CONFORMATIONAL TRANSITIONS OF FERRICYTOCHROME *c* IN STRONG INORGANIC ACIDS

Marek STUPÁK[a], Jaroslava BÁGEĽOVÁ[b1], Diana FEDUNOVÁ[b2], Marián ANTALÍK[b,c,*]

[a] *Department of Medical Chemistry and Biochemistry, Faculty of Medicine, P. J. Šafárik University, Tr. SNP 1, 040 66 Košice, Slovak Republic; e-mail: stupakm@yahoo.com*

[b] *Department of Biophysics, Institute of Experimental Physics, Slovak Academy of Sciences, Watsonova 47, 040 01 Košice, Slovak Republic; e-mail:* [1] *bagelova@saske.sk,* [2] *fedunova@saske.sk*

[c] *Institute of Chemistry, Department of Biochemistry, Faculty of Sciences, P. J. Šafárik University, Moyzesova 11, 040 01 Košice, Slovak Republic; e-mail: antalik@saske.sk*

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Conformational transitions of horse heart ferricytochrome *c* (ferricyt *c*) have been investigated in the presence of strong inorganic acids and their salts by optical absorption spectroscopy, magnetic circular dichroism and circular dichroism. In the presence of acids ($HClO_4$ or H_2SO_4, pH 2) or their salts (1 M $NaClO_4$ or Na_2SO_4, pH 2, 25 °C), the three ligation states of ferricyt *c* heme were identified. One is the high-spin state: His18-Fe-H_2O (40–50%), and two are the low-spin states: His18-Fe-Met80 (30–25%) and His18-Fe-His (30–25%). Under these conditions low temperatures facilitate native heme coordination of ferricyt *c*. Transition from low-spin to high-spin heme coordination of ferricyt *c* is complete in 1 M $HClO_4$ or 3 M H_2SO_4. At the concentration of $HClO_4$ and H_2SO_4 above 3 M, different behavior in spectral transitions of ferricyt *c* near the heme is observed. High-spin pentacoordinated ferricyt *c* with the heme ligand of His18-Fe is formed in 8 M H_2SO_4. This state is unstable at higher concentration of H_2SO_4 and porphyrin ferricyt *c* is formed. At $HClO_4$ concentration higher than 3 M, the new, until this time not observed heme coordination structure of ferricyt *c* originates.

Keywords: Protein structure; Ferricytochrome *c*; Heme ligands; Conformational transitions; Absorption spectroscopy; MCD; CD.

The influence of various salts on the structure of denatured proteins is very complex and not completely understood yet[1–3]. The presence of heme groups in hemoproteins makes possible a very sensitive analysis of the changes caused by various denaturing solvents in the protein environment. Cytochrome *c* (cyt *c*) is a particularly useful model, because its heme group is covalently attached to the polypeptide chain, which supports the reversibility as well as rapid kinetics of the observed conformational transitions[4].

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Theorell and Akeson[5] showed that cytochromes *c* exhibit several conformational transitions, which influence the vicinity of cyt *c* heme at acid pH. These transitions are caused by electrostatic repulsion of positive charges on individual amino acid residues of the protein. The more detailed studies have shown that while in the native state (pH 7) heme iron is coordinated with two strong-field protein ligands His18 (fifth coordination place) and Met80 (sixth coordination place), at pH below 3, the denaturation of protein and several spectral conformational states of ferricyt *c* are observed. The arrangement of heme ligands depends on cytochrome *c* species as well as on experimental conditions. The heme iron–Met80 bond is the most sensitive site to changes in structure of cyt *c*, caused by external conditions[6]. The p*K* value of disruption of this bond is equal to 2.4–3.8 for animal cytochromes *c* [5–8]; however, for cytochrome *c* from thermostable bacteria the p*K* value[9] is equal to 0.3. Denatured ferricyt *c* in acid conditions after increasing the acid concentration forms the molten-globule (MG) state, as a result of the electrostatic shielding of positive charges of protein with negative charges of the acid[3,10–12]. The MG state is characterized by only moderate expansion of the volume of molecules and by high content of α-helical secondary structure. The formation of the MG state depends on the type of anion of used acid[1,3,13,14].

In spite of the fact that there are numerous studies devoted to the conformational transition of ferricyt *c* at acid pH, it is not clear under which conditions the axial ligands, His18 and Met80, are released from heme iron, and what the differences are between high-spin penta- and hexacoordinated states[15–18] of this protein. The salt induced collapse of acid-denatured cyt *c* leads to a number of equilibria between high-spin and low-spin heme states and between two types of low-spin states. The equilibrium between these states is dependent on the concentration and/or size of the anions[13,14]. The aim of this paper is to characterize the conformational transitions of ferricyt *c* near heme, which are caused by strong acids, $HClO_4$ and H_2SO_4. The effect of corresponding anions of the used acids on properties of this protein was also investigated.

