# Dynamics of protein-protein interactions studied by paramagnetic NMR spectroscopy

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# Chapter 6

# A structural study of the complex of cytochrome $b_5$ and myoglobin with an artificial interface

**Abstract**

The interaction between the physiological complex of cytochrome $b_5$ and myoglobin was studied by Worrall *et al.*. The NMR study indicated that these proteins form a highly dynamic, transient protein complex with a dissociation constant of 200 μM. Prof. Hoffman and coworkers applied Brownian dynamics on these proteins and showed that the affinity of this complex can be enhanced by applying charge reversal mutations. The mutation of three negative residues to the positively charged residues on Mb (Mb (+6)) (D44K/D60K/D85K) not only increased the binding affinity of these proteins but also the rate of electron transfer between them. Here the interactions between the mutant Mb (Mb (+6)) and wt cyt $b_5$ were studied using $^{15}$N HSQC experiments and compared with the results of Brownian dynamics. The chemical shift perturbations of cyt $b_5$ upon addition of Mb(+6) are more than 20 times larger than for the wt Mb. The dissociation constant of cyt $b_5$ and Mb (+6) complex was determined by fitting the chemical shift changes using a 1:1 binding model and it was found to be 63 μM. Plotting of binding maps using the averaged chemical shift perturbations of cyt $b_5$ upon Mb(+6) binding indicated that the cyt $b_5$ residues responsible for binding are present in front face near the heme edge. Most of these residues are negatively charged residues and the NMR results match well with the Brownian dynamics. These results suggest that any dynamic low affinity complex can be strengthened by redesigning the surface charges on one or more binding partners using Brownian dynamics to guide the process. However, the chemical shift changes of cyt $b_5$ upon binding of Mb(+6) were strongly reduced in presence of 100 mM and completely abolished in presence of 250 mM NaCl. This suggests that similar to Cc/Mb(-6) complex, this protein complexes is stabilized only by electrostatic interactions and that short range interactions, such as H-bonds and the hydrophobic effects, are not optimized.

## Introduction

The physiological complex of cytochrome $b_5$ (cyt $b_5$) and myoglobin (Mb) has been studied extensively by many research groups around the world[206-213]. Mb uses its haem cofactor for storage of dioxygen (see chapter 4), whereas cyt $b_5$ is a electron transfer haem protein. Oxidation of Mb leads to formation of metmyoglobin (met-Mb), which is a physiologically inactive form. In order to bring the met-Mb back in its reduced form, the enzyme NADH-cyt $b_5$ reductase transfers two electrons from NADH to two molecules of cyt $b_5$ [214] and the cyt $b_5$ reduces met-Mb back to its physiologically active state[206,215,216].

Protein-protein complex formation involves at least two stages. A dynamic encounter complex is formed before the final, well-defined complex[217-220]. In this dynamic state proteins search each other's surface for the binding surface to form the final complex[221]. The encounter complex is mainly formed by long-distance electrostatic interactions, whereas the well-defined complex is stabilized by short-range charge interactions (salt bridges), hydrogen bonding, hydrophobic interactions and van der Waals contacts[221]. The dynamic nature of the encounter complex makes it impossible to study this state by X-ray crystallography, but it can be studied by NMR spectroscopy. Earlier studies in our group on various transient dynamic protein complexes[196,211,222,223] showed that in the encounter complex the proteins exhibit only very small chemical shift perturbations and often sample an extensive surface area. The encounter state is highly populated in weak, short-lived complexes, including many ET protein complexes. In these complexes, both the association ($k_{on}$) and dissociation ($k_{off}$) rate constants are usually higher than of static complexes.

Hoffman and co-workers showed, using laser-flash induced ET studies, that the complex between cyt $b_5$ and wt Mb is a highly dynamic and transient complex.[207-210] This conclusion was supported by Worrall *et al*. by using NMR chemical shift mapping, protein docking simulations and $^{15}N$ relaxation data.[224] In this study, $^{15}N$ HSQC titrations were performed in which Mb was titrated into the $^{15}N$ cyt $b_5$ sample. The chemical shift

perturbations of cyt $b_5$ upon addition of Mb were very small and spread over the surface of cyt $b_5$. To show that the shift changes were caused by complex formation, $^{15}N$ relaxation experiments were performed to determine the rotational correlation time. For the free cyt $b_5$ a value of 6.7 ns was found and it increased to 9.2 ns in the presence of met-Mb, confirming that complex formation occurs. Docking simulations suggested that cyt $b_5$ samples a large area of the Mb surface area, in a wide variety of docked confirmations. All these results supported the conclusion from the laser-flash kinetic studies that the complex between the Mb and cyt $b_5$ is purely dynamic in nature.

