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Secondary Structures Associated With Alkaline Transition of Horse Heart Ferricytochrome *C*: An FTIR Study

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ABSTRACT: The spectra of amide I region (1700-1600cm^{-1}) of horse heart ferricytochrome c at 20^{o}C are reported at low ionic strength at of pH values between 7.0 and 11.5 encompassing the alkaline transition. The mid-infrared spectra can probe the protein secondary structures. The Fourier transform infrared spectroscopic technique is used to investigate the changes in the conformationally sensitive amide I band of cytochrome c with pH and ionic strength. Analysis of these results supports the hypothesis that an increase in the non-repetitive secondary structure during alkaline transition is at the expense of regular secondary structures. These strongly suggest that structural switching and ligand exchange behaviour of ferricytochrome c is accompanied by conformational change in the protein backbone.
Keyword: Ferricytochrome c, Alkaline transition, Secondary structure, Infrared.

INTRODUCTION

Cytochrome c a small single domain heme protein belongs to the class of metalloproteins which serve as an electron shuttle in the respiratory chain of aerobic organisms. The heme group is covalently bound to the polypeptide chain and coordinated by histidine-18 and methionine-80 (Bushnell *et al.,* 1990). The importance of the natural function of this protein has stimulated numerous studies on its structure and dynamics (Jonathan *et al.,* 2005). The pH-dependence of cytochrome c has been studied by a wide variety of spectroscopic techniques including magnetic-resonance (Gadsby *et al.,* 1987), optical methods (Theorell and Akesson 1941; Greenwood and Wilson, 1971; Gadsby *et al.,* 1987; Lawal, 1999), and Raman spectroscopy (Davies *et al.,* 1977). All these methods show that ferricytochrome c undergoes a change in the ligation with a pKa of -9.35. This transition has been shown to be a complex process as circumstantially reported from stopped flow pH-jumped study (Pribic *et al.,* 1993 and Lawal *et al.,* 2000).

Alkaline transition was earlier studied by exploiting optical and electro paramagnetic spectroscopy (Wallace, 1984 and Gadsby et al., 1987) for qualitative assessment purpose. The ability for Fourier transforms infrared spectroscopy to measure the absorption envelope in amide I band of C=O stretching vibration characteristic of different secondary structure was a welcome development. This study is therefore aimed at reporting the secondary structural elements of ferricytochrome c prior and subsequent upon methionine displacement using Fourier transform Infrared spectroscopy.

MATERIALS AND METHODS

Sample preparations: Horse heart cytochrome c (C5772) tris(hydroxymethyl) aminomethane, potassium hexacyanferrate (III) (12643) and Sodium dithionite (985) were purchased from Sigma Chemical Co. (St. Louis MO), USA. The horse heart cytochrome c was used without further purification. For a H_2O-based solution, the protein was dissolved in 10mM tris buffer pH 7.0 at a concentration of 10µM/ml. The oxidized cytochrome c was obtained by treatment of a solution of cytochrome c with excess potassium ferricyanide. The oxidized cytochrome c was recovered by gel filtration chromatography on sephadex G-25 (separation range 1500-3000Da). The conversion of cytochrome c to ferricytochrome c was confirmed by optical spectroscopy between 500 and 600nm. The absence of twin peaks in the region confirmed the absence of ferrocytochrome c in the eluent. All other chemicals were from reputable Companies and were of the highest available qualities.

Infrared measurements and the amide I spectra analysis: Protein sample dissolved in

10mM buffer pH 7.0 (150µl) of cytochrome c was redissolved with 2ml of buffers of varying pH ranges (5.0-11.5 with an increment of 0.5) and mixed thoroughly in a cuvette. The exact pH of the solution in the cuvette was recorded; this pH value was taken as the true pH of the solution. This fresh solutions were prepared for infrared measurements in a sodium chloride cell (Thermo FH-01). Spectra were recorded at 20°C with a Thermo-Electro (Nicollet AVATAR) 333FT-IR spectrophotometer equipped with a mercury/cadmium/telluride detector and interfaced with PC data station. For each spectrum a 32 scans interferogram were averaged in a single beam mode with a 4cm^{-1} resolution and a 2cm^{-1} interval from the 4000cm^{-1} and 400cm^{-1} (see appendix I). Reference spectra were recorded under identical scan conditions with only buffer in the cell. Second–derivative spectra were obtained using the derivative function of OMNIC software (Nicolet) (Byler and Susi, 1986).