MATERIALS AND METHODS

Horse heart ferricytochrome *c*, type IV (Sigma Chemical Product) was used without further purification. All other chemicals were of analytical grade.

The absorption spectra were monitored with an HP 8452A diode array spectrophotometer and a UV 3000 Shimadzu spectrophotometer. Magnetic circilar dichroism (MCD) measurements were carried out with a Jasco J-500C spectropolarimeter, with electromagnet operated at 1.3 T. Circular dichroism (CD) spectra in far-UV region were obtained using a Jasco J-600

spectropolarimeter equipped with PC as data processor. Quartz cells with 1 mm light paths were used for measurements. Thermal titrations were performed in Peltier temperature-controlled cuvettes with accuracy ±0.5 °C. The protein concentration used was 5–10 μM for spectral measurements. The concentration of ferricyt *c* was determined spectrophotometrically at pH 7.0, using the molar absorption coefficient[19] ε_{410}^{ox} = 106 l mmol^{-1} cm^{-1}.

pH measurements. pH was determined using a HI 9017 pH-meter (Hanna Instruments Srl., Italy) coupled to a SG901C pH electrode (Sensorex, U.S.A.). The high salt concentrations influence the pH value monitored by glass pH electrode[20]. Therefore, the solution of protein with desired pH was prepared and its pH measured. Afterwards, the salt solution (prepared by addition of salt to buffer with the same pH) was added.

Calculation of percentage of various conformers. For calculation of amount of individual heme species we take as a reference three absorption spectra (300–800 nm) of cyt *c* in different conditions but equal protein concentration. (i) The spectrum at pH 2 and low ionic strength conditions corresponds to 100% of cyt *c* molecules in high-spin state – H_2O/His18 ligation. (ii) The spectrum at pH 7 and low ionic strength corresponds to 100% of cyt *c* molecules in low-spin state – His18/Met80 ligation. (iii) The spectrum in 9 M urea at pH 8.5 is assigned to 100% of cyt *c* molecules in another low-spin state – His18/His(26,33) ligation[17]. Using iteration method of factor analysis we obtained estimates of individual heme species in $HClO_4$, pH 2 and 1 M Na_2SO_4, pH 2 with statistical errors about 5%.

RESULTS

Conformational Transitions of Ferricytochrome c at Low pH

Absorption spectra: Figure 1 shows UV-VIS absorption spectra of ferricyt *c* under different conditions. For the native protein (Fig. 1, curve *1*), the intensity maximum of the absorption band in the Soret region lies at 408 nm. There are three weaker bands: β-band at 527 nm, shoulder at 560 nm (α-band) and the 695 nm charge transfer band. This spectrum is characteristic of low-spin configuration of ferricyt *c*, when His18 and Met80 are bounded to the heme iron[21]. Whereas the spectra of cyt *c* practically do not change in the pH region from 7.0 to 4.5 at room temperature, at more acid pH they are very sensitive to anion of the used acid[13,16]. A decrease in pH resulted in a shift of Soret and β-bands to a lower wavelength, disappearance of the 695 nm band and appearance of a new band in the 600–650 nm region. At low ionic strengths (< 20 mM) and in the presence of HCl, pH 1.8 there is a very intense band at 394 nm giving the molar absorption coefficient ca. 182 l mmol^{-1} cm^{-1} as well as weaker peaks at 500 and 620 nm (Fig. 1, curve *2*). A similar extinction coefficient was obtained by Robinson et al.[22] for pH 2. This spectrum is characteristic of the high-spin[16] state of heme iron with ligands His18 and H_2O.

Different situation is in presence of $HClO_4$, pH 2. In comparison with curve *2*, we observed a decrease of the 620 nm band intensity and a partial

