the loss of observable broadening for the

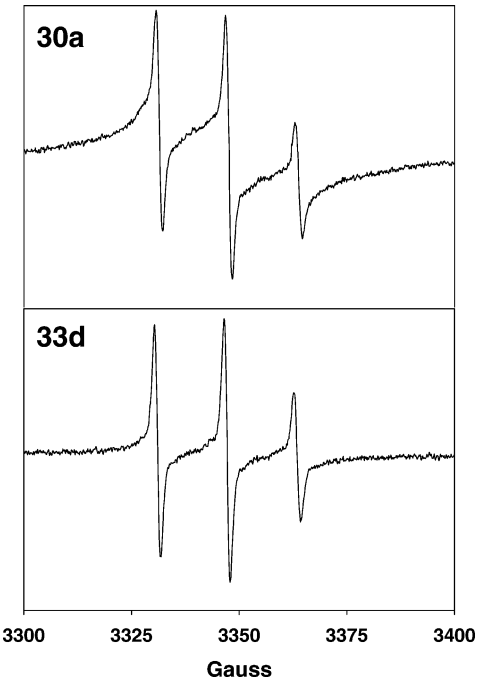

FIGURE 4: EPR spectra at room temperature of C30a and C33d, plotted at similar center peak heights.

peptide labeled at either site (Figure 9), indicating that the spectrum of completely labeled C473g is also broadened somewhat due to spin−spin interactions, although they are much weaker than for C480g. The resulting nonbroadened spectra of C473g and C480g in the presence of unlabeled peptide are similar to each other (Figure 9) with motional parameters (similar to the correlation time) of 1.3 and 1.5 ns, respectively. The smaller increase in intensity observed for C480g (Table 3) indicates that a 3 to 1 excess of unlabeled peptide was not enough to eliminate dimers containing two spin labels, and thus it did not completely eliminate broadening for C480g. Addition of 4 M Gdn·HCl eliminated dimerization and secondary structure, resulting in a large increase in intensity of the spectra of the peptide labeled at both sites (Table 3) and a further increase in mobility of the probe (not shown). The much greater broadening observed for C480g relative to C473g indicates that the labels in the peptide dimer are closer together for C480g than for C473g.

DISCUSSION

Despite several studies using EPR to determine structural information about coiled coils, little has been reported on the influence of Cys substitution in various heptad positions and spin labeling of those Cys on the stability of such structures. Furthermore, this technique has not been previously applied to dimeric coiled coils or to antiparallel coiled

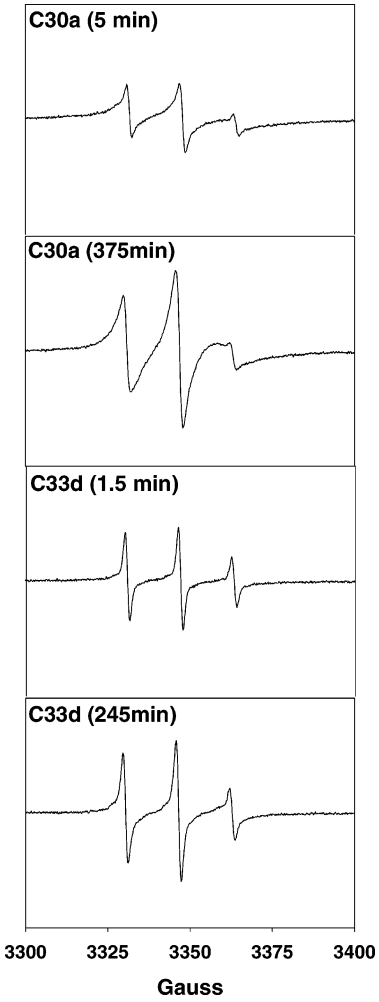

FIGURE 5: Effect on the spectra of C30a and C33d of exchange with unlabeled peptide at a mole ratio of unlabeled to labeled peptide of 3:1. Panels: C30a 5 min after addition of unlabeled peptide; C30a 375 min after addition of unlabeled peptide. Both are plotted normalized to the same amount of spin label to show the increase in intensity which occurs after exchange with unlabeled peptide and loss of exchange broadening. Panels: C33d 1.5 min after addition of unlabeled peptide; C33d 245 min after addition of unlabeled peptide. Both are plotted normalized to the same amount of spin label.