RESULTS AND DISCUSSION

In the present study, the secondary structure composition of native and alkaline ferricytochromec has been investigated using Fourier transform infrared (FT-IR) spectroscopy. The amide I band (1700-1600cm^{-1}), which is due primarily to the C=O stretching vibration of backbone peptide linkages, known to be sensitive to small variations in molecular geometry and hydrogen bonding pattern, is one of the most extensively studied amide vibrations (Krimm and Bandekar, 1986 and Surewicz and Mantsch, 1988). The results obtained indicate that while the overall secondary structural component of the native and oxidized cytochrome c remains similar, deligation of methionine-80 and subsequent binding of lysine 72(79) causes significant conformational changes in the regions ascribed to β-sheet and unordered(random coil) structures.

The pH-dependence of the Fourier transform infrared spectra for ferricytochrome c in 10mM tris buffer, pH 7.0, at 20°C and its second derivative is shown in Figures 2, 3 and 4. It has been well documented that the absorbance maxima of amide I infrared bands are determined by the predominant secondary structure in the proteins (Dong *et al.,* 1995). In H_2O the amide I band maximum of a protein with a predominant α-helix structure is generally found near 1656±2cm^{-1} , whereas the maximum of a predominant β-sheet structure occurs between 1643 and 1631cm^{-1} (Surewicz and Mantsch, 1988). The primary peaks are characterized between 1700 and 1600cm^{-1} that is the so called amide I band (Figure 1a and 1b).

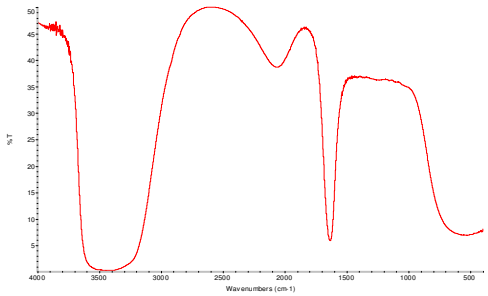

Figure 1: pH resolved infrared spectrum of ferricytochrome c at pH 7.0 in 10mM tris buffer pH (7.0) at 20°C.

The amide I mode is primarily a C=O stretching band. It may have some contributions from CN stretching and C-CN deformation, which in this work shows small but distinct changes at neutral pH compared to the alkaline species (pH 9.5). The band is composed of several underline components arising from various secondary structural contributions such as α-helix, β-sheet, β-turns and random coil (Miyazawa and Blout, 1960), and thus sensitive to the conformational changes of proteins (Susi and Byler, 1986). The spectrum obtained at pH (7.0) has a peak centered around 1635cm^{-1} which can be assigned to β-sheet (see Figure 1a and b). Other workers suggested that, band centered at 1635cm^{-1} could arise as a result of solvated α-helical structure that can disappear due to helix transition (Haris and Chapman, 1995; 1996; Xu and Keiderling, 2004). Complete amide I band assignments are summarized in Table 1. Standard approach as described by Susi and Byler, 1983, Byler and Susi, (1986); Surewicz and Mantsch, (1988) and Haris and Chapman, (1995) were used for assigning the peaks in this work.

Figures 2, 3 and 4 show typical infrared spectra upon second derivative transformed secondary structure determination of ferricytochrome c. Frequencies of intrinsic band components were resolved using the

above mentioned enhancement techniques. In contrast with single band observed in the primary spectrum, second derivative in the amide I region (1700 and 1600 cm^{-1}), was characterized by several bands (see Table 1). Naturally, these wave numbers are not precise and may deviate by as much as $4cm^{-1}$ (Van Holde *et al.,* 2006).

Table 1: Assignment for deconvoluted infrared amide I components of ferricytochrome *c*.

