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Characterization and Calculation of a Cytochrome *c*-cytochrome *b₅* Complex Using NMR Data*

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Abstract

To identify the binding site for bovine cytochrome *b₅* (cyt *b₅*) on horse cytochrome *c* (cyt *c*) cross saturation transfer NMR experiments were performed with ²H-¹⁵N cyt *c* and unlabelled cyt *b₅*. In addition, chemical shift changes of the cyt *c* backbone amide and side chain methyl resonances were monitored as a function of cyt *b₅* concentration. The chemical shift changes indicate that the complex is in fast exchange, and are consistent with a 1:1 stoichiometry. A *K_a* of $4 \pm 3 \times 10^5 \text{ M}^{-1}$ was obtained with a lower limit of 855 s^{-1} for the dissociation rate of the complex. Mapping of the chemical shift variations and intensity changes upon cross-saturation NMR experiments in the complex reveals a single, contiguous interaction interface on cyt *c*. Using NMR data as constraints a protein docking program was used to calculate two low energy model complex clusters. Independent calculations of the effect of the cyt *b₅* heme ring-current-induced magnetic dipole on cyt *c* were used to discriminate between the different models. The interaction surface of horse cyt *c* in the current experimentally constrained model of the cyt *c* - cyt *b₅* complex is similar but not identical to the interface predicted in yeast cyt *c* by Brownian dynamics and docking calculations. The non identical interfaces can largely be accounted for by the occurrence of different amino acids at the protein-protein interface and the dissimilar assumptions employed in the calculations.

Electron transfer reactions play an essential role in numerous important biological processes such as photosynthesis and respiration and typically involve the transport of electrons through a series of distinct protein-protein complexes. Interaction between cytochrome *c* (cyt *c*) and cytochrome *b₅* (cyt *b₅*) is an excellent model system to study biological electron transfer reactions. Both reactants are structurally well-characterized and amenable to computer and wet laboratory experiments. The experimental and theoretical investigation of the interaction between these two proteins has provided considerable insight into the manner in which these proteins recognize and bind to each other (reviewed in (1). The strong pH and ionic strength dependence of the cyt *c*- cyt *b₅* association (2–4) suggests that electrostatic interactions play an important part. The crystal structures of cyt *c* and cyt *b₅* reveal that the heme groups in both proteins are surrounded by a conserved set of charged amino acid residues. The surface at the predicted interface of cyt *c* has a number of lysine residues that complements the conserved negatively charged regions of cyt *b₅*. Based on modeling studies of the interaction of tuna cyt *c* and bovine cyt *b₅*, Salemm (5,6) proposed that four intermolecular complementary electrostatic interactions would result in formation of a 1:1 cyt *b₅*-cyt *c* complex of appropriate geometry for electron transfer. In the resulting model, there is displacement of several water

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molecules from the surface of each protein and the heme prosthetic groups of the molecules are oriented in nearly the same plane with a tilt of approximately 15°. As a result of this model, attention was focused on the experimental characterization [kinetic studies (2–4,7), hyperbaric spectroscopy (8–10), electrochemistry (11), site-directed mutagenesis (12–14), fluorescence quenching (15–17), potentiometric titrations (4), chemical cross linking (1,7,8,18) and theoretical modeling studies such as molecular and Brownian dynamics (3,13,19,20)] of the interaction of these two proteins. Subsequently, many of the features predicted by this model have been demonstrated.

However, the exact extent of the surface area of cyt *c* involved in the recognition of cyt *b*₅ has never been experimentally determined. Furthermore, which forces are contributing significantly to the stability of complex is still an open question. It has been suggested by several investigators that complex geometries different from the one described by Salemmé can be invoked to explain experimental results, and that more than one complex configuration might be present in solution (3,13). The model obtained by Northup et al. using Brownian dynamics simulation calculations on yeast iso-1-cyt *c* and bovine cyt *b*₅ (13) predicted that the two proteins approach each other with two different docking geometries. One of the complexes incorporated a similar set of salt bridges to those of the Salemmé model. However, the second complex which was a slightly translated complex compared to the first was significantly more stable and was dominated by an alternate set of salt bridges between the proteins.

There has been only limited success using crystallography to study electron-transfer complexes of moderate affinity that are dominated by electrostatic contacts. Even if the structure of a crystalline complex could be obtained it is likely it would represent only one of a number of productive associations. Therefore, the structural characterization of the cyt *b*₅-cyt *c* complex and other electron transfer complexes has been performed largely by NMR spectroscopy (20–24). Furthermore the recent publication of sophisticated programs has made it possible to conduct docking simulations using X-ray and NMR data. Mapping the perturbation of chemical shifts upon complex formation provides a highly sensitive tool for identifying the residues that play a role in inter-protein interactions. ¹H NMR spectroscopy has already provided evidence of an intermolecular interaction, first through hyperfine-shifted signals (25) and then through 2D ¹H NMR spectroscopy (26,27). Later, as ¹⁵N-enriched cyt *b*₅ and cyt *c* became available, HSQC experiments were performed in the presence of each other to monitor changes in the backbone chemical shift upon complexation (20,28). Recently, NMR studies have been carried out on ¹⁵N, ¹³C-labeled cyt *b*₅ which was titrated with unlabeled cyt *c* to monitor chemical shift changes in not only the backbone, but also the side chain methyl groups. Cross-saturation transfer experiments, which delineated only a single binding site on cyt *b*₅ for cyt *c*, have also been conducted (29).

In the current work, the NMR studies have been extended by monitoring a full titration series of ¹⁵N, ¹³C-labeled ferrous cyt *c* with the unlabeled ferrous cyt *b*₅ to identify residues on horse cyt *c* which interact with bovine cyt *b*₅. From these data, the association constant and the lower limit of *k*_{off} were calculated. The chemical shift changes induced in the cyt *c* upon titration with cyt *b*₅ were mapped onto the structure to identify the residues affected by complex formation. A recently developed computer program, HADDOCK (30), allowed us to use the NMR data to provide a model of the interaction surface. Our docking predicts the formation of salt bridges between cyt *c* Lys72 and cyt *b*₅ Asp60, cyt *c* Lys73 and cyt *b*₅ Glu56 and Asp60, cyt *c* Lys86 and cyt *b*₅ Glu43 and Glu44, cyt *c* Lys87 and cyt *b*₅ Glu48, cyt *c* Lys88 and cyt *b*₅ Glu44.

Docking in which surface residues are allowed to move coupled to NMR mapping is now a well-established method to study protein-ligand interactions and is a promising approach for drug design. Heteronuclear NMR and docking calculations have been used by other investigators to build structural models of complexes of electron transfer proteins for example

cytochrome *c*553-ferredoxin (31), cytochrome *c*553-[Fe] hydrogenase (32), cytochrome *b*₅-myoglobin (23) and the cytochrome *c* – cytochrome *f* complexes (33).

MATERIALS AND METHODS

Materials

Yeast extract and tryptone for *Escherichia coli* growth media were obtained from Fisher Scientific. Restriction endonucleases were from New England Biolabs (Beverly, MA). T4 DNA ligase and ligase buffer were from Stratagene. Qiagen minikit was used for DNA extraction. Chloramphenicol, glycerol and potassium phosphate were from Fisher Scientific. Carbenicillin, isopropyl β-D-thiogalactopyranoside, and δ-aminolevulinic acid were from RPI (Mount Prospect, IL). All chemicals used for media supplements and for the vitamin mix were purchased from Sigma. Sepharose CL-6B and G-75 resins were from Sigma. Deuterium oxide, isotope labeled Martek-9 media (¹⁵N, ¹³C-¹⁵N and ²H-¹⁵N), ¹³C-glycerol and ¹⁵N-ammonium sulfate were from Spectra Stable Isotopes (Columbia, MD). The plasmids pBTR horse cyt *c*, pBTR(horse cyt *c*), and pEC86 were kindly supplied by Dr. G. J. Pielak (University of North Carolina, Chapel Hill, North Carolina) and Dr. L. Thöny-Meyer (Institute of Microbiology, Zurich, Switzerland), respectively.

