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Cytochrome c_3-heteropolytungstate complex: a model for the interaction of the tetraheme cytochrome with its redox partners, ferredoxin and rubredoxin

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Complex formation between ferricytochrome c_3 and a large three-dimensional polyanion has been investigated in order to study the influence of surface electrostatic interactions on the structural and redox properties of the tetraheme cytochrome. This protein presents the property of interacting with different redox partners such as ferredoxin, rubredoxin, flavodoxin and hydrogenase in the electron transfer chain. The polyanion used, $[Bi_2W_{22}O_{76}H_2]^{12-}$ is a heteropolytungstate which possesses various properties (molecular weight, charge and size) in common with ferredoxin and rubredoxin. Circular dichroism and ^{1}H-NMR spectroscopic studies show that this entity interacts with the polytheme cytochrome with the same stoichiometry and brings to the same structural modifications on the cytochrome as ferredoxin or rubredoxin. This indicates that this synthetic molecule has the same interacting site on the cytochrome c_3 as its known redox partners. Association constants have been evaluated and show that the heteropolytungstate and the ferredoxin have a comparable affinity for the cytochrome c_3 (respectively $K = 2 \cdot 10^6$ and $6 \cdot 10^6$ M^{-1}). The polyanion induces a slight shift of redox potential observed by cyclic voltammetry: -20 mV for the Norway strain and -50 mV for the Hildenborough strain. In the latter case, our results suggest that the highest redox potential is preferentially affected by the complexation. From the similarity between the polytungstate and the Fe-S proteins in the cytochrome c_3 binding and their bulk electric and steric properties, we are tempted to conclude that the polyanion appears as a valid model for the study of the interaction between the cytochrome c_3 and its redox partners.

Introduction

Numerous studies have been developed to understand the factors that govern the rate and specificity of protein-protein electron transfer mainly in cytochrome *c* [1]. Hypothetical structures of both physiological or non physiological complexes have been inferred by computer graphic methods in the cases of cytochrome *c*/cytochrome *c* peroxidase [2], cytochrome *c*/cytochrome b_5 [3], cytochrome *c*/flavodoxin [4] and cytochrome *c*/photosynthetic center [5]. These models are supported by chemical modifications, chemical cross-linking studies and spectroscopic techniques such as nuclear magnetic resonance (NMR) [6,7] and circular dichroism (CD) [8,9]. The influence of electrostatic interactions on the structure and function of cytochrome *c* has been investigated by means of complex formation between ferricytochrome *c* and large three dimensional entities of tungsten atoms linked by negatively charged oxygen atoms, namely heteropolytungstates [10]. When complexed to the cytochrome *c*, they have been shown to mimic reasonably well the interactions between cytochrome *c* and negatively charged interfaces [11,12].

In an attempt to compare structure function relationship in tetraheme cytochrome c_3 with monoheme cytochrome *c*, the study of the electron transfer mechanism between *Desulfovibrio* cytochrome c_3 and its redox partners has been developed.

Desulfovibrio desulfuricans Norway cytochrome c_3 (M_r 13000) contains four low redox potential hemes (-165 mV, -305 mV, -365 mV and -400 mV) [13,14] localized in non equivalent protein environments as described by electron paramagnetic resonance (EPR) [15], NMR [16] and X-ray crystallographic

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studies [17,18]. *Desulfovibrio desulfuricans* Norway ferredoxin I is a (4Fe-4S) cluster ferredoxin (M_r 6000) exhibiting a redox potential of −374 mV [19,20]. Rapid kinetic studies of the electron exchange reaction between cytochrome c_3 and ferredoxin I [21] have shown the formation of an intermediate complex followed by a bidirectional electron transfer between heme and cluster. The cytochrome c_3/ferredoxin I complex has been studied by biophysical techniques. From microcalorimetric measurements [22] and ^{1}H-NMR experiments [23] a stoichiometry of one cytochrome c_3 molecule per ferredoxin subunit was found and an association constant $K = 1.3 \cdot 10^6$ M^{-1} (285 K, 10 mM Tris-HCl buffer, pH 7.7) was determined. It was also found that the methyl groups resonances of the two highest potential hemes of the cytochrome are affected by the complex formation. A three dimensional protein-protein hypothetical complex, in which the Fe-S cluster faces heme 4 (sequentially numbered from the amino-terminus) has been generated using interactive computer graphic methods [24]. A covalent crosslinked cytochrome c_3/ferredoxin complex has been obtained [25] and peptide mapping of the covalent complex has confirmed the hypothetical model [26].

