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Evolutionary alkaline transition in human cytochrome *c*

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Abstract Conformational transitions in cytochrome *c* (cyt *c*) are being realized to be responsible for its multi-functions. Among a number of conformational transitions in cyt *c*, the alkaline transition has attracted much attention. The cDNA of human cyt *c* is cloned by RT-PCR and a high-effective expression system for human cyt *c* has been developed in this study. The equilibrium and kinetics of the alkaline transition of human cyt *c* have been systematically investigated for the first time, and compared with those of yeast and horse cyt *c* from an evolutionary perspective. The pK_a value for the alkaline transition of human cyt *c* is apparently higher than that of yeast and horse. Kinetic studies suggest that it is increasingly difficult for the alkaline transition of cyt *c* from yeast, horse and human. Molecular modeling of human cyt *c* shows that the omega loop where the lysine residue is located apparently further away from heme in human cyt *c* than in yeast iso-1 and horse heart cyt *c*. These results regarding alkaline conformational transition provide valuable information for understanding the molecular basis for the biological multi-functions of cyt *c*.

Keywords Human cytochrome *c* · Alkaline transition pH-jump

Introduction

Cytochrome *c* (cyt *c*), an electron transporting protein, was recently found to play central roles in both pro-apoptotic (Kagan et al. 2005) and apoptotic (Liu et al. 1996) pathways. These findings sparked an intense interest aimed at understanding the molecular basis for the multi-functions of this ancient protein. Conformational transitions in cyt *c* are being realized to be responsible for its multi-functions. For instance, a conformational transition conferring the protein peroxidase activity is a must for cyt *c* to release from mitochondria and, therefore, for the execution of the following apoptotic steps (Kagan et al. 2005; Belikova et al. 2006; Sinibaldi et al. 2008). In addition, cyt *c* was found to undergo conformational changes when it interacts with some of its redox partners (Hildebrandt et al. 1990; Guo et al. 2004).

Among a number of conformational transitions in oxidized cyt *c*, a so-called alkaline transition has attracted much of attention (Rosell et al. 1998; Dopner et al. 1998; Assfalg et al. 2003; Martinez and Bowler 2004; Silkstone et al. 2005; Baddam and Bowler 2005a, b; Battistuzzi et al. 2007; Bandi et al. 2007; Shah and Schweitzer-Stenner 2008; Hagarman et al. 2008; Abriata et al. 2009). The alkaline transition is a pH-induced conformational change in which the axial ligand methionine-80 dissociates and is replaced by a lysine at high pH (Wilson and Greenwood 1996). The ligand exchange lowers the redox potential of cyt *c* dramatically (Rosell et al. 1998). Thus, the alkaline transition has been supposed to act as an electron transfer gate, and play a fundamental role in energy metabolism (Baddam and Bowler 2005a, b; Bandi et al. 2007; Davidson 2000). Recent studies also suggest that the alkaline conformer of cyt *c* may coincide with a late folding intermediate of this protein (Bai et al. 1995; Krishna et al. 2003; Hoang et al. 2003). Moreover, the alkaline transition of cyt *c* is considered to be involved in apoptosis (Liu et al. 1996; Jiang and Wang 2004; Degli Esposti 2004). Although significant progress has been made in understanding the nature of this conformational transition, the exact role of the alkaline transition *in vivo* is still unknown.

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Cyt *c* from some species, such as yeast and horse, are often selected for research. However, only a few investigations on human cyt *c* have been reported (Osheroff et al. 1980; Jeng et al. 2002; Olteanu et al. 2003; Rodriguez-Roldan et al. 2006). Although the pK_a value of human cyt *c*, which was obtained from human heart tissue, was measured over two decades ago (Osheroff et al. 1980), the thermodynamics and kinetics of the alkaline transition for human cyt *c* were never explored further. In the present study, we have cloned cDNA encoding human cyt *c* by RT-PCR for the first time, and developed a high level expression and purification system for human cyt *c*. The equilibrium and kinetics of the alkaline transition of human cyt *c* were systematically investigated. It is surprising that human cyt *c* shows remarkable differences in alkaline transition from cyt *c* of other species. The evolutionary alkaline transition of cyt *c* from yeast, horse and human has been investigated and discussed in this work.