Expression of ¹⁵N and ¹³C-¹⁵N labeled horse cyt *c*

Unless otherwise specified all DNA manipulations were performed as described (34). Horse cyt *c* was overexpressed in *E. coli* C41 cells (35) using the pLW01 plasmid containing the horse cyt *c* and yeast, *S. cerevisiae*, heme lyase gene (36). The pLW01-cyt *c*-heme lyase plasmid was constructed by excising the horse 1-cyt *c* and heme lyase genes from pBTR (horse cyt *c*) (a generous gift from Dr. Pielak) (37) and cloning it into the *Nco*I and *Hin*III restriction sites of pLW01 (36). The sequence of the cyt *c* and heme lyase gene was confirmed by double stranded sequencing of the genes. The pLW01 cyt *c*-heme lyase plasmid was transformed into C41 cells. Following overnight incubation at 37 °C a single colony was picked from a Luria Broth (LB) plate containing carbenicillin at a final concentration of 0.24 mM. The single colony was inoculated into 10mL of Terrific Broth (TB) containing 0.24 mM carbenicillin and incubated for 16 hours at 37 °C with shaking at 250 rpm. The cells were pelleted and gently resuspended in 10 mL of fresh Martek-9N or 9CN media for ¹⁵N and ¹³C-¹⁵N labeled media, respectively. One mL of the resuspended cells was subsequently transferred to 100 mL of Martek-9N or 9CN media. The Martek-9N and 9CN media contained 90 mL of the appropriate Martek solution, 9 mL of 1 M potassium phosphate buffer pH 7.2, 0.8 mL of 50% glycerol (¹³C-glycerol was used for ¹³C labeling), 0.24 mM carbenicillin, 0.2 g of (NH₄)₂SO₄ (¹⁵N ammonium sulfate used when ¹⁵N was required), 1 mM CaCl₂•7H₂O, 10 mM MgSO₄, 10 μM FeCl₃•6H₂O, 25 μM ZnSO₄•7H₂O, 20 μM MnCl₂•4H₂O, 500 μM δ-aminolevulinic acid, vitamin mix (25 μg of biotin, choline chloride, folic acid, niacinamide, pyridoxal•HCl, riboflavin, D-pantothenic acid and 500 μg of thiamine).

Optimal expression was obtained with 100 mL of culture volume in a 500 mL Erlenmeyer flask. The flasks containing 100 mL of the expression media were incubated at 35 °C with shaking at 250 rpm until the OD at 600 nm was 1.5–1.8. Isopropyl β-D-thiogalactopyranoside was added to 250 μM and the incubation was continued for 16 hours at which time the cells were harvested.

Expression of ²H-¹⁵N horse cyt *c* with and without δ-aminolevulinic acid

The pLW01-horse cyt *c*-heme lyase plasmid and C41 cells were used for expression in the presence of δ-aminolevulinic acid as described above except for the following modifications. *E. coli* C41 cells will not grow in 100% deuterated medium unless they have been “trained” to grow on increasing deuterium oxide concentrations. Transformed C41 cells were

successively grown on LB, 0.24 mM carbenicillin plates containing 10, 20, 30 and 50% deuterium oxide. A single colony from a plate made with 50% deuterium oxide was transferred into 10 mL of 100% deuterated TB medium containing 0.24 mM carbenicillin. The culture was incubated at 37 °C with shaking at 250 rpm for 20 hours. Growth is slower in medium containing 100% deuterium oxide. The cells were pelleted and resuspended in 10 mL fresh Martek-9DN medium containing the same ingredients (minerals, vitamins, etc.) described for expressing ^{15}N and ^{13}C - ^{15}N cyt *c*. One mL of the resuspended cells was transferred to 100 mL of Martek-9DN medium and incubated at 35 °C with shaking at 250 rpm until the absorbance at 600 nm was 1.5–1.8 $\approx$ 25 hours. Isopropyl β -D-thiogalactopyranoside was added to a final concentration of 100 μM and the incubation was continued for 37 hours at 30 °C with shaking at 200 rpm. The NMR saturation transfer experiments revealed that cyt *c* purified from these cells contained heme that was not completely deuterated. The heme contained a significant number of protons. Presumably the unlabelled heme precursor δ -aminolevulinic acid was incorporated directly into the heme.

In order to express cyt *c* containing deuterated heme the pLW01 horse cyt *c*-heme lyase plasmid was coexpressed with the pEC86 plasmid which contains the 8 genes required for cyt *c* heme maturation (*ccm*ABCDEFGH) (38). The plasmid was a generous gift from Dr. Thöny-Meyer. Coexpression of the pEC86 and pLW01-horse cyt *c*-heme lyase plasmids in C41 cells enabled us to express cyt *c* in good yields in the absence of unlabelled δ -aminolevulinic acid, thereby increasing the deuteration of the heme. *E. coli* C41 cells containing both plasmids were selected on LB plates containing carbenicillin (0.24 mM) and chloramphenicol (50 $\mu\text{g/mL}$) and grown as described in the previous paragraph with the addition of chloramphenicol and elimination of δ -aminolevulinic acid to the growth medium.

Purification of cyt *c*

The C41 cells from 1L of culture were harvested at the appropriate times. They were pelleted at 30000g at 4 °C for 15 minutes. The purification was conducted at 4 °C. The supernatant was discarded and the cells were resuspended in 60 mL of B-per (Pierce) with 3 protease inhibitor cocktail tablets (Roche) and 30 mg lysozyme. The solution was incubated on ice for 1 hour and then placed at –20 °C. Subsequently the cells were defrosted and then lysed by sonication. Cell debris was removed by centrifugation at 24000g for 30 minutes. The supernatant was removed and saved. The pellet was resuspended in 40 mL of 46 mM potassium phosphate buffer pH 7.0 at 4 °C (Buffer A). The sonication and resuspension procedure was repeated approximately four times until greater than 95% of the cyt *c* had been extracted from the original cell pellet. A CM-Sepharose CL-6B column (3 $\times$ 15cm) was pre-equilibrated with 200 mL of buffer A. The combined supernatants from the sonicated cells were pooled and diluted with water until the conductivity was equal to that of buffer A. The cyt *c* was loaded onto the column at $\approx$ 1 mL/min. The column was washed with buffer A until a yellow compound eluted ($\approx$ 400 mL buffer A). Cyt *c* was then eluted with a solution of buffer A and 0.5 M NaCl. The red fractions were pooled and concentrated to <10 mL in an Amicon concentrator fitted with a YM5 membrane. The protein was loaded onto a G-75 column (3 $\times$ 90 cm) pre-equilibrated with 1 L of 20 mM potassium phosphate buffer pH 7 at 4 °C (Buffer B). The cyt *c* was eluted with buffer B. The fractions containing more than 1 μM cyt *c* were pooled and concentrated to <5 mL. An extinction coefficient of 106.1 $\text{mM}^{-1}\text{cm}^{-1}$ at 410 nm was used to quantitate cyt *c* (39). A few grains of potassium ferricyanide (Sigma) were added to oxidize the protein. The cyt *c* was further purified by HPLC (Waters 650E) on a cationic S-10153 column (Biorad) using a gradient from 100% buffer A to 100% buffer D (10 mM potassium phosphate buffer pH 7 containing 1 M NaCl) over 55 minutes. The 280 nm peak at 25% buffer D and 75% buffer A was collected. The buffer in which cyt *c* eluted was exchanged to a 1 mM potassium phosphate, pH 7 solution. Two μg of the cyt *c* ran as a single band on a 15% SDS polyacrylamide gel. The protein was concentrated to 3 mM on an Amicon concentrator, filtered through a 0.22

μm sterile filter and stored at $-20\text{ }^{\circ}\text{C}$. A Biorad protein assay kit was used to measure the total protein concentration.

