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ARTICLE

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EPR studies of iso-1-cytochrome *c*: effect of temperature on two-component spectra of spin label attached to cysteine at positions 102 and 47

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Abstract Wild-type iso-1-cytochrome *c* from *Saccharomyces cerevisiae* containing naturally occurring cysteine at position 102 and mutated protein S47C (derived from the protein in which C102 had been replaced by threonine) were labeled with cysteine-specific methanethiosulfonate spin label. Continuous wave (CW) electron paramagnetic resonance (EPR) was used to examine the effect of temperature on the behavior of the spin label in the oxidized and reduced forms of wild-type cytochrome *c* and in the oxidized form of the mutated protein. The computer simulations revealed that the CW EPR spectrum for each form of cytochrome *c* consists of at least two components [a fast (F) and a slow (S) component], which differ in the values of the rotational correlation times $\tau_{R_\parallel}$ (longitudinal rotational correlation time) and $\tau_{R_\perp}$ (transverse rotational correlation time) and that the relative contributions of the F and S components of the spectra change with temperature. In addition, the values of the rotational correlation times ($\tau_{R_\parallel}$ and $\tau_{R_\perp}$) for the F component appear to change much more dramatically with the temperature than the respective values for the S component. A large difference between the behavior of the oxidized and reduced wild-type spin-labeled cytochromes *c* indicates that the temperature-induced unfolding of the protein in the region around C102 progresses more rapidly when cytochrome *c* is in the oxidized form.

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Introduction

Soluble *c*-type cytochromes provide an ideal focus to address questions concerning the conformational dynamics of proteins in solution. Among them, mitochondrial cytochrome *c*, because of its stability, solubility and ease of preparation, has become the subject of intense scrutiny using a wide range of experimental approaches and is considered as one of the most thoroughly characterized proteins (Margoliash and Schejter 1996).

In our studies, we have been using electron paramagnetic resonance spectroscopy (EPR) as a tool for probing the local dynamic structure of cytochrome *c*. In this approach, the placement of a spin label at a specific position within the protein allows us to monitor exclusively the chosen region of the protein, i.e. the local environment of the spin label (Hubbell et al. 1996; Mchaourab et al. 1996; Steinhoff and Hubbell 1996). The site specificity of the labeling can be achieved either by a combination of the chromatographic techniques to separate monoderivatives having the label attached to individual residues of the same type (e.g. lysines) or by the use of site-directed mutagenesis which introduces the unique attachment site (cysteine) into the desired position of the protein (SDSL). We employed chromatographic separations to modify mitochondrial horse cytochrome *c* (Turyna et al. 1998), while the SDSL technique was used in the case of iso-1-cytochrome *c* from *Saccharomyces cerevisaie* (Pyka et al. 1999).

The modification of horse cytochrome *c* on individual ϵ-amino groups of lysines showed that there were significant differences in the mobility of the spin labels attached to different lysines, even though all labeled residues exhibited a similarly high degree of surface exposure (Turyna et al. 1998). These results demonstrated

the high sensitivity of the spin labels in general and indicated that the surface of cytochrome *c* is not uniform but rather provides regions of different topology that may influence the tumbling of the attached probe in a highly distinct and specific manner. The modification of iso-1-cytochrome *c* on naturally occurring cysteine (C102 in the wild-type protein) or on the cysteine introduced into positions 47 or 85 (mutations S47C or L85C, respectively) revealed that the spin label attached to L85C differed from those attached to wild-type cytochrome *c* and S47C by having a two-component EPR spectrum (Pyka et al. 1999).

Taken together, the labeling of cytochrome *c* (both horse and yeast) at different positions appeared to produce EPR spectra with different contributions of the components of different mobility. Considering that the origin of these phenomena is not fully understood, we analyze here the EPR spectra recorded for wild-type iso-1-cytochrome *c* and the mutant S47C in the temperature range 4–46 °C. In addition, we examined the temperature-dependent behavior of spin-labeled ferri- and ferrocytochrome *c*. The reduced form of cytochrome *c* has not been examined in detail by means of spin labels, mainly due to the fact that it is experimentally challenging to provide the redox conditions that reduce the iron of heme *c* without reducing the attached spin label.