Materials and methods

Materials

Human NCI-H460 cells were provided by the Chinese Academy of Science. The plasmid pBTR2 was a gift from Professor A.G. Mauk. Pfu DNA ploymerase, T4 DNA ligase, dNTP and restriction enzymes were purchased from New England Biolabs. SuperScript II RNase H reverse transcriptase was from Invitrogen. The gel extraction kit and RNeasy Mini Kit were purchased from Qiagen. QuikChange Site-Directed Mutagenesis Kit was the product of Stratagene. The *E. coli* strain XL1-BLUE, BL21(DE3) pLysS and Rosetta(DE3)pLysS were obtained from Novagen. CM-52 and CM Sepharose Fast Flow resin were purchased from Whatman and Pharmacia, respectively. Horse heart cyt *c* was purchased from Sigma-Aldrich. All other reagents were of analytic grade.

RNA extraction and RT-PCR

Total RNA was extracted from human NCI-H460 cells using Trizol reagent and purified according to the manufacturer's instructions. Reverse transcription was carried out as follows: 5.0 μL total RNA (5.0 μg), 1.0 μL oligo-dT, 1.0 μL dNTP (10 mM), 4.0 μL 5×buffer, 2.0 μL DTT (100 mM) and 7.0 μL RNase-free water. The product was used as the template for PCR amplification. Specific PCR primers for human cyt *c* were designed:

Upstream primer: 5′-TCCCCCGGGCTTTAATC-CATGGGTGATGTTGAGAAAGG-3′

Downstream primer: 5′-CTAGCTAGCTTACTCAT-TAGTAGCTTTTTTG-3′

The PCR product was purified by gel extraction.

Plasmid construction

The plasmid pBTR-fd was constructed from the plasmid pBTR2 (Pollock et al. 1998) by removing one of the two *Nhe*I restriction sites. The purified human cyt *c* cDNA was digested with *Nhe*I and *Sma*I enzymes and cloned into pBTR-fd vector (pBTR-hCYC), and verified by DNA sequencing.

Expression and purification

A single and freshly transformed colony of *E. coli* Rosetta (DE3)pLysS containing the pBTR-hCYC vector was inoculated into 50 mL SB media with 100 mg/L ampicillin. After overnight incubation at 37 °C with vigorous shaking (300 rpm), 5 mL culture was used to inoculate 800 mL modified SB (tryptone: 10 g/L, yeast extract: 8 g/L, NaCl: 5 g/L, glycerin: 1.5 mL/L, $NaNO_3$: 2 g/L) with 100 mg/L ampicillin in a 1 l flask. These flasks were sealed by plastic wrap on caps and incubated for 12–15 h. Then, cells were harvested by centrifugation (Sorvall GS3 rotor, 6000 rpm, 10 min) and resuspended in lysis buffer (50 mM Tris-HCl, 1 mM EDTA, pH 7.0) adding DNaseI and lysozyme. Cells were stirred for 2 h then sonicated. The lysate was centrifuged and $(NH_4)_2SO_4$ was slowly added to the supernatant to a final concentration of 300 g/L. The supernatant was dialyzed overnight and applied into a CM-52 column equilibrated with 50 mM sodium phosphate buffer, pH 6.7. The pink protein was eluted with the same buffer plus 250 mM NaCl, and then oxidized with $K_3Fe(CN)_6$. After diluting with ddH_2O, the protein solution was further purified on a CM Sepharose Fast Flow column and was eluted using a linear NaCl gradient. Fractions with an A_{410}/A_{280} ratio higher than 4.5 were collected, concentrated and exchanged into ddH_2O. Finally, the protein solution was snap-frozen in liquid nitrogen and stored at −80 °C. The yeast iso-1 cyt *c* was expressed and purified as described by Pollock et al. (Pollock et al. 1998) Final proteins were >99% pure, based on imaging Coomassie blue stained SDS-PAGE (12%) gel.

