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The source of heterogeneity in the heme vicinity of ferricytochrome *c*

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Abstract

Heterogeneity in the heme vicinity of ferricytochrome *c* was reported to be detectable by a split of the NMR signal of the heme methyl 3 group [P.D. Burns and G.N. La Mar, J. Am. Chem. Soc. 101 (1979) 5844]. Using cytochrome *c* mutants and computer simulations of the native and mutated cytochromes, the source of this heterogeneity is found to originate from the His-33 residue motions. The H33F mutation abolished the NMR split and computer simulations of the H33F mutant revealed a narrower distribution of fluctuations of the radius of gyration, suggesting a more rigid structure due to the mutation. The stabilization of the mutant was further demonstrated by a reduction in the H33F mutant of 4 Kcal/mol in the calculated interaction energy between residue 33 and the rest of the cytochrome, in keeping with known experimental results [W. Qin, R. Sanishvili, B. Plotkin, A. Schejter and E. Margoliash, Biochim. Biophys. Acta 1252 (1995) 87].

Keywords: Cytochrome *c* chemistry; NMR; Molecular dynamics; H33F mutant

1. Introduction

Heterogeneity in the environment of heme methyl groups in cytochrome *c* was detected using proton NMR by a split of the peak of the methyl 3 group [1,2]. The split peak was attributed to two exchanging conformations of the cytochrome with a pK value of about 7 that was measured at −7°C using a 20% methanol solution [1,2].

Several residues were suggested to cause this heterogeneity including His-26, His-33, Phe-82, the salt bridge Lys-13-Glu-90 and residue 46 (that varies between different species) [1–4]. His-26

Abbreviations: TSP, 3-(Trimethylsilyl)propionic-2,2,3,3,-*d*4 acid, sodium salt

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was ruled out as the affecting factor by Moench et al. [4] and Angstrom et al. [5] since its low pK did not match the value found by Burns and La Mar [2].

In order to determine the source of the described heterogeneity we used site directed mutations in a couple of relevant residues, and compared the NMR proton spectra of native rat cytochrome *c* and two rat cytochrome *c* mutants, H26Q and H33F [6,7]. These two mutants are stable, possessing an alkaline pK and $T_{1/2}$ of disappearance of the 695 nm band, very close to that of native rat cytochrome [6,8]. Substituting phenylalanine for the His-33 in the rat protein causes a global stabilization resulting in an increase of the free energy of denaturation by urea, relative to the native cytochrome [8].

Computer simulations of the native and H33F mutant cytochromes *c* were performed in order to allow a finer analysis, on the atomic level, of the differences between the mutant and the native cytochrome and of the origin of the described heterogeneity. The simulations included a comparison of the fluctuations of the radius of gyration and the interaction energy of residue 33 with the rest of the protein, in the models of the native and mutant cytochromes. The computer simulations were made using the coordinates of horse heart cytochrome *c,* which has a sequence identity of 95% with that of rat cytochrome *c* [9,10].

2. Experimental procedures

2.1. Materials

Horse heart and rat cytochromes *c* were purchased from Sigma. Recombinant rat H26Q and H33F mutants were prepared as described elsewhere [6,7]. All cytochrome solutions (wild type and mutants) were prepared in deuterium oxide ($\sim 98\%$ D_2O). The pH was adjusted with NaOD; the pH values are the pH readings given without correction for the deuterium isotope effect or the minor temperature drift. Small amounts of potassium ferricyanide were added to the solutions to insure full oxidization of the cytochrome. The concentrations of the rat wild type and mutants solutions were 1 ± 0.5 mM and of the horse cytochrome solutions were 5 mM.

2.2. NMR

Proton NMR spectra were acquired on a Bruker ARX500 NMR spectrometer with a 5-mm QNP probe. Chemical shifts are given in ppm from TSP where the peaks of interest are assigned according to known horse cytochrome *c* assignments [11,12].

The exchange rate was estimated using a modification of the inversion transfer sequence [13,14] in which the selective 180° pulse (required for selective inversion of one of the exchanging peaks) is replaced by two 90° pulses separated by a delay:

$$90^{\circ} - \frac{1}{2\Delta f} - 90^{\circ} - \tau - 90^{\circ} - acq \qquad (1)$$

where τ is a variable interval, Δf is the separation in Hz between the two exchanging peaks and the carrier frequency is set on the inverted peak. Peaks that are not resolved enough for regular inversion transfer pulse sequence can be selectively inverted using the described modified pulse sequence.

2.3. Molecular dynamics

Molecular dynamics simulations of the native cytochromes *c* and the cytochromes *c* H33F mutant were carried out using the CHARMM molecular dynamics program (version 23) [15]. The models were based on the native horse heart cytochrome *c* X-ray structure [16] (Protein Data Bank [17] entry 1hrc) including six structurally preserved water molecules [16,18], embedded in a 25-Å sphere of TIP3P water molecules [19] using stochastic boundary conditions [20,21]. The total number of atoms in the simulation was 6279, including 1504 water molecules.

