Biophysical Chemistry 17 (1983) 193–202
Elsevier Biomedical Press

A SPIN LABEL STUDY OF CONFORMATIONAL CHANGES IN CYTOCHROME *c*

G.B. POSTNIKOVA, N.P. GORBUNOVA and M.V. VOLKENSTEIN

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Pushchino-on-Oka, Moscow Region, U.S.S.R.

Received 6th July 1982
Revised manuscript received 24th November 1982
Accepted 8th December 1982

Key words: Spin label; Cytochrome c; Conformational change

Spin-labeled pig heart cytochromes *c* singly modified at Met-65, Tyr-74 and at one of the lysine residues, Lys-72 or Lys-73, were investigated by the ESR method under conditions of different ligand and redox states of the heme and at various pH values. Replacement of Met-80 by the external ligand, cyanide, was shown to produce a sharp increase in the mobility of all the three bound labels while reduction of the spin-labeled ferricytochromes *c* did not cause any marked changes in their ESR spectra. In the pH range 6–13, two conformational transitions in ferricytochrome *c* were observed which preceded its alkaline denaturation: the first with pK 9.3 registered by the spin label at the Met-65 position, and the second with pK 11.1 registered by the labels bound to Tyr-74 and Lys-72(73). The conformational changes in the 'left-hand part' of ferricytochrome *c* are most probably induced in both cases by the exchange of internal protein ligands at the sixth coordination site of the heme.

1. Introduction

In order to understand the manner of functioning of protein molecules, detailed studies of their conformational properties are required. Incorporation of spin labels into a definite part of the molecule surface allows one to gain information concerning conformational changes in the region of interest induced by various factors. Similar testing of different parts of the structure enables one to describe, in principle, the conformational behavior of a rather large portion of the molecule or even a macromolecule as a whole.

The present work deals with the effects of pH, the valence and ligand states of the heme in pig heart cytochrome *c* on the mobility of the spin labels bound covalently to Met-65, Tyr-74 and to one of the lysine residues, Lys-72 or Lys-73. The chemical modification of the methionine residue was done using a paramagnetic analog of bromoacetate, and that of the tyrosine and lysine residues using a paramagnetic analog of *N*-acetylimidazole. The residues modified by the spin labels are situated in the left-hand side of the cytochrome *c* molecule, in the conventional representation of its structure shown in fig. 1.

No marked changes in the conformation of the region studied were found to occur on transition from ferricytochrome *c* to ferrocytochrome *c*. However, replacement of the sixth ligand of the heme, Met-80, in ferricytochrome *c* by the external ligand, CN^-, leads to a sharp increase in the mobility of all three labels, indicating a significant change in the conformation of this region. In the pH range 6–13, the spin labels registered two pH-induced conformational transitions in ferricytochrome *c* which preceded its alkaline denaturation. The first transition with pK 9.3 was caused by the replacement of Met-80 by another protein ligand, most probably Lys-79. This conformational change does not seem to be significant, since it was registered only by the label at the Met-65 position whereas the mobility of the two other labels at the position of Tyr-74 and Lys-72(73) remained unchanged. These labels registered a new conformational transition with pK 11.1. This transition was probably induced by ionization of the 'buried' Tyr-67 residue and resulted in substantial