From 1 L of cell culture it was possible to isolate 24 mg, 26 mg and 14 mg of pure ^{15}N , ^{13}C - ^{15}N and ^2H - ^{15}N labeled cyt *c* respectively with an overall yield from the cell culture averaging $\approx 25\%$. The molecular weight of the labeled proteins was determined by MALDI-TOF (Matrix Assisted Laser Desorption Ionization Time-of-Flight) Mass Spectrometry.

The molecular weights were $12448 \pm 12\text{ Da}$, $12638 \pm 12\text{ Da}$ and $13053 \pm 13\text{ Da}$ for the ^{15}N (99% enriched), ^{13}C - ^{15}N (96% enriched), ^2H - ^{15}N (99% enriched when grown with δ -aminolevulinic acid) cyt *c*, respectively. Interestingly, the ^2H , ^{15}N sample prepared in the absence of δ -aminolevulinic acid had a molecular weight of $12166 \pm 12\text{ Da}$ indicating it was only 97% labeled.

Preparation of ferrous cyt *c* samples for NMR

The reduced NMR samples were prepared under anaerobic conditions in a glove box. The required amount of isotope enriched cyt *c* and unlabelled cyt *b5* in 1 mM potassium phosphate buffer pH 7.0 were aliquoted into a microcentrifuge tube from a concentrated solution. A solution of sodium dithionite whose concentration was known was used to stoichiometrically reduce the cyt *b5* and cyt *c*. When the proteins were reduced, a 1.1–1.5-fold molar excess of dithionite was added. Depending on the sample the pH was then adjusted with KOH, KOD, HCl or DCl to pH7 on the pH meter. 1 mM potassium phosphate buffer in the appropriate solvent D_2O vs H_2O was added to 700 μL . The sample was transferred to a Wilmad NMR tube in the glove box and capped with a rubber stopper. Outside the glove box the glass NMR tube was sealed under positive pressure nitrogen with a propane-oxygen torch.

NMR experiments

The NMR experiments were carried out at $25\text{ }^{\circ}\text{C}$ on a Bruker Avance 600 spectrometer equipped with a triple resonance gradient probe unless stated otherwise.

^1H - ^{15}N fast-WATERGATE-HSQC spectra of ^{15}N , ^{13}C -enriched ferrous cyt *c* unbound and in complex with cyt *b5* in 1 mM potassium phosphate buffer at pH7 at different ratios of cyt *b5*/cyt *c* were recorded with a spectral width of 7692 Hz for ^1H and 1773 Hz for ^{15}N as an array of 4k by 200 complex points. ^1H - ^{13}C CT-HSQC spectra for ^{13}C / ^{15}N enriched ferrous cyt *c* alone and in complex with unlabelled cyt *b5* were collected with a spectral width of 8333.3 Hz and 4k complex points in the ^1H dimension, and with a spectral width of 10000 Hz and 480 complex points in the ^{13}C dimension.

A 3D-HNCA spectrum of ^{15}N - ^{13}C enriched ferrous cyt *c* was recorded with spectral widths of 7505 Hz, 1785 Hz and 7505 Hz for ^1H , ^{15}N and ^{13}C respectively on a Bruker Avance 500 spectrometer. A 3D- CCCCONH spectrum was recorded with spectral widths of 9615 Hz, 1825 Hz and 10562 Hz for ^1H , ^{15}N and ^{13}C respectively. A 3D-HCCCONH spectrum was recorded with spectral widths of 9615 Hz, 1825 Hz and 4200 Hz for ^1H , ^{15}N and aliphatic protons respectively.

The NMR spectra were processed using NMRPipe (40a) and analyzed with SPARKY software (40b). In general, the time domain data were zero-filled in the indirect dimensions and apodized with shifted sine bell or Lorentzian-to-Gaussian window functions. Linear prediction was applied to indirect heteronuclear dimensions acquired with constant-time evolution. Molecular representations were made with the program MOLMOL (41).

Analysis of chemical shift changes

Chemical shifts of cyt *c* in the complex with cyt *b*₅ were compared with the confirmed resonance assignments of cyt *c* alone. All NMR experiments were conducted in 1 mM potassium phosphate buffer pH7. The binding constant, *K*_a for cyt *c* and cyt *b*₅ was determined from the data using Equation 1:

$$\Delta\delta = \Delta\delta_{\infty} \left(-B - \sqrt{B^2 - 4ab} \right) / (2a) \quad [\text{Eq. 1}]$$

where

$$B = -[a + b + (1/K_a)] \quad [\text{Eq. 2}]$$

and *a* and *b* are the total concentrations of cyt *c* and cyt *b*₅, $\Delta\delta$ is the chemical shift change of a cyt *c* residue upon addition of cyt *b*₅. $\Delta\delta_{\infty}$ is the estimated chemical shift change for the state where $b/a \rightarrow \infty$ *K*_a is the equilibrium association constant assuming a bimolecular interaction.

Composite chemical shift differences for cyt *c* amide ¹H and ¹⁵N chemical shifts upon cyt *b*₅ binding were calculated using the equation: $\Delta\delta(NH) = [(\Delta\delta H^2 + (\Delta\delta N/5)^2)/2]^{1/2}$, where $\Delta\delta H$ and $\Delta\delta N$ are the difference in chemical shift observed between the bound and free states. The corresponding weighted averages for the side chain methyl groups were calculated using the equation $\Delta\delta(CH) = [(\Delta\delta H^2 + (\Delta\delta C/4)^2)/2]^{1/2}$, where $\Delta\delta H$ and $\Delta\delta C$ are the chemical shift differences observed for the methyl protons and carbons, between the bound and the free states. The composite shift differences were mapped onto the structure of cyt *c*.

The cross-saturation NMR experiments were performed on samples containing 0.8 mM ferrous ²H, ¹⁵N cyt *c* in 1 mM potassium phosphate, pH 7.0 in 1:1 H₂O:D₂O alone or with 0.96 mM cyt *b*₅ on a Varian Inova spectrometer at 18.1 T (800 MHz ¹H) as described by Shimada and coworkers (42). To maximize the magnetization transfer, the temperature was reduced to 15 °C. The saturation of aliphatic protons of cyt *b*₅ was achieved with a 2.4 sec train of iburp-2 pulses of 6 ms separated by 6 ms delays. Control experiments were carried out to ascertain that the H₂O resonance was not affected by the saturation field. Off-resonance experiments were collected with the center of the decoupling field shifted 20,000 Hz up-field. A high amide proton occupation on the target protein (cyt *c*) was used in order to obtain a sufficient signal-to-noise ratio. We have not observed any indications of amide-proton – amide proton spin diffusion on the target protein, which would have shown up as an extensive surface patch, and/or as saturation transfer to internal amide protons. Apparently our choice of solvent conditions and experimental parameters struck the right compromise between sensitivity and selectivity for this particular complex.

Calculation of a cyt *c*-cyt *b*₅ complex using NMR data

The HADDOCK docking software (30) was used to compute the lowest energy cyt *c*-cyt *b*₅ complexes using chemical shift mapping and cross saturation transfer data. HADDOCK CNS scripts were modified to accommodate the heme prosthetic group of cyt *c* and cyt *b*₅. The topology and parameter files for heme were generated using the software XPLO2D (43–45). The charge on the individual heme atoms was assigned using values from the toph19x.heme force field of XPLOR. All force constants in the parameter file were matched to parallhdg.pro of XPLOR, i.e. 1000 kcal mol⁻¹ Å⁻² for bonds and 500 kcal mol⁻¹ Å⁻² for angles, dihedrals and impropers. The chemical shift perturbation and cross-saturation data identifying the contact residues of cyt *c* in this work, along with data identifying the contact residues on cyt-*b*₅ (29), as derived from the Boolean cross section of chemical shift perturbation, saturation transfer mapping and change in relaxation parameters for cyt *b*₅ resonances, were used as input for the docking process with HADDOCK. The docking protocol consisted of three sequential steps.