Materials and methods

Preparation of spin-labeled cytochromes and EPR measurements

Site-directed mutagenesis of iso-1-cytochrome *c* from *Saccharomyces cerevisiae*, protein purification and spin labeling were performed as described by Pyka et al. (1999).

Oxygen was removed from the solution of spin-labeled cytochrome *c* by bubbling with argon. All the solutions used for reduction were oxygen free and kept under an argon atmosphere during manipulations. Reduction of cytochrome *c* was performed after Hodges et al. (1974), with the following modification. The solution of $Fe(EDTA)^{2-}$ was prepared by dissolving $FeCl_2$ in 50 mM sodium phosphate, pH 7.0, containing 10 mM EDTA under an argon atmosphere to obtain 10 mM $Fe(EDTA)^{2-}$ solution. Typically 0.2 mL of the solution was added to 0.2 mL of spin-labeled cytochrome *c* (protein concentration about 2×10^{-4} M). All the reagents were removed from the solution of spin-labeled cytochrome *c* by centrifugal filtration (Ultrafree-MC, Millipore).

The EPR spectra were recorded on Bruker ESP-300E spectrometer fitted with a 4103TM/9103 cavity. Immediately after the reduction procedure, spin-labeled cytochrome *c* was loaded into the gas-permeable TPX plastic sample capillary. The measurements were performed under a nitrogen atmosphere. The temperature-dependent EPR spectra were recorded in the range 4–46 °C using the Bruker nitrogen flow temperature controller. During the measurements, care was taken to avoid lineshape distortions that could arise from the experimental conditions. The modulation amplitude was 0.02 mT and the microwave power was 2 mW or 8 mW for the CW EPR spectra. In order to improve the signal-to-noise ratio, the scans were accumulated if needed.

Curve fitting and calculations

A modified version of the program developed by Robinson et al. (1999a, 1999b) was used to extract the Lorentzian components of the spectral linewidth. A two-component spectrum was fitted to the experimental data. A value of 0.50 obtained from a computer simulation for free spin-label for a single Gaussian broadening function was used.

The Lorentzian linewidths obtained from the computer simulations were used to calculate the values of the correlation times: $\tau_{R_\parallel}$, which is the shorter time tumbling about a diffusion axis that allows faster tumbling, and $\tau_{R_\perp}$, which is the longer tumbling time perpendicular to diffusion axis (after Marsh 1989), with the nitroxide *y*-axis as the rapid axial diffusion direction (Qu et al. 1997).

Results

Figure 1 shows the CW EPR spectra of spin-labeled wild-type iso-1-cytochrome *c* in the oxidized and reduced states (Fig. 1a and Fig. 1c, respectively), as well as the spectrum of spin-labeled ferricytochrome *c* mutated at position S47 (Fig. 1b) recorded at room temperature. In addition to the experimental spectra (solid lines), the results of theoretical lineshape simulations are shown (dotted lines). It followed from the computer analysis that in each case the experimental spectra contain at least two distinguishable components of different linewidth: a fast (F) component (narrow lines) and a slow (S) component (broad lines). The comparison of the simulations for one- and two-component models for wild-type ferricytochrome *c*, shown in Fig. 2a and Fig. 2b, respectively, indicated the latter model generates better fits than the former one. Similar results were obtained for wild-type ferrocytochrome *c* and the mutant S47C. Therefore, in what follows we analyzed all data using the two-component model.

Figures 3, 4, 5 describe the effect of temperature on the behavior of spin-labeled forms of cytochrome *c* (the wild-type ferri- and ferrocytochrome *c* and the mutant S47C in the oxidized state). The Lorentzian linewidths obtained from the computer simulations were used to calculate the values of the correlation times ($\tau_{R_\parallel}$ and $\tau_{R_\perp}$) for both the F and S components. The results of these calculations are shown in Fig. 3. For all three forms of cytochrome *c* the values of $\tau_{R_\parallel}$ and $\tau_{R_\perp}$ obtained for the F component change much more dramatically with temperature than the values of $\tau_{R_\parallel}$ and $\tau_{R_\perp}$ obtained for the S component, which seem to remain more stable within the tested temperature region. Furthermore, there is a large difference between the behavior of the oxidized and reduced spin-labeled cytochrome *c*. The dependence of wild-type ferricytochrome *c* shows two clear phases (low- and high-temperature regions), with a transition point around 24 °C, while in the case of ferrocytochrome *c* the phases, if they exist, are much less distinguishable. It seems possible that a transition point of the latter form is much higher and falls within the upper limit of the tested temperature region (approx. 40 °C). The dependence of S47C ferricytochrome *c* seems to show two phases like the wild-type ferricytochrome *c*; however, the transition point of S47C is slightly shifted towards higher temperatures and has a value of approx. 28 °C.