Frequency (cm^{-1})	Assignment
1628	β-Strand
1636	β-strand
1648	Unordered(Random coil)
1654	α-Helix
1669	β-Turn
1675	β-Turn
1685	β-Turn

Lower wave number region starts with very intensive amide I band. In the spectrum of ferricytochrome c is the band at $1654cm^{-1}$ with pronounced asymmetric shape that indicates fine structured amide I. This is generally considered to be characteristic of α-helical structure (Goormagtigh *et al.,* 2006). This band has been obtained by theoretical calculations (Krimm and Bandekar, 1986; Surewicz and Mantsch, 1993), in agreement with the experiments with peptides and proteins that are largely α-helical (Byler and Susi, 1986 and Surewicz and Mantsch, 1988 and Haris and Chapman, 1995). Interestingly, the α-helical content in both neutral and higher pH in this work was lower than the reported values (Jackson and Mantsch, 1995). This could be due to the presence of tertiary and/or long-range contacts which are reported to stabilize and increase the secondary structural content in proteins.

It is quite possible that the secondary structure of the proteins could be stabilized by the formation of non-local interactions upon binding with other proteins (Surewicz and Mantsch, 1988). The α-helical structure have been reported to be sensitive to its environment (Haris and Chapman, 1995), hence the difference in its absorption. Therefore, the difference in frequency can be attributed to the solvent effect of surrounding buffer, which lowers the band frequency maximum. The low-frequency region of the spectrum may distort the secondary and tertiary structure. The complex pattern of the spectra (Fig. 2, 3, and 4) results from a highly heme and is comprised of heme-peripheral substituent modes (Das *et al.,* 1998). It is worth noting that, in the case of myoglobin 35% is made up of helical structure. The apoprotein structures of cytochrome c, myoglobin or horseradish peroxidase have been reported to exhibit loose packing of the helices but regain structural rigidity (Vicent *et al.,* 1988 and Hughson *et al.,* 1990). However, the above phenomena can responsible for structural changes observed in the ferricytochrome c molecule.

Table 2: Parameters obtained by infrared second-derivative amide I spectra of native cytochrome c at different pH .

pH	α-Helix[a] $1654cm^{-1}$	Random coil[a] $1648cm^{-1}$	β-sheet[a] $1636cm^{-1}$	β-turn[a] $1685cm^{-1}$
5.0	25.90	17.99	9.30	46.70
5.5	26.20	26.94	25.22	21.62
6.0	32.70	31.55	5.58	29.15
7.0	40.60	28.46	15.20	15.80
7.5	39.51	30.82	16.00	13.62
8.0	46.03	43.40	1.07	9.64
8.5	43.34	28.90	7.23	20.62
9.0	36.47	20.73	15.28	27.60
9.5	40.04	22.40	6.60	30.60
10.0	43.60	23.64	4.15	28.76
10.5	27.63	18.13	4.83	49.54
11.0	25.81	18.25	5.32	50.82
11.5	32.43	16.52	12.51	38.60

[a]Percentage secondary structural assignment as reported by Byler and Susi (1986)

Assignation of other components in the amide I region is not so straightforward. There are at least two adequate origins (explanations) of remainder components. In general, amide I bands in the spectral regions between 1620cm^{-1} and 1640cm^{-1} can be attributed to β-sheet structures (Susi and Byler, 1986). Therefore, the bands centered at 1636 and 1628cm^{-1} can be assigned to β-sheet. Frequencies of these bands depend on hydrogen bonding strength in β-sheet structures, as well as on coupling of transition dipole (Haris and Chapman, 1995). An anti-parallel β-sheet structure can further be identified by presence of another band at 1675cm^{-1} region. However, this unusual component can be overlapped with the bands, which belong to vibrations of various types of turns and random coil.

The first group of bands arise from intramolecular C=O vibrations of β-sheet. The latter was found in denatured proteins, but it was not so common in native proteins. The 1628cm^{-1} band first reported in cancanavalin A (Arrondo *et al.,*1993) was assigned to peptides in the extended configuration, with hydrogen bonding pattern formed by peptide residues not taking part in intramolecular β-sheet but rather hydrogen-bonded to other molecular structures e.g. forming intermolecular hydrogen bonding in monomer-monomer interaction, while β-turns bands are assigned near 1685, 1675, and 1669cm^{-1}.