0301-4622/83/0000-0000/$03.00

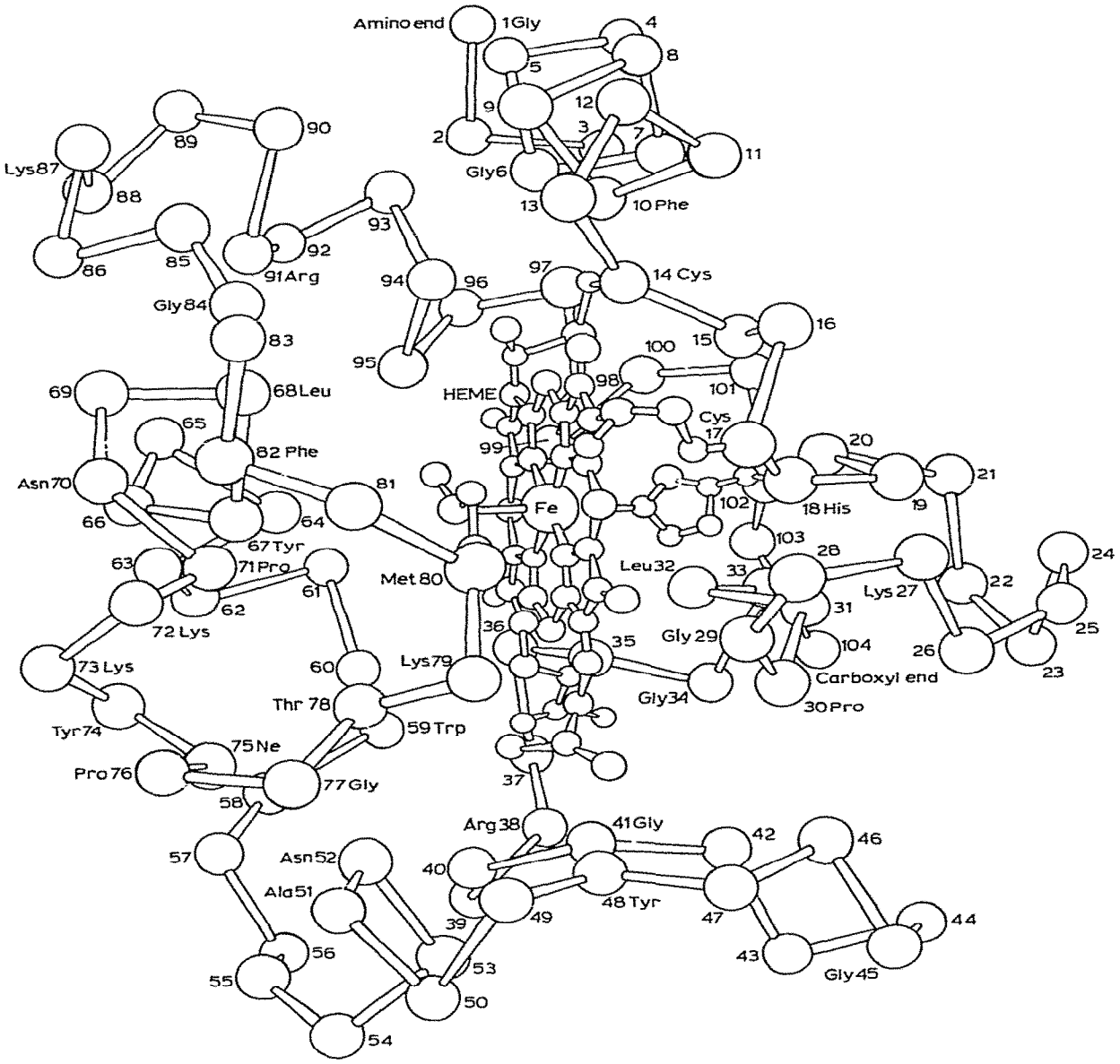


Fig. 1. Spatial structure of horse heart cytochrome c at a 2.8 Å resolution [1].

changes in the conformation of the left-hand side of the molecule.

Preliminary results of the present work have been reported in brief elsewhere [2].

2. Materials and methods

2.1. Materials

Ferricytochrome *c* from pig heart (Biomex, Poland) was dissolved in water, the pH of the solution was rapidly adjusted to 11.5–12.0 and after 5–10 min returned to pH 7. After extensive dialysis against 0.01 M Tris-HCl buffer, pH 8.5, the cytochrome *c* solution was passed through a DEAE-cellulose column equilibrated with the same buffer. The ferricytochrome *c* thus purified was additionally fractionated on CM-Sephadex C-25 with a stepwise gradient of phosphate buffer, pH 7, from 0.01 to 0.2 M. The main fraction of ferricytochrome *c* which was eluted with 0.2 M buffer and accounted for 90% of the applied protein was then freed from salt by extensive dialysis against double distilled water and lyophilized.

The spin labels, 2,2′,6,6′-tetramethyl-4-bromoacetoxypiperidine-1-oxyl (SL_1) and 1-(2,2′,5,5′-tetramethyl-3-carboxypyrroline-1-oxyl)imidazole (SL_2), were synthesized by methods described earlier [3,4].

KH_2PO_4 and Na_2HPO_4 were twice recrystallized to prepare phosphate buffer; KCN (Reanal, Hungary) and NaOH (purest grade) were used without additional purification.

2.2. Chemical modification

The reaction of ferricytochrome *c* with the label SL_1 was performed in 0.2 M phosphate buffer, pH 5, in the presence of a 10-fold molar excess of the reagent at room temperature according to Azzi et al. [5]. After 3 days, excess reagent was separated by gel filtration through a Sephadex G-25 column equilibrated with 0.01 M phosphate buffer, pH 7. The extent of modification of cytochrome *c* was approx. 10%.

Ferricytochrome *c* derivatives modified at Tyr-74 and Lys-72(73) by the label SL_2 were prepared as described earlier [4]. The reaction was carried out in the presence of a 30-fold molar excess of the reagent and 10^{-3} M imidazole in 0.02 M phosphate buffer, pH 7.5, at room temperature for 24 h. Low molecular weight substances were removed by gel filtration through a Sephadex G-25 column equilibrated with 0.01 M phosphate buffer, pH 7, then the reaction mixture was fractionated on CM-Sephadex C-25 with elution by a stepwise gradient of phosphate buffer, pH 7, from 0.01 to 0.2 M.