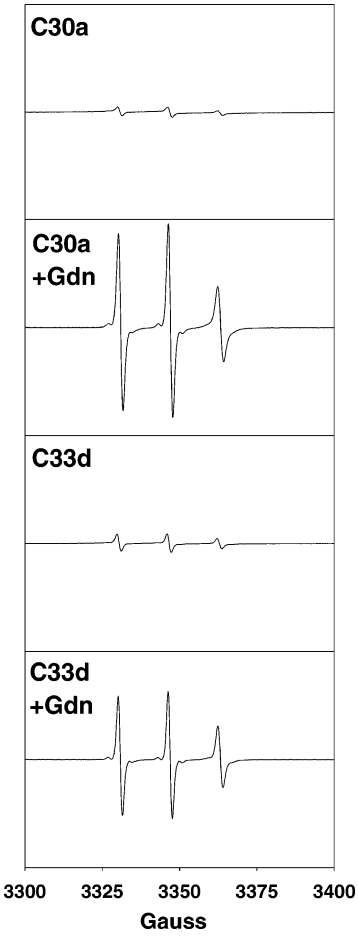

FIGURE 6: Effect on the spectra of C30a and C33d of addition of Gdn·HCl. Panels: C30a, no Gdn·HCl; C30a plus 3.7 M Gdn·HCl (Gdn). Both are shown plotted normalized to the same amount of spin label to show the large increase in intensity after denaturation with Gdn·HCl. Panels: C33d, no Gdn·HCl; C33d plus 3.5 M Gdn·HCl. Both are shown plotted normalized to the same amount of spin label.

coils. To characterize the effect of Cys subsitution and spin labeling on dimeric coiled-coil stability and to determine the orientation of the ProP peptide coiled coil, we performed Cys substitutions and spin labeling thereof on two different peptides which form dimeric coiled coils. Substitution of Cys for Leu at a or d positions in a 35-residue two-stranded parallel coiled-coil model peptide was shown to partially destabilize the coiled-coil structure (*10*). Therefore, Cys was substituted in a g position for residues E480 or K473 in the ProP C-terminal peptide, where we found that it had no effect on the stability of the coiled coil. For all peptides, the thermal and Gdn·HCl denaturation studies show that spin labeling the introduced Cys residues had a stabilizing effect on the coiled-coil structure. Thus spin-labeled Cys in these peptides may share the helix-supporting nature of unbranched aliphatic side chains found for spin-labeled Cys in monomeric helices (*9*). The disulfide bond joining the spin label to Cys is thought to interact with the peptide backbone (*20*). In addition, for the spin-labeled Cys in the g position, the spin label can lie across and interact with the hydrophobic core of the coiled coil. For example, adding a hydrophobic residue like Leu at position g has been shown to dramatically increase the stability of a GCN4-like coiled coil (*21*). Further, substitutions that increase stability have a greater effect near the center of a coiled coil than those closer to the ends because the ends of a coiled coil are more dynamic (*22*). This likely accounts for the greater increase in stability caused by spin labeling of Cys 480, near the center of the ProP peptide, compared to Cys 473, near the N-terminus (Figure 1b).

The EPR spectra of spin-labeled C30a and C33d show that coiled-coil formation caused exchange and dipolar broadening of the spectra in both cases although the effect was much weaker for C33d than C30a. A smaller fraction of the spin-labeled C33d resulted in a broadened spectrum than for C30a, and the spectrum of C33d was dominated by a nonbroadened spectral component. Since the CD spectra indicated that the spin-labeled peptides were fully helical in both cases, this may indicate that the end of the coil is somewhat unraveled, resulting in less probability of interaction of the spin labels at position 33, near the end of the peptide, than at position 30. Neither spin-labeled site was significantly motionally restricted by coiled-coil formation despite the fact that they are at the hydrophobic core of the coiled coil. This is likely due to the rapid tumbling of the