Two other models of electron transfer complexes based on X-ray structure of *Desulfovibrio vulgaris* cytochrome c_3/rubredoxin [27] and *Desulfovibrio vulgaris* cytochrome c_3/flavodoxin [28] have been described. The same heme, namely heme 4, appears equally to interact with the flavin of the flavodoxin and the iron cluster of the rubredoxin. These results suggest heme 4 is the interacting site of the molecule. The role of the three other hemes may be associated with either a specificity for different redox partners (like hydrogenases), a redox potential modulation or an electron storage [29]. Intramolecular electron transfer within the tetraheme cytochrome has been recently demonstrated [30].

With the aim of studying the influence of surface electrostatic interactions, we have investigated complex formation between *Desulfovibrio* ferricytochromes c_3 and an heteropolytungstate: $[Bi_2W_{22}O_{76}H_2]^{12-}$ [31]. The charge and the molecular weight of this polyanion is similar to the ferredoxin and rubredoxin. The resulting structural modifications of the various complexes have been characterized by circular dichroism, cyclic voltammetry and NMR spectroscopies.

Material and Methods

Cytochrome c_3 and ferredoxin I were purified as previously reported [32,33] from *Desulfovibrio desulfuricans* Norway strain, *D.d.* N. (NCIB 8310). Rubredoxin and cytochrome c_3 were purified from *Desulfovibrio vulgaris* Hildenborough strain, *D.v.* H. (NCIB 8303) as reported [34,35].

The heteropolytungstate $[Bi_2W_{22}O_{76}H_2]^{12-}$ (M_r 6500), hereafter named Bi_2W_{22}, has been prepared according to Ref. 31. Its concentration was checked by ultraviolet absorption using the following coefficient at 260 nm: $5.12 \cdot 10^4$ M^{-1} cm^{-1}. Complexation experiments between the cytochrome c_3 and the polyanion were performed in a non-binding Tris-cacodylate buffer at pH 7.6.

Circular dichroism

CD measurements have been carried out on a Jobin-Yvon Mark IV Dichrograph, on cytochrome c_3 solutions 13–32 μM in $2 \cdot 10^{-2}$ M Tris-cacodylate buffer at pH 7.6 unless otherwise specified.

NMR spectroscopy

For NMR experiments, the protein samples (except ferredoxin) were prepared by D_2O exchanging after successive lyophilizations. Ferredoxin I was concentrated in D_2O on a centricon microconcentrator Amicon. Ferricytochrome c_3 (10^{-3} M) ^{1}H-NMR spectra were recorded with increasing amounts of ferredoxin, polyanion and rubredoxin (from 0.5 to 2 excess). The experiments performed using ferredoxin and polyanion were done in Tris-cacodylate buffer 20 mM, those involving rubredoxin were done in phosphate buffer 10 mM, (pH 7.6) and at 35°C.

^{1}H-NMR spectra were recorded in the Fourier mode on a Bruker AM 200 spectrometer. The water line was irradiated during all experiments. Chemical shifts are in parts per million (PPM) from internal tetramethyl silane (TMS). Chemical shifts variation ($\Delta\delta$i) were obtained by the difference of chemical shifts of free and bound cytochrome c_3.

Titration curves were deduced from chemical shift variation for each cytochrome c_3 methyl line affected as previously reported [23], for at least three different experiments.

