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Stabilization of cytochrome b_5 by a conserved tyrosine in the secondary sphere of heme active site: A spectroscopic and computational study

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ABSTRACT

Heme proteins perform a large array of biological functions, with the heme group bound non-covalently or covalently. To probe the stabilization role of conserved tyrosine residue in the secondary sphere of heme site in heme proteins, we herein used cytochrome b_5 (Cyt b_5) as a model protein, and mutated Tyr30 to Phe or His by removal of Tyr30 associated H-bond network and hydrophobic interaction. We performed thermal-induced unfolding studies for the two mutants, Y30F Cyt b_5 and Y30H Cyt b_5, as monitored by both UV–Vis and CD spectroscopy, as well as heme transfer studies from these proteins to apo-myoglobin, with wild-type Cyt b_5 under the same conditions for comparison. The reduced stability of both mutants indicates that both the H-bonding and hydrophobic interactions associated with Tyr30 contribute to the protein stability. Moreover, we performed molecular modeling studies, which revealed that the hydrophobic interaction in the local region of Y30F Cyt b_5 was well-remained, whereas Y30H Cyt b_5 formed an H-bond network. These observations suggest that the conserved Tyr30 in Cyt b_5 is not replaceable due to the presence of both the H-bond network and hydrophobic interaction in the secondary sphere of the heme active site. As demonstrated here for Cyt b_5, it may be of practical importance for design of artificial heme proteins by engineering a Tyr in the secondary sphere with improved properties and functions.

1. Introduction

Heme proteins are a major class of metalloproteins, which perform a large array of biological functions, including electron-transfer, oxygen delivery, catalysis and signaling [1–11]. The heme group is usually associated with the protein polypeptide chain non-covalently (such as cytochrome b_5, Cyt b_5, and most cytochromes P450), or covalently by post-translational modifications [12]. For a non-covalently attached heme protein such as Cyt b_5 [13], the stability of the heme group is mainly contributed by the coordination of heme axial ligands and the hydrophobic interactions within the heme pocket [14]. Moreover, the protein stability is contributed by both hydrophobic interactions and hydrogen(H)-bonding interactions remote from the heme active site.

Among natural amino acids, the hydrophobic Tyr has a phenolic hydroxyl group, which is capable of acting as an H-bond donor or acceptor. Moreover, Tyr is conserved in abundant heme proteins, both in the heme active site, such as Tyr67 in cytochrome *c* (Cyt *c*) [15] and Tyr244 in heme-copper oxidase [16], and in the hydrophobic domain, such as Tyr74 in rat Cyt b_5 [17]. These Tyr residues are involved in forming diverse H-bond networks and play crucial roles in fine tuning the protein structure and function. For example, disruption of the Tyr67-associated H-bond network in Cyt *c* perturbs its tertiary structure, resulting in altered chemical and biological properties [18–20], and replacement of Tyr74 in rat Cyt b_5 with a Lys leads to a faster (6-times) dissociation rate of the heme group from the mutant compared to the wild-type (WT) Cyt b_5 [21].

In a recent study, we introduced a Tyr residue in the heme active site (at position 107) and in the hydrophobic site (at position 138) in myoglobin (Mb), respectively, which forms a distinct H-bond network involving water molecule and neighboring residues, fine tuning both ligand binding and protein stability [22]. In this study, we are interested in further probing the role of conserved Tyr residues in the secondary sphere of heme site in heme proteins, which might be as important as those in both the heme active site and the hydrophobic site. Based on the X-ray structure of both bovine and rat Cyt b_5, we noticed that there is a conserved Tyr30 located between core 1 (heme-binding domain) and core 2 (hydrophobic domain), which forms an H-bond network with water molecules and surrounding residues (Fig. 1) [13]. We envisaged that this H-bond network as well as the hydrophobic interactions might play important roles in stabilization of Cyt b_5. To confirm our speculation, we replaced Tyr30 with Phe or His residue by site-direct mutagenesis, and compared the stability of the two mutants, Y30F Cyt

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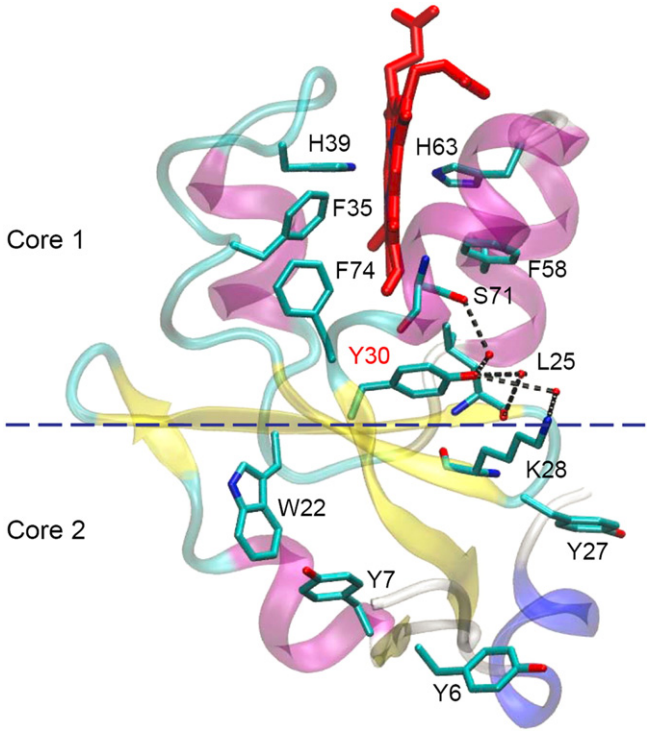

Fig. 1. Crystal structure of bovine Cyt b_5 (PDB entry 1CYO), showing the heme-binding core 1 and the hydrophobic core 2, as indicated by a dashed line. The heme group, Tyr30-associated H-bond network, and selected residues in the heme pocket and in the hydrophobic core 2, are shown for clarification.

b_5 and Y30H Cyt b_5, with that of WT Cyt b_5. Moreover, we performed molecular modeling studies for these mutants, which provides valuable insight into the stabilization role of the conserved Tyr30 in Cyt b_5.