Initially, the cyt *c* (2GIW) and cyt *b*₅ (1CYO) were positioned 150 Å apart from each other in space. Rigid body energy minimization (EM) was then performed: first, four cycles of orientational optimization were performed in which each protein in turn is allowed to rotate to minimize an artificial intermolecular energy function (see below). Subsequently, both translations and rotations are allowed, and the two proteins were docked by rigid body energy minimization. The intermolecular energy is the sum of electrostatic, van der Waals, and ambiguous intermolecular distance restraint (AIR) energy terms between contact residues on each molecule. 500 rigid body docking complex conformations are calculated at this stage. The best 50 solutions in terms of intermolecular energies were then refined. The second stage consists of three simulated annealing refinements in torsion angle space for each of the best 50 structures. In the first simulated annealing (1000 steps from 2000 to 50 K with 8 fs time steps), the two proteins are considered as rigid bodies and their respective orientation is optimized. In the second annealing (4000 steps from 2000 to 50 K with 4 fs time steps), side chains at the interface are allowed to move. In the third simulated annealing (1000 steps from 500 to 50 K with 2 fs time steps), both side chains and backbone at the interface are allowed to move to allow for some conformational rearrangements. The same 50 structures are then subjected to 200 steps of steepest descent energy minimization. The final stage consists of a gentle refinement in Cartesian space with explicit solvent. The final ensemble of 50 structures is analyzed in this paper.

Calculation of the chemical shifts expected from the docked structure and the effect of the heme ring current on the chemical shifts of its partner

A low energy model structure of the cyt *c*-cyt *b*₅ complex was analyzed with the software ShiftCalc (46–48) to determine how well the calculated chemical shifts from the model complex agree with the chemical shifts observed experimentally. We computed the amide proton chemical shifts for the NMR structure of reduced horse cyt *c* (2GIW), the crystal structure of bovine cyt *b*₅ (1CYO), and a model structure obtained from the docking program.

ShiftCalc was also used to evaluate the contributions of the magnetic dipole of the ring current of the heme of cyt *b*₅ on the chemical shifts observed in cyt *c* by comparing the chemical shifts predicted in the presence of holo cyt *b*₅ and apo cyt *b*₅ (cyt *b*₅ lacking the heme). A similar calculation was conducted to examine the effect of the ring current of the heme of cyt *c* on the chemical shifts of cyt *b*₅.

RESULTS AND DISCUSSION

Confirmation of the resonance assignments for horse cyt *c*

Assignments of backbone resonances and side chain resonances have been reported previously for the H26N, H33N double mutant of horse cytochrome *c* (49). Our results for the wild type protein used in this study, are similar to those previously reported except for a few resonances, mostly around the mutated region. The sequential backbone resonance assignments of those residues were obtained and others were confirmed using a single 3D HNCA experiment. Side chain assignments were confirmed from the analysis of 3D CCCCONH, HCCCONH and HCCH-TOCSY spectra.

Titration of ¹⁵N, ¹³C-enriched cyt *c* with unlabeled cyt *b*₅

Two-dimensional ¹H-¹⁵N HSQC and ¹H-¹³C HSQC spectra were used to monitor the titration of 0.8 mM ¹⁵N, ¹³C-labeled cyt *c* with unlabeled cyt *b*₅. A single resonance is observed for each observable amide of cyt *c* at any point during the titration indicating that the cyt *c* – cyt *b*₅ complex is in fast exchange on the NMR time scale between its unbound and bound forms. A superposition of the HSQC spectrum of free cyt *c* (red) and complexed with cyt *b*₅ (cyt *b*₅/cyt *c* = 1.2, green) is shown in Figure 1. Chemical shift perturbations and increases in line

width were observed in the spectrum of cyt *c* upon complex formation with cyt *b*₅. The linewidth of the ¹H-¹⁵N cross peaks increased over the range of the titration in a saturable manner, providing strong evidence for a stable intermolecular interaction. However, the increase in the change in chemical shift was followed by a decrease for all resonances upon further addition of cyt *b*₅. This behavior was attributed to an increase in the ionic strength of the solution which occurs upon addition of cyt *b*₅. The higher ionic strength weakens the electrostatic interactions between the two proteins and causes partial dissociation of the complex. This is consistent with earlier reports that have demonstrated that complex formation is acutely sensitive to ionic strength and pH (1). To decrease the contribution to the ionic strength from the protein source, the titration was repeated using a lower concentration of cyt *c* (0.1 mM) with cyt *b*₅ in molar ratio of cyt *c*: cyt *b*₅ varying from 0–15. An overlay of the HSQC spectra of 0.1 mM cyt *c* with and without cyt *b*₅ shows perturbation of same set of residues observed earlier. However in this case, the change in chemical shift increased in a saturable manner (Figure 2A).

The changes in the amide proton chemical shifts of cyt *c* at molar ratios of cyt *b*₅ ranging from 0–15 are shown in Figure 2A. The profile of chemical shift variations and intensity change as a function of cyt *b*₅ match well to what was expected on the basis of the formation of a 1:1 adduct. In contrast to our current results with bovine cyt *b*₅ and horse heart cyt *c*, the profile of chemical shift change on addition of yeast iso-1-cyt *c* to rabbit cyt *b*₅ by others suggested the possibility of formation of a complex of 2 moles of iso-1-cyt *c* with 1 mole of cyt *b*₅ at high cyt *c* to cyt *b*₅ ratios (50). Thus, the experimental result may vary depending on the source of the cyt *b*₅ and cyt *c* proteins.

Analysis of the chemical shift changes indicates an equilibrium association constant of $4 \pm 3 \times 10^5 \text{ M}^{-1}$ with a 1:1 stoichiometry. The uncertainty represents the variability of fitting amide proton chemical shift changes for five different residues.

A range of affinity constants for the cyt *c*-cyt *b*₅ complex can be found in the literature, depending upon the source of the proteins involved in the complexes and the differences in conditions under which the measurements are taken. The value of K_a obtained in this study is one order of magnitude larger than the one we reported when labeled cyt *b*₅ (0.8 mM) was titrated with unlabeled cyt *c* (29). The difference between the observed binding constants can be attributed to the overall lower ionic strength of the solution when labeled cyt *c* (0.1 mM) is titrated with cyt *b*₅. The interaction between the two cytochromes is strongly dependent on ionic strength. However, assessing the ionic strength for solutions containing the high concentrations of charged proteins required for these experiments in the presence of low salt concentrations is not reliable.

Two amide resonances (Ala 83 and Lys 88), which were absent in the spectrum of free cyt *c*, appeared when unlabeled cyt *b*₅ was added to the solution (Figure 2B). Comparison of two spectra with different concentrations of cyt *b*₅ indicate that Ala 83 and Lys 88 undergo chemical shift changes with the addition of cyt *b*₅. It thus appears that millisecond conformational fluctuations of these residues in free cyt *c* were perturbed by binding to cyt *b*₅. Milli- to microsecond motions have often been observed by NMR spectroscopy at protein surfaces that are destined to be interfaces in complexes (50).

The maximum chemical shift change, 136 Hz, occurring in the complex was observed for the amide proton of Thr89. This suggests a lower limit for the dissociation rate (k_{off}) of 855 s^{-1} ($k_{off} \gg 2\pi\Delta\delta$, where $\Delta\delta$ is the maximum observed difference in chemical shift between the free and complexed form of cyt *c*).

Chemical shift changes observed on cyt *c* upon complex formation

The chemical shift changes induced in cyt *c* by cyt *b*₅ binding were mapped using the composite chemical shift differences of the backbone amide ¹H and ¹⁵N resonances and are shown in Figure 3. The chemical shift perturbations of the amide protons are large relative to the chemical shifts of the amide protons observed for cyt *b*₅ (29). In a later section, we demonstrate this is likely due to the larger effect of the ring current of the solvent exposed heme of cyt *b*₅ on the resonances of cyt *c* as compared to the smaller effects of the largely buried heme of cyt *c* on the resonances of cyt *b*₅.

