Study on the Gas Phase Stability of Heme-binding Pocket in Cytochrome *Tb*$_5$ and Its Mutants by Electrospray Mass Spectrometry

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To elucidate the effect of various amino acid residues on the heme-binding pocket in cytochrome *Tb*$_5$, several residues were chosen for replacement by means of site-directed mutagenesis. Comparison of the mass spectrum between the F35Y mutant and the wild type shows that the relative abundance of holoprotein ion of F35Y is lower than that of the wild type in gas phase. It is concluded that mutation from Phe35 residue to tyrosine decreases the hydrophobic character of cytochrome *Tb*$_5$ heme pocket, which decreases the stability of heme-binding pocket. ESI-MS spectra of the mutants V61E, V61K, V61H and V61Y show various contribution of amino acid to the stability of heme-binding pocket. The small and non-polar residue Val61 was replaced with large or polar residues, resulting in enhancing the trend of heme leaving from the pocket. In addition, comparison of the mass relative abundance of holo-proteins among all the Val61-mutants, shows that their stability in gas phase appropriately submit the following order: wild type > V61H > V61E > V61K ≈ V61Y. The extra great stability of quadruple sites mutant E44/48/56A/D60A shows that reduction of electrostatic or hydrogen bond interactions among the residues locating in the outside region of the heme edge remarkably affect the stability of heme. The results of analyzing the oxidation states of heme iron in *Tb*$_5$ and its mutants by in-source-CAD experiment suggest that the charge states of heme iron maintain inflexible in mutation process.

Keywords electrospray, cytochrome *Tb*$_5$, mutants, gas phase, stability

Cytochrome b_5 (Cyt b_5) is found both as a component of the microsomal membranes and as a soluble form in erythrocytes. It plays an important role in biological systems, in which Cyt b_5 functions as an electron carrier, participating in a series of electron-transfer processes, including reduction of methemoglobin, fatty acid denaturation, and the cytochrome P450 catalytic cycle.[1-3] Cyt b_5 consists of two domains, a hydrophilic heme-containing domain and a hydrophobic one, which anchors the protein to the membrane.[4] The water-soluble domain is readily released from the microsomal membranes by mild proteolysis[5] and retains full activity of the cytochrome b_5.[6,7] Proteolysis of bovine liver microsomal cytochrome b_5 by lipase produces a hydrophilic heme-containing domain consisting of 93 amino acid residues (Ser1—Ser93), which is termed cytochrome *Lb*$_5$. When Cyt b_5 is treated with trypsin, a 84-residue (Ala3—Lys86) fragment is obtained and termed cytochrome *Tb*$_5$.[8] The three-dimensional structure achieved by X-ray crystallography reveals that the heme iron is ligated in a hydrophobic pocket by residues His39 and His63, greatly contributing to the molecular interaction between polypeptide and heme in cytochrome b_5, so are the four N-ε of pyrrols of the porphyrin.[8-11]

In the past decade, several papers were published concerning the investigation of the effects of the specific side chain of cytochrome b_5 on its structure and properties by using chemical modification[12] and site-directed mutage-

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Received April 1, 2002; revised June 17, 2002; accepted July 19, 2002.
Project supported by the National Natural Science Foundation of China (No. 20175034).

nesis.[13-18] Especially mutations at the residues in the heme-containing hydrophobic pocket demonstrated that they are important in maintaining the stability of holo-protein.

Studying the contribution of individual amino acids to the properties and functions of non-covalent protein complexes is a challenge. The advent of electrospray ionization (ESI) opened a new era in mass spectrometry by generating ion of intact proteins and their complexes in solution-phase.[19,20] ESI-MS is a rapid and highly sensitive method for providing information on structurally specific and biologically important interactions involving multimeric proteins, as well as receptor-ligand, antibody-antigen, enzyme-substrate, and other structurally specific liquid-phase association.[21] Initial studies have also used ESI-MS to investigate the thermodynamics for dissociation of such complexes by manipulation of solution conditions (*e.g.* the thermodynamics of the ribonuclease S, s-protein-s-peptide association) which were compared to known values obtained by more traditional solution techniques.[22] These results suggest that ESI-MS has potential for the study of such relatively weak but highly specific non-covalent molecular interaction, and thus may provide a major tool for biochemical research by enabling much faster screening and/or identification of such phenomenon.[22]