Parameters of protein complex formation were calculated by computing graphics of relative induced chemical shifts $\Delta\delta i_n/\Delta\delta i_f$ ($\Delta\delta i_n$ is the chemical shift variation for a ratio [ligand]/[cytochrome c_3] = n and $\Delta\delta i_f$ is the maximum induced chemical shift) as a function of [ligand]/[cytochrome c_3] ratio.

The K values were iterrated until the correlation coefficient and the residue value tend towards 1 and 0 respectively. The K value corresponding to the best correlation coefficient and the lowest residue was selected [36].

Electrochemical instrumentation

Cyclic voltammetry (C.V.) was performed in an inactinic cell containing 1–2 ml of solution at 20°C. The solution was desaerated by argon flushing and was maintained under argon during the experiment. The working electrodes were either glassy carbon electrode

(Tokai carbon) or a basal pyrolytic graphite electrode. The auxiliary electrode was made of a Pt wire. All potentials were referred to SCE. The electrode surface was polished carefully after each potential sweep on polishing discs with different diamond pastes varying from 15 μ to 1 μ. The C.V. apparatus was composed of a function generator (EGG PAR175) and a solid state home-made potentiostat implemented with a positive feedback IR drop compensation. The current potential curves were displayed on XY recorder Ifelec 2502.

Results

Circular dichroism

The CD spectrum of *D.d.* N. cytochrome c_3 in the Soret region consists of a strong narrow positive peak at 408 nm and a weak trough at 420 nm (Fig. 1). Upon stepwise addition of the heteropolytungstate to a 30 μM cytochrome c_3 solution in $2 \cdot 10^{-2}$ M Tris-cacodylate buffer at pH 7.6, a progressive vanishing of the trough occurs up to a 1:1 ratio. An analogous modification of the cytochrome c_3 CD is observed when it is mixed in a 1/1 ratio with ferredoxin I (Fig. 2). However, no change is noticeable in the optical absorption spectra.

The CD study gives an overall view of structural changes of cytochrome c_3 resulting from association. The Soret CD signal of native cytochrome c_3 is of a non conservative excitonic type: the relative intensities of the two components seems to depend on the origin of the protein [37]. Calculations have shown that the excitonic character of the signal cannot be solely accounted for by heme-heme coupling and that aromatic residues contribute to the signal [38]. Upon complexation with the polyanion as well as with ferredoxin and rubredoxin, the excitonic character of the signal is lost.

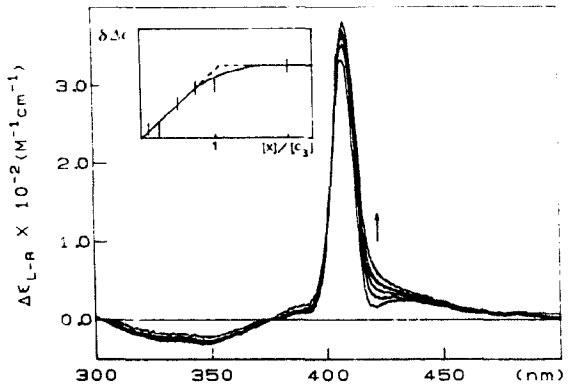

Fig. 1. Soret CD spectra of *D.d.* N cytochrome c_3 in the presence of increasing amounts of Bi_2W_{22}. [Polyanion]/[Cyt. c_3] ratios are 0; 0.25; 0.5; 0.75; 1 and 2 from bottom to top. Cyt. c_3:30 μM in Tris-cacodylate buffer 20 mM at pH 7.6. Inset: titration curve variations of $\Delta\epsilon$ at 421 nm versus concentration ratios.