As shown in Figure 4, the structure of cyt *c* is characterized by five α helices (3–13, 50–53, 63–69, 71–74, 89–101) connected by loops. The two axial iron ligands belong to the first and last loops. The largest composite chemical shifts are observed for Asp2, Lys13 in helix α ₁, Gln16 and Val20 in the loop connecting helices α ₁ and α ₂, Asn70 in the loop connecting α ₃ and α ₄, Lys72 in α ₄, Gly77, Ile81, Ile85, Lys86, and Thr89 in the loop connecting helices α ₃ and α ₄, and Arg91 in helix α ₅. As side chains commonly play a dominant role in molecular recognition, we have investigated the chemical shift changes for the side chain methyl protons of cyt *c* upon complexation with cyt *b*₅. The ¹H-¹³C HSQC spectrum of cyt *c* overlaid on that of the cyt *b*₅-cyt *c* complex is depicted in Figure 1B in the supplement. There are only a few side chain resonances which become perturbed on the addition of cyt *b*₅. These include side chains I81 γ , I81 δ , A83 β , I85 γ and I85 δ , all of them are located in the loop between α ₄ and α ₅.

The competitive paramagnetic difference spectroscopy experiments carried out by others (25), where [Cr(oxalate)₃]³⁻ and [Cr(CN)₆]³⁻ were used as competitors for cyt *b*₅, have been useful in delineating the interacting region of cyt *c* for cyt *b*₅. At pH 7.2, the main interaction surface on cyt *c* for cyt *b*₅ was shown to include Lys72, Ile81, Ala83, Phe82, and Ile85, which is consistent with our results. Comparison of the crystal structures of oxidized and reduced forms of horse heart cyt *c* reveals that Gln16, Lys87, Glu90 undergo significant larger conformational changes upon change in oxidation state. These residues have also been shown to be in contact with residues of cytochrome *c* peroxidase in the crystal structure of the cyt *c*-cyt *c* peroxidase complex (51).

Residues showing perturbations in the HSQC spectra are located near the solvent exposed heme and form a large single patch on the surface of cyt *c*. Among them, there are two of the three pairs of conserved positively charged lysines surrounding the solvent exposed heme, with no significant chemical shift changes for the other pair of conserved lysines, Lys27 and Lys79, and nearby residues.

Figures 5A–5D show the residues on cyt *c* implicated in binding cyt *b*₅ in the present work and from the models and experiments in the literature. The backbones of the three forms of cyt *c* were superimposed and all molecules are shown with the heme in the same orientation and residues are numbered as in horse cyt *c*. The interface region identified here for horse cyt *c* is different from that proposed by Salemm for tuna cyt *c* (5), and instead close to the alternative, more stable, structure predicted by Brownian dynamics simulations of Northup using yeast iso-1-cyt *c* (13). It is to be noted that the inter-protein interface of cyt *b*₅ in the Northup and Salemm model are very similar. A chemical shift mapping study is also available on the interaction between ¹⁵N-enriched reduced yeast iso-1-cyt *c* and ferrous rabbit cyt *b*₅ (52). The interprotein interface is close to, but different from, those experiencing the largest shifts in this work, presumably indicating a slightly different interface. In the above studies, rabbit cyt *b*₅ induces a large number of chemical shift changes which are primarily near the heme but also on the back side of yeast cyt *c* away from the heme. Nevertheless, the perturbed residues near the heme are the same in both the Banci and Northup model.

Cross saturation transfer NMR experiments of the cyt *b*₅-cyt *c* complex

The cross-saturation transfer experiment (42,53) is a way to reliably and directly identify residues in contact at the protein-protein interface. It provides more direct evidence of an interprotein interface than chemical shift mapping which may identify buried residues indirectly affected by binding. The cross saturation experiment utilizes an isotope-labeling scheme in which a protein (cyt *c*), whose interface residues are to be identified, is uniformly enriched with ²H and ¹⁵N. The enriched protein is complexed with a non-labeled target protein (cyt *b*₅) in a solvent containing a mixture of H₂O and D₂O. The partially protonated solvent will replace some of the exchangeable amide deuterons of cyt *c* with protons so that the correlation between amide nitrogen and amide proton can be observed. In an attempt to obtain ²H,¹⁵N-cyt *c* with a per-deuterated heme, a cyt *c* containing plasmid was coexpressed with a plasmid encoding all of the cyt *c* maturation genes. This enabled the cyt *c* to be expressed at significant levels in the absence of unlabelled δ-aminolevulinic acid (38). The NMR experiment consists of a steady state saturation of the aliphatic proton resonance spectral region followed by a ¹⁵N-¹H HSQC experiment (on-resonance experiment). Since the cyt *c* aliphatic and heme protons are replaced by deuterons, this irradiation results in selective saturation of the aliphatic protons of the non-labeled cyt *b*₅. Intra-molecular cross-relaxation carries the saturation from the aliphatic protons of cyt *b*₅ to other protons of cyt *b*₅. Amide protons on cyt *c* that are in the proximity of saturated cyt *b*₅ protons will become partially saturated through inter-molecular cross-relaxation over the interface of the complex. The intensity of the amide peaks (*I*_{on}) in the experiment in which the aliphatic hydrogens are saturated (on-resonance experiment) was compared with the intensity of the amide peaks (*I*_{off}) in the control off-resonance experiment where an “empty” proton NMR spectral region was selectively irradiated. Those cyt *c* ¹⁵N-¹H cross-peaks that show a decrease in intensity in on-resonance experiment, indicated by increase in (*I*_{off}/*I*_{on}), are considered to be at the intermolecular interface. The temperature of the experiments was lowered from 25 to 15 °C to increase the protein rotational correlation time and thus enhance the saturation transfer rate.

In a control on-resonance experiment where the aliphatic hydrogens on isolated ²H-¹⁵N labeled cyt *c* were saturated, we unexpectedly observed that several of NH resonances exhibited a decrease in peak height. Additional controls verified that the effect was not mediated through accidental irradiation of the water resonance. Apparently, the deuteration of the heme and aliphatics was not entirely complete.

Two strategies were employed to minimize the residual intra-molecular saturation transfer in cyt *c*. First, three separate experiments were carried out with saturation frequency at 0.2, 1.2 and 2.2 ppm with a very narrow bandwidth of 1 ppm to the sample of cyt *c* alone. The purpose was to find a condition where the saturation of heme protons of cyt *c* can be minimized.

The mean values of (*I*_{off}/*I*_{on}) for the amide protons of isolated cyt *c* were found to be 1.25, 1.40 and 1.41 for the saturation frequency centers at 0.2, 1.2 and 2.2 ppm respectively. It is clear that saturation at 0.2 ppm is the best to carry out the saturation transfer experiment. The second strategy employed was to plot (*I*_{off}/*I*_{on}) for cyt *c* in presence of cyt *b*₅ versus (*I*_{off}/*I*_{on}) for cyt *c* alone. Assuming that the residual intra-molecular saturation of cyt *c* amide protons will not be affected by the complex formation, the above plot can be used to separate the inter-molecular proton saturation transfer from the unwanted intra-molecular proton saturation. Figure 6A shows such a plot when the saturation frequency was set to 0.2 ppm. A linear fit of the data was carried out, yielding a line with a slope of 1.4 and correlation coefficient, *R*, of 0.95. The slope is expected to be larger than unity because (intra-molecular) cross saturation in cyt-*c* is more efficient in the larger complex (the cross saturation rate is proportional to the rotational correlation time). Several data points in Figure 6A are seen to deviate more than one standard deviation from the linear fit. These points all have a higher value of (*I*_{off}/*I*_{on}) corresponding to larger cross-saturation in the complex than in cyt *c* alone (note that the reverse is physically

improbable). The outliers, corresponding to residues Gln12, Lys13, Gln16, Phe82, Lys87, are thus likely to be at the interface of cyt-*c* with cyt-*b*₅. A similar plot of the independent data using a saturation frequency of 1.2 ppm also shows three outliers Gln12, Lys13 and Gln16, but not Phe82 and Lys87 (Figure 6B in the supplemental material). The data using a saturation frequency of 2.2 ppm shows no outliers, likely due to masking by the overall increase in cross saturation, as discussed above (data not shown).

