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Spectroscopic study on the communication between a heme a_3 propionate, Asp399 and the binuclear center of cytochrome *c* oxidase from *Paracoccus denitrificans*

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Abstract

The proton pumping mechanism of cytochrome *c* oxidase on a molecular level is highly disputed. Recently theoretical calculations and real time electron transfer measurements indicated the involvement of residues in the vicinity of the ring A propionate of heme a_3, including Asp399 and the Cu_B ligands His 325, 326. In this study we probed the interaction of Asp399 with the binuclear center and characterize the protonation state of its side chain. Redox induced FTIR difference spectra of mutations at the site in direct comparison to wild type, indicate that below pH 5 Asp 399 displays signals typical for the deprotonation of the acidic residue with reduction of the enzyme. Interestingly at a pH higher than 5, no contributions from Asp 399 are evident. In order to probe the interaction of the site with the binuclear center we followed the rebinding of CO by infrared spectroscopy for mutations on residue Asp399 to Glu, Asn and Leu. Previously different CO conformers have been identified for bacterial cytochrome *c* oxidases, and its pH dependent behaviour discussed to be relevant for catalysis. Interestingly we observe the lack of this pH dependency and a strong influence on the observable conformers for all mutants studied here, clearly suggesting a communication of the site with the heme-copper center and the nearby histidine residues.

Keywords: Cytochrom c oxidase; FTIR spectroscopy; Heme propionates; CO conformers

1. Introduction

Cytochrome *c* oxidase is the terminal enzyme of the respiratory chain and catalyzes the stepwise reduction of oxygen to water [1,2]. During this reaction cycle, electron and proton transfer steps are efficiently coupled to establish an electrochemical proton gradient which drives ATP synthesis. Four redox-active cofactors are involved in electron transfer. Cu_A acts as electron acceptor from cytochrome *c* and transfers the electron to heme *a*. Further electron transfer leads to the binuclear center, formed by heme a_3 and Cu_B, where oxygen is bound and reduced. At present, X-ray structures of the cytochrome *c* oxidase from *P. denitrificans* [3,4], bovine heart [5], *T. thermophilus* [6] and *R. sphaeroides* [7] are available. The architecture of the protein is well understood, however, the role of individual residues and their protonation states, as well as the discussed proton pumping and exit mechanism, are still under debate.

Recent electrostatic calculations postulate an important role for the histidines that coordinate Cu_B [8–11]. Further discussed

Abbreviations: FT-IR, Fourier-transform infrared; SHE′, standard hydrogen electrode at pH 7

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pumping sites are the ring D and ring A heme propionates of heme *a* and a_3 with data based mainly on kinetic experiments [12–16].

Asp399 (numbering in the text refers to the oxidase from *P. denitrificans)* is located in H-bonding distance to the ring A propionate of heme a_3, in proximity to the Cu_B ligands His 325, 326, and at the interface of subunits I and II (see Fig. 1). The residue was previously suggested to form part of the proton exit path [3]. Site-directed mutagenesis was used to probe the relevance of this residue for catalytic activity or proton pumping for the cytochrome *c* oxidase from *R. sphaeroides* [27–29] and *P. denitrificans* [30,31]. Quian et al. [27] concluded that the residue is not essential for proton pumping or for manganese binding in the *R. sphaeroides* system. For the cytochrome *c* oxidase from *P. denitrificans* three different variants have been studied: Asp399 to Asn, Glu and Leu [30,31]. From these data the acidic side chain does not seem to be essential, since the Asp399Asn mutant enzyme retains enzymatic turnover and proton pumping activity, a result that is in agreement with the observation that this position is an Asn in several oxidases [32]. In the Asp399Glu mutant the side chain length is increased and in the Asp399Leu mutant the possibility for hydrogen bonding is eliminated completely. Both mutants exhibit a decrease in turnover numbers to 38% and to 8%, respectively, as compared to wild type. Whereas the Asp399Glu mutant retains proton pumping at a wild type stoichiometry, the Asp399Leu mutant lacks proton pumping [30]. It can thus be concluded that the alteration of H-bonding significantly perturbs the local structure. Indeed the high degree of conservation [27,31,32] is striking and an exclusively structural role appears unlikely. This residue could, together with the histidines that coordinate Cu_B, be part of a cluster of residues that regulate proton proton movements above the binuclear center.