pH titration

Protein samples were prepared in the mixture of sodium phosphate and sodium borate buffers ([cyt *c*]=0.2 mM, ionic strength=0.1 M), and titrated with increasing amounts of 12 M NaOH at 25 °C. The progress of basification was monitored by Hewlett-Packard 8453 spectrometer. The pH values were measured directly in the cuvettes using a Schott

microelectrode (type 16PH) connected to an Orion pH meter (type 310P-02).

Stopped-flow kinetics

For the kinetics studies, protein samples (40 μM protein, 100 mM NaCl, pH 7 and unbuffered) were rapidly mixed with equal volume of buffer in a SF-61 DX2 stopped-flow apparatus (Hi-Tech, UK) thermostated at 25.0±0.1 °C. The following buffers were used: CHES (Sigma) (pHs 9.00, 9.10, 9.21, 9.29, 9.41, 9.51, 9.65, 9.78, 9.90 and 10.01) and CAPS (Sigma) (pHs 10.17, 10.37, 10.60 and 10.79). These buffers were prepared with sufficient NaCl to achieve the final ionic strength of 0.1 M. The decrease of absorbance at 695 nm was monitored.

Simulation of molecular structure

The initial coordinate for human cyt *c* was constructed based on the crystal structure of horse heart cyt *c* (pdb entry: 1hrc) (Bushnell et al. 1990). Water molecules in the crystal structure were retained in the simulation. Force field parameters for heme were described elsewhere (Autenrieth et al. 2004) and the peptide was simulated with CHARMM force field (Mackerell et al. 2004). The structure was first minimized for 1000 steps with conjugate gradient method, and equilibrated for 10 ps with the time step of 1 fs, then was further minimized for 60,000 steps. NAMD (Not just A Molecular Dynamics) program was used for simulations, and VMD (Visual Molecular Dynamics) program (Humphrey et al. 1996) was employed for structural analysis.

Results

Plasmid construction

It is reported that there are 49 pseudo cyt *c* genes in the human genome (Zhang and Gerstein 2003). Therefore, it is very difficult to clone cyt *c* gene from the human genome. Previously, human cyt *c* gene was converted from yeast cyt *c* with long primers (Jeng et al. 2002) or horse cyt *c* by site-directed mutagenesis (Olteanu et al. 2003). In the present study, human cyt *c* cDNA was cloned by RT-PCR for the first time, and was inserted into the pBTR-fd vector (pBTR-hCYC).

Expression system construction

The pBTR-hCYC vector was first transformed into *E. coli* BL21(DE3)pLysS strain for expression. However, no expression of human cyt *c* was detected. The human cyt *c* gene contains some codons which are rarely used in *E. coli*. Thus, we transformed the pBTR-hCYC vector into a rare codon optimizer strain Rosetta(DE3)pLysS. It was found that human cyt *c* was highly expressed.

A variety of expression conditions were investigated to enhance the yield of human cyt *c*. For example, the addition of a small amount of $NaNO_3$ into SB medium effectively elevated the expression level. Besides, sealing flask caps by plastic wrap significantly improved the yield. Probably, lower oxygen level is more suitable for the holo-cyt *c* formation. Through the procedures described above, we successfully obtained human cyt *c* with the yield of 20 mg per liter culture, much higher than that of the expression systems reported previously (8 mg/L (Olteanu et al. 2003), 10–15 mg/L (Jeng et al. 2002)).

pH titrations (equilibrium studies of the alkaline conformational transition)