recovery of the 695 nm band. The spectrum of ferricyt *c* diluted with $HClO_4$, pH 2 (Fig. 1, curve *3*), suggests the presence of the various states: low-spin state (His18-Fe-Met80; ~30%), high-spin conformer (His18-Fe-H_2O; ~40%) and another low-spin state (His18-Fe-His; ~30%). The contribution of individual states was estimated from the band intensities at 620 and 695 nm (see Materials and Methods). A nearly equal absorption spectrum was observed for ferricyt *c* in H_2SO_4, pH 2.0 (not shown). We assume that the presence of the low-spin forms is a result of a strong influence of SO_4^{2-} or ClO_4^- anions on the protein leading to the formation of the MG state. The similar absorption spectrum has been observed for ferricyt *c* in a solution of 1 M Na_2SO_4, in H_2SO_4, pH 2.0 (Fig. 1, curve *4*). At 25 °C and in the pH region between 2.5 and 1.0, the contribution of high-spin form (His-Fe-H_2O) is approximately 50% and the rest includes two low-spin forms, 25% His18-Fe-Met80 and 25% His-Fe-His. A further decrease in pH completes this conformational transition from the low-spin to the high-spin form. From Fig. 1 it is evident that the spectra of ferricyt *c* in 1 M $HClO_4$, pH ~ 0 (curve *5*) or in 3 M H_2SO_4, pH < 0 (curve *6*) are very similar to the spectrum in HCl, pH 1.8 (curve *2*). The intensive band at 398 nm (ε = 170 l mmol^{-1} cm^{-1}), β-band at 500 nm and a band at 622 nm are observed.

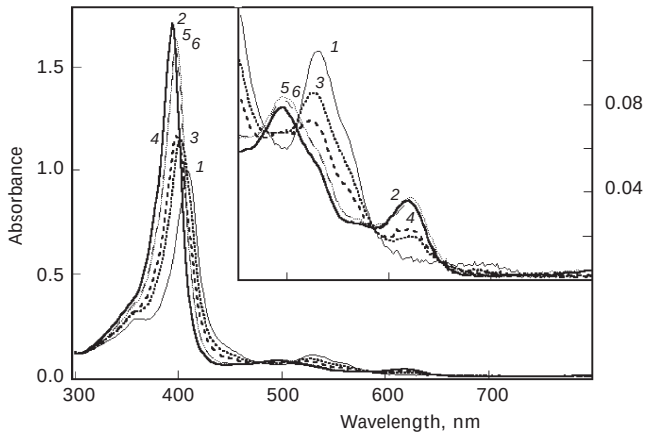

FIG. 1

UV-VIS absorption spectra of ferricyt *c* (9.5 μM) at 25 °C in: 10 mM phosphate, pH 7.0 – thin full line (*1*); HCl, pH 1.8 – thick full line (*2*); $HClO_4$, pH 2 – thick dotted line (*3*); 1 M Na_2SO_4, H_2SO_4, pH 2 – thick dashed line (*4*); 1 M $HClO_4$ – thin dotted line (*5*); 3 M H_2SO_4 – thin dashed line (*6*)

MCD spectra: In Fig. 2 MCD spectra of ferricyt *c* in various conditions are presented. The native cyt *c* (pH 7) has very intensive positive and negative bands in the Soret region (Fig. 2, curve *1*), in accord with previous data[23]. The intensities of both bands are reduced and both the maximum and minimum are blue-shifted in the presence of HCl, pH 1.8 (Fig. 2, curve *3*). The intensity of the negative band in the region 500–600 nm decreases and shifts to lower wavelengths. This spectrum is characteristic of the high-spin state of ferricyt *c* in which heme iron is ligated by His18 and H_2O, as has been shown by absorption spectroscopy (Fig. 1, curve *2*). In the presence of 1 M Na_2SO_4, pH 1.8 (Fig. 2, curve *2*), the intensity of positive and negative bands are increased compared with that in HCl, pH 1.8 and the maximum is shifted towards the bands obtained in 10 mM phosphate, pH 7.0. Along with that, the intensity of the negative band in the region 500–600 nm moved up closely to the intensity corresponding to the native state (Fig. 2, curve *1*). This indicates that a definite fraction of cyt *c* molecules was restored to the low-spin state (His18/Met80). In 1 M $HClO_4$ and in 3 M H_2SO_4 (curves *4* and *5*), the intensity of both negative and positive bands are very similar to those obtained for cyt *c* in HCl, pH 1.8 (curve *1*), which indicates a similar coordination of ligands on heme iron, i.e. His18-Fe-H_2O [17]. The presence of high-spin form is also shown in Fig. 1, curves *2*, *5* and *6*.