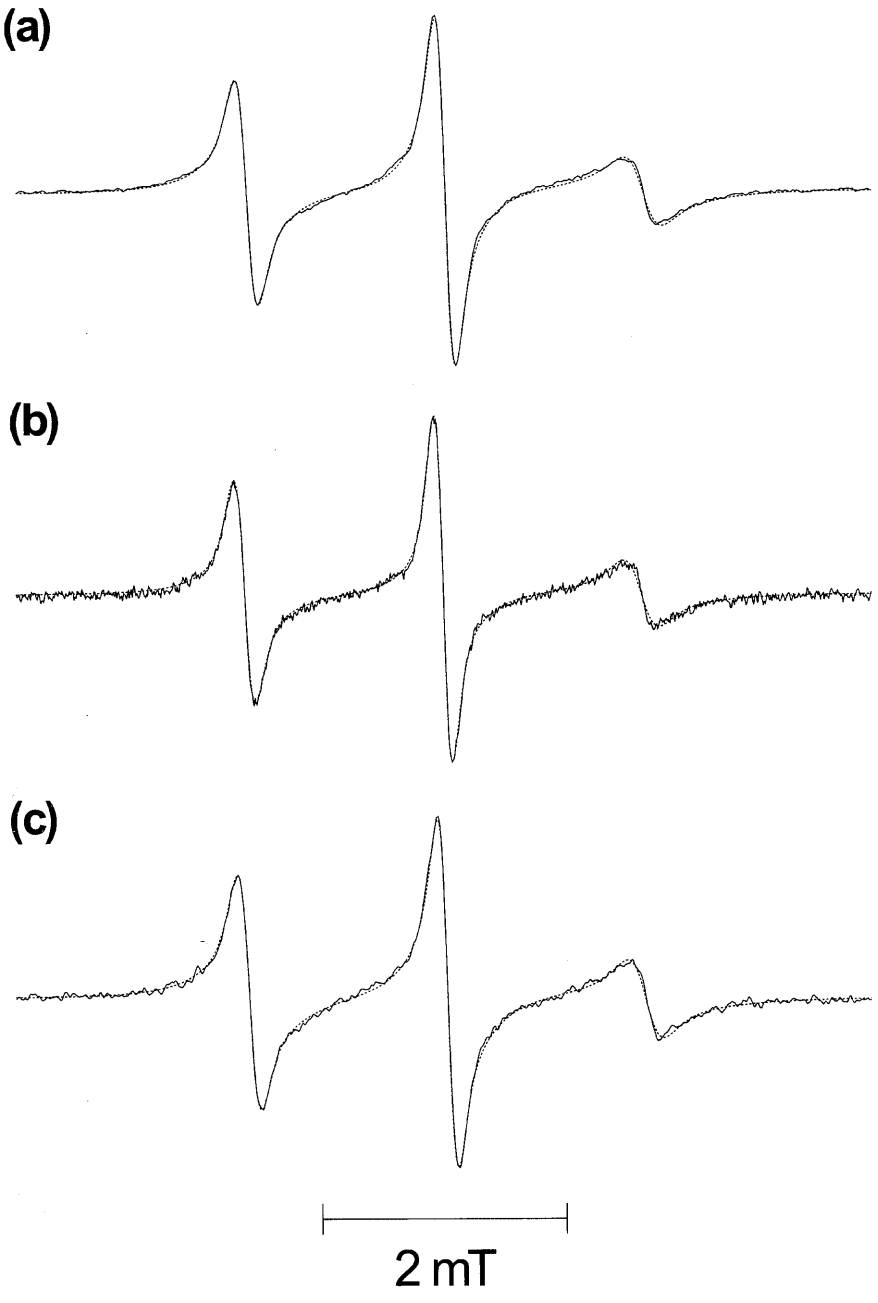

Fig. 1 The experimental (*solid lines*) and computer simulated (*dotted lines*) CW EPR spectra for the spin-labeled yeast iso-1-cytochrome *c*: wild-type cytochrome *c* (C102) in the oxidized state (**a**), cytochrome *c* mutated at the position S47 in the oxidized state (**b**) and wild-type cytochrome *c* in the reduced state (**c**) recorded at room temperature