Taken, together, the cross saturation data provides strong evidence that Gln12, Lys13 and Gln16 are at the interface. The evidence for Phe82 and Lys87 is weaker. However, all five residues comprise a subset of residues identified with chemical shift mapping data. As such, the cross saturation data provide direct support that the interface is indeed located at the residues inferred from shift mapping.

Calculation of a cyt *b*₅-cyt *c* complex using NMR constraints

The program HADDOCK (High Ambiguity Driven protein-protein Docking) enables one to dock two proteins using constraints obtained from biochemical or biophysical interaction data. We used the data obtained from our cross saturation and chemical shift perturbation NMR experiments on both cyt *c* and cyt *b*₅ to calculate low energy complexes of the proteins (29). The NMR structure of horse cyt *c* (2GIW) and the crystal structure of bovine cyt *b*₅ (1CYO) were used as the starting structures. Briefly, in the first stage of the program the two proteins are docked as rigid bodies, where the docking interface is defined by a combination of experimental NMR restraints and intermolecular energy. In the second stage the side chains and the backbone at the interface are allowed to move and adopt an optimal conformation. The final stage is refinement of the complex in explicit water, allowing also backbone adaptation. According to the established criteria of the HADDOCK program, those residues that are solvent exposed and that show chemical shift perturbation or decrease in peak height during saturation transfer or change in dynamics upon binding were considered to be “active” residues for constraining the docking interface. The “passive” residues correspond to the residues that are surface neighbors of the active residues and also have a high solvent accessibility. On the basis of NMR titration and saturation transfer data (this work and (29) and solvent accessibility of residues, 12 amino acids of cyt *c* (Asp2, Lys13, Gln16, Lys72, Gly77, Ile81, Ala83, Ile85, Lys86, Lys87, Lys88, and Thr89) and 10 residues of cyt *b*₅ (His26, Gly38, Gly41, Gly42, Glu44, Glu59, Asp60, Val61, Gly62, and Ser64) were used as “active” ambiguous intermolecular restraints. By displaying these amino acids on the surface of the free structures, we defined 7 “passive” amino acids for cyt *c* (His26, Arg38, Pro44, Gly56, Lys60, Glu62 and Lys79) and 4 “passive” amino acids for cyt *b*₅ (Glu37, Pro40, Val45, Glu48). Considering the proposed importance of the C6-propionate group of heme of cyt *b*₅ in the formation of the complex (5,54–56), the ambiguous distance restraint file was modified to include the carboxylic oxygens of heme-6 propionate as active atoms excluding other parts of the heme. During the torsion angle space simulated annealing and the refinement in water, the amino acids at the interface are allowed to move to optimize the interface packing. The interfacial residues that are allowed to move consisted of residues in the ambiguous distance restraint file of both molecules.

Figure 7 shows the intermolecular energy as a function of the root mean square deviation (RMSD) of the backbone atoms from the lowest energy structure for the 50 structures. Two clusters could be identified based on pair wise backbone RMSD values using a 1.0 Å cut-off. The first cluster contains the overall lowest energy structure as well as the largest number of structures. The statistical analysis of the clusters is summarized in Table 1. The average RMSD to the lowest energy structure in cluster A is 0.911 and 12.46 Å for models in cluster A, and B, respectively, indicating substantially different conformations for the clusters. The difference in the average value of intermolecular energies for cluster A and B, –526 and –386 kcal

mol^{-1} , respectively, is too small to serve as a selection criterion. For both clusters, the major contribution to $E_{\text{intermolecular}}$ is from $E_{\text{electrostatic}}$ signifying the importance of electrostatic interactions in complex formation. The average value of buried surface areas for the two clusters are 1152 and 1042 $\text{\AA}^2$ respectively. Also these measures were not sufficiently different to discriminate between the clusters.

Information concerning the distance between the heme groups in the cyt *c*-cyt *b*₅ complex has been sought through fluorescence quenching experiments involving fluorescent derivatives of cyt *c* in which the prosthetic group is modified by the replacement of the central iron atom with zinc or by complete removal of the central metal atom (1). The Fe-Fe distance estimated in both these studies for the cyt *c*- cyt *b*₅ complex was about $\sim 18 \text{ \AA}$ while the heme planes are approximately perpendicular. The average Fe-Fe distance in cluster A is 16.75 $\text{\AA}$, and in cluster B 18.62 $\text{\AA}$. Both of these are in good agreement with the fluorescence data. Regrettably this measure does not discriminate between the two clusters.

Calculation of the chemical shifts expected from the docked complex

In an attempt to discriminate between the clusters, we selected the best structure of each cluster and calculated what can be predicted for the amide proton chemical shift changes upon docking in each complex. Using the software ShiftCalc (46–48), we obtained a correlation between the sequence location and sign of the predicted amide proton chemical shifts and the experimentally observed chemical shifts for the best structure of cluster A (panel A & B of Figure 8). The correspondence is much less for the best structure of cluster B, especially for the region around residue 13 (panel C of Figure 8). Interestingly the computational analysis shows that the chemical shifts observed in cyt *c* upon complexation with cyt *b*₅ are almost exclusively caused by the proximity of the edge of the heme of cyt *b*₅. For cluster A, the ring current-induced magnetic dipole on the cyt *b*₅ heme adds to the magnetic field sensed by the amide protons of cyt *c* in the areas around residues Ile85 and Lys13 leading to an NMR frequency increase (shifts to higher ppm as observed). For cluster B, it is predicted that the inter-molecular ring current effect would lead to shifts to lower ppm values for the area around Lys13. Ring current shielding analysis is well-established in NMR spectroscopy, and the direction of the shifts is predicted very reliably. Hence, our calculations suggest that cluster A is the best representation of the complex.

Interestingly, the ShiftCalc calculation also predicts the experimental observation of a much larger chemical shift change for the amide protons of cyt *c* upon complexation with cyt *b*₅ (this study) as compared to the chemical shift change for the amide protons of cyt-*b*₅ upon complexation with cyt *c* (29). This is understandable when considering the fact that $\cong 11\%$ of the heme in cyt *b*₅ is solvent accessible and can closely approach its interaction partner while the heme of cyt *c* is almost entirely embedded in the protein matrix (only 3.5% of the cyt *c* heme is solvent accessible) and remains distant from cyt *b*₅ in the complex.

Comparison with other work

The average RMSD positional difference when superimposing all atoms in the best ten complexes (all fall in cluster A), are 0.90 and 1.01 $\text{\AA}$ for the interface residues of cyt *c* and cyt *b*₅, respectively. The slightly higher RMSD for cyt *b*₅ compared to cyt *c* may indicate a more diffuse nature of the interaction surface of cyt *b*₅. This may be another reason for the smaller magnitude of chemical shift changes observed when isotope enriched cyt *b*₅ is titrated with cyt *c* (29). Three solutions (all of cluster A), based on the lowest intermolecular energy, were selected to represent the models of the cyt *c* – cyt *b*₅ complex.