In the study presented here, we propose the interaction of the site in the vicinity of the ring A heme propionate, namely Asp399 and the nearby histidines that coordinate Cu_B with the binuclear center. By means of a combined electrochemical and FTIR spectroscopic approach the protonation state of the residue is studied. We monitor infrared spectroscopically the rebinding of CO for variants on residue Asp399 in order to reveal the interaction of the propionate side chain with the active centre. Infrared spectroscopy is a well established technique for the characterization of the binuclear center from cytochrome *c* oxidase [17–20] and several other oxygen binding proteins [21–25]. In this approach the CO rebinding to the reduced binuclear center after a light flash is followed infrared spectroscopically. The spectra obtained provide informations on the direct environment of the binuclear center. Multiple CO stretch bands are found that could be associated with different conformational states of the enzyme. In the case of bacterial oxidases, these CO conformers-exhibit a pH dependent behavior [19] which is lost in the mutant enzymes.

2. Materials and methods

Cytochrome *c* oxidase from *P. denitrificans* was prepared as described previously ([31] and references within). The CO adduct of cytochrome *c* oxidase was obtained by reduction with a phosphate-buffered $Na_2S_2O_4$ solution, followed by incubation with CO gas for 30 min [36]. 3-4 μl of the sample were used to fill a cell with a pathlength of 10 μm and two CaF_2 windows. For electrochemistry samples at a final concentration of app. 0.3 mM in 100 mM cacodylate buffer (pH 4.8 and 5.5) or 200 mM phosphate buffer, with 100 mM KCl and 0.15 % dodecylmaltoside were used. Enzymatic activity at pH 4.8 was found comparable to the value at pH 7 (Aimo Kannt, Ph.D. thesis, Frankfurt). For mediators and electrode modification see Ref. [37]. The spectroelectrochemical cell was used as previously reported in Ref. [38] and the redox difference spectra were recorded and processed as previously described in Ref. [37]. Potentials quoted in the text refer to Ag/AgCl electrode; please add 208 mV for SHE′.

3. Results and discussion

3.1. Ox-red FTIR difference spectra of cytochrome c oxidase from P. denitrificans

Electrochemically induced FTIR difference spectra of wild type cytochrome *c* oxidase from *P. denitrificans* were previously analyzed in detail [37,39,40]. The difference signals observed represent the total of the molecular changes concomitant with the redox reaction of all cofactors including conformational changes, charge redistributions at the cofactor sites and coupled protonation reactions.

3.1.1. Asp399Asn

Fig. 2 shows the ox-red FTIR difference spectra of the wild type (dotted line) and the Asp399Asn mutant enzyme (full line) at pH 5.5 (A) and pH 4.8 (B). The direct comparison of the ox-red FTIR difference spectra of wild type with the Asp399Asn

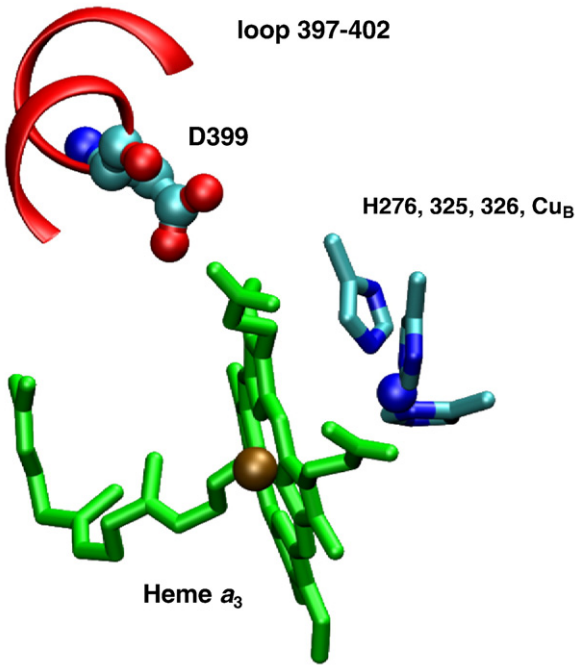

Fig. 1. Position of D399 in the structure of subunit I of cytochrome *c* oxidase from *P. denitrificans* (PDB pdb1ar1). The binuclear heme a_3 –Cu_B center is represented together with Cu_B ligands His276, 325 and 326. Both D399 side chain oxygen atoms are located within hydrogen bonding distance (3.35 □). The software used for visualization was VMD (Ref [52]).