As shown in Fig. 4, the relative contributions of the F and S components of the spectra of wild-type ferri- and ferrocytochrome *c* and the mutant S47C in the oxidized state show a clear dependence upon the temperature. In all cases the same tendency can be observed: increasing temperature decreases the contribution of the S component. Moreover, the splitting into two temperature regions seems to correlate with the biphasic behavior of the F component shown in Fig. 3. The two regions can be clearly recognized for ferricytochrome *c* (both wild-type and S47C). The low-temperature region (between 4 and 24 or 28 °C for wild-type or S47C, respectively) is characterized by a progressive decrease of the contribution of the S component to the benefit of the F component. At high temperatures above the transition point (over 24 or 28 °C for wild-type or S47C, respectively) the contribution of both S and F components remains constant.

In contrast to ferricytochrome *c*, the contribution of the S component for ferrocytochrome *c* decreases continuously over the entire tested temperature region (4–46 °C) and, similar to what was observed with the F component for this form (Fig. 3), the transition point seems to fall within the upper limit of the tested temperature region (approx. 40 °C).

The data shown in Fig. 4 were used to calculate the activation energy (E_a) for exchange between the two motional states of the spin label in all three forms of the

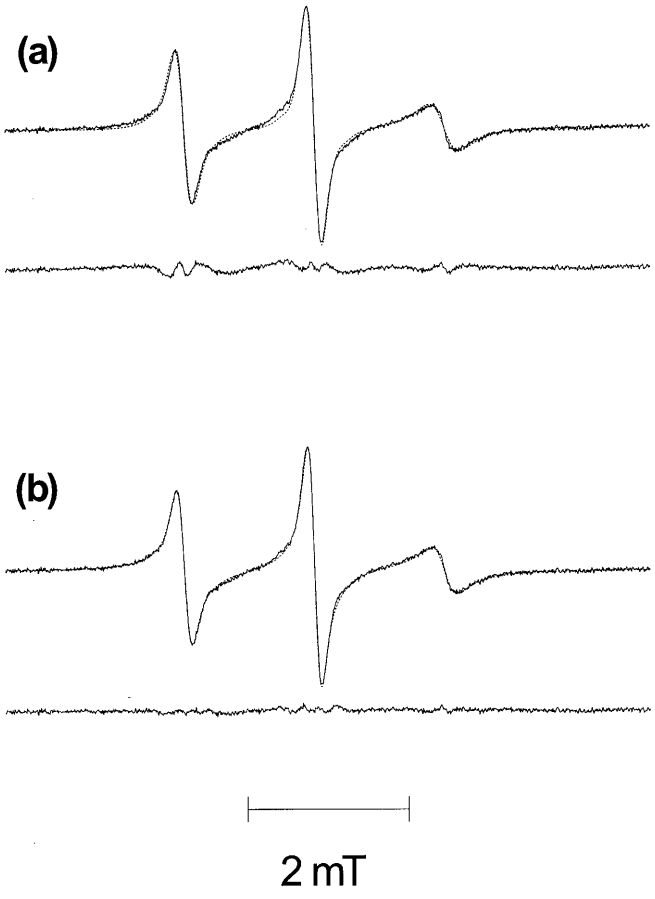

Fig. 2 Comparison of the simulated CW EPR spectra for one-component (**a**) and two-component (**b**) models (see text for details)

protein. The regions with a linear dependence were fit to the equation (Alonso at al. 2000):

$$\ln N_S/N_F = \ln A + E_a/RT \quad (1)$$

where A is a pre-expotential factor that reflects the ratio between the number of S and F components.

The values of E_a, determined from the slope coefficient of the curves shown in Fig. 4, are 10 ± 1 kcal/mol for the wild-type ferricytochrome *c*, 7 ± 1 kcal/mol for ferricytochrome S47C and 6 ± 1 kcal/mol for wild-type ferrocytochrome *c*. The values obtained are somewhat similar and appear to be associated with local environmental changes in the vicinity of the spin label (e.g. alteration in the pattern of hydrogen bonds and/or vibration modes of the polypeptide chains) rather than with the changes in the overall structure of the protein.

