¹H-n.m.r. evaluation of the ferricytochrome *c*–cardiolipin interaction

Effect of superoxide radicals

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The interaction between ferricytochrome *c* and cardiolipin was investigated by ¹H n.m.r. at 270 MHz. From the phospholipid-induced changes of the protein spectral features it is concluded that the first 2 equivalents of cardiolipin cause a conformational change at the lower part of the solvent-exposed haem edge, involving a rearrangement of the hydrogen-bond interactions of propionate 6, thus partly accounting for the lowered redox potential of cytochrome *c* in the presence of cardiolipin. The increased value for the p*K* of the alkaline isomerization of ferricytochrome *c* shows that cardiolipin stabilizes the native structure of the protein, indicating that the oxidized form assumes ferrocytochrome *c*-like properties. Peroxidation of cardiolipin by superoxide radical ions drastically decreases the protein binding to this phospholipid. The implications of this finding, and the likelihood of the ternary cytochrome *c*–cardiolipin–cytochrome *c* oxidase complex, for the binding of cytochrome *c* to cytochrome *c* oxidase *in vivo*, are discussed in relation to peroxidative damage following ischaemia and reperfusion.

INTRODUCTION

Several membrane enzymes require specific phospholipids for maximum activity. Cardiolipin has thus been implicated as being essential for the activity of cytochrome *c* oxidase (Robinson & Capaldi, 1977; Robinson *et al.*, 1980; Marsh & Powell, 1988), the terminal enzyme of the respiratory chain. However, it is now known whether it is directly so or indirectly through an enhanced binding of cytochrome *c* to the cardiolipin–cytochrome *c* oxidase complex (Vik *et al.*, 1981). Cytochrome *c*, on the other hand, has been shown to interact strongly and specifically with cardiolipin (Nicholls, 1974; Brown & Wüthrich, 1977*a,b*; De Kruijff & Cullis, 1980; Rietveld *et al.*, 1983; Huang & Kimura, 1984; Devaux *et al.*, 1986), and studies of the phospholipid distribution in the mitochondrial membrane show that more than 50% of the cardiolipin is found on the cytoplasmic cytochrome *c*-binding side of the inner membrane in the mitochondrion (Cheneval *et al.*, 1985). This interaction is primarily electrostatic in nature, involving lysine residues on the surface of cytochrome *c*, but other forces are likely to contribute to the binding.

Although the interaction between cytochrome *c* and various phospholipids has been extensively studied, little attention has been given to conformational aspects of the protein molecule itself in the cytochrome *c*–lipid complex. However, a recent resonance-Raman study (Vincent & Levin, 1986) indicated that cardiolipin induces conformational changes affecting the haem group such that the oxidized form mimics the characteristics of the ferrous form. This investigation was followed up by an e.p.r. study (Vincent *et al.*, 1987) of ferricytochrome *c*, revealing a shift of the *g*_z-value from 1.25 to 1.54 in the protein–lipid complex, from which it was concluded that

a conformational change of the haem group occurs. This finding was suggested to account for the observed fall in redox potential from 260 mV to 220 mV following formation of the complex between cytochrome *c* and cardiolipin (Kimelberg & Lee, 1970).

An explanation, at the molecular level, of the conformational changes leading to the observed cardiolipin-induced spectral shifts of ferricytochrome *c* has yet to be offered. The aim of the present study was therefore to investigate the interaction between cardiolipin and ferricytochrome *c* by ¹H n.m.r. in order to shed further light on the structural mechanism behind previous observations. The present approach yields new evidence for the conformational variability of ferricytochrome *c*, thus extending and linking previous knowledge concerning the interaction between this protein and cardiolipin.

Additionally, since cardiolipin is vulnerable to peroxidative damage caused by ischaemia and subsequent reperfusion (Smith *et al.*, 1980; Okayasu *et al.*, 1985; Feher *et al.*, 1987; B. Soussi, unpublished work), we also investigated the effect of superoxide radical anions on the cardiolipin–cytochrome *c* interaction to illuminate further the requirement of this lipid for cytochrome *c* binding to the mitochondrial inner membrane and hence possibly also to cytochrome *c* oxidase.