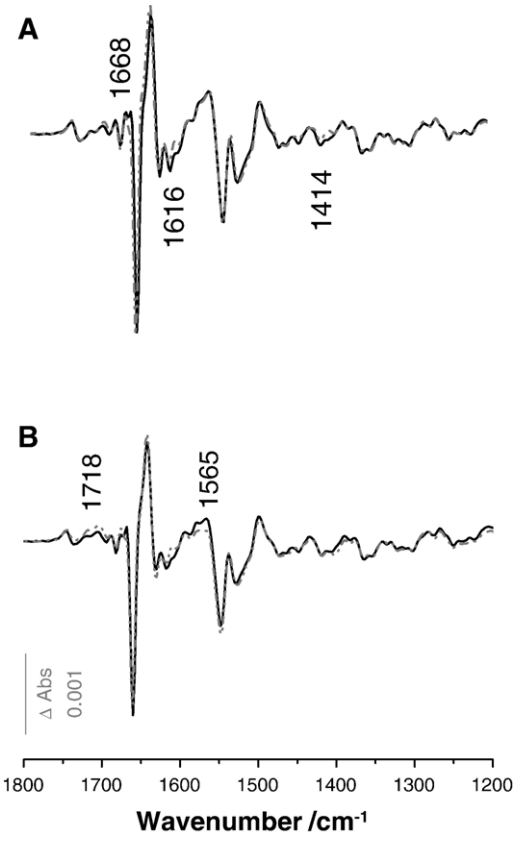

Fig. 2. Oxidized-minus-reduced FTIR difference spectra of wild type (dotted line) and Asp399Asn mutant enzyme (full line) at pH 5.5 (A) and pH 4.8 (B).

mutant enzyme (Fig. 2A) at pH 5.5 shows only small differences. Additional signals can be observed at 1668 cm^{-1} and at 1616 cm^{-1}, accompanied by a variation at 1414 cm^{-1}. On the basis of model compound spectra of the amino acid Asn in aqueous solution [41,42,50], a tentative assignment of the signals at 1668 cm^{-1} to the ν(C=O) mode, at 1616 cm^{-1} to the $\delta(NH_2)$ vibration and, possibly, at 1414 cm^{-1} an involvement of the ν(C-NH_2) vibration, can be suggested. Furthermore an influence of the mutation on the vibration of the protonated acidic group from the ring A propionate of heme a_3, previously assigned to a mode at 1676 cm^{-1} is not excluded. The redox-dependent changes of at least two of the four heme propionates in cytochrome *c* oxidase from *P. denitrificans* were found by FTIR-spectroscopy and specific ^{13}C labeling of the heme propionates [43] and specifically attributed by site-directed mutants in hydrogen bonding distance to the individual heme propionates [44] confirming the effect seen here.

No contribution from Asp 399 itself is evident in the spectra at pH 5.5 (Fig. 2A), since in characteristic spectral range for protonated (1710-1760 cm^{-1}) or deprotonated aspartic acids (1590-1540 cm^{-1} and 1450-1370 cm^{-1}), these modes are essentially identical to the wild type spectra. At pH 4.8 (cf. Fig. 2B) however, variations in this spectral range are clearly observed in comparison of the Asp399Asn mutant to wild type, indicating that the residue is addressed by the redox reaction at a pH below 5.5.

The different pH dependency of WT and Asp399 redox induced FTIR difference spectra are depicted in the double difference spectra in Fig. 3. The data was obtained by subtracting the wild type spectra at pH 5.5 from those at pH 4.8 for wild type (3A) and for the Asp399Asn mutant respectively (3B).