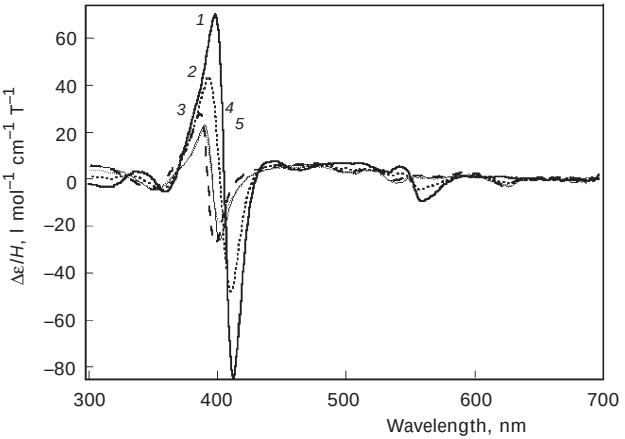

FIG. 2

MCD spectra of ferricyt *c* (5 μM) at 25 °C in: 10 mM phosphate, pH 7.0 – thick full line (*1*); 1 M Na_2SO_4, H_2SO_4, pH 1.8 – thick dotted line (*2*); HCl, pH 1.8 – thick dashed line (*3*); 1 M $HClO_4$ – thin full line (*4*); 3 M H_2SO_4 – thin dotted line (*5*)

CD spectra: In Fig. 3 CD spectra of native cyt *c* at pH 7, acid-denatured cyt *c* by HCl at pH 2 and in the presence 1 M H_2SO_4 and 1 M $HClO_4$ are illustrated. The high acid concentration leads to a recovery of cyt *c* secondary structure. CD spectra correspond to spectra of the MG state of cyt *c* in 1 M Na_2SO_4 or 1 M $NaClO_4$ at pH 2 (not shown). So at high acid concentration, presence of corresponding anion minimizes the intramolecular charge repulsion and supports the formation of the MG state such as at high salt concentrations[3].

pH-titration curves: pH-titration curves for ferricyt *c* measured as the absorbance changes at 398 nm under various conditions are presented in Fig. 4. A cooperative transition from the low-spin to high-spin state is observed in 2 mM acetate, titrated with HCl, with a p*K* value of transition equal to 2.5 (curve *1*). Titration of ferricyt *c* in 1 M $NaClO_4$ with $HClO_4$ shows two transitions (Fig. 4, curve *2*). The first is characterized by p*K* = 4.3 and it is the transition from the low-spin (His18-Fe-Met80) to the mixed-spin state (His18-Fe-Met80, His18-Fe-His, His18-Fe-H_2O). The second transition, with p*K* = 1.5 corresponds to the transition from the mixed-spin state to high-spin state. 1 M Na_2SO_4 shifts the first transition to p*K* = 2.4 (Fig. 3, curve *3*). The transition is fully completed below pH 0, as can be seen in Fig. 1 (curve *6*).

It is evident that perchlorate, as one of the most intensive chaotropic ions, destabilizes the cytochrome *c* structure near the heme more effec-

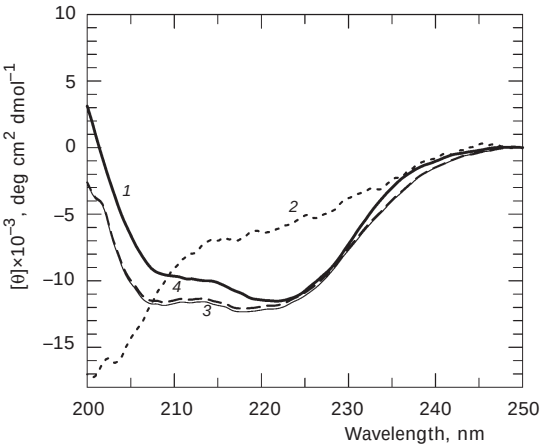

FIG. 3
CD spectra in the far-UV region of cyt *c* (10 μM) in various conditions: pH 7 – thick line (*1*); pH 2, HCl – dashed line (*2*); 1 M $HClO_4$ – thin line (*3*); 1 M H_2SO_4 – long dashed line (*4*)

tively, and sulfate, as a strong cosmotropic ion, stabilizes this region of the protein to acid titration.

The destabilization of native structure near heme is only partial under these conditions (1 M Na_2SO_4, pH 2, 25 °C) (ca. 25% of cyt *c* molecules remains in low-spin state). Figure 4 (curve *4*) illustrates that a decrease in temperature to –5 °C results in reduction of the Soret band. In addition, an increase in the 695 nm band as well as a decrease in the 620 nm band were observed after decreasing temperature from 25 to –5 °C (not shown).

Conformational Transition of Cytochrome c at High Acid Concentrations

An increase in H_2SO_4 or $HClO_4$ concentration above 3 M brings additional changes around the heme region of cyt *c*. The absorption spectra (Fig. 5a) show that the intensity of the Soret band considerably decreases, the band of the high-spin state is slightly red-shifted and its intensity moderately decreases in 8 M H_2SO_4. The band at 695 nm is not present in the spectra under these conditions. Similarly, MCD spectra (Fig. 5b) show that an increase in H_2SO_4 concentration (8 M) induces a considerable decrease in the intensity of both negative and positive peaks of cyt *c*, as well as the shift of the maximum and minimum to 395 and 411 nm, respectively, compared with spectrum of cyt *c* in 3 M H_2SO_4.