The molecular interface of the lowest energy solution of cluster 1 is shown in Figure 9A. Although the interaction surface of horse cyt *c*, which was used in the present study, is similar

to the one predicted by Northup et al. (13), using yeast iso-1-cyt *c*, the electrostatic pairing of residues is different. While Northup and coworkers proposed that the most frequently encountered docking geometries involved hydrogen bonding interactions between Lys13, Lys87, Lys86, and trimethyl lysine 72 of yeast cyt *c* and Glu48, Glu56, Asp60, and heme-6-propionate of cyt *b*₅ respectively, our docking predicts the formation of salt bridges between cyt *c* Lys72 and cyt *b*₅ Asp60, cyt *c* Lys73 and cyt *b*₅ Glu56 and Asp60, cyt *c* Lys86 and cyt *b*₅ Glu43 and Glu44, cyt *c* Lys87 and cyt *b*₅ Glu48, cyt *c* Lys88 and cyt *b*₅ Glu44 (shown in Figure 10). Other interactions present are hydrogen bonds between HG1 of Thr89 (cyt *c*) and OE1 and OE2 of Glu 44 (cyt *b*₅), the ϵ -amino group of the Lys13 (cyt *c*) and the carbonyl oxygen of Gly41 (cyt *b*₅), the ϵ -amino group of the Gln16 (cyt *c*) and heme-6-propionate (cyt *b*₅). Important hydrophobic contacts are between residues Lys13, Gln16, Ile81, Phe82, Ala83, Lys86, and Lys87 of cyt *c* and Gly41, heme, Asp60 and Val61, Val61 and heme, Asn57 and Val61, Glu44 and Gly 42, Glu44 of cyt *b*₅, respectively. The distance between the two irons is 16.05 Å° whereas the two heme planes are nearly perpendicular.

The interaction surface of solution 2 is somewhat different than structure 1. While Lys72 of cyt *c* can make salt bridges with both Glu56 and Asp60 of cyt *b*₅, the amide group of Ala83 and carbonyl oxygen of Gly84 of cyt *c* are in hydrogen bonding distance with the carbonyl oxygen of Asp60 and the amino hydrogen of Asn57 of cyt *b*₅. There are important hydrophobic contacts in the cyt *c* – cyt *b*₅ interface between residues Lys72 of cyt *c* and Asp60 of cyt *b*₅, between Ile81 of cyt *c* and Asp60, Val 61, and Gly62 of cyt *b*₅, between Phe82 of cyt *c* and the heme of cyt *b*₅, Ala83 of cyt *c* and Asp60 of cyt *b*₅ Lys 86 of cyt *c* and Glu 44 and Val 45 of cyt *b*₅, between Lys87 of cyt *c* and Glu44 of cyt *b*₅, between Lys86 of cyt *c* and Val45 of cyt *b*₅. The interaction surface of solution 3 is similar to that of solution 2.

The molecular interface of the lowest energy solution of cluster 2 is shown in Figure 9B. The molecular interface of lowest energy solution of cluster 2 consists of salt bridges between cyt *c* Lys13 and cyt *b*₅ Asp60, Lys72 and cyt *b*₅ Glu44, cyt *c* Lys79 and cyt *b*₅ Glu48, cyt *c* Lys86 and cyt *b*₅ heme-6-propionate. Other interactions present in solution 1 are hydrogen bonds of cyt *c* Lys87 with cyt *b*₅ Ser64 and Gly62. Important hydrophobic contacts are between residues cyt *c* Ile81 and cyt *b*₅ Glu44, Glu48 and Val45, cyt *c* A81 and cyt *b*₅ Gly41 and Gly 42, cyt *c* Ile 85 and cyt *b*₅ Val61, cyt *c* G84, K86 and K87 and cyt *b*₅ heme. The molecular interface of the three solutions in cluster 1 is consistent with the earlier observation of the importance of charged residues of cyt *b*₅ 43, 44, 48, 56, 60 and the heme 6-propionate in the complex formation. Replacement of the charged residues 43, 44, 48, and 60 of cyt *b*₅ with their neutral analogues (8) and of 44, 48, 54 and 60 (as well as multiple replacements) to Ala by site directed mutagenesis results in an increase in the dissociation constant of the complex (12,57). However, removal of Glu44, Glu48, Asp60 and modification of the heme propionate yields only a 14 % decrease in free energy of association to cyt *c* (9). This observation may be explained by the fact that Glu44 can transfer its interactive function to its neighbor Glu43, while Asp60 can handover its interactive function to Glu59 and vice versa as observed in the case of solution 1, 2 and 3. Dimethyl-esterification of the heme-propionates of cyt *b*₅ diminishes the binding and electron transfer rate indicating the importance of heme propionates in the complex formation (3). Measurement of the rate of interprotein electron transfer rates and binding constants with numerous cyt *b*₅ mutants by McLendon, Huang and colleagues is consistent with our proposed binding interface (57). Their extensive studies led the investigators to conclude that electrostatic interactions participate primarily in maintaining the stability of the complex whereas the hydrophobic residues flanking the heme regulate the electron transfer rates.

The molecular interfaces of these structural models are consistent with the importance of cyt *c* lysine residues, Lys72, Lys73, Lys86, Lys87 and Lys88, in the formation of the complex. These residues also contribute to a large molecular dipole which interacts with an oppositely charged molecular dipole on cyt *b*₅ to precollisionally orient the two proteins (1). Other cyt *c*

residues involved in hydrogen bonds are Lys13, Thr89. The important hydrophobic residues are Ile81 and Phe82. The above findings may be relevant for the physiological function of cyt *c*. For example, in the crystal structure of the cyt *c*-cyt *c* peroxidase complex these same residues have been reported to be involved in interprotein interactions. Indeed, Gln16 of cyt *c* was seen to be in close contact with Ala193 of cyt *c* peroxidase, and Lys 87 of cyt *c* was proposed as a hydrogen bond donor to Asp 34 of the peroxidase.

Very recently, another group has used NMR chemical shift perturbations and docking methods to propose a model for the interaction of bovine ferricyt *b*₅ with yeast iso-1-ferri and ferrocyt *c* (58). While the equilibrium dissociation constant, and general chemical shift perturbation patterns are similar to our case, their docking results are compatible with a complex, described by a rather diffuse intermolecular interface. We determined, in contrast, a cluster with essentially one major structure, which may be the average of a dynamic complex. There can be several reasons for this. First, we are working with different species of cyt *c*. A close look at the cyt *b*₅ interface of horse and yeast cyt *c* (Figure 5A and 5B) reveals some differences. Importantly, residues 86–89 constitute a basic patch on the cyt *c* surface. Each patch contains three lysines, while the fourth residue is a glutamic acid in the yeast protein and a threonine in the horse cytochrome. This amino acid substitution will increase the negative charge on the yeast patch and render it less attracted to the anionic surface of cyt *b*₅. As a consequence the binding interface may be destabilized and more diffuse in the yeast protein. Important hydrophobic residues like Ile81, Ala83 and Ile85 in horse cyt *c* are replaced by Ala 81, Gly 83 and Leu 85. Cyt *c* residues 8, 9, 12, 13, and 15 which are part of the binding interface are not conserved between the 2 sources of cyt *c*. Residues 8, 9, 12, 15 are involved in the yeast cyt *c* while only residues 12 and 13 of horse cyt *c* are at the interface. Secondly, we have incorporated more data (saturation transfer, chemical shift perturbation of methyl group protons, and calculation of the chemical shifts predicted by the model complex with ShiftCal). Thus it is possible to define the interface on cyt *b*₅ and cyt *c* more precisely during computing. In turn, this required use of a smaller number of “passive residues” in the calculation of the docked complex and likely accounts for the major difference between the extent of our interface and that of Ubbink and coworkers. They used a total of 26 active and 43 passive ambiguous intermolecular restraints in their calculations (58). In contrast our HADDOCK calculations employed a total of 23 active but only 11 passive restraints. Passive residues are surface residues which were not identified experimentally, but are included in the calculations because they are in direct contact with the active residues. Fewer passive residues allow a more precise interface determination. In addition, the carboxylate oxygens of the cyt *b*₅ heme-6-propionate were considered to be active residues in our calculation in contrast to the calculation by Volkov et al., where the 5-CH₃ and 6- α -porphyrin CH₂ were deemed active (58). Third, our titration of reduced horse cyt *c* with high concentrations of reduced bovine cyt *b*₅ did not reveal any evidence of a 1:2 complex as reported by Volkov et al., for their system. Therefore, there was no need to invoke a ternary complex with higher stoichiometry in our system.