From the specific effects on certain haem resonances it is concluded that a conformational change at the lower part of the solvent-exposed haem edge of ferricytochrome *c* takes place as a result of cardiolipin binding to the protein. This change most likely involves perturbations of the hydrogen-bond interactions of the outer propionate side chain, which in turn would explain the lower redox potential found for the protein–lipid complex. Peroxidation of cardiolipin drastically decreases binding of this lipid to the protein.

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EXPERIMENTAL

Horse heart cytochrome *c* was purified on ion-exchange chromatography (Brautigan *et al.*, 1978) and its concentration calculated from $\epsilon_{\text{red-ox}} = 21.1 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ at 550 nm. Bovine heart cardiolipin and egg-yolk phosphatidylcholine were purchased from Sigma Chemical Co., and $^2\text{H}_2\text{O}$ was from CIBA-GEIGY. All other substances were of analytical grade.

Cardiolipin vesicles and phosphatidylcholine vesicles were prepared separately in $^2\text{H}_2\text{O}$ solutions and sonicated for $3\frac{1}{2}$ min at power level 4 in a Branson model B-12 Sonifier. The sonication was carried out in a glass tube held in an ice bath and under N_2 atmosphere. Freeze-dried ferricytochrome *c* was then added to 0.5 ml of the phosphatidylcholine-vesicle solution followed by pH adjustment. The pH is given as the meter reading and is designated pH*. The sonicated cardiolipin vesicles were subsequently gradually added to this solution. Initial ferricytochrome *c* concentrations ranged from 5 to 7 mM. In cases where superoxide was used, solid KO_2 was added to the phosphatidylcholine/cardiolipin mixture, in the presence of O_2 , yielding final concentrations ranging from 1 to $10 \mu\text{M}$. The pH* was adjusted to 7 before addition of the protein, whereafter a final adjustment of the pH* was made.

Optical spectra were measured with a Beckman Acta M4 spectrophotometer. ^1H -n.m.r. spectra were obtained on a Bruker WH 270 MHz spectrometer with a 20 kHz spectral width, a 50° flip-angle and a recycle time of 0.4 s in the quadrature detection mode. Chemical shifts are quoted relative to 4,4-dimethyl-4-silapentane-1-sulphonate, but the residual water peak at 4.70 p.p.m. was used as internal reference.

RESULTS

Fig. 1 shows the normal ferricytochrome *c* spectrum together with literature peak assignments for the paramagnetically resolved resonances (Santos & Turner, 1987, and references cited therein).

Figs. 2(a)–2(c) show the effect of cardiolipin on the ferricytochrome *c* ^1H -n.m.r. spectrum at neutral pH*,

and in Fig. 3 the excess linewidth of the downfield resonance at 34.5 p.p.m., assigned to haem methyl group 8 (see Fig. 1), is shown as a function of increasing amounts of cardiolipin. The spectral features are not significantly broadened in the presence of phosphatidylcholine liposomes only, indicating that this neutral lipid does not specifically interact with ferricytochrome *c* (Brown & Wüthrich, 1977b). Cardiolipin, however, induces, after an initial lag phase, a dramatic broadening, which at a molar ratio of approx. 1.7:1 (i.e. with 7.2 mg of cardiolipin added), is 6-fold. Most of the paramagnetically resolved resonances show a similar behaviour. It is also to be noted that previous studies indicate that all cardiolipin molecules are available for cytochrome *c* binding when added in unilamellar vesicular form (Rietveld *et al.*, 1983).

It has earlier been shown by freeze-fracture studies that addition of ferricytochrome *c* to cardiolipin vesicles causes a number of changes, including bilayer stacking, formation of hexagonal H_{II} phase and particulate structures (Rietveld *et al.*, 1983). In addition, ^{31}P n.m.r. reveals that a considerable fraction of the phospholipids undergo isotropic motion (Rietveld *et al.*, 1983). Furthermore, the increased diameter of the H_{II} structures in mixed-phospholipid systems (Verkleij, 1984) indicates that in our case fusion of the phosphatidylcholine vesicles and cardiolipin vesicles occurs (see below also). The smooth increase in linewidths and unaltered resonance lineshapes shown in Fig. 2 may thus be explained by ferricytochrome *c* binding to increasingly larger cardiolipin-phosphatidylcholine aggregates of various sizes, yielding a sum of superimposed resonances from both bound and free protein forms, under slow exchange conditions, for each resonance. That slow exchange behaviour on the n.m.r. time scale prevails for the protein exchange between the bound state and bulk solution is evident from the low K_D ($35 \mu\text{M}$) and the fact that the protein-phospholipid complex can be separated by centrifugation or ultrafiltration (Rietveld *et al.*, 1983). In principle a small amount of high-spin haem iron might account for the observed line broadenings. This is not the case, since the Fe-S charge-transfer band at 695 nm did not change significantly and no band appeared around