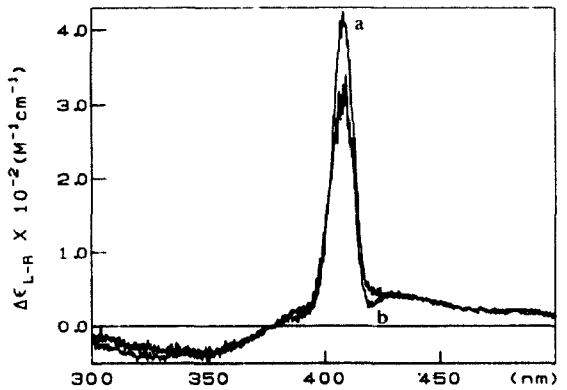

Fig. 2. Soret CD: (a) mixture of cytochrome c_3 and ferredoxin at 1/1 concentration ratio, 25 μM in phosphate buffer 20 mM at pH 7.6 (b) summation of cytochrome c_3 and ferredoxin spectra. Cyt. c_3:50 μM in H_2O, Fd:50 μl in 0.04 M phosphate buffer at pH 7.6.

indicating that the π electrons coupling within cytochrome c_3 is perturbed.

The plot of differences $[\Delta\epsilon_{complex} - \Delta\epsilon_{c3}]$, measured at 420 nm versus the [polyanion]/[cytochrome c_3] concentration ratios reveals the formation of a 1/1 complex, with an association constant K equal to $(2 \pm 1) \cdot 10^6$ M^{-1}. An analogous titration experiment run with ferredoxin I leads to a K value of $(6 \pm 2) \cdot 10^6$ M^{-1}, this value is in good agreement with the previous one obtained from microcalorimetric experiments [22]. We have checked that the interaction of Bi_2W_{22} with cytochrome c_3 from *D.v.* H. leads to the same CD changes.

NMR spectroscopy

^{1}H-NMR spectra of ferricytochrome c_3 from *D.d.* N. have been obtained for various reduction states [16], allowing the correlation between the heme redox potential values and heme methyl resonances in the 30 to 12 ppm region of the oxidized protein spectrum. Such an assignment was used to determine the effect of ferredoxin complexation on ferricytochrome c_3 [23].

To elucidate the structural role of the four hemes in cytochrome c_3, we have reiterrated such experiments with rubredoxin and the polyanion. Fig. 3 presents ^{1}H-NMR spectra of ferricytochrome c_3 (10^{-3} M) (Fig. 3a) in presence of rubredoxin (Fig. 3b), ferredoxin (Fig. 3c) and the polyanion Bi_2W_{22} (Fig. 3e). The induced chemical shifts of few resonances show a selective modification of heme methyl resonances of the cytochrome depending on the ligand. In all cases the heme methyl resonances at 14.64, 14.07 and 13.79 ppm were affected by the presence of rubredoxin, ferredoxin or polyanion. Ferredoxin I and polyanion induce shifts of heme methyl line at 20.01 ppm. Titrations or complex formations were carried out for each ligand as

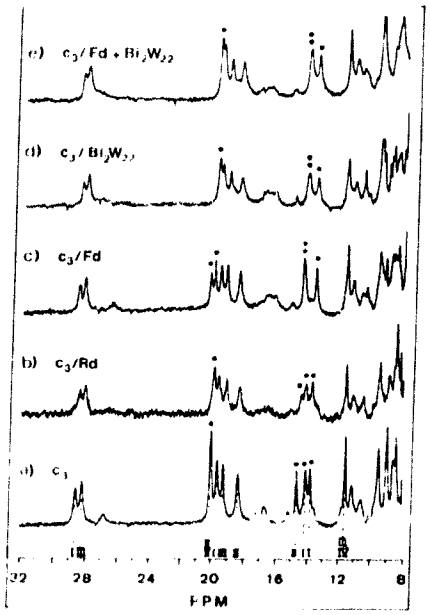

Fig. 3. ^{1}H-NMR spectra of free *D.d.* N ferricytochrome c_3 (1 mM) (a) and complexed ferricytochrome c_3 with *D.v.* H rubredoxin (b), *D.d.* N ferredoxin I (c) or heteropolytungstate Bi_2W_{22} (e), respectively, at 2 mM concentration. The c_3/Fd I complex (from c) in presence of two equivalents of heteropolytungstate Bi_2W_{22} (d). The spectra were recorded at 35°C, p^{2}H 7.6, with the water line irradiated. The affected resonances are marked.