However, cytochrome c has been reported to contain little β-structures (Provencher and Glockner, 1981; Goormaghtigh *et al.,* 2006). It has been previously suggested that, the band assigned to β-sheet and β-turns are attributable to transition resulting to extended chains connecting the α-helices (Byler and Susi, 1986) or to helix-helix interaction (Reisdorf and Krimm, 1996). The 1648cm^{-1} band can be assigned to unordered structure (random coil) (Provencher and Glockner, 1981). The infrared spectrum of proteins can distinguish between different types of hydrogen bonds within a protein, as well as their approximate length. This enable the technique detect the change of this hydrogen bonds network and hence overall to the change in the secondary structural elements of the proteins.

A quantitative estimate of secondary structure of ferricytochrome c was carried out using the infrared second derivative spectra (IR-SD) (Table 2). The results show that α-helical and random coil contents of ferricytochrome c was (40.60 and 28.82%) at neutral pH (7.0) while at pH 9.5 it was (40.04 and 20.73%) . There was a significant decrease of non-repetitive secondary structure between the results at neutral pH (7.0) and in the neigbourhood of alkaline transition at pH 9.5. Although it was in agreement the work reported result by Dong and Lam (2005), that unfolding of cytochrome c due to α-helix structure was as a result of acid effect. The β-turns content was higher at neutral pH (15.80%) as against (30.60%) in the neigbourhood of alkaline transition of ferricytochrome c at pH 9.5.

Quantitative analysis confirmed that the random coil structure is one of the most abundant secondary structures in this work. These findings provide direct evidence for extensive conformational rearrangements as a result of alkaline transition, even though the relative contribution of different secondary structures expressed in broad general terms (particularly α-helix) were little affected. Various β-turn conformation are additional secondary structures that generally exist in globular proteins (Chou and Fasman, 1977). Acid-induced unfolding of cytochrome c occurs in a single cooperative transition accompanied by the loss of axial Met-80-heme ligation under low ionic strength conditions (Tonge *et al.,* 1989). Simple treatment of cytochrome c by potassium ferricyanide for the purpose of fully oxidizing it could prevent achieving the maximum unfolded state, due to strong interaction between the ferricyanide ions and cytochrome c (Dong and Lam, 2005). The cytochrome c in this work reveals a much regular secondary structural elements. The second derivative spectra of the protein continue to change after transition, indicating that the structure of alkaline transition is associated with the increase in β-turns, α-helical and decrease β-sheet and random coil structure in neugbourhood of alkaline transition (pKa 9.5). The relative amounts of α-helix, β-sheet, β-turns and random secondary structures of cytochrome c estimated by analysis of the infrared second-derivative spectra (IR-SD) (presented in table 2) shows a remarkable consistency in amide I stretch frequency for each type of secondary structure. The infrared amide I band, the most informative spectral region, provides mainly

the conformational information on the protein secondary structures such as α-helix, β-sheet, β-turns and random coil (Krimm and Bandekar, 1986, Susi and Byler, 1986; Reidorf and Krimm, 1996).

However, information from the tertiary structural levels may also provide conformational information especially the ones derived from spectral changes in the β-turns region (1660 and 1688cm^{-1}). Unlike other secondary structures the β-turns structures are closely associated with the tertiary structure of proteins (Dong *et al.,* 1995; Dong *et al.,* 2002). Conformational changes in the tertiary structural level are undoubtedly accompanied by structural changes at β-turns, which in turn will be reflected in the spectral changes at the region assignable to the β-turns (Dong and Lam, 2005). Significant amount of β-turns at neutral pH (7.0) was lower than the value obtained in the neigbourhood of alkaline transition at pH 9.5.

The spectral changes at β-turn region associated with conformational changes at tertiary structural levels may be due to it percentage content alteration, changes in band frequencies, half bandwidth and/or numbers of band components. It is clear from the result that the transition of ferricytochrome c has affected the β-turn structures in both content and number of components (figures 1, 2 and 3). A significant increase in the percentage content of β-turn was observed in the spectrum of β-turn band and it exhibited 3cm^{-1} difference at 1685 cm^{-1} than that of the native state. This is in agreement with results reported by near UV CD (Goto *et al.,* 1990; Qureshi *et al.,* 2003), hydrogen exchange/2D NMR (Jeng *et al.,* 1990), X-ray scattering (Cinelli *et al.,* 2001).