Recently, Hoffman and co-workers showed that optimizing the surface charges on Mb leads to an increase of the affinity as well as the electron transfer rate between cyt $b_5$ and Mb[188,191,225,226]. For this purpose, three acidic surface residues surrounding the horse Mb haem, Asp44, Asp60, and Glu85, were mutated to Lys, creating Mb(+6). In their experiments the haem was substituted by Zn deuteroporphyrin (ZnD) to enable the laser-flash ET measurements. In some cases, the two negative charges on the propionate groups were neutralized by using Zn deuteroporphyrin dimethyl ester, increasing the Mb charge by an additional +2, denoted as ZnMb(+8). The increased affinities and ET activity were interpreted as evidence that the mutations created a better defined binding site close to the Mb haem group. The detailed structural characterization of this engineered complex has not been done. It is an open question whether the electrostatic interactions are still dominating, though more localized in this complex.

To address this question in this chapter the protein-protein interactions between bovine cyt $b_5$ and horse Mb are studied by NMR spectroscopy, with a focus on charge-optimized variants of Mb. The objective of the work is to establish the influence of the redesigned surface of Mb on the degree of dynamics in the complex. Mb has been described in more detail in chapter 4. Below some background information of cyt $b_5$ is provided.

*Cytochrome $b_5$*

Two genes have been identified that code for cyt $b_5$ iso-forms. One gene product is targeted to the membrane of the endoplasmic reticulum, known as microsomal or Mc cyt $b_5$, and the other to the outer mitochondrial membrane, called OM cyt $b_5$ [227,228]. Both Mc and OM $b_5$ are attached to their respective membranes by a hydrophobic C-terminal domain. The soluble, haem binding N-terminal domain in both iso-forms is separated from the membrane by approximately 20 polar residues, called the linker region. The soluble N-terminal domain of OM $b_5$ is a 9 kDa protein. The first crystal structure of haem bound bovine cyt $b_5$ was solved by Argos and Mathews[229]. The Mc cyt $b_5$ soluble domain consists of two hydrophobic cores, which are separated by a five-stranded β-sheet (Fig. 6.1). Core 1 consists of four α-helices with the haem coordinated by His 39 and His 63. Core 2 consists of two α-helices.

Cyt $b_5$ functions in many biosynthesis pathways, like lipid and cholesterol biosynthesis[230]. Cyt $b_5$ is also involved in the reduction of proteins, like cytochrome P450, met-Hb, and the aforementioned met-Mb. Reduction of met-Hb and met-Mb is very important in keeping these $O_2$-carriers in physiologically active forms.

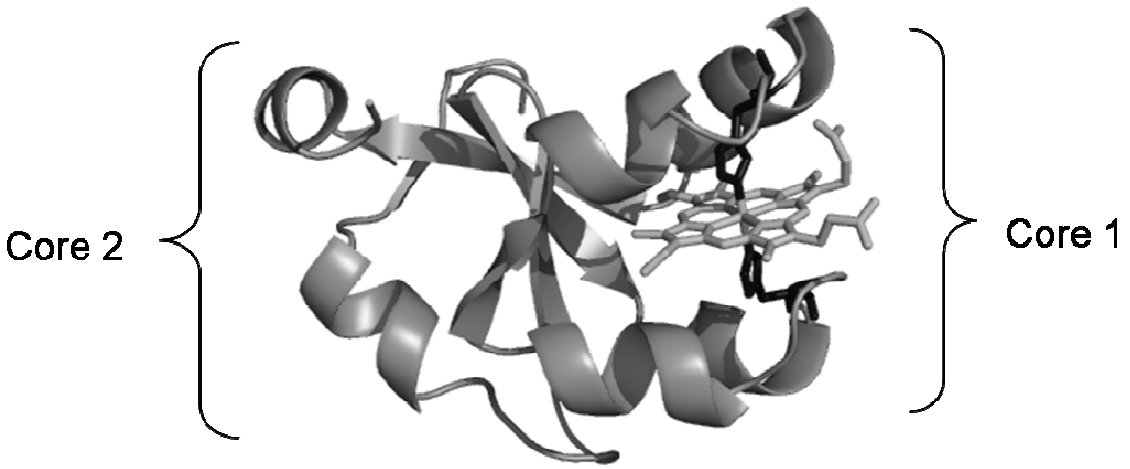


**Figure 6.1.** Three dimensional structure of mitochondrial bovine cyt $b_5$ (PDB entry 1CYO[231]). The two hydrophobic cores are separated by a five-stranded β-sheet. The haem (sticks) is bound in core 1, surrounded by a bundle of four α-helices and coordinated by His 39 and His 63 (blue). Core 2 consists of two α-helices.