The UV absorbance at 695 nm of human cyt *c*, characteristic of heme-Met80 coordination, was recorded during pH titration to investigate the alkaline isomerization. As a comparison, the yeast iso-1 and horse heart cyt *c* were also studied. Addition of increasing amounts of NaOH caused a progressive decrease of the extinction at 695 nm, reflecting the displacement of ligand Met80. Changes in the absorbance of the three cyt *c* at 695 nm with increasing pH are compared in Fig. 1, representing the transition from the native state to the alkaline conformation. Apparently, the three cyt *c* show different equilibrium behavior. The curves were further analyzed using an expression for a two state transition,

$$\mathrm{pH} = \mathrm{p}K_a - \log\left[\frac{A_{695} - A^{H}_{695}}{A^{L}_{695} - A_{695}}\right] \quad (1)$$

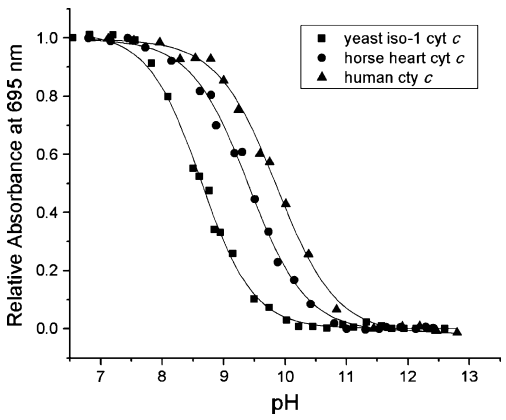

Fig. 1 Absorbance changes at 695 nm as a function of pH values for yeast iso-1, horse heart and human cyt *c* (25 °C, ionic strength=0.1 M, [cyt *c*]=0.2 mM). Each point represents the average of at least three determinations. (■) yeast iso-1 cyt *c*; (●) horse heart cyt *c*; (▲) human cyt *c*

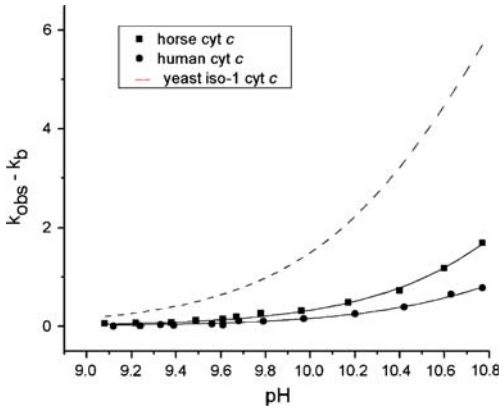

Fig. 2 pH-jump kinetics of yeast iso-1, horse heart and human cyt *c*. Experimental conditions were as described under "Materials and Methods". The pH indicated is the pH after mixing. Each point represents the average of at least three determinations. (■) horse heart cyt *c*; (●) human cyt *c*. The *dashed line* is the curve calculated from the published kinetic constants of yeast iso-1 cyt *c* by Pearce et al. (1989)

where A^H_{695} is the absorbance of the alkaline form and A^L_{695} is that of the native form at 695 nm (Davis et al. 1974). The result shows that the apparent pK value (pK_a) of human cyt *c* (pK_a=9.9) is higher than those of horse heart cyt *c* (pK_a=9.4) and yeast cyt *c* (pK_a=8.6).

Apparently, human cyt *c* is the most unfavorable among the proteins investigated to undergo conformational transition induced by alkaline pH. Furthermore, it should be noted that the pK_a of human cyt *c* is also higher than that of horse heart cyt *c* although they share ~90% sequence identity. In order to deepen our understanding of this finding, we characterized and compared the kinetics of the alkaline transition of both human and horse heart cyt *c*.

pH-jump kinetics (kinetics of the alkaline conformational transition)

