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QUARTERLY

Probing iso-1-cytochrome *c* structure by site-directed spin labeling and electron paramagnetic resonance techniques[o]

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A cysteine-specific methanethiosulfonate spin label was introduced into yeast iso-1-cytochrome *c* at three different positions. The modified forms of cytochrome *c* included: the wild-type protein labeled at naturally occurring C102, and two mutated proteins, S47C and L85C, labeled at positions 47 and 85, respectively (both S47C and L85C derived from the protein in which C102 had been replaced by threonine). All three spin-labeled protein derivatives were characterized using electron paramagnetic resonance (EPR) techniques. The continuous wave (CW) EPR spectrum of spin label attached to L85C differed from those recorded for spin label attached to C102 or S47C, indicating that spin label at position 85 was more immobilized and exhibited more complex tumbling than spin label at two other positions. The temperature dependence of the CW EPR spectra and CW EPR power saturation revealed further differences of spin-labeled L85C. The results were discussed in terms of application of the site-directed spin labeling technique in probing the local dynamic structure of iso-1-cytochrome *c*.

Cytochromes *c* are electron transfer proteins that contain the heme prostethic group covalently bound to the protein matrix. In mitochondria they function as mobile electron carriers connecting the cytochrome bc_1 complex with the cytochrome *c* oxidase. In general, cytochromes *c* are among the best characterized proteins. High resolution three-dimensional structures has been obtained for cytochromes *c* from several species including those from horse [1], rice [2], tuna [3], yeast [4–6]. Solution structure obtained through NMR for yeast cytochrome *c* provided independent information on the mobility of the

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Abbreviations: CW, continuous wave; EPR, electron paramagnetic resonance; SDSL, site-directed spin labeling; SOE, splicing by overlap extention; TPX, methylpentene polymer; YPD, yeast extract-peptone-dextrose; YPG, yeast extract-peptone-glucose.

backbone and side chains fluctuations [7-9]. A variety of other techniques have extensively been used to characterize the physiological properties of cytochromes *c* and the interactions with their electron-transfer partners (reviewed in refs. [10 and 11]).

The availability of detailed structural information on iso-1-cytochrome *c* from *Saccharomyces cerevisiae* makes this protein an excellent model for studying structure-function relationship using site-directed mutagenesis. Moreover, the protein proved to be highly amenable to genetic manipulations making it possible to analyze the effects of mutations both *in vitro* and *in vivo* (reviewed in refs. [12 and 13]). In this study we used iso-1-cytochrome *c* from *S. cerevisiae* for selective labeling with the spin label probe and successive EPR analysis.

The spin-labeling technique combined with site-directed mutagenesis is capable of providing structural and dynamic information about selectively targeted regions of the protein. The most common approach, called site-directed spin labeling (SDSL), uses site-directed mutagenesis to replace the chosen amino acid of the protein with cysteine – a suitable attachment site for the cysteine-specific spin label (reviewed in ref. [14]). This approach has successfully been used in studies with colicin E1 [15], bacteriorhodpsin [16, 17], rhodopsin [18], T4 lysozyme [19], diphtheria toxin [20], lactose permease [21], and human carbonic anhydrase [22].

In SDSL for the selectivity purposes, the protein should not contain any natural cysteines, otherwise they need to be first replaced by residues of similar size and properties (usually threonine or serine). In this context, iso-1-cytochrome *c* from *S. cerevisiae* appears as a good candidate for SDSL studies since it contains only one naturally occurring cysteine (at the position 102) with free SH group accessible to the spin label. As early as since 60-th, this particular group was used for labeling of cytochrome *c* in studies concerning the interaction of this protein with the membranes and its redox partners, and the protein folding ([23] and references therein, [24-28]). Furthermore, many structural studies on iso-1-cytochrome *c* were done using the single mutant C102T (cysteine 102 replaced by threonine) and its derivatives (reviewed in ref. [29]). The C102T mutation has been shown to make the protein more amenable to biochemical and biophysical investigations without changing its structural or functional properties [30]. Thus, the mutant C102T provides a suitable background for SDSL.