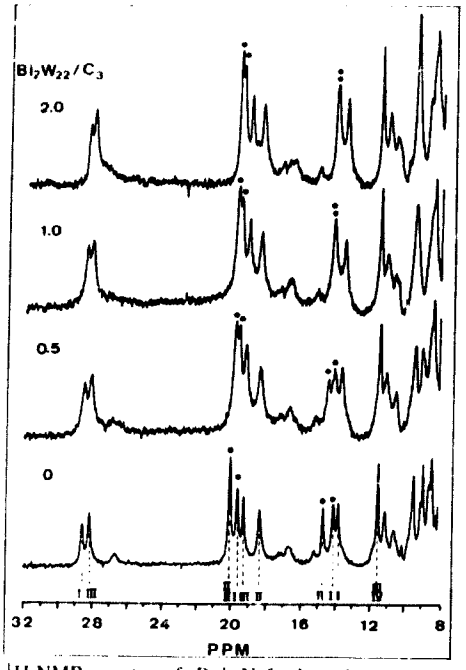

Fig. 4. ^{1}H-NMR spectra of *D.d.* N ferricytochrome c_3 (1mM) in presence of increasing concentrations of heteropolytungstate Bi_2W_{22}. The spectra were recorded at 35°C, p^{2}H 7.6, with the water line irradiated. The affected resonances are marked.

previously described [23]. The titration of the cytochrome c_3 complexation with the polyanion is illustrated by Fig. 4. Analysis of the induced shifts by computing $\Delta\delta i$ as a function of the ratio polyanion/cytochrome c_3 reveals the formation of a 1/1 complex between one molecule of cytochrome c_3 and one molecule of polyanion. Such experiments were also done for the titration of cytochrome c_3/ferredoxin I and cytochrome c_3/rubredoxin complex formation.

Fig. 6 summarizes the titration curves obtained for the three complexes respectively for the 20.01, 14.66, 14.07 and 13.79 ppm resonances. As indicated in Fig. 5, the relative induced shifts of each affected resonance are the same for a complex formation, the chemical shift variation for a methyl line is not the same for the various complexes (Table I). The unlike values are probably due to different effect on the heme methyl environments for each cytochrome c_3 partners. In Fig. 6 the titration curves show a reproducibility of the complex formation parameters for each partner according to the K values.

From redox potential titration experiments [16], the present ^{1}H-NMR results show that as for the ferredoxin/cytochrome c_3 complex formation [23], the two highest redox potential hemes (−165 and −305 mV) are affected by the complexation of polyanion or rubredoxin on cytochrome c_3. These data lead us to suppose that the three partners (ferredoxin, rubredoxin and polyanion) have the same interacting site on cytochrome c_3. We have undertaken competition experiments between ferredoxin and polyanion as the affinity

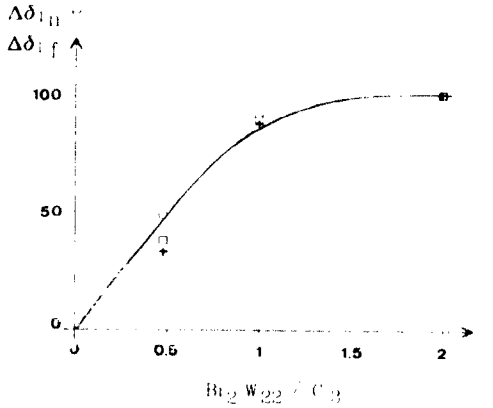

Fig. 5. Titration curve plotted from relative chemical shifts evolution of the different resonances at 13.79 (+), 14.66 (□) and 20.03 (*) ppm of the ferricytochrome c_3 ^{1}H-NMR spectra affected by the addition of increasing concentrations of Bi_2W_{22}.