2.3. Spectral methods

ESR spectra were measured on a home-made spectrometer EPR-2 (3 cm) equipped with a multichannel analyser LP-4050 (Finland) at room temperature. Registration conditions were: klystron power, 1.6 mW; amplitude of high-frequency modulation 1 G; RC, 0.1 s. The concentration of the protein-bound iminoxyl radicals was estimated by comparing the intensities of central components in the ESR spectra of modified cytochromes *c* at pH 12 and of a free radical reference solution. The rotatory correlation times (τ_c) of the bound labels were calculated from the formula for weakly immobilized radicals [6]:

$$\tau_c = 6.65 \Delta H_{+1} \left[\sqrt{\frac{J_{+1}}{J_{-1}}} - 1 \right] \times 10^{-10} \text{ s}$$

where J_{+1} and J_{-1} are the heights of the low- and high-field components, respectively, of the ESR spectrum in arbitrary units, and ΔH_{+1} is the line width of the low-field peak in gauss.

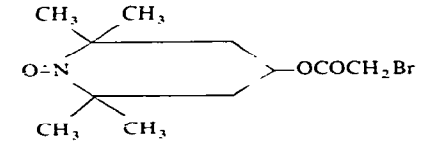
The concentration of cytochrome *c* was measured on a spectrophotometer (Specord UV VIS, G.D.R.) using $\epsilon_{550} = 27\,700$ M^{-1} cm^{-1}. The pH titration was carried out in 0.01 M phosphate buffer in the presence of 0.2 M KCl in order to eliminate the effects of the charge environment on ESR spectra of the labels. The pH values were measured with a home-made pH-meter (PY-01) with an accuracy of ± 0.05 pH units.

3. Results and discussion

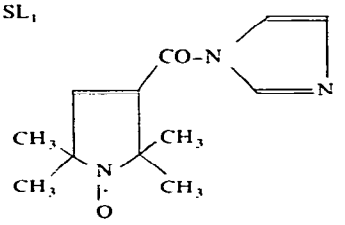
3.1. Characteristics of the spin-labeled cytochromes c

According to Azzi et al. [5], under modification conditions, the alkylating spin label SL_1 binds only to Met-65 of ferricytochrome *c*. Any other products of the modification of cytochrome *c*, as

the amino acid analysis shows, are not formed. The low yield of the spin-labeled cytochrome *c* ($\approx 10\%$) seems to be due to some steric hindrance to the reagent reacting with Met-65.



SL_1



SL_2

Ferricytochrome *c* spin labeled at Met-65 does not differ from the intact protein, as judged by the properties of the heme complex, the structure of the heme environment and the conformation of the molecule as a whole [5,7]. Both native and spin-labeled cytochromes *c* have similar absorption spectra in the Soret and visible regions. They possess also the same chromatographic mobility when fractionated on CM-Sephadex C-25 so that they cannot be separated by this procedure. Boswell et al. [8] have shown that chemical modification of Met-65 causes an extremely small perturbation to a part of the molecule close to the site of modification.

The imidazolide spin label SL_2 reacts with ferricytochrome *c* under the studied conditions to form two singly modified derivatives, one on the Tyr-74 residue, SL_2(Tyr-74)-cytochrome *c*, and the other on one of two adjacent residues Lys-72 or Lys-73, SL_2(Lys-72/73)-cytochrome *c*. The preparation and properties of these spin-labeled derivatives are described in detail elsewhere [4]. As determined by optical spectroscopy, circular dichroism and high-resolution NMR techniques, the binding of the label SL_2 in both cases does not produce any marked perturbations in the structure of the heme crevice and in the total conformation of the protein moiety though there are small structural changes at the modification sites. Like intact ferricytochrome *c*, the modified derivatives show in the NMR spectra a transition with pK 9.3 induced by replacement of Met-80 at the sixth ligand position of the heme by another protein ligand. We have shown that the thermal stability of ferricytochrome *c* at temperatures up to 60°C is unaffected by acylation of Tyr-74 or Lys-72(73) by the label SL_2 [9].

3.2. ESR spectra of spin-labeled cytochromes c. Effects of the heme iron reduction, addition of cyanide and variation in pH of the medium

Fig. 2 (b–d) presents the ESR spectra of ferricytochrome *c* modified by the labels SL_1 and SL_2 at Met-65, Tyr-74 and Lys-72(73); the values of τ_c are $(5.1 \pm 0.5) \times 10^{-10}$, $(7.1 \pm 0.7) \times 10^{-10}$ and $(6.4 \pm 0.6) \times 10^{-10}$ s, respectively. In all cases the covalent binding gives rise to weak immobilization of the radicals. The movement of the label SL_2 bound to the tyrosine and lysine residues is restricted to a greater extent than that of the label SL_1 attached to the methionine residue, since SL_1 has a longer and a more flexible 'leg' (see left column). When comparing the two SL_2 derivatives of cytochrome *c*, the rotatory diffusion of the radical SL_2 bound to Tyr-74 is more impeded.

Upon reduction of SL_1(Met-65)-cytochrome *c*, SL_2(Tyr-74)-cytochrome *c* and SL_2(Lys-72/73)cytochrome *c* by $LiBH_4$ in 0.1 M phosphate buffer, pH 7, the rotatory correlation times of the labels remain unaltered within the limits of experimental error, and are $(4.8 \pm 0.5) \times 10^{-10}$, $(6.9 \pm 0.7) \times 10^{-10}$ and $(6.5 \pm 0.6) \times 10^{-10}$ s, respectively. Similar results were obtained on reduction of ferricytochrome *c* spin labeled at Met-65 with oxymyoglobin in 0.01 M phosphate buffer; the derivatives of ferricytochrome *c* modified at Tyr-74 and Lys-72(73) cannot be reduced with oxymoglobin [10].