In this work iso-1-cytochrome *c* was labeled with the cysteine-specific methanethiosulfonate spin label at three different positions (see Fig. 1). Apart from the wild-type cytochrome *c* labeled at cysteine 102, two constructs with the spin label at positions 47 and 85 were generated. The two constructs derived from the mutant C102T and contained a cysteine residue introduced into position 47 (the mutation S47C) or into position 85 (the mutation L85C). All three forms of the protein were characterized in terms of the SDSL applicability in probing the local dynamic structure of cytochrome *c*.

MATERIALS AND METHODS

Materials, yeast strain and DNA. The *S. cerevisiae* strain GM-3C-2 (a strain deficient in both isotypes of yeast cytochrome *c*) was used as a background for mutagenesis [31]. DNA isolations were carried out with columns and buffers from Qiagen, Inc. The mutagenic oligonucleotides and sequencing primers were synthesized using Cyclone^{TM} Plus DNA Synthesizer (MilliGen Biosearch, Division of Millipore). DNA sequencing was performed on 373A DNA Sequencer (Applied Biosystem). All materials for growth media were from Difco. Growth media were prepared by standard recipes [32].

Site-directed mutagenesis. Plasmid pING4 (a generous gift from Dr. Michael Smith from the University of British Columbia) consisting

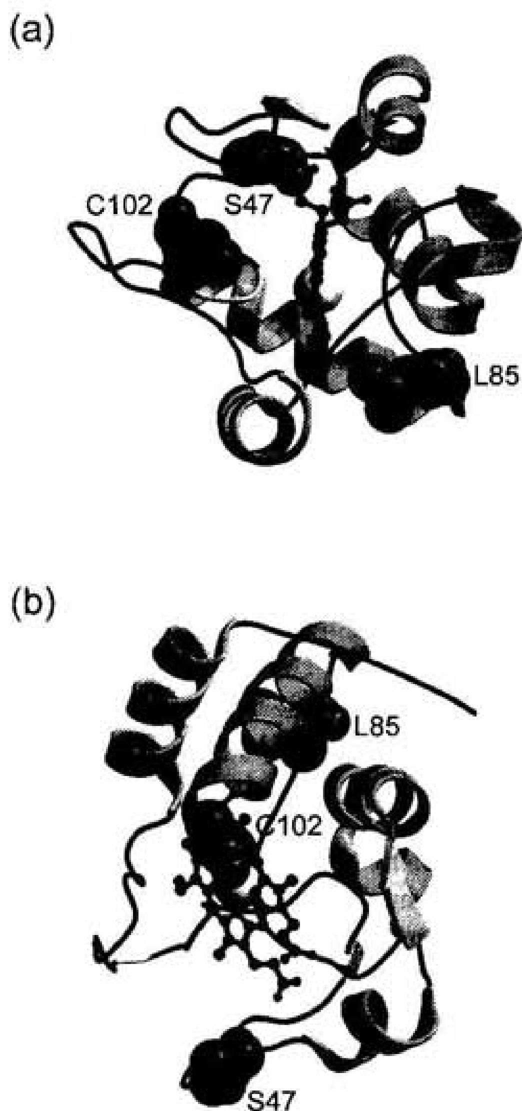

Figure 1. Ribbon diagram of three-dimensional structure of yeast iso-1-cytochrome *c* (protein data bank entry 1YCC).