2. Materials and Methods

2.1. Protein Preparation

The gene for lipase-solubilized bovine liver microsomal Cyt b_5 was expressed and purified as described previously by Yao et al. [23] The Y30F Cyt b_5 and Y30H Cyt b_5 gene were constructed using the QuickChange Site Directed Mutagenesis Kit (Stratagene). The mutations were confirmed by DNA sequencing assay. The mutants were expressed and purified using the same procedure as that for WT Cyt b_5. Sperm whale Mb was expressed using the Mb gene of pMbt7-7 and purified using the procedure described previously [24]. Apo-Mb was prepared using the method of Teale F.W. [25]. The protein concentration of WT Cyt b_5 and its two mutants were determined with an extinction coefficient of $\varepsilon_{413} = 117\ mM^{-1}\cdot cm^{-1}$ [26]. The concentration of apo-Mb was determined with an extinction coefficient of $\varepsilon_{280} = 15.2\ mM^{-1}\cdot cm^{-1}$ [27]. UV–Vis spectra were collected on an Agilent 8453 spectrometer in 100 mM potassium phosphate buffer (pH 7.0) at 25 °C.

2.2. Thermal Denaturation Studies

Thermal denaturation of WT Cyt b_5 and its mutants was performed with a Jasco 1500 spectropolarimeter equipped with a MCB-100 mini circulation bath. The protein (20 μM) was dissolved in potassium phosphate buffer (100 mM, pH 7.0) using a cuvette with a path length of 1 cm. The temperature was increased stepwide over the range of 25–90 °C. At each temperature, the protein sample was allowed to equilibrate for 10 min before the UV–Vis (250–700 nm) and CD spectra (250–500 nm) were recorded. The denaturation midpoint (T_m) was calculated by fitting the absorbance of Soret band *versus* the temperature to the two-state Boltzmann function (Eq. (1)):

$$A = A_2 + (A_1 - A_2)/\left(1 + e^{(T - Tm)/dT}\right) \quad (1)$$

Here, A is the absorbance of Soret band; A_1 and A_2 are the initial and final absorbance of Soret band, respectively; T is the temperature.

2.3. Heme Transfer Studies

Kinetic analysis of heme transfer from WT Cyt b_5 and its mutants to apo-Mb was carried out on an Agilent 8453 spectrometer using a tandem mixing cell with a path length of 2 × 0.438 cm, as described by Hargrove and Olson [28]. The temperature was controlled at 25 °C with a circulating bath instrument. The reaction was initiated by rapidly mixing equal volumes of Cyt b_5 and apo-Mb, with a final concentration of 2 μM and 4 μM, respectively, in 10 mM sodium acetate buffer (pH 5.5). The change in absorbance due to heme transfer from Cyt b_5 to apo-Mb was monitored every 60 s at 405 nm, the maximum difference between Cyt b_5 and Mb in ferric state. The heme transfer reaction can be treated as a first-order reaction, and the kinetic trace can be described mathematically by the equation (Eq. (2)) [29]:

$$\Delta A_t = \Delta A_{eq}\left(1 - e^{-kt}\right) \quad (2)$$

Here, ΔA_t is the increase in absorbance at time t, ΔA_{eq} is the increase in absorbance at equilibrium, and k is the rate constant for the heme transfer.

The activation free energy ($\Delta G^{o\neq}$) was calculated from the equation (Eq. (3)) [30]:

$$\Delta G^{o\neq} = -RT \ln(hk/k_b T) \quad (3)$$

where R is the gas constant, T is temperature, h is the Plank constant, k is the experimental rate of heme transfer, and k_b is the Boltzmann constant.

2.4. Molecular Modeling Studies

The initial structure of Y30F Cyt b_5 and Y30H Cyt b_5 were constructed based on the X-ray structure of bovine liver microsomal Cyt b_5 (PDB entry 1CYO [13]) using program VMD 1.9 (Visual Molecular Dynamics) [31]. The psfgen plug-in of program NAMD 2.9 (Nanoscale Molecular Dynamics) [32] was used to add hydrogen atoms and assign charges according to pH 7. The protein was then solvated in a cubic box of TIP3 water, which extended 10 Å away from any given protein atom. Counter ions (Na^+ and Cl^-) were further added to obtain the physiological ionic strength of 0.15 M by using the autoionize plug-in of VMD 1.9. The resulting system was minimized with NAMD 2.9 using 50,000 minimization steps with conjugate gradient method at 0 K, and equilibrated for 10,000,000 molecular dynamics steps (1 fs per step, 10 ns in total) at 300 K, then further minimized for 50,000 steps at 0 K. The trajectory data was saved every 5000 steps. Visualization and data analysis were done with VMD 1.9.

3. Results and Discussion

3.1. Comparison of Thermal Stability

As purified, both Y30F Cyt b_5 and Y30H Cyt b_5 mutants exhibit the same UV–Vis spectrum in ferric state (Soret band, 413 nm; visible bands, 532 and 560 nm, Fig. 2) to that of WT Cyt b_5, which suggests that the mutation in the secondary sphere does not alter the conformation of the heme active site. To evaluate the stabilization role of conserved Tyr30 in the secondary sphere, we performed thermal-induced