Fig. 1. Ferricytochrome *c* ^1H -n.m.r. spectrum

Assignments taken from the literature (Santos & Turner, 1987, and references cited therein) are as follows: peaks A, B and H, haem CH_3 groups 8, 3 and 5 respectively; peak C, His-18 C-4; peaks D and G, $\alpha\text{-CH}_2$ of propionate 7; peak E, His-18 $\beta\text{-CH}_2$; peak F, Met-80 $\beta\text{-CH}_2$; peak I, thioether 2 CH_3 ; peak J, Leu-68 $\delta\text{-CH}_3$; peak K, Gly-29 $\alpha\text{-CH}$; peak L, Pro-30 $\delta\text{-CH}_2$. The extreme upfield part of the spectrum has been omitted. The spectrum was acquired at 270 MHz, in $^2\text{H}_2\text{O}$ (pH* 7.0 and 303 K).

Fig. 2. Effect of cardiolipin on the spectral features of ferricytochrome *c*

Parts of the 270 MHz ^1H -n.m.r. spectrum of ferricytochrome *c* as a function of increasing amounts of cardiolipin (pH* 7.0 and 303 K). Spectrum *A* is of 6.2 mM ferricytochrome *c* in the presence of phosphatidylcholine liposomes (25 mg/ml); spectra *B*–*E* were recorded after addition of 2.4, 3.6, 4.8 and 7.2 mg of cardiolipin in vesicular form respectively. The samples were mechanically shaken in order to ensure thorough mixing before spectra were recorded. Dilution of the protein concentration was compensated for by making a corresponding increase in the number of free induction decays collected. Spectra have been artificially broadened by 10 Hz and baseline-corrected.

620 nm, where high-spin haem groups absorb (result not shown). Furthermore, the Met-80 methyl resonance accordingly only experienced the general broadening seen in the n.m.r. spectrum.

However, even at the low molar ratios used in this study specific effects are observed in Figs. 2(b) and 2(c). The haem resonance at 10.1 p.p.m., assigned to methyl group 5 on pyrrole ring III (see Fig. 1), is reduced in intensity as the cardiolipin concentration is raised and is barely discernible at the 1.7:1 ratio. Concomitantly, a new very broad resonance centred around 15 p.p.m. appears, which most probably corresponds to the resonance at 10.1 p.p.m. in a magnetically different environment. This shift probably occurs as a result of altered charge interactions at propionate 6 due to a conformational change that is completed at a 2:1 ratio (see the Discussion section).

Furthermore, the resonance at 11.4 p.p.m. disappears at the same rate as the haem methyl resonance at 10.1 p.p.m. This resonance has been assigned to one of the $\alpha\text{-CH}_2$ protons of the inner propionate side chain (see Fig. 1). The second proton gives rise to a resonance at 18.7 p.p.m., which, interestingly, only experiences the broadening generally observed throughout the spectrum. It thus appears that a highly localized effect is observed

at the inner propionate side chain, possibly involving steric interactions. A third specific effect is observed in the upfield region of the spectrum. Apart from the overall broadening observed for the resonances shown in Fig. 2(c), an additional broad resonance at approx. -6.5 p.p.m. gradually appears with increasing amounts of cardiolipin. The size of the shift suggests that it must be of contact origin from a group located on the haem group.