Two orientations of the molecule are presented. The mutated residues and the heme iron are shown as a space-filling model, the heme plane is shown as a ball-and-stick model. The distances between heme iron and the α-carbon atoms are 14 Å for C102, 13 Å for S47 and 12 Å for L85. The Figure was generated by the programs MOLSCRIPT [41] and RASTER3D [42].

of the coding regions for *Leu2* and Amp^r selectable markers as well as the origins of replication for yeast and *Escherichia coli* was chosen as a mutagenic shuttle vector [33]. A 1 kb *Sma*I/*Hind*III fragment derived from this plasmid includes the complete yeast iso-1-cytochrome *c* gene (with introduced C102T mutation) together with its transcriptional regulatory elements.

The *Sma*I/*Hind*III fragment from pING4 [33] was inserted into pUC18 and in this form used as a template for PCR. Site-directed mutagenesis was performed using a gene splicing by overlap extension (SOE) method patterned after Horton *et al.* [34]. The mutagenic primers were (mismatch positions are underlined):

5′- GTGTAGCAATACCCTTCAGCTTGAC - 3′ and
5′- CTGAAGGGTATTGCTACACAGATGCCA - 3′
(for mutation S47C);
5′- CTTCTTGCACCCACCAAAGGCCATCT - 3′ and
5′- CTTTGGTGGGTGCAAGAAGGAAAAAGA - 3′
(for mutation L85C)

The flanking universal primers were:
5′- ATGGCCAGGCAACTTTAGTGCTGACAC - 3′ and
5′- GGGGGAGGGCGTGAATGTAAGCGTGAC - 3′.

The PCR amplifications were carried out in a 1605 Air Thermo-Cycler (Idaho Technology) in 50 μL with 2.5 units of Taq DNA polymerase for each reaction. The first stage of SOE consisted of 20 cycles (15 s at 94°C, 15 s at 55°C, 20 s at 72°C). The expected products were extracted from 1% agarose gel and used as a template in the second stage of PCR (5 cycles of 15 s at 94°C, 15 s at 55°C and 28 s at 72°C followed by 10 cycles of 15 s at 94°C, 15 s at 57°C and 28 s at 72°C). The final full-length mutated PCR product was extracted from 1% agarose gel, digested with *Nsi*I and *Eco*RV and ligated with *Nsi*I/*Eco*RV digested pUC18. The ligation mixture was transformed into Nova Blue competent cells (Novagen). The plasmids with the correct mobility on 1% agarose gel were chosen for sequencing. The whole amplified *Nsi*I/*Eco*RV fragments were sequenced and the plasmids with confirmed desired mutation (L85C or S47C) and without any other random mutations were digested with *Sma*I and *Hind*III and cloned into pING4.

Introduction of mutated cytochrome* c *genes into yeast. The plasmid pING4 containing the mutated gene was isolated into 50 μL of water from a 5 mL bacterial culture. A sample of this DNA was used to transform

yeast strain GM-3C-2 [31]. Yeast cells were made competent for transformation using a lithium acetate procedure of Ito *et al.* [35] with the modifications patterned after Soni *et al.* [36]. Ten percent ethanol was used as a factor stimulating transformation efficiency. The yeast cells containing the plasmid were selected on the basis of acquisition of the plasmid-borne *Leu2* marker by growth on SC agar plates lacking leucine and containing dextrose as a sole carbon source [37].

Analysis of mutants. In order to confirm the identity and integrity of the mutated genes after their introduction into yeast, plasmid DNA was isolated from GM-3C-2 cells by the method described in ref. [33], re-introduced into bacteria cells and isolated from bacteria for DNA sequencing. In order to test that the yeast phenotype was plasmid-based, transformed yeast colonies were subjected to a curing procedure [37, 38]. The colonies were first grown for 2 days on YPD medium. One hundred microliter of 10^{-4} and 10^{-5} dilutions of that culture was spread onto YPD plates. Single colonies were then tested for growth on YPD, YPG and SD Leu$^-$ plates to monitor the loss of plasmid-based phenotypes. Transformed yeast colonies were tested for growth on glycerol or lactate as a sole carbon source to check whether the gene product (mutated protein) is functional and can reduce cytochrome *c* oxidase.