On the other hand, an increase in $HClO_4$ concentration above 3 M causes a decrease in the Soret band intensity of ferricyt *c*, β-band is shifted to

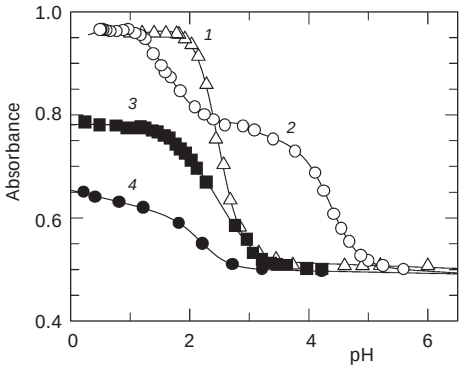

FIG. 4

Titration curves of ferricyt *c* measured as the absorbance change at 398 nm in: 2 mM acetate, titrated with HCl, 25 °C – △ (*1*); 1 M $NaClO_4$, titrated with $HClO_4$, 25 °C – ○ (*2*); 1 M Na_2SO_4, titrated with H_2SO_4, 25 °C – ■ (3); 1 M Na_2SO_4, titrated with H_2SO_4, –5 °C – ● (4). Concentration of cyt *c* 6.1 μM

higher wavelengths, an intensive α-band is formed at 556 nm and the intensity of the band at 620 nm is significantly lowered (Fig. 6). The configuration around heme at high concentrations of $HClO_4$ differs from that observed in H_2SO_4. As we know, the conformer with these spectral characteristics has not been observed up to now.

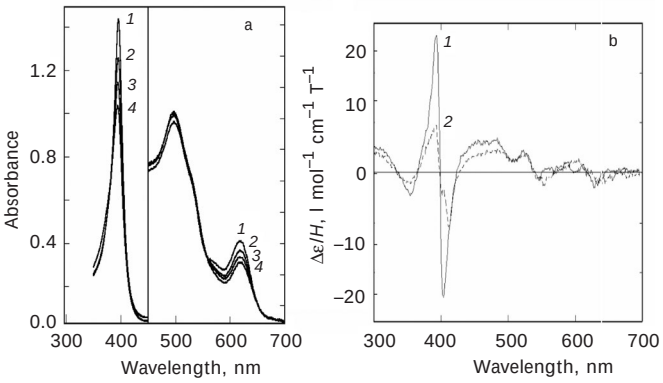

FIG. 5

a UV-VIS absorption spectra of ferricyt *c* (9.5 μM) at 25 °C and various concentrations of H_2SO_4: 3 M (*1*), 5 M (*2*), 6.4 M (*3*), 8 M (*4*). b MCD spectra of fericyt *c* (5 μM) at 25 °C and various concentrations of H_2SO_4: 3 M (*1*), 8 M (*2*)

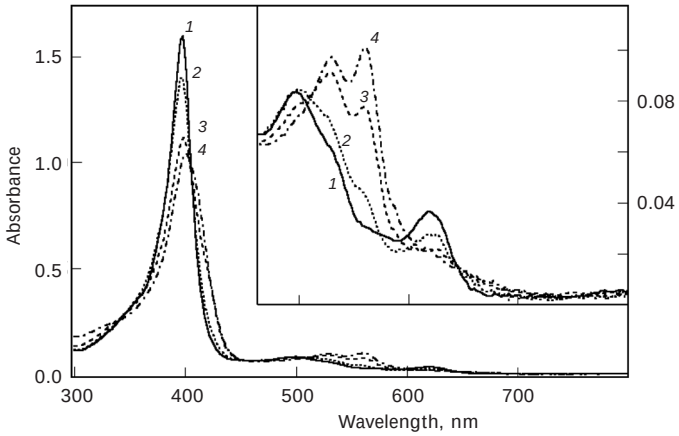

FIG. 6

UV-VIS absorption spectra of ferricyt *c* (9.5 μM) recorded for 1 min after mixing at 25 °C and various concentrations of $HClO_4$: 3 M (*1*), 5.7 M (*2*), 8.5 M (*3*), 10 M (*4*)

Whereas ferricyt *c* spectra in $HClO_4$ (<3 M, 25 °C) and 8 M H_2SO_4 are stable and the reversibility of the transition to the native form due to increasing pH to 7 is preserved (not shown), the higher concentration of $HClO_4$ (>3 M) partially destroys the heme region of cyt *c* (Fig. 7, curve *2*). The concentration of H_2SO_4 above 8 M causes the formation of porphyrin cyt *c* (Fig. 7, curve *1*), as we suggest from comparison of these spectra with the spectra of porphyrine cyt *c* showed by other authors[24].