Replacement of Met-80 by CN^- in ferricytochrome *c* causes a sharp increase in the mobility of all the three labels (Fig. 2e–g) which approaches the mobility of a free label in solution, $\tau_c = 3 \times 10^{-11}$ s (Fig. 2a). For cyanide complexes of SL_1(Met-65)-cytochrome *c*, SL_2(Tyr-74)-cytochrome *c* and SL_2(Lys-72/73)-cytochrome *c*, the

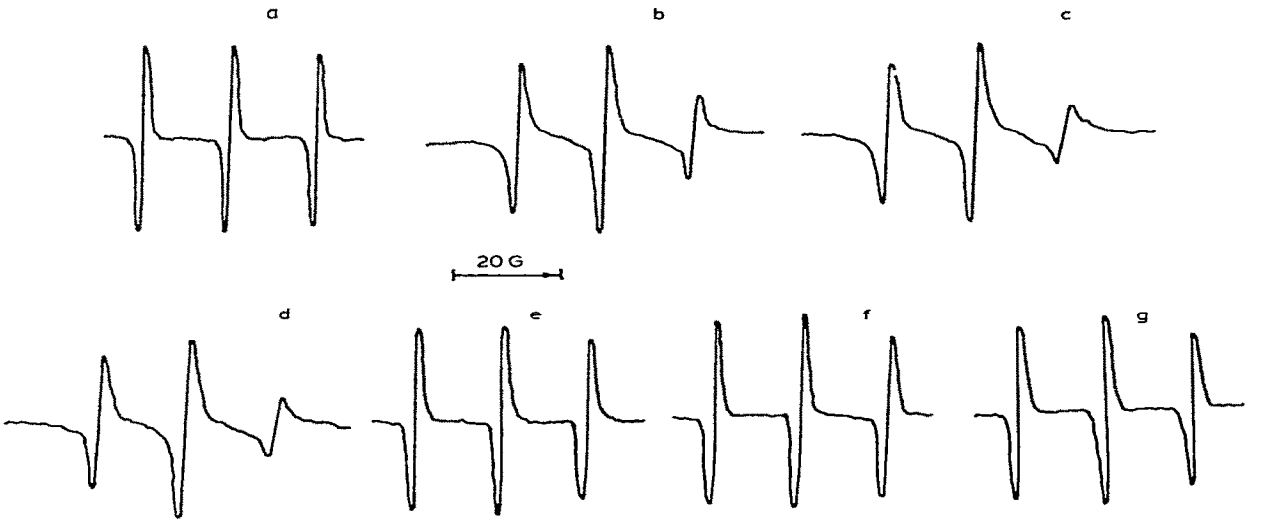


Fig. 2. ESR spectra of the free iminoxyl radical in solution (a), of the ferricytochrome *c* derivatives spin labeled on Met-65, Tyr-74 and Lys-72(73) (b–d), and of cyanide forms of these derivatives (e–g); 0.1–0.2 M phosphate buffer, pH 7.

τ_c values are $(7.0 \pm 1) \times 10^{-11}$, $(5.8 \pm 0.6) \times 10^{-11}$ and $(4.3 \pm 0.5) \times 10^{-11}$ s, respectively. It should be noted that in weak buffer solutions, the addition of KCN to a concentration of 0.1 M produces a shift to alkaline pH values. Because of this, all the measurements were taken in 0.2 M phosphate buffer, pH 7.

Fig. 3 (a–c) demonstrates the pH-dependent changes in the parameter characterizing the rotatory mobility of the labels in spin-labeled ferricytochromes *c* over the pH range 6–13. Also given are the values of this parameter for the reduced and cyanide forms of the studied spin-labeled cytochromes *c* at pH 7.

As seen from fig. 3a, over the pH range 6–10 the spin label at the Met-65 position registers a conformational transition with p*K* 9.3. In the same pH range, the spin labels at the positions of Tyr-74 and Lys-72(73) experience no marked alterations in mobility (Fig. 3b and c). However, at pH > 10, the latter two labels register a conformational transition with pH 11.1. That the observed increase in the mobilities is not related to hydrolysis of the radical SL_2 is confirmed by the absence of any changes in the ESR spectra of sperm whale myoglobin modified with the same radical SL_2 [11]. In the present work the occurrence of the hydrolysis products in the titrated samples at high pH values was tested by rapid back-titration to neutral pH values.

3.3. Transition from ferricytochrome c to ferrocytochrome c

It has been shown that a variation in the valence state of the heme iron produces changes in the stability of the cytochrome *c* molecule to temperature, pH and denaturing agents, in the ion-exchange properties of the protein as well as in the ability of cytochrome *c* to react with specific chemical reagents, antibodies and its partners in the mitochondrial respiratory chain, cytochrome *c* oxidase and cytochrome *c* reductase (refs. 1 and 12 and references cited therein).