Protein expression and purification. The mutated proteins were expressed by growing the transformed yeast cells to saturation on glycerol based medium YPG. Iso-1-cytochrome *c* was isolated and purified according to the procedure described by Cutler *et al.* [30]. The ensuing chromatographic runs were carried out on FPLC (Pharmacia/LKB). The final purification of cytochrome *c* was achieved on a HR5/5 MonoS column (Pharmacia/LKB) using 50 mM sodium phosphate, 2 mM β-mercaptoethanol, 1 mM EDTA, pH 7.2, as an elution buffer and linear gradient running from 0 to 0.5 M sodium chloride in 90 min, at a flow rate 1 mL/min. After dialysis against 50 mM sodium phosphate, 2 mM β-mercaptoethanol, 1 mM EDTA, pH 7.0, the protein was concentrated and lyopilized.

Spin labeling and EPR measurements. The cytochrome *c* was dissolved in 50 mM sodium phosphate, pH 7.0. The stock solution of methanethiosulfonate spin label, [(1-oxyl-2,2,5,5-tetramethyl-Δ^3-pyrroline-3-methyl)-methanethiosulfonate], (Reanal, Budapest, Hungary), was prepared in acetone and added to cytochrome *c* at a ratio 3 mole of spin label/1 mole cytochrome *c* (final concentration of acetone was 5%, v/v). The reaction was carried out at room temperature in darkness for 60 min. After the reaction a few drops of freshly prepared potassium ferricyanide was added to obtain the oxidized form of spin labeled cytochrome *c*. Unreacted spin label and potassium ferricyanide were removed from the reaction mixture by gel filtration on Sephadex G-10 equilibrated with 50 mM sodium phosphate, pH 7.0. The resulting samples of labeled cytochrome *c* were used for EPR measurements.

The EPR spectra were recorded on Bruker ESP-300E spectrometer fitted with TM_{110} cavity. Temperature was stabilized using the Bruker temperature controller. The gas-permeable TPX plastic sample capillary was used for the measurements under nitrogen atmosphere. During the EPR measurements care was taken to avoid line-shape distortions that could arise from experimental conditions. A modulation amplitude was 0.02 mT, microwave power was 2 mW or 8 mW for CW EPR spectra and from 1 mW to 200 mW in power saturation measurements. In order to improve the signal-to-noise ratio, the scans were accumulated if needed. The temperature dependent EPR spectra were recorded in the range from 4°C to 48°C.

For the saturation curves, the vertical peak-to-peak amplitudes of the central line of the CW EPR spectrum (A_0) were measured. The amplitudes were plotted as a function of square root of incident microwave power and fitted to the function [16]:

$$A_0 = N(\Lambda P^{1/2}) / (1 + \Lambda^2 P[\gamma^2 T_1 T_2])^{1.5} \quad (1)$$

where: A_0 is vertical peak-to-peak amplitude of the central line, N is an adjustable parameter, Λ is a constant equal to 1, P is incident microwave power, γ is electronic giromagnetic ratio, T_1 is spin-lattice relaxation time, T_2 is spin-spin relaxation time.

The $P_{1/2}$ (the power where the amplitude of the central line is reduced to half of its unsaturated value) was calculated from the equation [16]:

$$P_{1/2} = (2^{2/3} - 1) / (\Lambda^2[\gamma^2 T_1 T_2]) \quad (2)$$

using a value of $[\gamma^2 T_1 T_2]$ obtained from the fitting procedure (see above).

RESULTS AND DISCUSSION

Characterization of mutants

In this study iso-1-cytochrome *c* from *S. cerevisiae* was mutated at two positions, S47 and L85, by constructing two forms of the protein with cysteine residue at respective positions (mutants S47C and L85C). The expression system was based on the strain deficient in both isotypes of yeast cytochrome *c* (*S. cerevisiae* strain GM-3C-2) and a shuttle vector pING4 [33]. GM-3C-2 cells transformed with pING4 containing the mutation S47C or L85C were tested for growth on glycerol (YPG) and lactate. Growth rates similar to that of the wild-type (C102T) iso-1-cytochrome *c* were observed indicating that functional iso-1-cytochromes *c* were expressed.