In order to confirm the presence of two components of the CW EPR spectra of the spin-labeled cytochromes used in this study, additional measurements in 50% sucrose were performed. As representatively shown in Fig. 5, the S component clearly emerges from the spectra recorded at different temperatures and is most detectable at lower temperatures.

Discussion

In this study, three forms of spin-labeled iso-1-cytochrome *c* were analyzed using EPR techniques and computer EPR spectra simulations. These forms included wild-type cytochrome *c* labeled in the oxidized and reduced states and the mutated form of cytochrome

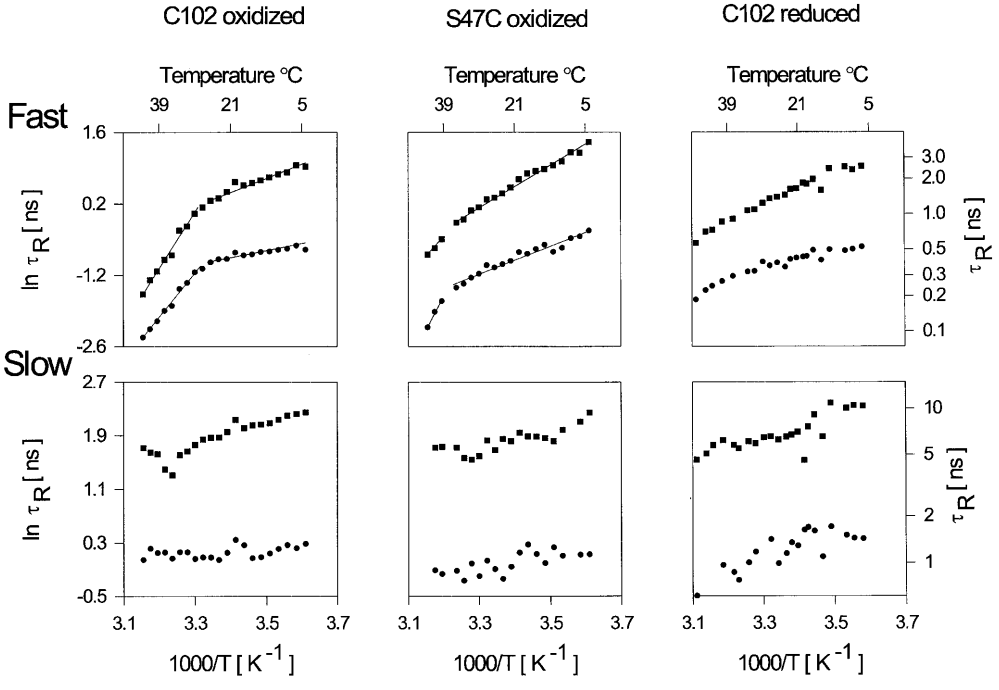

Fig. 3 The values of the rotational correlation times [$\tau_{R_\parallel}$ (●) and $\tau_{R_\perp}$ (■)] as a function of temperature obtained from the computer simulations for cytochrome *c* (the wild-type ferri- and ferrocytochromes *c* and the mutant S47C in the oxidized state) for the fast (F) and slow (S) components

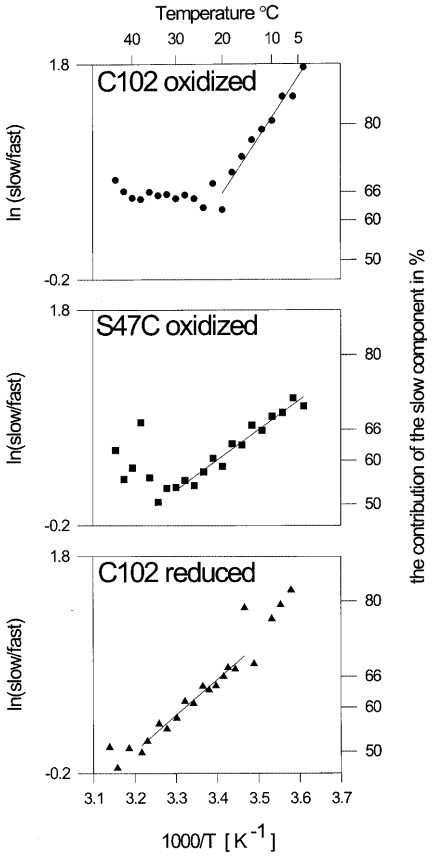

Fig. 4 The relative contributions of the fast (F) and slow (S) components of the spectra for cytochrome *c* (the wild-type ferri- and ferrocytochromes *c* and the mutant S47C in the oxidized state) as a function of temperature