Conclusion: It has been observed that pH have affected ditrisbution of secondary structures. For example, an increase in the percentage random coil structures was at the expense of β-sheet as the pH was changed from neutral to 9.5. This indicated that changes in the conformation-sensitive amide I spectra due occur in the alkaline form of ferricytochrome may be critical for its electron transfer function.

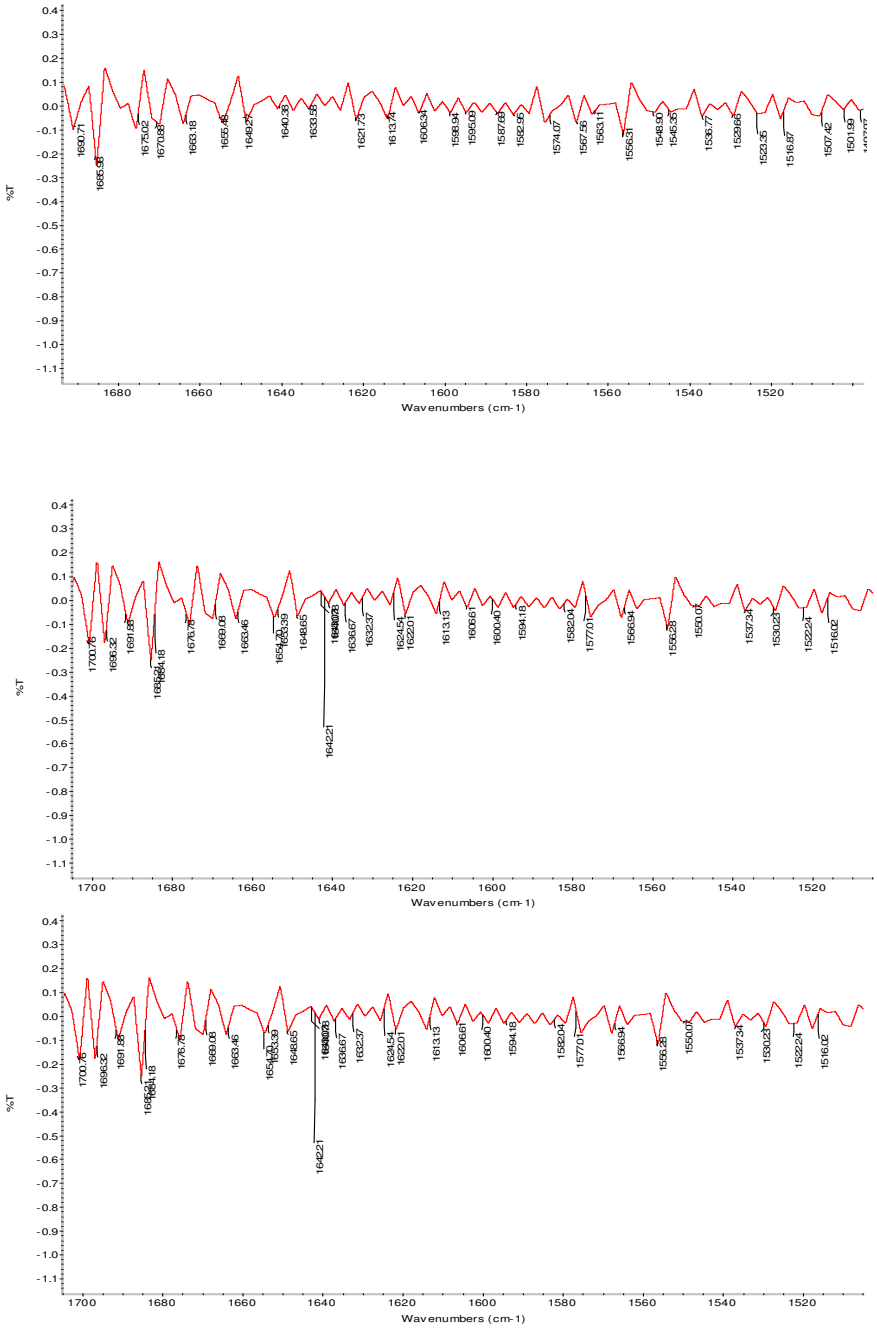

Figure 2: Infrared spectra of ferricytochrome c in the upper, middle and lower forms representing second derivative spectrum of amide I region (pH 7.0 7.5 and 8.5).