The origin of the differences between the two redox forms of cytochrome *c* has long been obscure. The point is that the X-ray analysis of crystals of ferricytochrome *c* and ferrocytochrome *c* from tuna heart at a resolution of 2 Å [13] and 1.8 Å [14] has not revealed any substantial redox

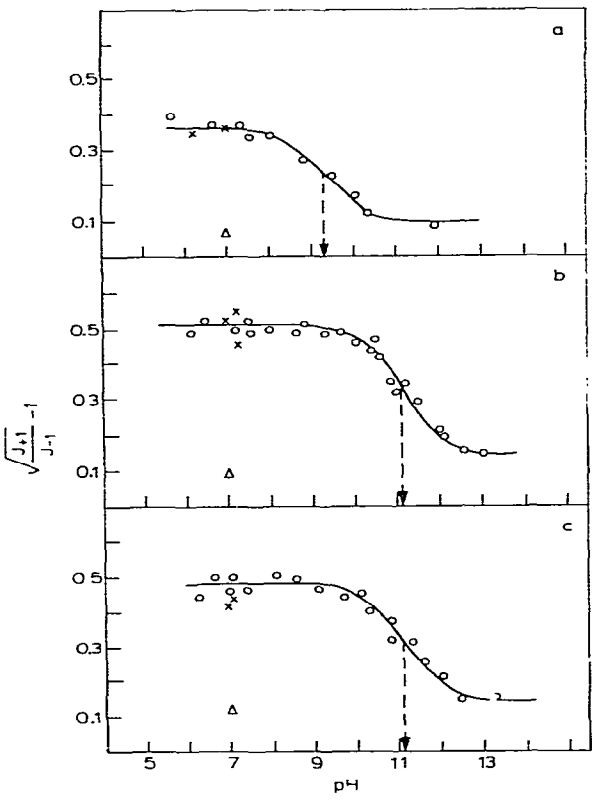


Fig. 3. pH-dependent changes in rotatory mobility of the labels in spin-labeled ferricytochromes c (○——○); 0.01 M phosphate buffer. (a) SL_1(Met-65)-cytochrome c, (b) SL_2(Tyr-74)-cytochrome c, (c) SL_2(Lys-72/73)-cytochrome c. (x) The corresponding values of the parameter characterizing mobility of the labels after reduction of the spin-labeled ferricytochromes c by $LiBH_4$ (a–c) or by oxymyoglobin (a); 0.01–0.1 M phosphate buffer, pH 7. (△) The same after addition of 0.1 M KCN to SL-ferricytochromes c (0.2 M phosphate buffer, pH 7).

conformational changes at the periphery of the molecule. Small conformational differences were found only in the lower part of the heme crevice in the environment of the buried H_2O molecule hydrogen bonded to Asn-52, Tyr-67 and Thr-78 [14].

The NMR [12] and chemical data [15] evidenced, however, that in solution there are some differences in the conformation of the oxidized and reduced molecules at the bottom of the cytochrome c structure where Lys-39, Lys-53, Lys-55 and Ile-57 are situated (see fig. 1). According to NMR data, the region in the vicinity of Ile-57 where the main antigenic site in cytochrome c is known to be localized is more flexible than other parts of cytochrome c.

A recent refinement of the two X-ray structures of tuna cytochrome c at high resolution made with an increased accuracy, and their detailed comparison [16] did reveal a small concerted shift of the side chains and even the main chain at the bottom of the molecule along with conformational changes on the Met-80 side of the heme. The differences in the atom postions of the two redox forms of cytochrome c did not exceed 0.1 Å.

In the present work no redox-dependent changes in mobility of the spin labels bound to Met-65, Lys-72(73) and Tyr-74 of cytochrome c were reliably established.

The Met-65 and Lys-72(73) residues of cytochrome c are separated from the region discussed above by a rather large distance (≈ 15 Å), therefore the spin labels attached to them may not 'feel' the events occurring there. On the contrary, Tyr-74 is situated very close to the region mentioned: the distance to Lys-55 is about 5.7 Å and to Ile-57 approx. 4 Å. The spin label at Tyr-74 must have registered conformational changes in the surroundings of Ile-57 if these changes were the case. The similar τ_c values for both ferri- and ferro-SL_2-(Tyr-74)-cytochromes c indicate that redox conformational changes at the bottom of the molecule in solution, if any, are very small. This results agrees with the X-ray analysis data for the crystals [16]. It should be taken into account that introduction of the label by itself gives rise to small local perturbations in the cytochrome c structure [4].