DISCUSSION

The heme and its axial ligands are essential for structure stabilization and function of cytochrome *c*. The axial ligands of heme iron in native ferricyt *c* are Met80 and His18. This conformational state was designated[5] as state III. One of the most sensitive sites of ferricyt *c* to the environment is the bond between Met80 and heme iron[25].

Increasing the proton concentration with HCl to pH 2 (low ionic strength), changes in the heme ligation are observed. At room temperature Met80 is released from iron[8,26] (pK = 2.5). This transition occurs in a very narrow pH range (the number of associated protons is 3), this implies that a majority of ferricyt *c* molecules is in the native state even at pH 3 (Fig. 1, curve *2*). In some cases, native conformation of heme up to pH 0.8 has been observed (cyt *c* 552 from *T. thermophilus*, pK ~ 0.3)[9].

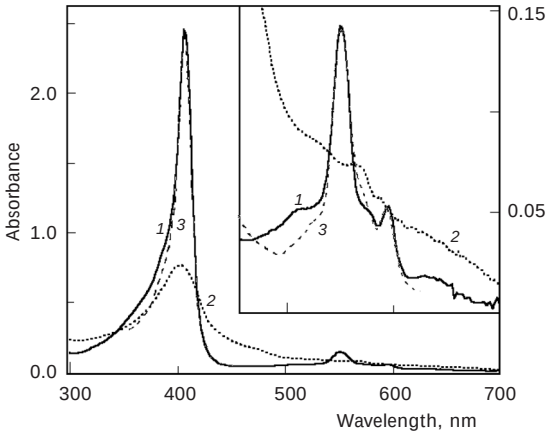

FIG. 7

UV-VIS absorption spectra of ferricyt *c* (9.5 μM) in: 10 M H_2SO_4 after 24-h incubation – thick full line (*1*); 10 M $HClO_4$ after 5 min, at 25 °C – dotted line (*2*); porphyrin cyt *c* (12 μM), 100 mM NaCl, 10 mM phosphate, pH 0.8 – dashed line (*3*), data taken from Zentko et al.[24]

It is well known that the presence of high concentrations of salts induces the formation of the MG state[3,27,28]. It was shown that ferricyt *c* contains a comparable amount of native-like secondary structure[22] in 1 M $HClO_4$. We confirmed similar fact in 1 M $HClO_4$ and in 1 M H_2SO_4 as it is ilustrated in Fig. 3. We can assume that ferricyt *c* is still in the MG state even under conditions where pH values are equal or less than zero also in the presence of other acids. Using acid titration of cyt *c* at 1 M concentrations of salts, we can notice more aspects of the pH effect on stability of ferricyt *c* near the heme. As can be seen in Fig. 4, the titration curve of ferricyt *c* in the pH region from 5 to 0 has two transitions in the presence of 1 M $NaClO_4$ (curve *2*). We observed a similar shape of acid titration curve also for 1 M NaCl (not shown). First transition for ferricyt *c* in 1 M $NaClO_4$ and 1 M Na_2SO_4 has apparent p*K* value equal to 4.3 and 2.4, respectively. The apparent p*K* value of the second transition at high salt concentration is equal to ~1.5 and <0, respectively.

The first transition suggests that the higher ion concentrations affect some attractive interaction between oppositely charged groups, which stabilize the native structure of ferricyt *c*. We assume that the ion bridge between Glu90 and Lys7 near the heme crevice is probably responsible for this phenomenon. The salts in 50–100 mM concentrations induce a weakening of this interaction and this results in a population of cyt *c* molecules with absent ligation of Met80 to the heme iron. The second transition observed at pH 2 suggests that a high salt concentration stabilizes Met80–heme iron bond due to shielding of positively charged groups. This transition is performed at room temperature at pH ca. 0 by $HClO_4$ titration. The secondary structure does not significantly change and the protein is in the MG state during the transition.

The loss of native ligands of heme starts at pH 5 (p*K* = 4.3) in 1 M $NaClO_4$. However, the high concentration of ClO_4^- has also a stabilization effect on the fraction of cyt *c* molecules, and total loss of native heme ligation of cytochrome *c* is observed at pH ~ 0. Using H_2SO_4, the native heme ligation is completely lost in 3 M H_2SO_4 at 25 °C. The stabilization effect of sulfate (1 M) in comparison with ClO_4^- is evident also from the fact that the loss of native ligands starts at pH 3 (p*K* = 2.4). In this pH region and at high concentrations of SO_4^{2-}, low temperatures facilitate native heme ligation. A similar effect was observed by Oerlich et al.[16]. Using resonance Raman spectroscopy, they showed, that cyt *c* in solutions with high salt concentration and at low temperatures (2 M NaCl, pH 0.8 and temperature from –60 to –140 °C), has only a low-spin hexacoordinated heme configuration.