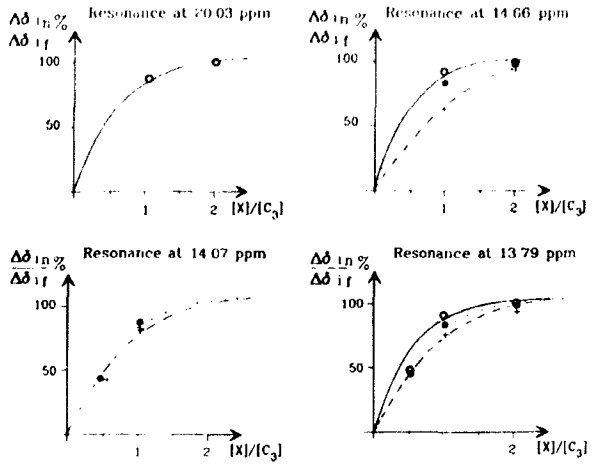

Fig. 6. Titration curves plotted from relative chemical shifts evolution of the different resonances of the *D.d.* N ferricytochrome c_3 ^{1}H-NMR spectra affected in presence of increasing concentrations of polyanion Bi_2W_{22} (———), ferredoxin (· · · · · ·) and rubredoxin (- - - - - -).

constants, evaluated by CD experiments at $6 \cdot 10^6$ M^{-1} and $2 \cdot 10^6$ M^{-1}, respectively, are in the same range. Fig. 3d shows the ^{1}H-NMR spectrum of ferricytochrome c_3/ferredoxin complex (ratio 1:2) in presence of two equivalents of polyanion Bi_2W_{22}. The resulting spectrum is not the addition of the effects observed in Fig. 3c and 3e. Table I shows that the deduced values of the shifts induced by the addition of polyanion to cytochrome c_3/ferredoxin preformed complex are the result of partially polyanion bound and partially released ferredoxin according to their respective estimated association constant. A competition process for a unique interacting site would agree with such an experiment.

Complexation experiments have been carried out between the cytochrome c_3 from *D.v.* H. and the rubredoxin or the polyanion. The same methyl resonances are affected by the two complex formations. The titration of the induced shifts in the both cases reveals the formation of a 1:1 complex with a higher affinity in the cytochrome c_3/Bi_2W_{22} complex than in the cytochrome c_3/rubredoxin one.

Cyclic voltammetry

Both cytochromes c_3 from *D.d.* N. and *D.v.* H. which have different isoelectric points (7.6 and 10.5) and various redox potentials were studied by electrochemistry.

For *D.d.* N. cytochrome c_3 at pH 7.6 in Tris-cacodylate buffer, when adding the polyanion Bi_2W_{22} in stoichiometric quantity, the two reduction waves obtained on polished basal pyrolytic graphite electrode disappear showing an inhibition to direct reduction at the electrode. Decreasing the pH to 5.5 renders the reduction de novo possible in the presence of the polyanion. A slight shift to negative value of the potential peaks (−20 mV) is detected (Fig. 7a). The same phenomenon is observed on glassy carbon electrode, too. We interpret the redox behaviour of the *D.d.* N. cytochrome c_3 at pH 7.6 as an electrostatic inhibition of the negatively charged complexes (cytochrome c_3/polyanion, 1:1) to the negatively charged electrode due to its low isoelectric point. When decreasing the pH of the buffers the number of the negative charges decreases and the electron transfer is restored.

TABLE I

Comparison of the relative chemical shifts of the D.d. N ferricytochrome c_3 ring methyl lines in the ^{1}H-NMR spectra induced by the total complexation of rubredoxin, ferredoxin I, polyanion Bi_2W_{22}, and by the addition of two equivalent of Bi_2W_{22} on the c_3/Fd complex

The more affected resonances are italicized.

$\Delta\delta_{ii} = (\delta_{i\,\text{completely complexed}} - \delta_{i\,\text{free}})$.