However, studying the non-covalent complexes by ESI-MS, a primary situation must be considered. Just to say, we should convince that the gaseous ion of complexes observed in the mass spectrum whether or not correspond to the molecular interaction present in solution prior to electrospray and are not formed during the electrospray process.[23] There are several ways to provide evidence to convince that. For example, the solution condition (*e.g.* pH, add of organic solvent, temperature *etc.*) should produce a corresponding change in the mass spectra. In addition, since non-covalent specific associations should be readily dissociated under more severe interface condition, adjustment of the interface conditions should more severely affect the intensity of the ions which are formed by specific complexation with defined stoichiometry than that of the ions due to random aggregation. Further evidence for the observation of a specific association by ESI-MS can be derived from the ability to chemically modify one component of the complex which affects the complex formation in solution and results in a corresponding change to the mass spectra.

A series of ESI-MS results presented here show that the polypeptide and heme in cytochrome Tb_5 combine together with molecular interaction including van der Waals forces, electrostatic forces, hydrophobic interaction *etc*. And further study shows that there are some similarities and differences for the stability of protein complexes in solution in comparison to those of their gaseous ions formed by electrospray ionization.

Experimental

Experiments were performed on a PE Mariner ESI time-of-flight spectrometer. The original pneumatically assisted turbo-ionspray source was replaced by a micro-electrospray source (SCIEX, USA). Injection flow rate of source was set to 5 μL/min. Needle voltage was 3500—3800 V. All measurements in maintaining the observation of holo-protein ion were performed at a nozzle potential of 70 V. The data were acquired by Mariner Instrument Control Panel for positive ion mode from 600 to 2500 amu, and dealt with by Data Explorer. The other conditions were as follows: nozzle temperature 140 ℃; flow rate of curtain gas 1.5 L/min; flow rate of neublizer gas 0.2 L/min.

The cytochrome Tb_5 and its mutant F35Y, V61H, V61K, V61Y, V61E, E44-48-56A-D60A were prepared as previous.[14] The ratios of the absorbance at 412 nm to that at 280 nm of wild type cytochrome Tb_5 and its mutants were all above 5.7, suggesting that they were pure enough for further analysis. To prevent dissociation of non-covalent heme-binding proteins, solutions were prepared by dissolving proteins in pure water to a final concentration of 10 μmol/L. External calibration was performed with egg white lysozyme (kindly provided by professor Zhang Zunjian). Methanol purchased from Sigma, HPLC grade.

Result and discussion

Determination of stability in F35Y *mutant molecular recognition*

The crystal structure of Phe35Tyr shows that its three-dimensional structure is similar to the wide type (Fig. 1). The side chain of phenylalanine35, which is located at the wall of hydrophobic pocket, points to the heme. As we mutate it to Tyr, the side chain of amino

residue 35, phenyl is replaced with phenyl hydroxyl. So the spatial sterical effect and hydrophile effect shall increase, which should have destabilized the hydrophobic pocket that was showed in the electrospray mass spectrum of F35Y (Fig. 2) mutant. The relative intensity of holo-protein ion of F35Y is weaker than that of wild type.

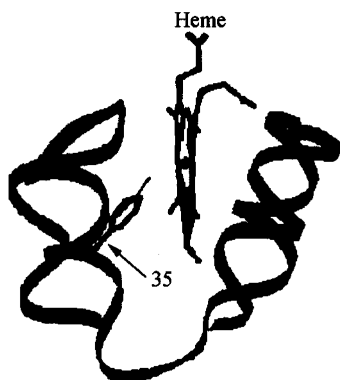

Fig. 1 Stereoscopic Cα drawing of cytochrome Tb_5 F35Y superimposed with that of cytochrome Tb_5. F35Y is shown as a thin line and cytochrome Tb_5 as a thick line.[24]

However, the stability experiments of F35Y in solution show that the F35Y is more stable than the wide type cytochrome Tb_5. Although the mutation from the residue Phe35 to tyrosine increases hydrophilicity of the pocket, forcing the pocket to expose the heme to solvent, in fact it increases the interactions between the tyrosine and the heme by hydrogen bond with a water molecule (solvent effect). The result of the X-ray crystallography shows that it is much closer between side chain of amino residue 35 and carboxyl of heme in F35Y than that in wild type. However, because we just analyze the gas phase character of cytochrome Tb_5 ion, where there is no solvent effect, the result of mass spectrometry is different from that of the solvent stability experiment.