A curing procedure was used to test whether the cytochrome *c* phenotype was not due to chromosomal reversion. The *Leu2* gene is carried on the yeast expression vector pING4 and complements a genetic deficiency of the GM-3C-2 cell line. In both mutants the loss of the cytochrome *c* phenotype was linked to the loss of the leucine biosynthesis phenotype indicating that cytochrome *c* was expressed from the plasmids.

Mutated plasmids were also isolated from the transformed GM-3C-2 cells and the entire coding region of the cytochrome *c* gene was resequenced. In each case only the expected point mutations were observed.

Protein preparations examined by SDS/polyacrylamide gel electrophoresis and Coomassie blue staining showed in each case only one band of purified cytochrome *c*. Typically a 3 L culture of the yeast yielded 60 g wet mass of cells giving 6–10 mg of purified iso-1-cytochrome *c*.

EPR characteristics of wild-type and mutants of iso-1-cytochrome c

In the present study three different structural positions of yeast iso-1-cytochrome *c* were monitored by a spin label probe. The attachment sites for the spin label, defined by the position of natural C102 in the native protein and by the positions of the introduced mutations at positions 85 and 47 are depicted in Fig. 1. In choosing L85 and S47 for mutations the following aspects were taken into account. First, together with C102 each position, when labeled, probes different region of the protein. Second, an attempt was made to keep the distance between the labeled residue and the heme iron as short as possible, keeping in mind that placing cysteine too close to the heme attachment sites may perturb the folding pattern and the proper incorporation of the heme ring. Third, the positions with different degree of solvent exposure were considered: in the three-dimensional structure of iso-1-cytochrome *c* both L85 and C102 are partially buried within the protein interior, while S47 is positioned on the surface of the protein [4].

The CW EPR spectra of spin labeled wild-type ferricytochrome *c* and two mutated forms S47C and L85C are shown in Fig. 2. At room temperature, in each case immobiliza-

tion of the spin label can be clearly recognized, demonstrating that the binding of the protein did occur (Fig. 2, row b). The spectra of wild-type cytochrome *c* (C102) and S47C appear to be similar indicating that the spin label at these positions may have the same level of mobility. On the other hand, L85C is characterized by a spectrum having much broader lines, which can be attributed to the different plexity could arise either from two different conformations of the spin label or two different local conformations of the protein in the probed region [16, 39].

In contrast to L85 which is located in the region between β-turn (residues 75–78) and α-helix (residues 87–102), C102 is the penultimate C-terminal amino acid and may, therefore, have higher conformational flexibility.