Figure 3: Infrared spectra of ferricytochrome c in the upper, middle and lower panels representing second derivative spectrum of amide I region (pH 7.0, 9.0 and 9.5).

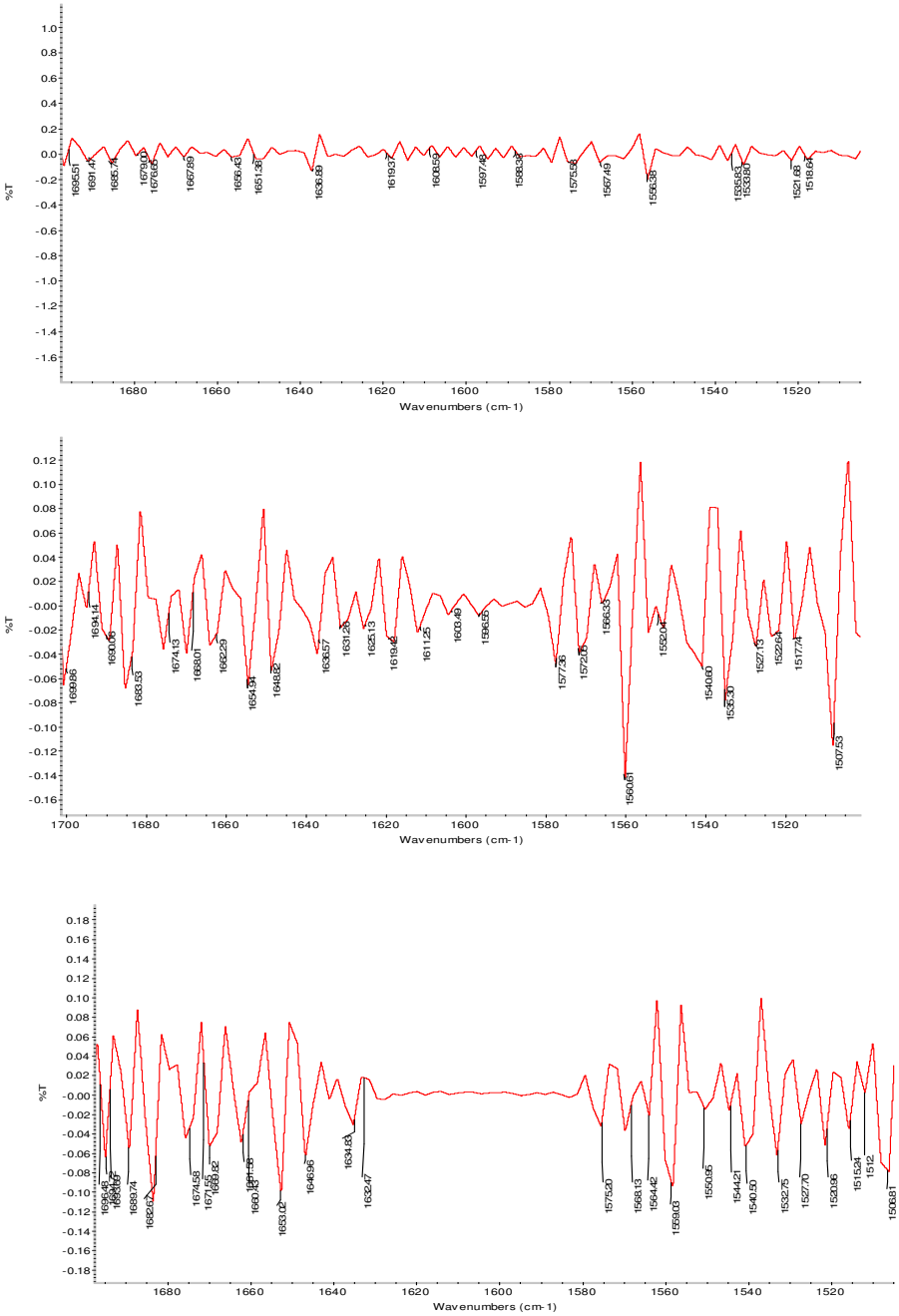

Figure 4: Infrared spectra of ferricytochrome c in the upper, middle and lower forms representing second derivative spectrum of amide I region (pH 7.0 10.0 and 11.5).

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