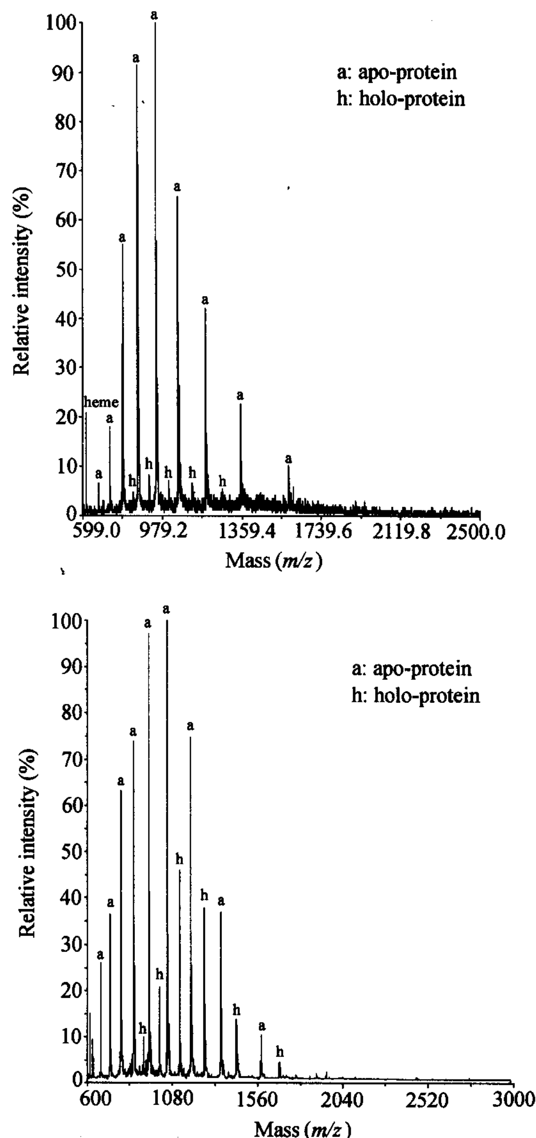

Fig. 2 Positive ion ESI-MS of cytochrome Tb_5 and its mutant in pure water. F35Y mutant is shown at the upper spectra, and the wild type of cytochrome Tb_5 at the lower spectra. For both spectra the nozzle potential were set at 70 V.

Comparison of stability in various of Cyt Tb_5 valine61 *mutants*

To elucidate the role played by Val61 of cytochrome Tb_5, this residue was chosen for replacement with tyrosine (Val61Tyr), histidine (Val61His), glutamic acid (Val61Glu), and lysine (Val61Lys) by means of site-directed mutagenesis. In the wild type cytochrome Tb_5 structure, the side chain of Val61 is located in the heme-exposed edge region and points to the heme. The mutation from this small hydrophobic residue to a large or hydrophilic residue not only forces the side chain of this residue to point away from the heme-binding pocket because of steric hinder but also significantly influences the hydrophilicity of the pocket. For example, as mutating the valine61 to histidine, the side chain of His61 imidazole ring would decrease the distance between the residue and heme (Fig. 3). To avoid the unreasonably close contact between heme and amino residue 61, the hydrophobic pocket has to enlarge, making the heme expose to the sol-

vent. The mutation from valine61 to tyrosine not only increases the volume of side chain but also opens a solvent channel to the pocket, resulting in reduction of hydrophobic interaction and the heme is easy to leave from pocket. The spectrum of ESI-MS (Fig. 4) suggests that the above explanation be reasonable. The relative abundance of holo-protein ion of all these mutants are weaker than that of wild type. The same phenomena of the mutants V61E and V61K were observed, which were explained similar to the mutant V61H and V61Y. Moreover, from the comparison with relative abundance of holo-protein ions between all four mutants, the stability of them in gas phase by electrospray mass spectrometer was possibly submitted to the following order: wide type > V61H > V61E > V61K ≈ V61Y.