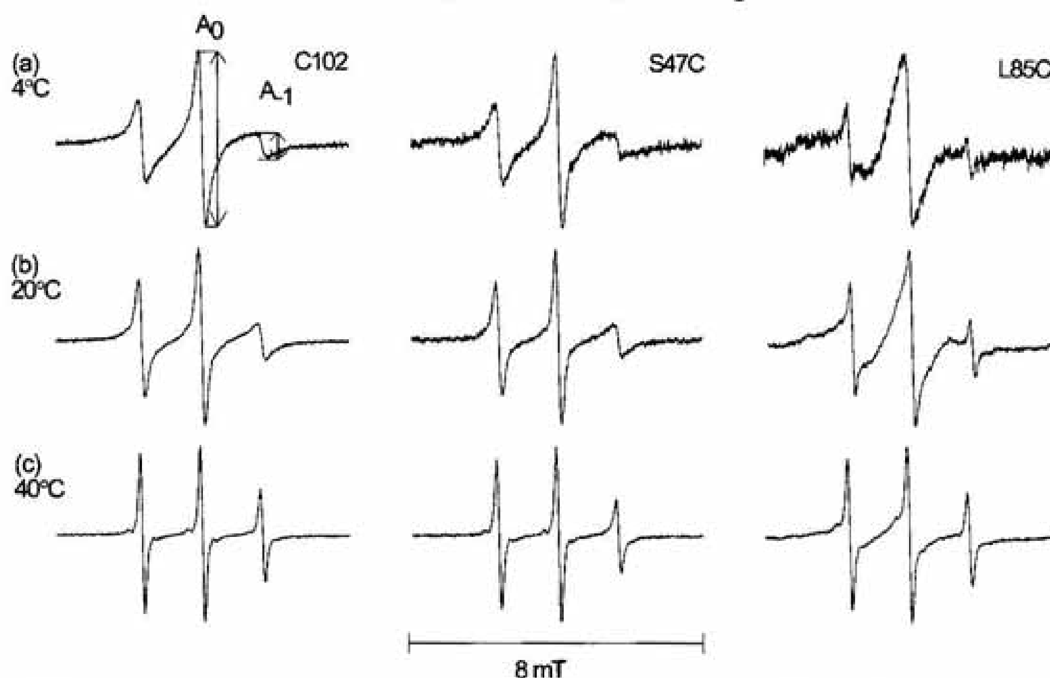

Figure 2. CW EPR spectra for the spin-labeled yeast iso-1-cytochrome *c*, wild type (C102) and two mutants (S47C, L85C), recorded at three temperatures: 4°C (a), 20°C (b) and 40°C (c).

Vertical peak-to-peak amplitudes for the central line (A_0) and high-field line (A_{-1}) were taken for A_{-1}/A_0 calculation (see text for details).

pattern of the mobility of spin label at the position 85 and, most probably, results from the limitations of the spin label tumbling caused by steric factors. This may by related to the local structure of the region around L85. Although the sulfhydryl group of cysteine introduced to the position 85 is apparently accessible to the spin label, the moiety of the residue remains in the highly rigid region, which in turn may force the label to accommodate the more restricted position. Furthermore, the complexity of its movement seems to be reflected by the two components of different mobility (fast and slow) that can be easily distinguished in the spectrum of L85C. This complexity ... Thus, although in the three-dimensional structure of iso-1-cytochrome *c* the sulfhydryl group of C102 appears to be inaccessible to the solvent, the incorporation of the spin label at this position may induce some perturbations and unwinding of the C-terminal α-helix exposing C102 more considerably to the surface of the protein. Such a change has been modeled for spin labeled wild-type iso-1-cytochrome *c* [28] and provides one possible explanation for higher mobility of the spin label attached to C102 in comparison to its mobility at position 85. It is also possible that in solution C102 is naturally more exposed on the surface of the protein when compared to its lo-

cation shown by the X-ray structure. Consequently, only minor structural changes occur upon binding of the label to C102. In fact, the EPR spectrum of wild-type cytochrome *c* is similar to that observed in the mutant S47C, where the labeled position is on the surface of the protein. This suggests that vicinities of both C102 and S47C provide similar conditions for the mobility of the spin label.

Figure 3 shows the influence of temperature on the CW EPR spectra of wild-type and mutants S47C and L85C in the range between 4°C and 48°C, expressed as the ratio of the amplitudes A_{-1}/A_0. Additionally, representative spectra for the two outermost temperatures (4°C and 40°C) are shown in Fig. 2 (rows a and c). In each case a clear effect of temperature on the mobility of the label is observed with the following tendency: highly immobilized spin label at low temperature shows a progressive increase in mobility with increasing temperature (increase in the A_{-1}/A_0 ratio). Such dependence is consistent with the process of unfolding of the protein occurring during the thermal melting: when the protein unfolds the attached spin label is likely to tumble more freely. Since this effect was observed for each labeled protein, it can be concluded that regardless of the attachment site, the label remained sensitive to changes in the tertiary fold of the protein.