Repeating the experiment in Fig. 2 with cardiolipin vesicles only reveals that the ferricytochrome *c* resonances experience approximately half the general broadening within the same concentration range (results not shown). This observation confirms the supposition that fusion of the phosphatidylcholine vesicles and cardiolipin vesicles occurs in the former case. Furthermore, the specific effects seen in Fig. 2 are not discernible when ferricytochrome *c* is titrated with cardiolipin in the absence of phosphatidylcholine. This fact indicates that most of the observed line broadening in this case is a result of non-specific binding to the protein, caused by the proximity of a large number of cardiolipin molecules. However, in the presence of phosphatidylcholine as well, a more even distribution of cardiolipin is obtained, which approaches a physiologically more relevant situation. Similar results

have been obtained for cytochrome *c* binding to phosphatidylcholine/phosphatidylserine mixtures (Devaux *et al.*, 1986).

The addition of a salt was avoided in the present study, since it was found that 0.1 M-NaCl induced stacking of the lipid fragments and/or further fusion, resulting in a general line-broadening in the ferricytochrome *c* spectrum such that a large fraction of the protein becomes n.m.r.-invisible, as evidenced by the resonance intensity loss. The remaining unbound protein fraction experiences a substantially reduced line-broadening. This residual broadening can mainly be ascribed to the increased viscosity of the sample (results not shown). This observation, however, strengthens the conclusion that the protein binds to cardiolipin under slow exchange conditions.

Fig. 3 also shows the excess linewidth of the downfield haem methyl resonance 8 at 34.5 p.p.m. as a function of increasing amounts of cardiolipin at pH* 9.5. It is seen that above 3 mg of added cardiolipin the induced broadening is reduced by approx. 2-fold, indicating a substantially diminished binding of the protein to the lipids. This observation must undoubtedly be due to the diminished charge of cytochrome *c* at this pH*, reinforcing previous conclusions that the interaction is primarily of an electrostatic nature. The specific effects observed at neutral pH* are present also at this pH*, but are difficult to monitor because of spectral overlap between the neutral and alkaline forms of ferricytochrome *c* (Andersson *et al.*, 1980). Furthermore, the intensities of the neutral and alkaline forms of the protein at pH* 9.5 were approximately equal, indicating that the pK* for the alkaline isomerization of ferricytochrome *c* had been displaced from 9.0 (Andersson *et al.*, 1980) to 9.5.

Fig. 4 shows the effect of superoxide radical anions on the interaction between cardiolipin and ferricytochrome *c*, at neutral pH*, as monitored by the two most downfield haem methyl resonances. Similar experiments were carried out with different amounts of KO_2 (1–10 μM) in order to determine a suitable concentration range. It is

Fig. 3. Ferricytochrome *c*–cardiolipin binding

Excess linewidth of the haem methyl 8 resonance (34.5 p.p.m.) at pH* 7.0 (■) and pH* 9.5 (●) as a function of increasing amounts of cardiolipin. Experimental conditions are the same as those in Fig. 2.

Fig. 4. Effect of superoxide radical ions on the ferricytochrome *c*–cardiolipin complex

Downfield region of the ferricytochrome *c* spectrum showing haem methyl resonances 3 and 8. Spectrum *A*, in the presence of lecithin liposomes (25 mg/ml); spectrum *B*, with 3.6 mg of vesicular cardiolipin added; spectrum *C*, the same as for spectrum *B* except that the phosphatidylcholine/cardiolipin mixture was allowed to react with 3.8 μM - KO_2 before the addition of the protein. Spectra were recorded at pH* 7.0 and 303 K.

seen that the broadening produced by 3.6 mg of cardiolipin (Fig. 4, spectrum *B*) is almost completely abolished by allowing 3.8 μM - KO_2 to react with the lipids before the addition of the protein (Fig. 4, spectrum *C*). Structurally intact cardiolipin is thus required for the binding of ferricytochrome *c* to mixed phosphatidylcholine/cardiolipin aggregates.

Despite the precaution of adding the protein subsequent to the superoxide, approx. 15% of the protein was found to be in the ferrous state after the spectrum was completed, which partly explains the reduced amplitudes in spectrum *C* in Fig. 4 as compared with spectrum *A*.

DISCUSSION

At neutral pH ferricytochrome *c* carries a positive charge of approx. 8, and is therefore expected to interact strongly with a negatively charged membrane surface, as borne out by a number of studies (Nicholls, 1974; Brown & Wüthrich, 1977a,b; De Kruijff & Cullis, 1980; Rietveld *et al.*, 1983; Huang & Kimura, 1984; Devaux *et al.*, 1986; Vincent & Levin, 1986; Vincent *et al.*, 1987). However, only a couple of these (Vincent & Levin, 1986; Vincent *et al.*, 1987) have focused on conformational aspects of the cytochrome *c* molecule itself.