The alkaline transition of cyt *c* is an intricate process which is now widely recognized to be consistent with a two-step mechanism (Davis et al. 1974). Firstly, deprotonation of one or more unidentified "trigger group (s)" induces a fast transition from native conformation to an intermediate state (Barker and Mauk 1992; Rosell et al. 1998; Martinez and Bowler 2004; Shah and Schweitzer-Stenner 2008; Hagarman et al. 2008). Then, the intermediate form transits to the alkaline conformer in which the axial ligand Met80 is replaced by lysine (Barker and Mauk 1992; Ferrer et al. 1993; Rosell et al. 1998; Dopner et al. 1998). A minimal model can be used to describe this mechanism

$$\mathrm{C-H(native)} \overset{K_H}{\rightleftharpoons} \mathrm{C(transient)} \underset{k_b}{\overset{k_f}{\rightleftharpoons}} \mathrm{NC(alkaline)}$$

in which K_H stands for the dissociation constant of the first step, while k_f and k_b are the forward and reverse rate constants in the second step, respectively.

The rate constants of change in absorbance at 695 nm (k_{obs}) were obtained by fitting the stopped-flow curves to a first-order exponential function. The plots of the constants (k_{obs}-k_b) versus pH are depicted in Fig. 2. The value of k_b which gave the curve with minimal error was used. The data can be fitted by the function below, as described by Davis et al. (Davis et al. 1974)

$$k_{obs} - k_b = \frac{k_f * K_H}{K_H + 10^{-\mathrm{pH}}} \quad (2)$$

The kinetic parameters for alkaline transition of horse heart and human cyt *c* are shown in Table 1. The pK values for the alkaline isomerization calculated by the kinetic parameters (pK_H+pK_C) (K_C=k_f/k_b) correlate well with the pK_a values obtained by pH titration experiments. Interestingly, while cyt *c* from horse and human have similar K_H values, their parameters differ markedly in the second step, which represents the coordination of the lysine residue to heme iron. k_b value of human cyt *c* is a bit bigger than horse, while k_f is no more than a half that of horse cyt *c*, and hence the K_C of human cyt *c* is only one third of that of horse.

Horse and human cyt *c* have a high amino acid similarity and only have one different amino acid in the omega loop (residues 71 to 85) where the lysine residues (Lys72, Lys73, Lys79) are located. However, there are obvious differences in alkaline isomerization between the two proteins, as indicated by our experiments. To better understand this, molecular modeling was employed to simulate the structure of human cyt *c* based on the crystal structure of horse heart

Table 1 Kinetic parameters for alkaline transition from pH-jump experiments

Protein	$k_b \times 10^2$ (s^{-1})	k_f (s^{-1})	K_C	pK_H	pK_H+pK_C	pK_a [b]
Yeast iso-1 cyt *c* [a]	2.9 (4)	13.4 (7)	460 (40)	10.9 (1)	8.3 (3)	8.6
Horse heart cyt *c*	4.8	10.5±0.2	218.4±4.2	11.5±0.1	9.2±0.1	9.4
Human cyt *c*	5.9	4.0±0.1	68.6±1.7	11.4±0.1	9.6±0.1	9.9

[a] Parameters from Reference (Pearce et al. 1989). The standard deviations of the values are indicated in parentheses.

[b] Parameters from pH titration experiments in this work.