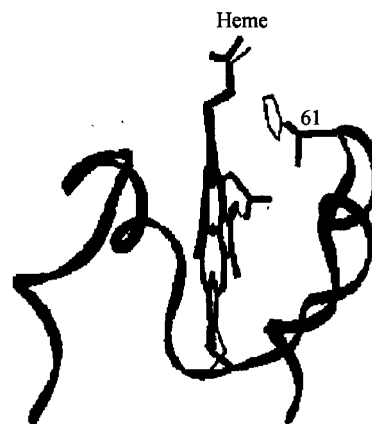

Fig. 3 Stereoscopic Cα drawing of cytochrome *Tb*$_5$ V61H superimposed with that of cytochrome *Tb*$_5$. V61H is shown as a thin line and cytochrome *Tb*$_5$ as a thick line.[25]

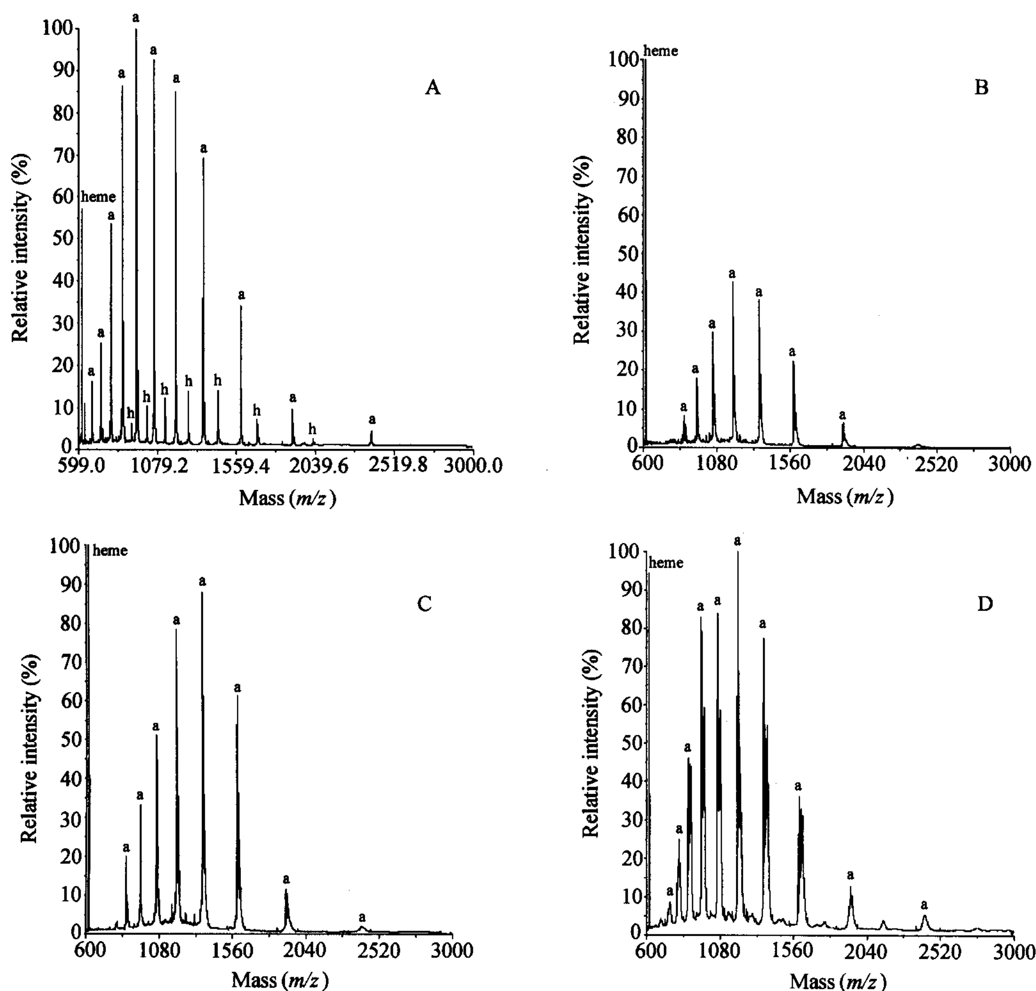

Fig. 4 Positive ion ESI-MS of cytochrome *Tb*$_5$ valine61 mutants at the same nozzle potential 70 V. The spectra of V61H, V61K, V61Y, V61E are shown from A to D, respectively. The labeling scheme for all spectra is as follows: a = apo-protein and h = holo-protein.

Unfortunately, this order is different from the stability order in solution experiments: wide type > V61E > V61Y > V61H > V61K. Possibly, it could be explained by the fact that the electrostatic interaction is stronger in gas phase, which has been reviewed by Loo.[24] Because histidine is easy to form positive charge in solution, it establish a salt "bridge" with carboxylic group of heme propionate which turns much tighter in gas phase as its surrounding solvent shell is stripped away. However, as we mutate the 35th amino residue with lysine, the MS spectrum results suggest that there be much lower stability to the gaseous ion of that mutated protein. We think it is contributed to the longer side chain of lysine which is difficult for the pocket to keep the interaction between amine group of lysine and carboxylic group of heme in gas phase.