Heme from	Redox potential (mV)	Free-ferri cytochrome c_3 ring methyl (ppm)	$\Delta\delta_{ii}$ of total bound ferricytochrome c_3 with (ppm)			
			Rd	FdI	Bi_2W_{22}	FdI + Bi_2W_{22}
I	−165	28.58	−0.03	−0.09	−0.18	0.20
		19.62	−0.04	−0.08	−0.03	0.10
		14.07	*+0.10*	*+0.23*	*+0.55*	*+0.52*
		13.79	*−0.12*	*−0.22*	*−0.24*	*−0.25*
II	−305	*20.01*	*−0.02*	*+0.16*	*−0.33*	*−0.29*
		20.01	*−0.02*	*−0.10*	*−0.53*	*0.29*
		18.34	0.00	−0.03	+0.07	+0.04
		14.64	*−0.24*	*−0.28*	*−0.58*	*−0.52*
		28.17	−0.02	−0.06	0.01	0.11
III	−365	19.25	−0.01	−0.08	0.18	0.18
		11.53	0.00	+0.04	+0.06	+0.09
IV	−400	11.53	0.00	+0.04	+0.06	+0.09

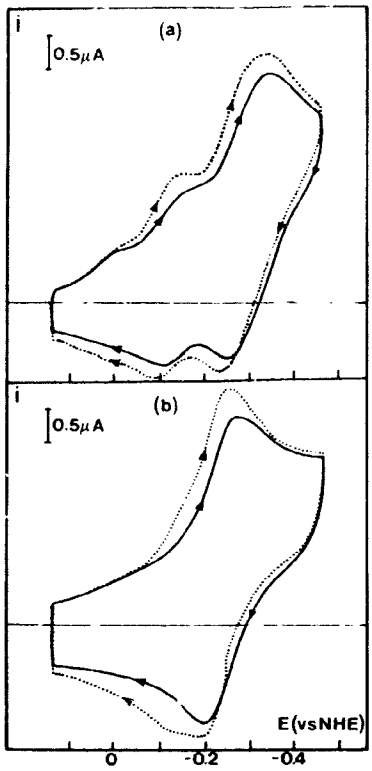

Fig. 7. (a) *D.d.* N ferricytochrome c_3 ($6 \cdot 10^{-5}$ M) in a Tris-cacodylate buffer 20 mM (pH 5.5), cyclic voltammetry on a glassy carbone electrode starting 0.25 V to −0.45 V vs. NHE, $v = 10$ mV/s. *D.d.* N ferricytochrome c_3 alone (·····), *D.d.* N ferricytochrome c_3 + Bi_2W_{22} (1/1) (———).(b) *D.v.* H ferricytochrome c_3 ($4 \cdot 10^{-5}$ M) in a Tris-cacodylate buffer 27 mM at pH 7.6, cyclic voltammetry on a glassy carbone electrode starting 0.25 V to −0.45 V vs. NHE, $v = 20$ mV/s. *D.v.* H ferricytochrome c_3 alone (·····), *D.v.* H ferricytochrome c_3 + Bi_2W_{22} (1:1) (———).

With the *D.v.* H. cytochrome c_3 in the same buffered medium (pH 7.6) in the presence of Bi_2W_{22} a more pronounced shift (−50 mV) is observed (Fig. 7b). C.V. reduction wave 1 is less pronounced in *D.v.* H. than in *D.d.* N. cytochrome because of the slight difference in redox-potential of the hemes [39]. This reduction step disappears or is overlapped with the second reduction wave in the presence of Bi_2W_{22}. This indicates a decrease of the highest redox potentials in agreement with the NMR results.