3.4. Replacement of Met-80 by CN^-

In cytochromes of the c type, as distinct from other heme-containing proteins, both axial ligands in a six-coordinated heme complex, His-18 and Met-80, belong to the polypeptide chain of the protein. There is still not sufficient information as to what role the binding of the sixth protein ligand, Met-80, to the heme plays in the maintenance of the native conformation of the protein. The data available evidence that the replacement of Met-80

by the external ligands, imidazole, azide or cyanide, affects insignificantly, if at all, such integral characteristics of cytochrome *c* as viscosity, α-helix content, stability to denaturing agents and temperature [1,17]. It has been also reported [18] that the fluorescence of the single Trp-59 residue localized in the depth of the heme cavity and being in contact with the heme increases insignificantly, about 1.5-times, upon replacement of Met-80 by cyanide.

Based on the data available, it may be concluded that the binding of Met-80 to the heme is not essential for the maintenance of the integrity of the deapest part of the heme cavity and the cytochrome *c* structure as a whole. However, the possibility cannot be excluded that the break of the link between the sixth protein ligand and the heme may result in a disturbance of the conformation and a destabilization of some part of the molecule next to the ligand-bound amino acid residue [17]. In favor of this assumption are the data of chemical modification of cyanoferricytochrome *c* where, as distinct from ferricytochrome *c*, Met-80 can be easily attacked by anionic reagents, since, the heme cavity seems to be less compact [7].

The present study of spin-labeled cytochromes *c* shows that replacement of Met-80 by cyanide is accompanied by pronounced changes in the conformation of the region where Met-65, Tyr-74 and Lys-72(73) residues are situated, i.e., in a segment of the polypeptide chain adjacent to Met-80. In spin-labeled cyanoferricytochromes *c* the mobility of all the three spin labels increases markedly (7–15-times) and approaches that of a free label in solution. This may point to some destabilization and enhanced flexibility of the left-hand side of cytochrome *c*. The conclusion is supported by our observations that in the presence of 0.1 M KCN the melting temperature of ferricytochrome *c* decreases by about 3°C and the denaturing concentration of urea by about 1 M, as determined by circular dichroism and microcalorimetry methods [9].

3.5. pH-induced conformational transitions

According to classical studies by Theorell and Åkeson [28], there are five pH-dependent conformational states in ferricytochrome *c* as measured by optical spectroscopy [1]. The transition from one state to another corresponds to dissociation of one proton. In the alkaline region two transitions are observed (pK values of the transitions are given beneath the arrows):

$$\mathrm{III} \xrightarrow[9.35]{H^-} \mathrm{IV} \xrightarrow[12.76]{H^+} \mathrm{V}$$

Scheme 1

State III is the native low-spin form of cytochrome *c* with Met-80 as the sixth ligand; state IV is also a low-spin form of the protein, from the absorption spectrum of which, however, the band at 695 nm is lacking, since Met-80 is replaced by another protein ligand; state V is the denatured cytochrome *c* form in which the site of the sixth ligand is occupied by OH^-.

In the present work two conformational transitions in ferricytochrome *c* were observed in the pH range 6–12: the first with pK 9.3 which corresponds to the transition III–IV in scheme 1 and the second with pK 11.1 which is absent in it.

The spectral transition III–IV has been widely studied using different methods and it has been shown that the new ligand that replaces Met-80 in state IV of cytochrome *c* is a lysine residue deprotonated with pK 9.35, most probably Lys-79 [1]. According to Dickerson et al. [21], replacement of Met-80 by Lys-79 must give rise to a shift of the polypeptide chain in the region of residues 78–83, which might affect the environment of the loop made of residues 70–82. It should be noted, however, that the assignment of Lys-79 is not conclusive [19,20]. From the analysis of the spatial structure of cytochrome *c*, Lys-72 or Lys-73 and Lys-13 cannot be completely excluded. Some authors even suggest that not lysine but some other residue replaces methionine at the sixth coordination site at pH 9.3 [1,20].

According to our data Lys-79 rather than Lys-72 or Lys-73 coordinates to the heme in state IV of ferricytochrome *c*. Actually, in the pH range 6–10, the spin labels at the Tyr-74 and Lys-72(73) positions do not register any conformational changes

(fig. 3) which would be the case if the orientation of the neighboring lysine residue were changed. Besides, as appears from NMR evidence, ferricytochrome *c* spin labeled at Lys-72(73) undergoes a normal spectral transition with p*K* 9.3 as does the native protein [4].

Investigations of spin-labeled cytochromes *c* enable us also to draw some conclusions as to what kind of changes occur in the cytochrome *c* conformation when Met-80 is replaced by another protein ligand, which regions of the structure are involved in these perturbations and how great they are.

As we could see, the conformational transition with p*K* 9.3 was registered only by the spin label at the position of Met-65 but not of Tyr-74 and Lys-72(73). At the same time, displacement of Met-80 by the external ligand CN^- caused substantial changes in mobility of all three labels. Hence, replacement of Met-80 by Lys-79 produced smaller perturbations in the structure of the left-hand side of cytochrome *c* than does the breakage of the heme iron-polypeptide chain bond. The change in τ_c of the spin label at Met-65 is the same in both cases, indicating that this site in the cytochrome *c* structure is probably sensitive to the reorientation of the side chain of Met-80. Since no conformational changes are observed during the III-IV transition in the vicinity of residues 72–74 included in the loop 70–82 mentioned above, it is obvious that the reorientation of Lys-79 upon coordination to the heme involves a smaller part of the polypeptide chain near Lys-79.