The more stable four sites mutant E44/48/56A/D60A

The four residues Glu44, Glu48, Glu56 and Asp60, locating at the outside of heme-binding pocket, were believed to take part in the formation of region with 9 negative electric charge. The mutation from them to alanines decreases the negative charge to −5, resulting in the decrease of repulsion between negative electric charge, which make the pocket reduce. The heme embeds in the pocket much more tightly.

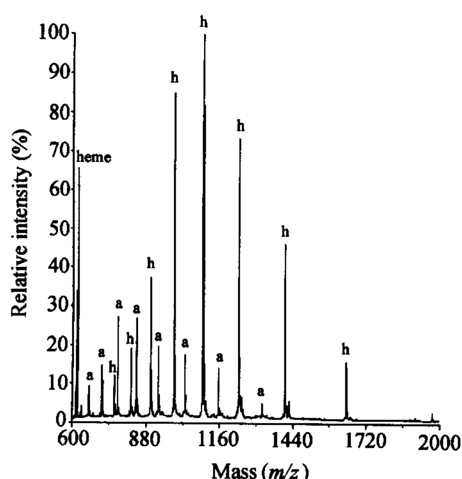

Fig. 5 Positive ion ESI-MS of four sites mutant at nozzle potential 70 V. The labeling scheme for all spectra is as follows: a = apo-protein and h = holo-protein.

Moreover, the side chain of Glu and Asp in wild type cytochrome Tb_5 all extend to the solvent, forming the hydrogen bond by water molecular in solvent. As mutated to alanines, the interaction among side chains and water, including the hydrogen bond interaction among various side chains disappear. The negative charge region tend to close, driving the heme embed in the pocket deeply, which form the new hydrophobic region by side chains of alanines. In fact, the conformation of mutant protein turns compact, which is proved by the ESI-MS spectrum (Fig. 5) where the multi-charge distribution of four sites mutant holo-protein tend to lie in the high mass region more seriously than that of wild type. It is because the protein with compact conformation decreases the amount of basic amino acid residue exposing to outside that it cannot plus the same amount of proton with the protein with relax conformation.

Determination of the iron oxidation state in heme

Marshall *et al*.[26] have obtained the unambiguous metal atom oxidation state in an intact metalloprotein by matching experimental and theoretical isotopic abundance mass distributions of one or more holo-protein charge states. In addition, infrared multiphoton irradiation of the Fe(III)-holo-proteins releases Fe(III)-heme from each of the noncovalently bound Fe(III) heme proteins, suggesting that the charge state of heme does not change in the dissociation process. According to this fact, we can determine the iron oxidation state of the heme in cytochrome Tb_5 mutants.[25]

By the method of in-source CAD experiment, increasing the nozzle potential from 70 V to 100 V, all the holo-proteins release the heme from the pocket, observing clearly the heme ion in the mass spectrum. If the iron oxidation state in heme is 2, the net charge of whole heme with two carboxylic group is zero, so it plus a proton to form the heme ion $(M+H^+)$ 617 mass-to-charge. However, the most abundant heme fragment mass-to-charge we observed in MS spectrum is 616(M^+), single-charge, identifying the iron oxidation state as Fe(III) in the heme because the heme can form the ion without adding the proton in that time. Thus, we safely conclude that the iron oxidation of heme iron did not change in the mutagenesis, which is the same as that of wild type cytochrome b_5.

Conclusions

The results obtained in this study provide three factors, which affect the stability of molecular interaction between polypeptide and the heme in cytochrome Tb_5. First, if the amino acid residue in pocket is mutated from hydrophobic to hydrophilic, the stability of holo-protein decrease. Second, mutation from the amino acid with small side chain to that with large side chain, resulting in the unreasonable close contact to the heme, should decrease the stability of holo-protein. Finally, electrostatic force of amino acid residue in negative charge region and hydrogen bond interaction of side chains at the outside of heme-binding pocket, that turn much weaker makes the three-dimensional conformation tend to reduce, affect the stability of holo-proteins importantly.

Furthermore, we also perceived different stabilizing order of complexes between in gas phase and in solution. So a cautionary note for the use of ESI/MS gas phase data to characterize the stability of non-covalent interactions in solution was proposed.

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(E0204013 CHENG, B.)