While the assignment of the spectra of native cytochrome *c* to configuration of heme ligands is possible due to the known 3D structure, this is not yet the case when conformational transitions of cyt *c* are induced by several factors (pH, temperature, ionic strength, denaturants). In the acid pH region (low ionic strength, pH ~ 2), the existence of high-spin conformational state of cytochrome *c* was observed by several spectral methods[8]. The state with identical spectrum was also observed for structurally engineered cytochrome *c* at pH 7.0, where the Met80 was substituted by Ala [29]. The authors suggest that the sixth ligand of heme iron is H_2O and the fifth is His18. A similar spectrum was obtained[30] also for cytochrome *c′* at pH 1.5. As it is shown in Fig. 1, we observed a similar spectrum as for cyt *c* in HCl, pH 2 for cytochrome *c* in 1 M $HClO_4$ or 3 M H_2SO_4. This suggests that the bond between His18 and heme iron is preserved even at such extreme concentrations of acids. This ligand configuration of cyt *c* at high concentrations of acids is supported by MCD measurements. The MCD spectra of cytochrome *c* (Fig. 2) show high similarity with respect to intensity and position of the bands for all displayed conditions (low ionic strength, HCl, pH 2; 1 M $HClO_4$; 3 M H_2SO_4). The result confirms our suggestion that heme iron of cyt *c* is ligated by His18 and H_2O under these conditions. This corresponds to the structure assigned by Theorell and Akeson[5] to the state II of ferricyt *c*.

New absorption spectrum of cyt *c* was observed using higher concentration of H_2SO_4 (>3 M) (Fig. 5a). Comparing the measured spectrum of cyt *c* in 8 M H_2SO_4 with the known spectra of cyt *c′* [31,32] and myoglobin[33], where the 3D structure is known, we suppose that the penta-coordinated high-spin state of cyt *c* with His as fifth ligand is present.

The conformer of cyt *c* observed in concentrated $HClO_4$ is different from that in 8 M H_2SO_4. Whereas in the Soret region a decrease in the intensity of absorption band was also observed, a rise of the α-band and decrease of the 630 nm band occur (Fig. 5b). The detailed characterization of this state is prevented by its low stability.

In concentrated H_2SO_4 (>8 M), heme of cyt *c* is destroyed also in another way. The iron is released from heme and porphyrin cyt *c* is formed. The observed spectrum is identical with that of porphyrin cyt *c* [24].

On the basis of the presented results it is possible to propose the following scheme (Fig. 8) for conformational transitions in the vicinity of heme and in 1 M sulfate:

Native state[5] of cyt *c*, state III, is present in the pH region from 7 to 3. In the pH region from 3 to pH corresponding to 3 M H_2SO_4 or 1 M $HClO_4$, three different heme ligations are present: the native low-spin state His18/