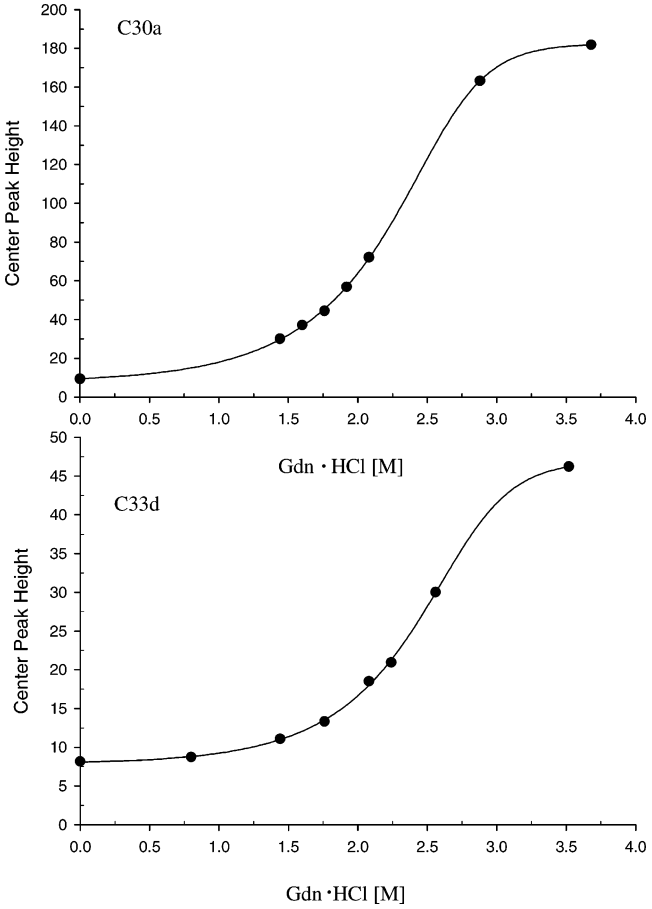

FIGURE 7: Dependence of intensity of the EPR spectrum (height of center line) of C30a and C33d on concentration of Gdn·HCl. A smooth curve (solid line) was fit to the data, and the Gdn·HCl concentration at which the height was increased by 50% of the maximum observed was determined.

Table 2: Impact of Spin Labeling on Coiled-Coil Stability for the ProP Peptide[a]

peptide	peptide concn (μM)	$T_{1/2}$ (°C)[b]
native sequence	95	28
C480g	120	26.5
C473g	105	28
C480g spin labeled	95	37
C473g spin labeled	75	32

[a] Based on the sequence of residues 468−496 of the C-terminus of ProP. [b] Thermal denaturation measured by CD spectroscopy.

small peptide. However, the spin label bound to C30a had less motion than that bound to C33d.

The g positions chosen for Cys substitution in the C-terminal ProP peptide help to distinguish between the parallel and antiparallel orientation, since g positions are on the same side of the helical coil if antiparallel and on the opposite side if parallel (*23*). Similar broadening should occur for parallel homodimers comprised of either C480g or C473g. In contrast, for antiparallel homodimers, greater broadening will be seen for C480g than for C473g. For this spin label bound to Cys in a helical peptide, a fit of experimental data to calculated distances showed that the nitroxide radical is 6.7 Å from the helix axis (*24*). If the dimer were parallel, the nitroxides bound to either C480 or C473 would then be about 16.6 Å apart, using a value of 4.9 Å for the supercoil radius of a dimer (*25*). This is close

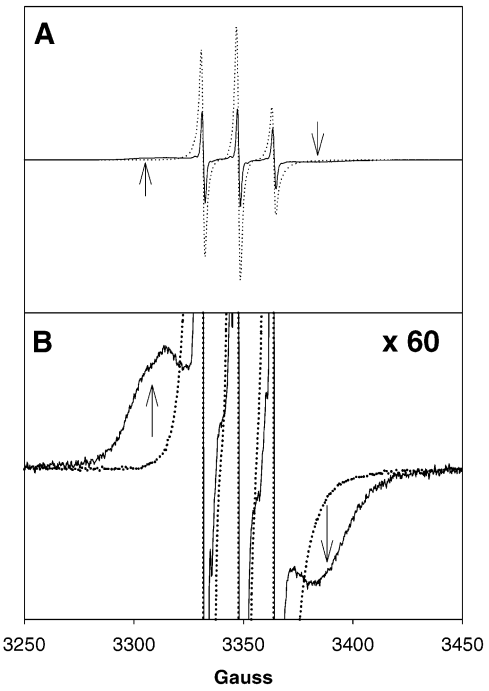

FIGURE 8: EPR spectra at room temperature of C473g (dotted line) and C480g (solid line) in 50 mM potassium phosphate buffer, pH 7, containing 0.1 M KCl, plotted normalized to the same amount of spin label. The same spectra are shown amplified 60× in the lower panel. Broad peaks with large splitting are indicated by arrows on the low- and high-field sides and were seen only for C480g.