SUMMARY

In the present work, chemical shift mapping and cross-saturation experiments were used to identify the residues of horse cyt *c* involved in the binding interface with bovine cyt *b*₅. Our results show that the association between ferrous cyt *c* and ferrous cyt *b*₅ occurs in a 1:1 stoichiometry. This is in contrast to a previous study of a complex between reduced yeast iso-1-cyt *c* and reduced rabbit cyt *b*₅ (52) which report the formation of a 2:1 adduct at high concentrations of cyt *c*.

Mapping chemical shift variations and intensity changes upon cross-saturation transfer on the solution structure of cyt *c* reveals a single interaction patch on cyt *c*. This patch is not consistent with the earlier Salemme model which involves tuna cyt *c* residues Lys27 and Lys79. These

residues are far from the interfacial region as derived from the experimental NMR data. Our NMR data reveal the involvement of Lys13, Lys72, Lys86, Lys87 and Lys88, corroborating the model proposed by Northup with yeast *cyt c* (13). NMR data from the current experiments, as well as NMR data delineating the interaction interface of *cyt b₅*, was used in the protein docking program HADDOCK to calculate a structural model for the complex. From the protein docking program, we obtained two different clusters of possible complex structures. One of these clusters could be ruled out on basis of ring current calculations. The three best structural models in the remaining cluster represent the *cyt c* – *cyt b₅* complex in solution. The average buried surface area is $1152 \pm 50 \text{ \AA}^2$. The iron-iron distance is $16 \text{ \AA}$, in contrast to the $20 \text{ \AA}$ obtained in a docking model for yeast iso-1-*cyt c* and rabbit *cyt b₅* (13).

Although the interaction surface of *cyt c* in our experimentally constrained model of *cyt c*-*cyt b₅* is similar to the one predicted by Northup (13), the electrostatic pairing of residues are different suggesting that similar but nonidentical complexes are formed by homologous proteins from different species.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

NMR	Nuclear Magnetic Resonance
HSQC	Heteronuclear single quantum coherence
Cyt <i>c</i>	Cytochrome <i>c</i>
Cyt <i>b₅</i>	the soluble domain of cytochrome <i>b₅</i>
EM	Energy minimization
AIRs	Ambiguous interaction restraints
RMSD	Root Mean Squared Deviation

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Figure 1. Superposition of the amide region of the ^1H - ^{15}N HSQC spectra of 0.8 mM ^{15}N - and ^{13}C labeled ferrous cyt *c* alone (red) and in the presence of unlabeled ferrous cyt *b*₅ (green) with a cyt *c*: cyt *b*₅ ratio of 1.2. The cross-peaks are labeled with sequence specific assignments.

Figure 2.

(A) Titration curves showing changes in chemical shift for a select number of amide protons of ^{15}N - ^{13}C labeled cyt *c* (0.1 mM) upon addition of unlabeled cyt *b*5. The curves represent the best fit of the individual data using equation 1 of the text.

(B) A region of the ^1H - ^{15}N HSQC spectra of ^{15}N - and ^{13}C labeled ferrous cyt *c* showing the appearance and shifting of A83 and K88 upon the addition of cyt *b*5.

Figure 3. Composite chemical shift difference, as defined by equation 1, of the ^{15}N and ^1H backbone amide resonances of cyt *c* upon complexation with cyt *b*₅. The bottom line represents the mean chemical shift change, and the top line at 0.021 ppm represents the mean value plus one standard deviation. Residues with composite chemical shift changes greater than 0.021 ppm are considered significantly affected upon complex formation and are color coded in Figure 4.

Figure 4.

Ribbon representation of the solution structure of horse heart ferrous cyt *c* (PDB Accession nr 2GIW). The residues that exhibited a composite chemical shift difference of the amide group greater than the mean value plus one standard deviation and Lys88 and Ala83 which appeared and then shifted (see Fig 3) are mapped as blue spheres and labeled. The heme is colored red and the bonds connecting the heme to the axial ligands and covalent bond with two cysteines are shown in black. The figure was prepared with MolMol (41).

Figure 5. Display of the surfaces of different species of cyt *c* showing interfacial residues predicted from (A) cross saturation transfer and chemical shift mapping results in the present work on horse cyt *c* (B) chemical shift mapping results from an earlier study by others on yeast iso-1-cyt *c* (52) (C) Salemme model on tuna cyt *c* (5) (D) Northup model on yeast trimethyllysine iso-1-cyt *c* (13). Positive, negative, polar and apolar interfacial residues are colored cyan, green, yellow and dark grey respectively. The figure was prepared with MolMol (41).

Figure 6.

The intensity ratio $(I_{\text{off}}/I_{\text{on}})$ of complexed cyt *c* amide proton resonances without (I_{off}) and with (I_{on}) the saturation of aliphatic protons, *versus* the same ratio for uncomplexed cyt *c*. The saturation center was at 0.2 ppm. The saturation bandwidth was 1 ppm in both cases. The middle line represents the mean chemical shift change, and the top and bottom line signify the mean value plus/minus one standard deviation.

Figure 7.

The intermolecular energies for the 50 best cyt *c*-cyt *b*₅ complexes in water *versus* the RMSD difference of the interface atoms from those of the lowest energy complex. The intermolecular energy corresponds to the sum of the ambiguous intermolecular restraints, van der Waals, and electrostatic energy terms. The nonbonded energies were calculated with OPLS parameters using a 8.5 $\text{\AA}$ cut-off. The complexes were computed with HADDOCK (30). The clustering was based on the pairwise backbone RMSD using a 1 $\text{\AA}$ cut-off.

Figure 8.

Comparison of the (A) experimentally determined chemical shift changes for the amide protons of 0.8 mM cyt *c* upon addition of 1.2 mM cyt *b*₅, with the calculated amide proton chemical shift change (46–48) for (B) the lowest energy structure of cluster 1, and (C) the lowest energy structure of cluster 2. The clusters are defined in Figure 7.

Figure 9.

The lowest energy cyt *c*-cyt *b*₅ complex of cluster 1 and cluster 2. Positive, negative, polar uncharged and hydrophobic interfacial residues are colored cyan, green, yellow and dark grey respectively. In the bottom panel, cyt *c* and cyt *b*₅ are rotated along their y- axes by 90° in opposite directions to display their interface.

Figure 10.

Stereo drawing of the Ca trace of the lowest energy cyt *c*-cyt *b*₅ complex in cluster 1. It illustrates the predicted salt bridges between the lysines of cyt *c* and the acidic glutamates and aspartates of cyt *b*₅.

Table

Statistical analysis of 50 lowest energy structures

	RMSD (Å) ^a	RMSD min (Å) ^b	Number of structures	E _{inter}	E _{elec}	E _{vdw}	E _{air}	Buried Surface Area Å ²
Cluster 1	0.91±0.2	0.91±0.2	27	-526.6 ±28.6	-501.1 ±83.2	-34.3 ±5.3	8.8 ±1.2	1152.2 ±59.3
Cluster 2	0.72±0.3	12.5±0.1	9	-386.8 ±28.6	368.6 ±27.6	-27.4 ±4.4	9.2 ±0.9	1042.1 ±42.7

^a = Average rmsd and standard deviation from the lowest energy structure of the cluster.

^b = Average rmsd and standard deviation from the lowest energy structure of all calculated structures.

E_{inter} is the intermolecular energy. It is the sum of E_{elec} (electrostatic energy), E_{vdw}, (van der Waals energy) and E_{air}, (the energy of the ambiguous intermolecular restraints).