A broad positive mode at 1718 cm^{-1} and strong negative modes at 1556 and 1402 cm^{-1} dominate the double difference spectra of the wild type (Fig 3A). The spectra for the pH step from 4.8 to 5.5 clearly show the spectral feature, which is expected to occur with the protonation/deprotonation of acidic groups [41,50]. The signal at 1718 cm^{-1} arises from a ν(C=O) mode of the protonated form upon oxidation of the enzyme, and the modes at 1556 and 1402 cm^{-1} from the $\nu(COO^-)^{s/as}$ vibrational mode of the reduced protein. The asymmetric form of the prominent signal at 1556 cm^{-1} suggests that more than one acidic group is involved here. In the corresponding data, analogously obtained for the Asp399Asn mutant (Fig. 3B), these modes are significantly decreased, indicating that the signals partially arise from Asp399. We attribute the modes in the double difference spectra to the protonation of Asp399 upon oxidation at pH 4.8. The remaining signals may originate from other acidic residues addressed by the redox reaction. A pK value of just below 5 for Asp399 can be deduced from this data, somewhat higher than for aspartic acid in aqueous solution (pK=3.9).

In summary, Asp399 appears deprotonated at a pH>4.8. At a pH<4.8 the residue takes up a proton in the oxidized state and H-bonding to the protonated heme propionate is no longer supported.

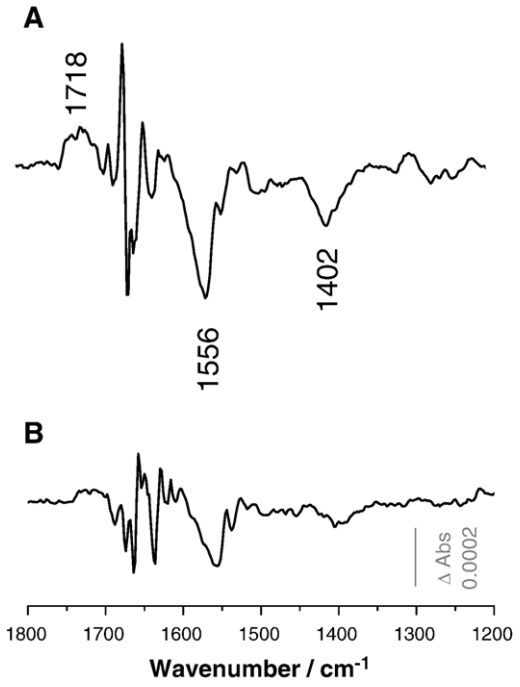

Fig. 3. pH dependency of WT and Asp399Asn mutant displayed in double difference spectra obtained by subtracting the wild type spectra at pH 5.5 from 4.8 (A) and the Asp399Asn mutant respectively (B) for a potential step from −0.5 to 0.5 V.

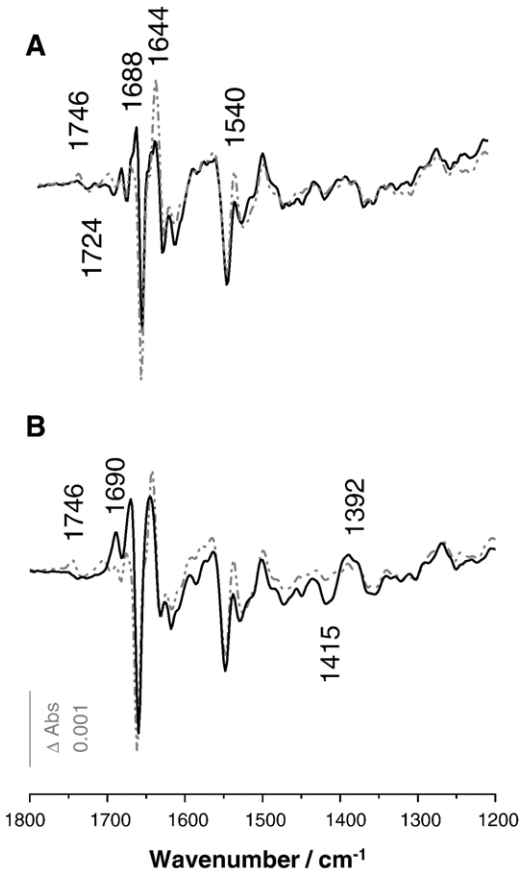

Fig. 4. Oxidized-minus-reduced FTIR difference spectra of the Asp399Glu mutant enzyme (full line, A) in direct comparison to wild type (dotted line, A) and of Asp399Leu (full line, B) in direct comparison to wild type (dotted line, B).