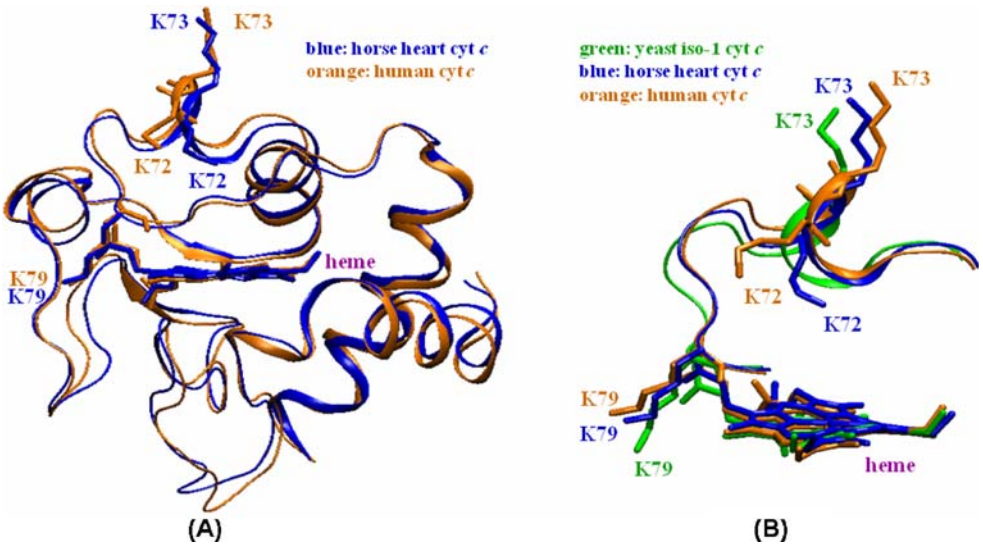

Fig. 3 **a** Molecular models of horse heart (blue, pdb entry: 1hrc) and human (orange) cyt *c*. The model of the human cyt *c* is energy-minimized structure based on the horse heart cyt *c* created with VMD program. The structures were aligned according to the heme group. **b** The enlargement of three structures (yeast iso-1 cyt *c*, green, pdb entry: 2ycc; horse heart cyt *c*, blue; human cyt *c*, orange) at the 70 to 80 side chains and the amino acids at position 72, 73 and 79. Lys72 of yeast iso-1 cyt *c* is not shown in the figure as is trimethylated and unable to coordinate to heme

cyt *c* (Fig. 3). It was found that the omega loop (residues 71 to 85) is apparently further away from heme in human cyt *c* than in horse and yeast iso-1 cyt *c*. The distance from nitrogen of Lys72 to heme iron is 8.9 Å in horse and 10.9 Å in human cyt *c*. The distance from nitrogen of Lys73 to heme iron is 19.1 Å in horse and 20.3 Å in human cyt *c* (Table 2). Probably, the longer N-Fe distance in human cyt *c* makes it more difficult to achieve an alkaline conformational transition.

Discussion

Cyt *c* is an indispensable and ubiquitous protein that is found in all organisms, including animals, plants and bacteria. Although thoroughly studied as an electron carrier in the fundamental metabolic process of oxidative phosphorylation, only recently was it found to play central roles in both pro-apoptotic and apoptotic pathways, opening the door to death (Liu et al. 1996; Kagan et al. 2005). These finding sparked an intense interest aimed at understanding the molecular basis for the multi-functions of this ancient protein.

The central goal of most studies is to understand the function of human proteins, elucidating human physiology, evolution and disease. As human proteins are unavailable in large amounts and high purity, cyt *c* from some other species, such as yeast and horse, are used as a substitute. Here we have developed a high-level expression system for human cyt *c*.

Although cyt *c* is highly conserved among eukaryotes, with even yeast and human sharing about 60% Aa identity, recent finding showed that cyt *c* underwent a period of unusual rapid protein evolution in the primate lineage that ultimately led to humans (Evans and Scarpulla 1988; Grossman et al. 2001; Schmidt et al. 2005). Considering the overall slow rate of evolution in vertebrates, it is reasonable to make an inference that almost every amino acid replacement in the primate lineage evolution is probably involved in the functional behavior of cyt *c* (Grossman et al. 2001). Indeed, Rodríguez-Roldán et al. have only recently reported that human cyt *c* exhibits remarkable differences when interacting with cytochrome *c* oxidase compared to Arabidopsis and horse cyt *c* (Rodriguez-Roldan et al. 2006). They ascribed the variance to the amino acid changes along evolution. In this paper, we have examined equilibrium and kinetics of alkaline transition of cyt *c* from yeast, horse and human. The results provide evidence supporting the view that human cyt *c* also shows remarkable differences in alkaline transition from cyt *c* of other species. Thus, we hope that our work will stimulate researchers to take note of the unattended variance between human cyt *c* and cyt *c* from other species, which are used as a substitute for human cyt *c* in the research involving human subjects.