Discussion

The heteropolytungstate Bi_2W_{22} has been used to get insight into the interacting process of the tetraheme cytochrome c_3 from *Desulfovibrio desulfuricans* Norway with some of its redox partners such as ferredoxin and rubredoxin. Both CD and ^{1}H-NMR experiments have shown that a 1:1 complex is formed between cytochrome c_3 and the polyanion with a high affinity. From CD experiments in $2 \cdot 10^{-2}$ M Tris-cacodylate buffer at pH 7.6 an association constant of $K = 2 \cdot 10^6$ M^{-1} has been deduced. This value is in the same range as the one we found for the cytochrome c_3/ferredoxin complex: $K = 6 \cdot 10^6$ M^{-1}.

NMR and electrochemical experiments carried out with both cytochromes c_3 from *D. vulgaris* Hildenborough and *D. desulfuricans* Norway show that the two highest potential hemes are affected by complexation with polyanion, ferredoxin and rubredoxin. Moreover, the NMR spectrum of a solution of the 1:1 cytochrome c_3/ferredoxin preformed complex added to one equivalent of Bi_2W_{22} shows that the polyanion replaces ferredoxin in the complex. This competition process confirms that ferredoxin and Bi_2W_{22} have the same interacting site on cytochrome c_3 and indicates that this polyanion and the ferredoxin have a comparable affinity for the cytochrome c_3 as suggested by the K values deduced from CD experiments. These different experiments lead us to propose the cytochrome c_3/polyanion complex as a model for the interaction of the tetraheme cytochrome with its redox partners (ferredoxin and rubredoxin).

The question is why an heteropolytungstate, negatively charged molecule without any expected specificity presents such a high affinity for cytochrome c_3? From cytochrome c_3 X-ray structure, it can be established that the distribution of positively and negatively charged residues at the surface of the isolated molecule is asymmetric, suggesting the existence of an important dipole moment [18]. An electrostatic analysis of each heme environment shows that the electrostatic potential field of heme 4 differs drastically from that of the other hemes [24]. Thus, heme 4, positively surrounded, would strongly attract a negatively charged face of a redox partner. An analogous binding of polyanions on a lysine cluster has been observed on cytochrome *c* [10,40].

To understand the interacting process between cytochrome c_3 and ferredoxin or rubredoxin, molecular modelling studies have been done. Two models have been proposed: one for cytochrome c_3/ferredoxin complex from *Desulfovibrio desulfuricans* Norway [24] and the other for cytochrome c_3/rubredoxin complex from *Desulfovibrio vulgaris* Hildenborough [27]. In both cases, the redox partner was docked to each exposed heme of the cytochrome c_3 and each docked structure was manipulated in order to maximize the interaction between its acidic groups and the lysine residues surrounding the cytochrome c_3 heme crevices. Visual inspection of the four models shows that the most favorable complex was the one in which the partner is facing heme 4.

This crevice possesses a diameter of about 26 Å allowing the approach and the fixation of an approxi-

mately spheric molecule presenting a diameter approaching the crevice one.

These modelling experiments demonstrate that, to interact with cytochrome c_3 at the heme 4 crevice, its redox partners must possess particular electrostatic and size properties. The fact that the polyanion forms a high affinity complex with the cytochrome c_3 at the same site than ferredoxin and rubredoxin, can be explained by common characteristics with these two proteins. Ferredoxin and rubredoxin are small globular proteins of approx. 6000 Da, presenting a spheric form with a diameter of about 24 Å for ferredoxin (value deduced from *Peptococcus aerogenes* ferredoxin structure). They are negatively charged entities: 15 negative charges versus 2 positive ones for ferredoxin (pI = 3.9), 10 negative charges versus 4 positive ones for rubredoxin (pI = 4.65). On the other hand, the polyanion has an ellipsoid shape with a long and a short diameter of 18 and 11 Å (Michelon, M. and Hervé, G., unpublished data), it carries 12 negative charges that are evenly distributed on its surface.

These properties allow the polyanion to strongly interact with cytochrome c_3 and thus it appears as a valid model for the study of the interaction between the cytochrome and its redox partners. The observations presented herein allow to say that, in reconstituted complex formation, the interacting process with polyanion mimics the protein-protein recognition on electrostatic and topology properties.

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