Both native cytochrome *c* and its H33F mutant, including their water spheres, were gradually heated to 280 K over a period of 5 ps and then equilibrated at 280 K for 50 ps. The systems were then simulated for 200 ps at 280 K from which conformations were sampled every 0.5 ps. A total

of 400 conformations was collected from the molecular dynamics trajectories of each system. All simulations were performed using the SHAKE algorithm, a dielectric constant of $\varepsilon = 1$ and a 10-Å energy cutoff.

3. Results

In order to enhance the observation of the split in the NMR heme methyl-3 peak of cytochrome *c*, we minimized the exchange rate between the two exchanging conformations by reducing the temperature. Under conditions of high pH values (pH reading of 9.4) and 5°C, rat cytochrome *c* exhibits a split of the heme methyl-3 peak (Fig. 1a), similar to that observed in many species by Burns and La Mar and with the same 2:1 integral ratio [2]. The same split is observed for the rat H26Q mutant (not shown) indicating heterogeneity in the heme environment and thus ruling out the attribution to this residue of the splitting phenomenon, in agreement with earlier conclusions [4,5]. For the rat H33F mutant (Fig. 1b) the methyl 3 peak in the spectrum is similar in height and width to that of the methyl 8 peak and does not exhibit any special split or broadening.

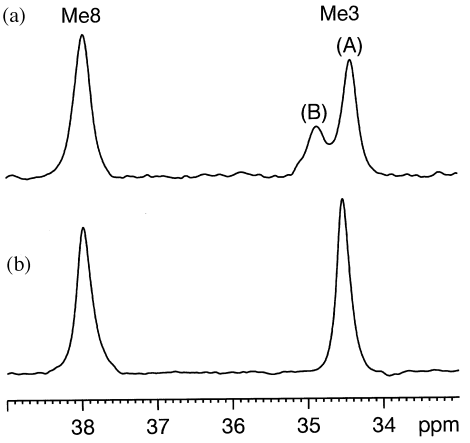

Fig. 1. ^{1}H-NMR spectra (500 MHz, deuterated solutions, 5°C, pH reading: 9.4) of the low field section, containing heme methyls 3 and 8 peaks, of (a) rat ferricytochrome *c* and (b) rat ferricytochrome *c* H33F mutant. The split peaks of Me3 are designated by (A) and (B). Chemical shifts are given in ppm from TSP following previous assignments [11,12].

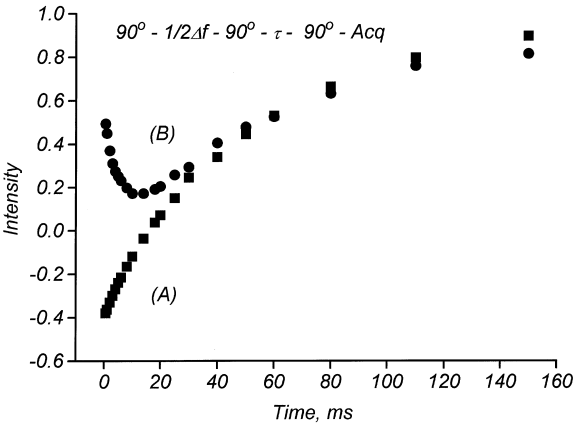

Fig. 2. Inversion transfer experiment performed on the methyl-3 peak of horse ferricytochrome *c* (500 MHz, deuterated solution, 5°C, pH reading: 9.5). The Me3 (A) signal was inverted and the measured intensities of the (A) (■) and (B) (●) Me3 signals are given as a function of the recovery time, τ. In the inversion transfer sequence [13,14] the selective 180° pulse is replaced by two 90° pulses separated by a delay of $1/2\Delta f$, where Δf is the distance in Hz between the two exchanging peaks and the carrier frequency is set on signal (A).

The exchange between the two cytochrome *c* conformations was substantiated using an inversion transfer experiment [13,14], applying the modified pulse sequence given in the experimental section. The inversion transfer sequence was applied on the split peak of the heme methyl-3 of horse cytochrome *c* at 5°C and pH reading of 9.5. From the initial slope of the resulting (B) curve [13] (see Fig. 2) we estimated an exchange rate of 90 s^{-1}.

Molecular dynamics simulations of the native and mutated H33F cytochromes *c* were carried out using the CHARMM molecular dynamics program [15]. The models were based on the horse cytochrome *c* X-ray coordinates [16], obtained from the Protein Data Bank [17], embedded in a 25-Å water sphere. The phenylalanine side-chain in the H33F mutant was modeled based on the His-33 coordinates of the native cytochrome. The systems were simulated for 200 ps at 280 K and a sample of 400 conformations was collected from the resulting molecular dynamics trajectory.

Two properties were chosen to assess the effect

of the mutation on the relative rigidity of the proteins: (1) the interaction energy of residue 33 with the protein; and (2) the fluctuations of the protein radius of gyration.