The kinetic parameters for the alkaline transition obtained from pH-jump experiments are collected in Table 1. From the kinetic parameters, it is interesting to note that the K_H values of horse and human cyt *c* are nearly identical (within experimental error), while the K_C values of the proteins differ greatly.

K_H is the ionization constant for the ionizable groups triggering the alkaline conformational transition. Some

Table 2 The distance between the N_ζ of the residue lysine (K72, K73, K79) and heme iron in yeast iso-1 (pdb 2ycc), horse heart (pdb 1hrc) and human cyt *c*

Protein	K72 (Å)	K73 (Å)	K79 (Å)
Yeast iso-1 cyt *c*	/	18.86	10.35
Horse heart cyt *c*	8.91	19.10	10.86
Human cyt *c*	11.18	20.30	11.49

groups, such as Tyrosine-67, a heme propionate carboxylate, the proximal histidine, or a buried water molecule have all been suggested to act as the trigger (Baddam and Bowler 2006). These highly conserved groups were preserved during primate evolution. Thus, it is plausible that the ionization constants (K_H) of cyt *c* from different species are quite similar.

K_C, determined from k_f/k_b, is the conformational equilibrium constant for alkaline transition. Interestingly, the K_C value of human cyt *c* is only one-third of that of horse cyt *c*, and one-seventh of that of yeast cyt *c*. To better understand the difference in K_C values, molecular modeling was employed to simulate the structure of human cyt *c*, and then structures of yeast, horse and human cyt *c* were aligned and compared according to the heme group (Fig. 3). A significant difference among the molecules comes from the surface omega loop (residues 71–85). Clearly, the omega loop is further away from heme in human cyt *c* than in horse and yeast iso-1 cyt *c*. It has been suggested previously that this loop (residues 71–85) protects the face of the heme that is misligated in the alkaline transition of cyt *c* (Hoang et al. 2003). Taken together, it could be concluded that this surface omega loop was driven away from heme along primate evolution, and thus the alkaline conformational transition is more difficult to achieve in human cyt *c* compared to cyt *c* from other species.

Interestingly, the surface omega loop was recently found to be one of the fastest unfolding units in cyt *c*, and participate in the dominant step in a stepwise unfolding pathway (Bai et al. 1995; Krishna et al. 2003). Notably, Kagan et al. have also reported that the partially unfolded cyt *c*, with a weaken or ruptured heme-Met80 bond, acquires high peroxidase activity (Kagan et al. 2005; Belikova et al. 2006). The catalytic cyt *c* peroxidate (CL) and its oxidized form (oxidized-CL) are required for release of pro-apoptotic factors from mitochondria, and for execution of the subsequent apoptotic steps (Kagan et al. 2005; Liu et al. 1996; Belikova et al. 2006; Sinibaldi et al. 2008). Taken together, this omega loop, which was driven away from heme during rapid protein evolution in the primate lineage, seems to be pivotal in cyt *c* for the functional transition from electron transport to apoptosis. Baddam et al. have suggested that the alkaline conformational transition could act as an "electron transfer gate", and may serve to modulate the flow of electrons through the electron transport chain (Baddam and Bowler 2005a, b). Probably, the "gate" is also involved in apoptosis. Although the driving force governing the rapid transition of the primate cyt *c* molecule to its current human form remains elusive, it is evident that the threshold for alkaline transition was elevated along this evolution. Understanding the driving force for this elevation is an interesting issue that needs further investigation.

In this paper, we have investigated the alkaline conformational transition from an evolutionary perspective, and some interesting biological consequences may emerge from our studies. For decades, much attention has been paid to the alkaline conformational transition of cyt *c*. However, unfortunately, until now there is no clear evidence pointing to the correlation between the alkaline transition and the conformational changes of cyt *c in vivo*. "How does alkaline or alkaline-like conformational change happen physiologically and what is its biological role" are attractive questions which require more attention and need to be explored in more details. We anticipate that our work will provide a better understanding of these issues, and advance our understanding of the molecular basis for the biological multi-functions of cyt *c*.

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