Of particular interest is a new alkaline conformational transition with p*K* 11.1 that is registered by the spin labels but is absent in the scheme 1.

Kihara et al. [22], who studied izomerization of ferricytochrome *c* in the pH range 8.5–12 by the absorption stop-flow method, were the first to suggest the occurrence of a transient cytochrome *c* form differing from states III and IV. Based on the changes in the ultraviolet region, the authors proposed that ionization of the tyrosine residue is involved in the formation of this transient species. However, they considered the final species to be the same at pH values both below and above 10 with Lys-79 as the sixth ligand.

Later, by high-resolution NMR spectroscopy at 220 MHz, Morishima et al. [23] found that in neutral and alkaline solutions there are four conformers of ferricytochrome *c* differing in the ligand state of the heme: native form (pH 4–9), lysine form (pH 9–11), 'strained-lysine' form (pH 11–12.5) and hydroxide form (pH 12.6). The nature of the Lys-79-iron coordination in the strained-lysine form was considered to differ from that in the lysine form, since at pH > 11 the ionization of the buried Tyr-67 takes place followed by breakage of its hydrogen bond with Thr-78. The ionization p*K* of Tyr-67 is known to amount to 11–11.4 [24,25].

Studies of spin-labeled cytochrome *c* derivatives have provided convincing evidence for the formation of a new conformer of ferricytochrome *c* in the pH range 10–12.5. There is no doubt that this isomerization of cytochrome *c* is induced by ionization of Tyr-67 because the pH interval of the conformational transition corresponds to ionization of one group and the p*K* value of the transition coincides with the ionization p*K* of this buried Tyr residue. As distinct from the transition with p*K* 9.3, the conformational changes in this case appear to embrace the whole left-hand side of the molecule, since they were registered by the spin labels at Tyr-74 and Lys-72(73) at a distance of more than 15 Å from Tyr-67. The increase in mobility of the spin labels which accompanies the pH-induced transition with p*K* 11.1 is similar to that observed when Met-80 is replaced by cyanide. The conformational changes in both cases seem to be analogous, which also follows from the circular dichroism data [17].

Thus, based on the results of the present work and on the literature data, conformational events occurring in ferricytochrome *c* at neutral and alkaline pH values prior to denaturation may be described as follows [2]. As the pH increases from 6 to 10, deprotonation of the ϵ-NH_2 group of Lys-79 with p*K* 9.3 and replacement of Met-80 by this lysine take place. The conformational change in the cytochrome *c* structure induced by such exchange of the protein ligands does not seem to be very large. It involves mostly the close proximity of Lys-79 and besides a part of the structure near Met-65 which is sensitive to the orientation of the side chain of Met-80.

The reorientation of Lys-79 will obviously affect the position of the neighboring Thr-78 residue which is hydrogen bonded to the internal Tyr-67 and to one of the -COO^- group of the heme [1,13]. Partial or complete disturbance of these hydrogen bonds due to the III–IV transition provides conditions favoring the ionization of the buried Tyr-67 which was impossible earlier but now takes place with pK 11.1.

The Tyr-67 residue is localized in the immediate vicinity of the heme ($r = 4.8$ Å) and plays a significant role in stabilization of the heme crevice from the side of the sixth ligand and the structure of the whole left-hand part of cytochrome c. Therefore, its ionization in the pH range 10–12.5 leads to substantial conformational changes and, probably, to some destabilization of this region. It should be noted that in this pH range unfolding of the polypeptide chain of cytochrome c does not take place [17]. The structure of other regions also remains essentially unaltered, which is evidenced by the fact that the spectral and fluorescence characteristics of Trp-59 localized in the deepest part of the heme cavity do not vary significantly [17,18] and that the ionization of the Tyr-48 and Tyr-97 residues localized in the bottom and right-hand parts of the structure is observed simultaneously with the alkaline denaturation of the protein at pH > 12.5 [24,25].

Thus, the alkaline isomerization of ferricytochrome c with pK 9.3 provides preconditions for the conformational transition with pK 11.1 and the latter, in turn, for global changes of the conformation during the alkaline denaturation process.

This interpretation of the different conformational states of ferricytochrome c at alkaline pH values [2] is supported by the data on pH-induced isomerization of ferricytochrome c obtained by circular dichroism and optical absorption stop-flow methods [26]. In the work by Hasumi [26] there is evidence that during the transition with pK 11.1, as also occurs with pK 9.3, an interconversion of the sixth ligand takes place. At present, it remains unclear which group coordinates to the heme at pH > 10 replacing Lys-79. From the analysis of the spatial structure of cytochrome c it follows that the most probable candidate is the ionized phenolic hydroxyl group of Tyr-67. The ideas that Tyr-67 may coordinate to the heme at alkaline pH values, even at pH < 10, have been put forward by several authors [1]. Model studies on heme complexes as well as the existence of mutant hemoglobins in which the proximal or distal histidine is replaced by tyrosine [27] have served as the basis for such assumption.