Although ^1H n.m.r. is one of the most powerful techniques available for this purpose, its use has not been reported previously, probably because of experimental difficulties. Consequently, we chose to monitor the effects

Fig. 5. Schematic view of cytochrome *c*

The interactions between propionate 6 and Thr-49, Thr-78 and Lys-79 are shown. Also shown are the position of the ϵ -nitrogen atoms of Lys-39, Lys-53 and Lys-55 residues that may participate in the interaction with cardiolipin. Propionate 7 has been omitted for clarity.

of cardiolipin on ferricytochrome *c*, since the oxidized form of the protein is conformationally more sensitive than its reduced counterpart (Dickerson & Timkovich, 1975) and has well-resolved resonances due to the paramagnetic haem iron.

Conformation of the cytochrome *c*-cardiolipin complex

The results in Fig. 2 demonstrate that ferricytochrome *c* binds cardiolipin with a stoichiometry of 1:2, indicating that specific binding sites exist on the protein for this phospholipid, most probably at the bottom of the cytochrome *c* molecule (Brown & Wüthrich, 1977b). Binding of cardiolipin to these sites suggests that conformational change occurs at the lower part of the solvent-exposed haem edge, as evidenced by the perturbation of the haem methyl 5 resonance. This conclusion is in agreement with a recent e.p.r. study, which reveals a shift of the g_x -value from 1.25 in the uncomplexed form of protein to 1.54 in the presence of cardiolipin (Vincent *et al.*, 1987). Since it has been shown that the g -tensor has its x -axis along the N-1-Fe-N-3 direction in the haem plane (Taylor, 1977), the same phenomenon is most probably being observed by the two techniques. A further indication that the region at the lower part of the solvent-exposed haem edge is conformationally labile comes from the temperature-dependence of the haem resonances. The temperature-dependence of the haem methyl 5 resonance, in the absence of cardiolipin, thus shows a reversed behaviour, when compared with the other haem methyl resonances, in that it does not shift towards its diamagnetic position with increasing temperature as expected for paramagnetically shifted resonances (Ångström *et al.*, 1982). It is probable that the dynamic equilibrium from which this observation stems represents a conformational change similar to the one induced by cardiolipin.

The nature of this conformational change most probably involves a local re-arrangement of charges at the propionate side chain on pyrrole ring III. The carboxy group on this moiety participates in a number of hydrogen-bond interactions with residues Thr-49, Thr-78 and Lys-79 (Takano & Dickerson, 1981), as shown in Fig. 5. Disruption of any of these charge-charge interactions, and in particular the one involving Lys-79, should affect the extent to which spin density from the unpaired electron is delocalized into pyrrole ring III, and hence also the contact shift of the haem methyl 5 resonance at 10.1 p.p.m. The observed spectral changes in the n.m.r. spectrum can thus be ascribed to altered charge interactions and not to the conformation change as such. The observation that ferricytochrome *c* upon being complexed with cardiolipin assumes characteristics closely related to those of the reduced form, as revealed by resonance Raman (Vincent & Levin, 1986), suggests that cardiolipin also induces structural changes in the oxidized form of the protein similar to the redox-linked conformational changes (Bosshard & Zürrer, 1980; Takano & Dickerson, 1981). This conclusion is supported by our observation that the pK for the alkaline isomerization of ferricytochrome *c*, a transition not found for the reduced form, increases in the presence of cardiolipin, as would be expected if the lipid induces ferrocytochrome *c*-like features in the oxidized form of the protein. However, the absence of shift perturbations on haem methyl resonances 3 and 8 argues against the possibility that the conformation of the haem group itself has undergone any change, as has been suggested by Vincent & Levin (1986).

The origin of the resonance appearing at -6.5 p.p.m. is unclear, but it must stem from a group located on the haem group. It has earlier been observed that ionization of propionate 7 of different bacterial cytochromes *c* results in a shift not only for the haem methyl 8 resonance but also for the methyl 3 resonance (Moore *et al.*, 1984). Thus a local change in charge at pyrrole ring IV is transmitted to the diametrically situated pyrrole ring II. If a corresponding situation prevails in the present case, groups(s) located on pyrrole ring I may be responsible for the resonance at -6.5 p.p.m.