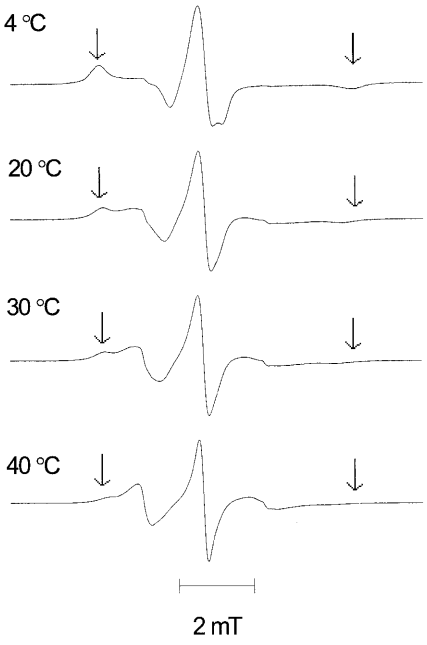

Fig. 5 CW EPR spectra for the spin-labeled wild-type yeast iso-1-cytochrome *c* in the oxidized state in 50% sucrose as a function of temperature. The slow component is denoted by *vertical arrows*

c (mutant S47C) labeled in the oxidized state. As shown in Fig. 1, the CW EPR spectra of all forms appear to be rather similar, indicating that both labeled positions (C102 in the wild-type, and C47 in the mutant S47C) provide similar conditions for the motion of the attached spin label. Moreover, the lineshape of the spectra is characteristic for the label located on the surface of the protein. While the three-dimensional structure of iso-1-cytochrome *c* shows that S47 is indeed exposed on the surface of the protein, the SH group of C102 appears to be rather inaccessible to the solvent (Louie and Brayer 1990). However, C102, as the penultimate C-terminal amino acid, may have high conformational flexibility. Thus, the incorporation of the spin label at this position may induce some perturbations and unwinding of the C-terminal α-helix, exposing C102 considerably more to the surface of the protein. Such a change has been modeled for spin-labeled iso-1-cytochrome *c* (Qu et al. 1997) and provides one possible explanation for the mobility of the spin label attached to C102, comparable to that observed in the mutant S47C. It is also possible that, in solution, C102 is naturally more exposed on the surface of the protein when compared to its location shown by the X-ray structure (Louie and Brayer 1990). Consequently, only minor structural changes occur upon the binding of the label to C102.

The results of computer simulations (Figs. 1 and 2) suggest the presence at least of two components of different mobility [fast (F) and slow (S)] in the EPR spectra of both wild-type ferri- and ferrocytochrome *c* and the mutant S47C. The broader of the spectral components (S) corresponds to a transverse rotational correlation time ($\tau_{R_\perp}$) of about 8 ns at room temperature, while the other more mobile component (F) has a transverse rotational correlation time ($\tau_{R_\perp}$) of about 2 ns at room temperature. The two components, having close values of the parameters, completely overlap and are fairly distinguishable in the experimental spectra recorded under standard conditions (room temperature, sodium phosphate buffer). However, increasing the viscosity and lowering the temperature enhances the splitting of the two components, which under certain conditions become clearly visible (Fig. 5). This provides additional indication that the spectra are composed of more than one component.

It should be noted that in our previous study the two-component CW EPR spectra were also obtained for spin-labeled horse mitochondrial cytochrome *c* (Turyna et al. 1998) and yeast iso-1-cytochrome *c* labeled at position 85 (the mutant L85C) (Pyka et al. 1999). In some cases the presence of more than one component clearly emerges from the spectrum (the mutant L85C and horse cytochrome *c* labeled at K72); in others it cannot be clearly recognized without computer simulations (horse cytochrome *c* labeled at K86 or K25). Considering that the complexity of the movement of the spin label attached to different residues of both horse and yeast

cytochrome *c* may result from the restrictions provided by the protein local environment, it seems remarkable that the spin label is able to detect such subtle differences even when it is attached to the surface-exposed residues of the globular protein in solution.