Our findings on spin-labeled ferricytochromes c as well as the changes observed in CD spectra in the ultraviolet and Soret regions at pH > 10 [26] are in favor of the replacement of the sixth ligand by ionized Tyr-67. Indeed, the ionization of Tyr-67 followed by a break of its hydrogen bond with Thr-78 and reorientation of the aromatic ring due to ligation with the heme may be the reason for the observed changes in ESR and CD spectra, though the experimental data obtained cannot be considered as a direct proof of such coordination.

Acknowledgement

The authors are grateful to R.I. Artyukh for help with ESR measurements.

References

1 R.E. Dickerson and R. Timkovich, in: The enzymes, 3rd edn. ed. P.D. Boyer, 11, part A (Academic Press, London, 1975) p. 397
2 G.B. Postnikova, R.I. Artyukh, N.P. Gorbunova and M.V. Volkenstein, in: Magnetic resonance and related phenoma. Proceedings of the 20th congress Ampere, Tallin, 1978, eds. E. Rundla, E. Lippmaa and T. Saluvere (Acad. Sci. Estonian SSR, Tallin, 1979) p. 522.
3 R.I. Artyulh, G.B. Postnikova, S.G. Kamzolova and B.I. Sukhorukov, Biokhimija 37 (1972) 902.
4 G.B. Postnikova, N.P. Gorbunova and A.G. Sukhomudrenko, Mol. Biol. (Moscow) 12 (1978) 1112.
5 A. Azzi, A.M. Tamburro, G. Farnia and E. Gobbi, Biochim. Biophys. Acta 256 (1972) 619.
6 A.N. Kuznetsov, A.M. Wasserman, A.I. Volkov and N.N. Korst, Chem. Phys. Lett. 12 (1971) 103.
7 E. Stellwagen, Biochemistry 7 (1968) 2496.
8 A.P. Boswell, G.R. Moore, R.J.P. Williams, C.J.A. Wallace, P.J. Boon, R.J.R. Nivard and G.I. Tesser, Biochem. J. 193 (1981) 493.
9 G.B. Postnikova, N.P. Gorbunova, T.V. Barnova and A.G. Sukhomudrenko, Proceedings of the 5th all-union symposium on chemistry and physics of proteins and peptides, Baku, 1980, p. 116.

10 G.B. Postnikova, N.P. Gorbunova, R.I. Artyukh, Proceedings of the 11th IUPAC international symposium on chemistry of natural products, Bulgaria, 1978, eds. N. Mazekov, I. Ognyanov, A. Ozahavats, V. Tarpanov, A. Trifonov and R. Vlagov (Bulgarian Acad. Sci., Sofia, 1978) b.1, p. 87.
11 R.I. Artyukh, B.P. Atanasov and M.V. Volkenstein, Mol. Biol. (Moscow) 13 (1979) 103.
12 G.R. Moore and R.J.P. Williams, Eur. J. Biochem. 103 (1980) 523.
13 T. Takano, B.L. Trus, N. Mandel, G. Mandel, O.B. Kallai, R. Swanson and R.E. Dickerson, J. Biol. Chem. 252 (1977) 776.
14 T. Takano and R.E. Dockerson, Proc. Natl. Acad. Sci. U.S.A. 77 (1980) 6371.
15 H.R. Bosshard and M. Zürrer, J. Biol. Chem. 255 (1980) 6654.
16 T. Takano and R.E. Dickerson, J. Mol. Biol. 153 (1981) 95.
17 Y.P. Myer, Biochemistry 7 (1968) 765.
18 T.Y. Tsong, J. Biol. Chem. 249 (1974) 1988.
19 H.T. Smith and F. Millet, Biochemistry 19 (1980) 1117.
20 H.R. Bosshard, J. Mol. Biol. 153 (1981) 1125.
21 R.E. Dickerson, T. Takano, O.B. Kallai and L. Samzon, in: Structure and function of oxidation-reduction enzymes, eds. Å. Åkeson and A. Ehrenberg (Pergamon Press, Oxford, 1972) p. 69.
22 H. Kihara, S. Saigo, H. Nakatani, K. Hiromi, M. Ikeda-Saito and T. Iizuka, Biochim. Biophys. Acta 430 (1976) 225.
23 I. Morishima, S. Ogawa, I. Yonezaca and T. Iizuka, Biochim. Biophys. Acta 495 (1977) 287.
24 J.A. Rupley, Biochemistry 3 (1964) 1648.
25 R.K. Cupta and S.H. Koeing, Biochem. Biophys. Res. Commun. 45 (1971) 1134.
26 H. Hasumi, Biochim. Biophys. Acta 626 (1980) 265.
27 E.W. Ainscough, A.W. Addison, D. Dolphin and B.R. James, J.A. Chem. Soc. 100 (1978) 7585.
28 H. Theorell and Å. Åkeson, J. Am. Chem. Soc. 63 (1941) 1804.