The analogy with the ionization of haem propionate 7 is also apt in partly explaining the decrease in redox potential (40 mV; Huang & Kimura, 1984) caused by cardiolipin. Ionization of this group results in a 65 mV fall in the redox potential of *Rhodospirillum rubrum* cytochrome *c*₂ (Moore *et al.*, 1984). This particular protein lacks Arg-38, which in mitochondrial cytochromes *c* forms a salt bridge with haem propionate 7, with the result that the ionization can be observed in the physiological pH range. Chemical removal of Arg-38 in horse heart cytochrome *c*, on the other hand, decreases its redox potential by as much as 90 mV (Moore *et al.*, 1984). These two observations strongly suggest that a re-arrangement of the charge interactions at propionate 6 may be responsible for the cardiolipin-induced decrease of the redox potential of cytochrome *c*. An additional cause for the observed fall in potential may stem from different affinities of the reduced and the oxidized forms of the protein for cardiolipin.

The finding that ferricytochrome *c* specifically binds cardiolipin up to a 1:2 ratio has important implications for the binding of this protein to cytochrome *c* oxidase. Previous studies have shown that two or three cardiolipin

molecules are tightly bound to the oxidase and are required for maximal activity of this enzyme (Robinson & Capaldi, 1977; Robinson *et al.*, 1980; Fry & Green, 1980; Vik *et al.*, 1981). It is also relevant to note that cytochrome *a*, the primary electron acceptor of the oxidase, is located on subunit II, where cardiolipin is attached and cytochrome *c* is bound. Whether optimal function of cytochrome *c* oxidase is related to the interaction between this protein and cardiolipin or to the formation of a ternary complex between these two components and cytochrome *c* has as yet not been established. However, the stoichiometries of cardiolipin binding to cytochrome *c* and to the oxidase suggest that the latter may be the case. A recent magnetic-c.d. study on the interaction between ferricytochrome *c* and cytochrome *c* oxidase shows that a conformational rearrangement, involving changes in the haem-protein contacts of the former (Weber *et al.*, 1987), much of the same nature as described above, takes place. The possibility that tightly bound cardiolipin in part may be responsible for the observed effects was not considered, but, however, it cannot be ruled out in the light of the present results.

Effect of superoxide radical ions

Relatively little information is available concerning the mechanisms behind mitochondrial dysfunction with reference to enzyme activities. It has been reported, however, that *in vitro* lipid peroxidation results either in a generalized effect along the mitochondrial inner membrane or in a specific effect on cytochrome *c* oxidase (Bacon *et al.*, 1986). Observations that more than 60% loss in the activity of cytochrome *c* oxidase in response to ischaemia and subsequent reperfusion was accompanied by a 55% loss in the mitochondrial cardiolipin content (B. Soussi, unpublished work) suggest that this alteration in the enzyme activity is due to cardiolipin peroxidation. This conclusion is supported by the experiments showing the exclusive ability of cardiolipin to reconstitute the activity of delipidated cytochrome *c* oxidase (Fry & Green, 1980; Robinson *et al.*, 1980; Vik *et al.*, 1981). Our findings are in line with the hypothesis implicating cardiolipin in the H⁺-pumping function of the oxidase by conformational change facilitation (Marsh & Powell, 1988), which is consistent with the reaction cycle and the mechanism of electron-gating in cytochrome *c* oxidase (Brzezinski & Malmström, 1986, 1987). Furthermore, the greatly decreased binding of ferricytochrome *c* to mixed phosphatidylcholine-cardiolipin aggregates, as a result of attacks by superoxide radical ions, demonstrates the absolute requirement of structurally intact cardiolipin for binding to occur (Fig. 4).

Since oxygen free radicals are generated in response to ischaemia followed by reperfusion (Feher *et al.*, 1987; Burton, 1988; Halliwell, 1989), it seems likely that mitochondrial dysfunction in part is related to a decreased cytochrome *c* oxidase activity, ultimately because of cardiolipin peroxidation (Smith *et al.*, 1980; Okayasu *et al.*, 1985) causing a diminished cytochrome *c* binding to this enzyme.

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