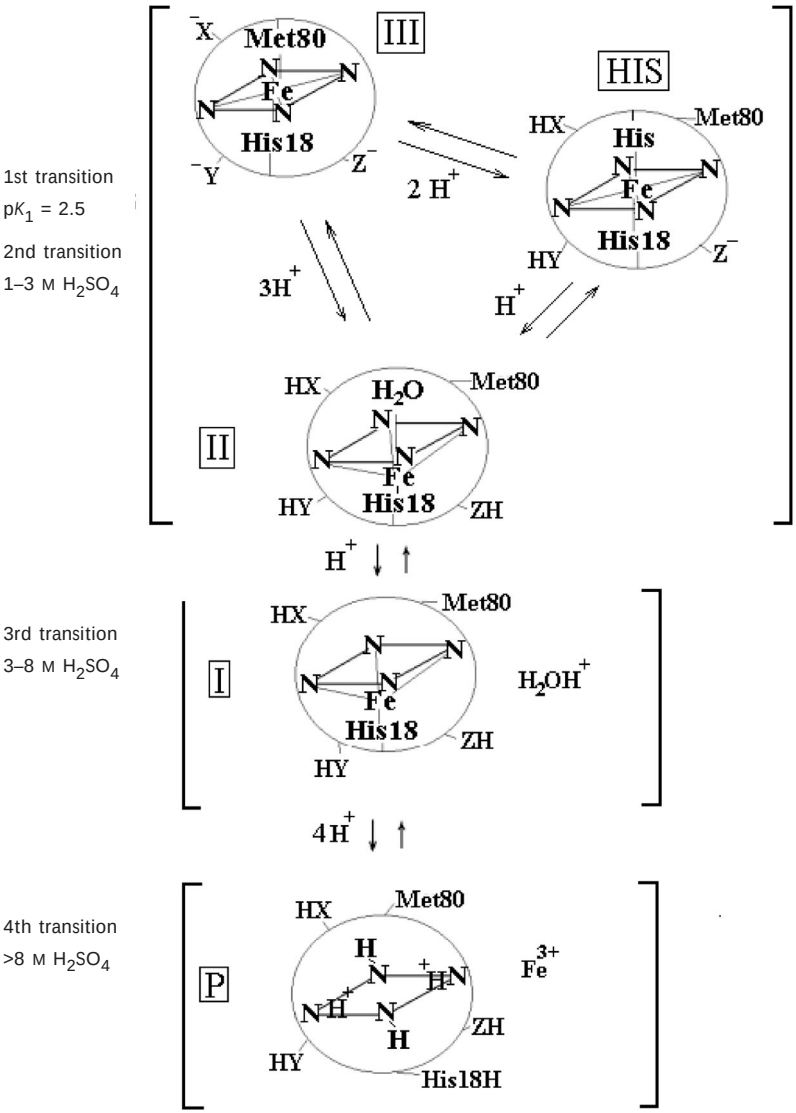

FIG. 8

Conformational transitions of ferricyt *c* in 1 M Na_2SO_4 titrated with H_2SO_4 at 25 °C. X, Y, Z are proton binding groups, which initiate the conformational transition. We did not estimated p*K* constants in higher acid concentration due to the difficulties of pH measurement by glass electrode in strong acids. The values in brackets indicate acid concentration interval in which conformational transition occurs

Met80, another low-spin state His18/His26,33 (state HIS), and high-spin state His18/H_2O (state II). The increase of H_2SO_4 concentration (decrease of pH) induces four conformational transitions. The first transition is characterized by macroscopic pK_1 constant equal to 2.5 and the second transition occurs at H_2SO_4 concentrations from 1 to 3 M. The population of state III and state HIS decreases and in 3 M H_2SO_4 cyt *c* is mainly in the high-spin state (state II). Further increase in H_2SO_4 concentration leads to the formation of high-spin pentacoordinated species (state I), with His18 as the only axial ligand, this third transition occurs in 3–8 M H_2SO_4 concentration range. At concentrations of H_2SO_4 above 8 M, the nitrogens of porphyrin bind protons and state P is formed (fourth transition).

The first and second conformational transitions of cyt *c* are observed also in the presence of increasing amount of $HClO_4$ (pK_1 = 4.3 and pK_2 = 1.5). A further increase in $HClO_4$ concentration (>3 M) does not lead to the formation of state I, but a new, so far unknown conformer is formed (Fig. 6); it is not included in the scheme.

The fact that various anions have different effects on conformational transitions of cyt *c* at high concentrations of acids is observed also for NaCl. At 1 M NaCl and pH 2 the mixed-spin state[10] of cyt *c* was found. A further decrease in pH to 0 (by adding HCl) led to the direct conversion of most of the cyt *c* population to state I, excluding the conversion of HIS and III states to state II (ref.[15]). In these different conditions (1 M HCl and 8 M H_2SO_4) pentacoordinated state occurs with His18 as fifth ligand.

It is noticeable that His18 is very tightly bonded to the heme iron as follows from spectral measurements of cyt *c* titration with strong acids. The release of His18 from the heme iron is associated with the binding of proton at concentrations above 8 M of H_2SO_4. The p*K* constant of the ligated His18 release is more than 6 units lower than that for free histidine, whereas for His33 or His26 it is only about one unit lower. We suggest that such strong binding of His18 to the heme iron is mainly a result of spatial approach to heme facilitated by Cys17, which is covalently bonded to heme.

In the present paper we have used extreme acid concentrations, which are far from physiological conditions. In spite of this fact, removing an acid (8 M H_2SO_4; 3 M $HClO_4$) from cyt *c* solution leads to recovery of native cyt *c* structure at pH 7. We assume that the observed cyt *c* conformers with characteristic spectra could be helpful in understanding the conformational changes occurring under more frequently used conditions for cyt *c* treatment (e.g. 9 M urea, 6 M guanidine hydrochloride at pH 2)[34,35] where the precise conformational state of cyt *c* is not yet understood (manuscript in preparation).

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