3.1.2. Asp399Glu

The ox-red FTIR difference spectra of the Asp399Glu mutant enzyme show strong perturbations as compared to wild type (Fig. 4). In the amide I range the mode at 1688 cm^{-1} increase and signals at 1666 and 1644 cm^{-1} decrease. The signal at 1540 cm^{-1} strongly decreases, and modes between 1400 and 1300 cm^{-1} vary in intensity. These variations reflect structural perturbations probably caused by the loss of hydrogen bonding; i.e. the change in side chain length caused by the mutation from Asp to Glu. Furthermore, it can be observed that signals at 1746/1724 cm^{-1} are decreased. This signal was previously attributed to Glu278 in cytochrome *c* oxidase from *P. denitrificans* [37] and related cytochrome oxidases [45–49]. A simple explanation would be that a structural perturbation upon mutation induces the deprotonation of Glu278. It should be noted, however, that the Asp399Glu mutant shows a considerable residual catalytic activity of 38 % and retains proton pump activity [31]. The structural changes observed here can thus not be essential for catalytic activity. In an alternative explanation, the introduced glutamic acid contributes with a negative mode at 1746 cm^{-1} and with a positive mode at 1724 cm^{-1}, overlapping the Glu278 modes. This would imply that Glu399 has a higher pK than Asp399, and that hydrogen bonding to the protonated ring A propionate of heme a_3 is impaired, leading to the structural perturbations and the decreased enzymatic turnover (38% as compared to wild type).

3.1.3. Asp399Leu

The ox-red FTIR difference spectra of the Asp399Leu mutant shows major shifts (Fig. 4B), which most likely originate from the absence of H- bonding. Importantly the difference signals at 1748/1734 cm^{-1} are absent that were previously assigned to the protonated amino acid Glu278 reorganizing upon redox reaction [37,45–49].

The Asp399Leu mutant was previously found to exhibit an uncoupled behavior: in intact cells as well as in proteoliposomes, no proton pumping takes place, but electron transfer still persists to a certain degree [30], an effect explained by the deprotonation of Glu278 seen here.

3.2. Light-induced FTIR difference spectra in the 2100 - 1900 cm^{-1} range

At cryogenic temperatures, CO photolytically dissociates from CO-poisoned heme a_3 and remains bound to Cu_B [18]. The low temperature FTIR difference spectra of the CO adduct are well established for the characterization of the binuclear site and reveal possible distortions of the heme copper center in mutants [17–21]. In Fig. 5 the light-minus-dark difference spectra at 140 K of the wild type, the Asp399Asn, the Asp399Glu and the Asp399Leu mutant oxidase are given for pH 6 (black line) and pH 8 (gray line). The stretching frequencies of CO bound to heme a_3 (negative signals) can be found at 1966 and 1952 cm^{-1} for wild type and at similar positions for the mutant enzymes. The respective stretching frequencies of CO bound to Cu_B (positive signals) can be seen at 2063 and at 2042 cm^{-1} for the wild type, and correspondingly for the mutants. We note that the Asp399Glu and

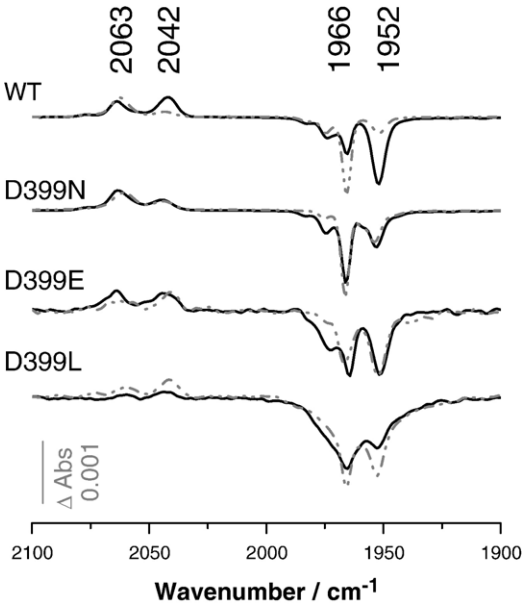

Fig. 5. Light-minus-dark FTIR difference spectra of the CO displacement at 140 K of wild type, Asp399Asn, Asp399Glu and Asp399Leu mutant cytochrome *c* oxidase for pH 6 (black line) and pH 8 (gray line) from 2100 to 1900 cm^{-1}. The spectra were normalized on the CO mode of the heme.