The comparison of the thermal melting profiles shown in Fig. 3 emphasizes again the difference in behavior between the labeled mutant L85C and, both the wild-type and S47C. The profiles of wild-type and S47C are similar and show two distinct phases in the tested region of temperatures with the transition point around 28°C. On the other hand, the profile of L85C is less steep and the two phases, if they do exist, are much less distinguishable. This effect may suggest that the spin label detects some subtle differences in the unfolding pattern of the region in the vicinity of L85. It may also be a result of the changes in the dynamic equilibrium between the two possible conformations of the spin label. It should be noted,

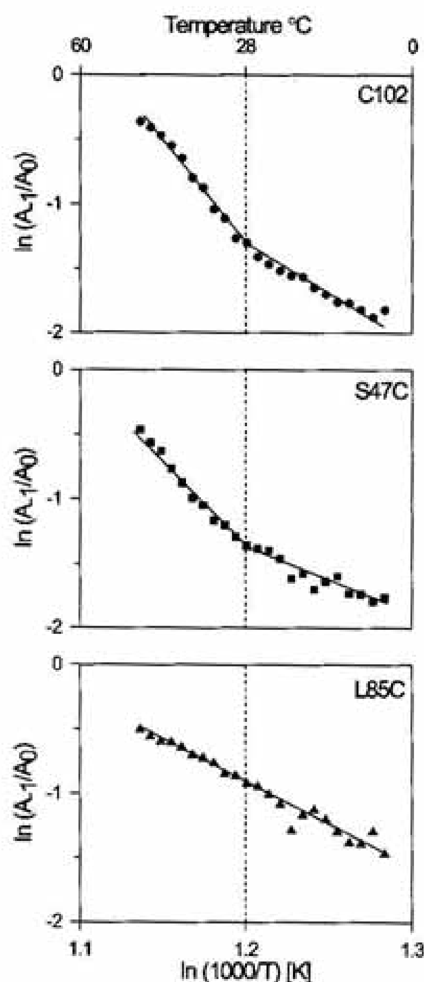

Figure 3. Thermal melting of the spin-labeled yeast iso-1-cytochrome c wild type C102 (●) and two mutants: S47C (■), and L85C (▲).

The A_{-1}/A_0 ratios are plotted as a function of temperature. Transition points are denoted by dashed lines.

however, that in either case the interpretation of this result is obscured by the fact that we were not able to resolve the two components of the spectra, and as a consequence, the A_{-1}/A_0 ratios obtained for L85C reflected mainly the changes in the fast component.

Figure 4 shows the power saturation curves for the wild-type and two mutants recorded under argon atmosphere. The fitting of the experimental curves yielded the following values of $P_{1/2}$ (the power at which the amplitude of the central line is reduced to half of its unsaturated value): 81 ± 1, 87 ± 1, 132 ± 5 mW for the wild-type, S47C, and L85C, respectively. From this comparison it is apparent that spin-labeled L85C becomes saturated at

higher incident microwave power than the wild-type and S47, which both show similar saturation behavior. Since $P_{1/2}$ depends on the relaxation times, T_1 and T_2, its value reflects both the mobility of the spin label and the interaction with the fast relaxing agent. In this context, the higher $P_{1/2}$ of L85C suggests that the protein environment surrounding L85 may somehow facilitate the relaxation process (our experimental conditions, i.e. argon atmosphere, eliminated fast-relaxing agents from solution, including the most common one – oxygen).