Table 3: Spin−Spin Interactions in Coiled Coils of Spin-Labeled C480g and C473g Inhibited by Denaturation or Dilution with Unlabeled Peptide

condition	relative amplitudes[a]	
	C480g	C473g
benign buffer, pH 7.0	18.6	86
4 M Gdn·HCl	70.4	109
unlabeled/labeled (3:1)	43	134

[a] Central peak amplitudes of the EPR spectra of the ProP C-terminal peptides; spectra were measured at room temperature.

enough for spin−spin interactions. In an antiparallel dimer, the two C473 in the two strands would be about 28.5 Å apart and the two C480 in the two strands would be about 7.5 Å apart, assuming 1.5 Å translation per residue for a rigid α-helix of 5.41 Å pitch. The latter is close enough for strong spin−spin exchange and for rotational dipolar relaxation to significantly broaden the lines.

Significant spectral broadening was observed for spin-labeled C480g, indicative of rotational dipolar relaxation and a distance between the nitroxides of less than 9 Å, indicating that the peptide forms a coiled-coil dimer. This degree of broadening did not occur for the peptide spin-labeled at C473, indicating that the orientation of the dimer is predominantly antiparallel. However, some broadening did occur for the peptide spin-labeled at C473 as revealed by the increase in height on dilution with unlabeled peptide. This was probably due to exchange interactions rather than rotational dipolar relaxation, since a spectral component with large hyperfine splitting was not observed even after amplification of the spectrum. The interspin distance between two nitroxides bound to Cys could have an uncertainty of up to 12 Å due to motion of the nitroxide about the bonds linking it to

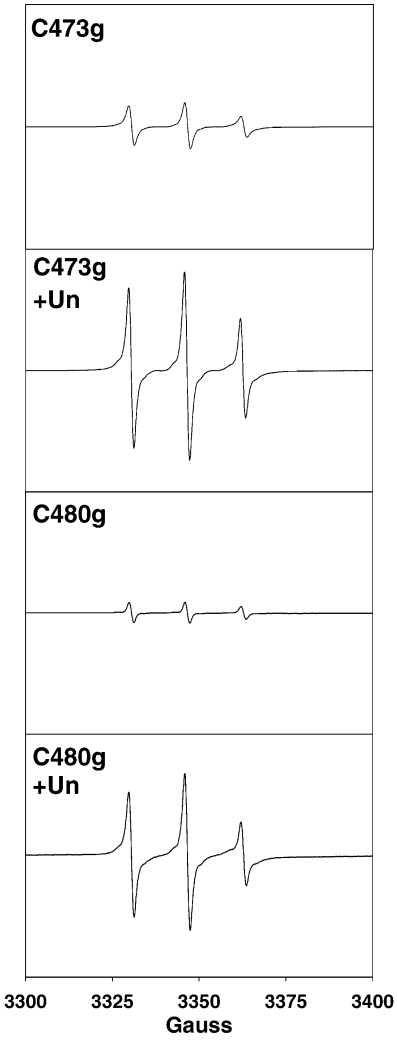

FIGURE 9: Effect on the spectra of C473g and C480g of exchange with unlabeled (Un) peptide at a mole ratio of unlabeled to labeled peptide of 3:1. Spectra with and without unlabeled peptide in each case are plotted normalized to the same amount of spin label to show the increase in spectral intensity on exchange with unlabeled peptide.

the disulfide (*19*, *20*). Thus the interspin distance along the helix axis between spin-labeled C473 in an antiparallel dimer could be as small as 16.5 Å. This is close enough for intermediate spin−spin exchange interactions and could lead to the smaller degree of broadening seen for the peptide spin-labeled at C473 in an antiparallel orientation. Formation of an antiparallel dimer by residues 468−497 of ProP containing the wild-type sequence has recently been confirmed by Zoetewey et al. (*26*) using NMR spectroscopy. They further showed that the antiparallel orientation was stabilized by salt bridges between R488 and residues D478 and D475.

In summary, we have used peptides with judiciously placed and spin-labeled Cys substitutions to detect both parallel and antiparallel, dimeric coiled-coil formation by EPR spectral broadening. A peptide replica of the C-terminus of ProP containing spin-labeled Cys at position 473 or 480 formed a dimeric coiled coil with stability comparable to that of the wild-type peptide, and its orientation was shown to be antiparallel, as predicted (*14*). This approach will now be applied in an effort to detect coiled-coil formation and dimerization of intact ProP in proteoliposomes.

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