Asp399Leu mutants have a significantly lower affinity to CO binding, accompanied by downshifts of the midpoint potentials in these mutants (data not shown). Photolysed CO may therefore leave the protein, in contrast to wild type and the Asp399Asn mutant enzyme. The broadened modes indicate an opening of the structure in the site, especially for the Asp399Leu mutant, allowing some orientational variation of the CO bonding.

CO bound to fully reduced cytochrome *c* oxidase exhibits two molecular conformations, termed α– and β-form, previously reported for the aa_3 oxidase from *R. sphaeroides* [19]. Signals at 1966/2063 cm^{-1} were assigned to the α–form and the signals at 1952/2042 cm^{-1} to the β-form of the wild type cytochrome *c* oxidase from *P. denitrificans* [36]. The pH dependency of the conformers was previously suggested to originate from a residue with a pK value of 7.3 in the vicinity of the heme copper center [19]. Here we describe a mutant without the pH dependency of the conformers, indicating a strong change in the binding pocket induced by the mutation located 8 Å from the His ligands of Cu_B. The pK value around 5 for Asp 399 was determined from the electrochemically induced FTIR difference spectra for the fully oxidized and reduced form, as described above and it does not correspond to the value around 7 correlating with the pK of the CO conformers (Ref. [20]). Asp 399 is not the direct origin of the pH dependency of the CO conformers, but it clearly interacts with it.

4. Conclusions

Light induced FTIR difference spectra generated by photolysis of the CO adduct to the heme a_3 -Cu_B center show that the reaction center is essentially unaffected in the Asp399Asn mutant. However, the pH dependency of CO conformers, which is determined by a protonatable group in the wild type enzyme, is absent. The pK of the unidentified residue giving rise to the pH dependency of the CO conformers was also reported around 7 [20]. Interestingly, we find that Asp399 is protonated below pH 5 and thus have to exclude this residue as the direct cause. Most likely the nearby Cu_B ligands His325 or His326 are indirectly perturbed in these mutants, thus indicating that either one of these residues is protonatable. This is interesting in the light of earlier discussions on the localization of the proton pathway or protonatable groups in the vicinity of the binuclear center [3]. It is possible that a protonatable residue in the vicinity of the center where oxygen is bound and reduced is involved in the catalytic function. On this basis it was previously suggested that the pH dependency of the CO conformers might be catalytically relevant and Das et al. [26] suggested Tyr280 to be involved in this effect. More recently FTIR spectroscopic studies on the CO rebinding in ba_3 oxidase from *T. thermophilus*, led to the conclusion that the highly conserved couple Asp399 ring A heme propionate is crucial for the mechanism of the protein [33–35].

On the basis of our data we conclude that Asp399 is protonated at a pH of about 5 in the oxidized state. This is in contrast to previous electrostatic calculations [51] that report Asp399 to be protonated at a pH of 7. Interestingly, these calculations also indicate that the proton is shared with the heme propionate located in hydrogen bonding distance to Asp399. A possible scenario would include that Asp399 and the ring A heme a_3 propionate share a proton at a pH higher than 5. Below this pH the shared proton is stabilized more efficiently by Asp399, inducing typical infrared signals for a protonated acidic residue. We note, that this is also in accordance with the line shape of the signal, that is quite broad and typical for a C=O group experiencing large conformational freedom. Importantly the high enzymatic and proton pumping activity of the Asp399Asn mutant, and its loss of pH dependency, clearly shows that the structural flexibility giving rise to these CO conformers, is not a prerequisite for the proper function of the enzyme. However, a hydrogen bond from the Asp99 side chain to the heme propionate appears absolutely crucial, since its absence (Asp399Leu) induces a loss in proton pumping capability. This residue is one of the most highly conserved residues [27,30–32] among heme copper oxidases, even in more distant members of this superfamily, such as the ba_3 oxidase from *T. thermophilus,* where the D pathway is not conserved [6,33–35]. A possible role would be the communication of this residue and the cluster that includes the histidines that coordinate Cu_B, for the regulation of proton translocation.

Acknowledgements

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