The influence of the temperature on the behavior of all three forms of spin-labeled cytochrome *c* used in this study (expressed as the changes in the values of the correlation time) shows that unfolding the protein (Fig. 3) causes the spin label to move more freely. This is mainly reflected in a decrease of the values of the correlation times ($\tau_{R_\parallel}$ and $\tau_{R_\perp}$). In our model, such dependency can be clearly observed only for the F component. In the case of the S component, the correlation times seem to show the same tendency but the dependency is much weaker. It should be noted, however, that while the absolute values of $\tau_{R_\parallel}$ and $\tau_{R_\perp}$ for the F component are within the regime for which the model is applicable (fast-motion regime), the values of $\tau_{R_\parallel}$ and $\tau_{R_\perp}$ for the S component are rather within the slow-motion regime for which the model is less reliable. Thus, the results of the simulations for the S component should be interpreted with more caution.

The most interesting observation with regard to the dynamic structure of cytochrome *c* is that the oxidized and reduced states of cytochrome *c* show different temperature dependency of $\tau_{R_\parallel}$ and $\tau_{R_\perp}$ for the F component (Fig. 3). In the high-temperature region the values of $\tau_{R_\parallel}$ and $\tau_{R_\perp}$ for the F component decrease much more dramatically for ferricytochrome *c* than for ferrocytochrome *c*, pointing towards some oxidation-state-related differences in the temperature-induced unfolding of the protein in the region around C102. As the decrease in the value of $\tau_{R_\parallel}$ and $\tau_{R_\perp}$ reflects higher mobility of the spin label, the differences in the above dependencies indicate that this process may progress more rapidly in ferricytochrome *c* than in ferrocytochrome *c*. To our knowledge, these results represent the first report on the differences in the behavior of spin-labeled cytochrome *c* associated with its oxidation state. They suggest that the conformational mobility of the region around C102 depends on a redox state of the protein with an increased flexibility characterizing ferricytochrome c. This agrees well with other studies on the redox-state-related changes in conformational dynamics of cytochrome *c* which find the protein backbone (C-terminal helix in particular) to be more dynamic in the oxidized state (Berghuis and Brayer 1992; Banci et al. 1997; Fetrow and Baxter 1999). It should be noted that our comparison of the two states of cytochrome *c* is based on the label attached to the same position within the protein. This makes the contribution of the multiple conformational states of the spin label itself rather negligible.

On the other hand, the profiles of the F component for the two oxidized forms of cytochrome *c* labeled at two distinct positions (C102 and S47C) also show some significant differences: the transition point is shifted towards higher values in the S47C mutant (Fig. 3). These changes indicate that unfolding of different regions of the protein influences the motion of the spin label in a highly distinct and specific manner. However, in this case the data should be interpreted with more caution since they concern the label that is not attached to the same position of the protein. Consequently, the possibility of contributions from multiple conformational states of the spin label itself should also be taken into account.

In the light of experimental data on the liganding properties of heme proteins (Angström et al. 1982; Moore and Pettigrew 1990), it is possible that the thermal denaturation of cytochrome imposed by our experimental conditions induces a transition in the state of the heme iron (from low- to high-spin form), resulting from the change in its sixth axial ligation. However, the occurrence of such a transition can be neither verified nor analyzed by our model, given that the contribution from dipolar coupling between the spin label and the spin state of the heme iron is expected to be the same for each linewidth of the spectrum. Consequently, it will not influence the obtained values of the correlation times.

As shown in Fig. 4, the relative contribution of the F and S components of the spectra strongly depends upon temperature. Furthermore, a temperature-independent region can be clearly distinguished in the case of oxidized cytochrome *c* (both wild-type and S47C). As the occurrence of these two regions strongly correlates with the behavior of the corresponding F components (see Fig. 3), we are tempted to speculate that these regions may reflect two transitional states of the protein. The explanation of the nature of the occurrence these states will be addressed in further studies.

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