Table 1 shows the averaged interaction energies between residue 33 and the rest of the cytochrome molecule. The interaction energy of the H33F mutant is 4 Kcal/mol lower than that of the native cytochrome. From this table, one sees that the excess stabilization energy originates in nearly equal parts from van der Waals interactions and electrostatic interactions (Table 1).

Fig. 3 depicts the distribution of fluctuations of the radius of gyration for both simulations. The radius of gyration for the native and H33F mutant were 12.822 ± 0.045 Å and 12.842 ± 0.035 Å, respectively. The two fluctuation profiles, fitted to a Gaussian distribution, are clearly different with 2σ of 0.077 ± 0.002 Å for the H33F mutant and 0.092 ± 0.003 Å for the native cytochrome. The difference in the fluctuation profiles indicates that the magnitude and distribution of the fluctuations in the native cytochrome are significantly larger than in the H33F mutant. The narrow distribution of fluctuations, as seen for the H33F mutant, corresponds to a more rigid structure.

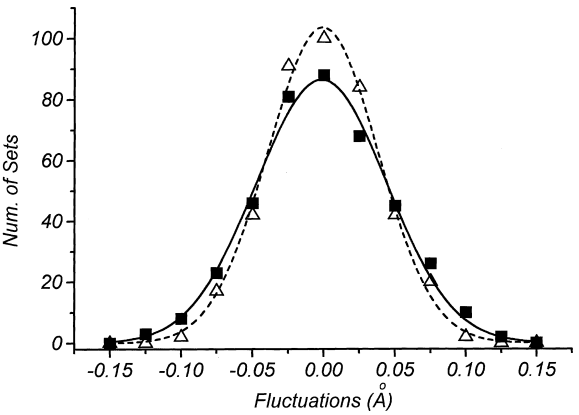

Fig. 3. The distribution of the fluctuations of the radius of gyration for the native cytochrome (squares) and the H33F mutant (triangles). The models included the native protein and the H33F mutant immersed in a 25 Å TIP3P water sphere (containing 1504 water molecules). The systems were equilibrated for 50 ps at 280 K. Than, 400 coordinate sets were collected from a 200 ps molecular dynamics trajectory using a 1 fs time step. The radius of gyration for the native and H33F mutant were 12.822 ± 0.045 Å and 12.842 ± 0.035 Å respectively. The fluctuations were collected using 0.025 Å bins. The fitting resulted in centered, equaled area (10.0 ± 0.2) Gaussians with 2σ of 0.077 ± 0.002 Å for the H33F mutant (dashed) and 0.092 ± 0.003 Å for the native cytochrome (solid line).

4. Discussion

The experimental and theoretical results reported above clearly demonstrate that the factor determining the presence of cytochrome *c* in two different conformations, observed by NMR, is indeed histidine 33.

Since the split in the methyl 3 peak remains constant, with a ratio of about 2:1, at pH values that are two pH units higher than the pK of histidine deprotonation [2] (see Fig. 1), the change of protonation state of histidine 33 cannot explain the formation of the two cytochrome conformations. Furthermore the exchange rate of 90 s^{-1} at 5°C, presented in this paper, and the value of 1000 s^{-1} found by Burns and La Mar at 25°C [2], are much slower than those expected for a normal deprotonation process, and must result from a structural rearrangement transmitted through the protein backbone, or through the hydrogen bonds network.

We propose that the histidine 33 side chain, regardless of its protonation state, shifts between two structural conformations which, in turn, are transmitted to the heme surroundings; a possible mechanism of transmitting the changes in the His-33 vicinity to the heme environment was suggested by Moench et al [4].

The additional stabilization of the H33F mutant, experimentally demonstrated by Qin et al. [8], is in keeping with the decrease of interaction energy, between Phe-33 and the rest of the cy-

Table 1
The interaction energy between residue 33 (His or Phe) and the rest of the cytochrome *c* molecule

	Interaction energy (Kcal/Mol)		
	Total	van der Waals	Electrostatic
Native	-50.5 ± 3.9	-11.2 ± 2.0	-40.7 ± 4.1
H33F	-54.5 ± 3.5	-13.4 ± 2.1	-42.5 ± 4.0
Difference	4	2.2	1.8

tochrome, observed in our simulations. Furthermore, the stabilization is also reflected in the significant reduction in protein motions, shown by the narrowing of the distribution of the radius of gyration fluctuations, described above. The mutant structure appears to gain rigidity because Phe-33 fixes the cytochrome structure in a single, more stable conformation, resulting in a single methyl-3 peak in the NMR spectrum.

We conclude, therefore, that events affecting the structure of cytochrome *c* around position 33, travel along or across the protein fabric towards the opposite side of the heme, causing changes in the behavior of atoms located in the latter region. A similar mechanism of intramolecular communication between different areas of the protein surrounding the heme was already implied by the behavior of mutants of P30 and H26, as described elsewhere [7,8,22].

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