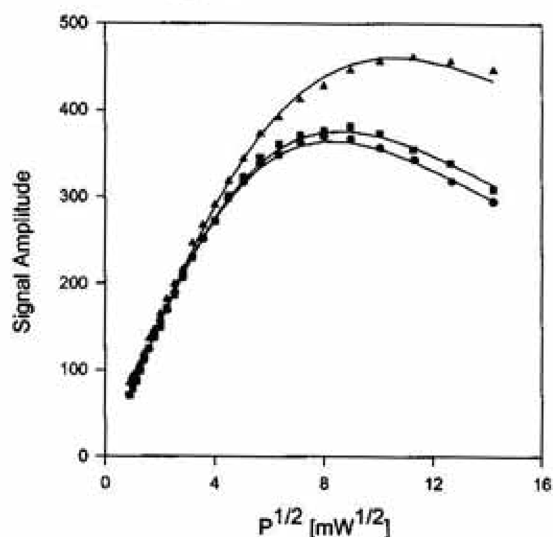

Figure 4. Saturation curves for the spin-labeled yeast iso-1-cytochrome *c*, wild type C102 (●) and two mutants S47C (■), L85C (▲).

The normalized values (arbitrary units) of the vertical peak-to-peak amplitude for the central line (A_0) are plotted against values of the square root of the incident microwave power. The solid lines represent theoretical curves obtained from computer fitting of the experimental data to equation 1.

The saturation behavior of three labeled derivatives shows again that spin label attached to cysteine at position 85 behaves differently from those attached to cysteine at positions 102 or 47. It is remarkable that the peculiar EPR characteristics obtained for L85C concerns a position occupied by the highly conserved residue L85 [40] that was shown to play a dually important structural role. This residue, being a part of the outer protein surface, forms also a portion of the internal hydrophobic heme-binding pocket [40].

Our previous study with spin-labeled horse mitochondrial cytochrome *c* showed that there were significant differences in the mobility of the spin labels attached to different lysine residues [39]. Considering the line shape of CW EPR spectra at room temperature, the mobility of the spin label attached to K25 in horse cytochrome *c* seems to correspond to the mobility of the spin label observed in wild-type iso-1-cytochrome *c* and mutant S47C, while the spin labels attached to K8, K86, and K87 in horse cytochrome *c* seem to tumble more freely. On the other hand, the spectrum of labeled K72 in horse cytochrome *c* resembles the spectrum of mutant L85C in that it is composed by a clearly distinguishable fast and a slow component. This comparison emphasizes that in both systems (i.e. horse cytochrome *c* labeled at lysine residues and iso-1-cytochrome *c* labeled at cysteine residues) the spin label is very sensitive to local structural properties of the probed region, even if the modified positions are located on the surface of the protein.

CONCLUDING REMARKS

The method of SDSL provides the strategy for investigating the changes in protein structure. The main advantage of this method is the possibility to obtain detailed information directly from the attachment site of the spin label. At the same time, the spin label may, however, be sensitive to the global structural changes of the whole protein. Considering that these two levels of influence may be superimposed in the observed EPR spectra, the interpretation of the results requires careful analysis and the straightforward conclusions may not always be attainable. Nevertheless, several methodological approaches can be used to overcome these difficulties. One of them involves the comparative studies based on a series of derivatives spin-labeled at differ-

ent positions. In such a situation, the comparison of the series of the EPR spectra may allow us to extract the specific local structural information from all the background information contained in the spectra [16, 17].

Along the lines of this reasoning, in this study we introduced the spin label into yeast iso-1-cytochrome *c* at three different positions. One labeled position (L85C) differed significantly in the CW EPR spectrum, thermal melting, and CW EPR power saturation from two other positions (C102, S47C). This phenomenon may reflect the structural properties of the probed regions providing a promising starting point for further investigations.

The mutants of iso-1-cytochrome *c* were prepared by one of the authors in the laboratory of Dr. Wayne L. Hubbell from the University of California in Los Angeles. Therefore, we would like to express our gratitude to Dr. Hubbell for financial support, hospitality and many valuable discussions. We are also grateful to Dr. Michael Smith from the University of British Columbia for providing us with the shuttle plasmid pING4 used in this study. Krzysztof Murzyn's assistance in preparation of Fig. 1 is gratefully acknowledged.

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