## Materials and Methods

*Proteins*

The proteins cyt $b_5$, wt Mb, met-Mb D44K/D60K/D85K ZnD-ester Mb (Mb+8) and met-Mb D44K/D60K/D85K ZnD Mb (Mb+6) were kindly provided by Mr. Ethan Trana, Ms. Nadia Petlakh, Dr. Judy Nocek and Prof. Brian Hoffman (Northwestern University, Evaston, IL).

*NMR sample preparation*

For ferric cyt $b_5$ and met-Mb, protein concentrations were determined from the absorbance of peaks at 414 nm ($\varepsilon$ =117 $mM^{-1}$ $cm^{-1}$ [232]) and 408 nm ($\varepsilon$ = 188 $mM^{-1}$ $cm^{-1}$ [233]), respectively. Samples contained 10 mM KPi pH 6.0, with or without 100 mM NaCl, and 6% $D_2O$ for the lock. Mb was concentrated to a stock concentration of 2 to 3 mM and the pH of the samples was adjusted to 6.00 ± 0.05 by addition of small aliquots of HCl or NaOH.

*NMR titration experiments*

[$^{15}N$, $^{1}H$] HSQC NMR experiments were recorded at 298 K on a Bruker Avance III 600 MHz NMR spectrometer, equipped with a TCI-Z-GRAD cryoprobe. The spectra were acquired with 1024 and 124 complex points in direct and indirect dimensions, respectively, with an interscan delay of 1 s. An inverse titration was attempted for Mb(+8) by titrating $^{15}N$ labelled cyt $b_5$ into the unlabeled Mb(+8). For Mb(+6) direct titrations were performed by titrating met-Mb into a sample of $^{15}N$ labelled cyt $b_5$ to a 7-fold excess. In each titration first a $^{15}N$ spectrum of free cyt $b_5$ was recorded. The pH of the sample was checked before and after the titration. The data were processed using nmrPipe software[154] and analysed using CCPN Analysis 2.1.5 software[155]. Assignments of cyt $b_5$ were available in the group, from former co-worker Dr Worrall.

*Chemical shift perturbation analysis*

Chemical shift perturbations of cyt $b_5$ upon Mb binding were analysed using the standard 1:1 binding model described in chapter 1.

**Results**

The interaction between ferric cyt $b_5$ and the Mb(+8) variant was studied by an inverse $^{15}$N HSQC titration experiment. In such experiments, the observed compound (cyt $b_5$) is the titrant. In the presence of Mb(+8) the resonances of cyt $b_5$ are broadened and shifted, indicating complex formation (Fig. 6.2). Initially, the titrations were performed in 10 mM KPi buffer at pH 6.0. Unfortunately, the stability of Mb(+8) was poor, so only a couple of titration points could be recorded. The stability of the protein was improved by adding 100 and 250 mM NaCl. The interactions between the proteins appeared to be severely reduced by adding 100 mM NaCl as judged from the line widths and chemical shift perturbations of cyt $b_5$ resonances. In the presence of 250 mM NaCl no interaction could be detected (Fig. 6.3). This suggests that electrostatic interactions play a dominant role in the complex formation between the cyt $b_5$ and Mb(+8).

The Mb(+6) variant was more stable than Mb(+8), so a direct titration could be performed in which unlabeled met-Mb(+6) was titrated into a sample of $^{15}$N labelled ferric cyt $b_5$ to a final ratio of 7:1 of Mb(+6) to cyt $b_5$. Large chemical shift perturbations were observed upon addition of the mutant met-Mb, in particular in the $^{1}$H dimension (Fig. 6.4). The shift changes are proportional to the fraction of bound cyt $b_5$, indicating that binding and dissociation are fast on the NMR time scale. Thus, the exchange rate, $k_{ex}= k_{off}+k_{on}$[cyt $b_5$], can be estimated to be >>1300 $s^{-1}$ on the basis of the largest shift observed.

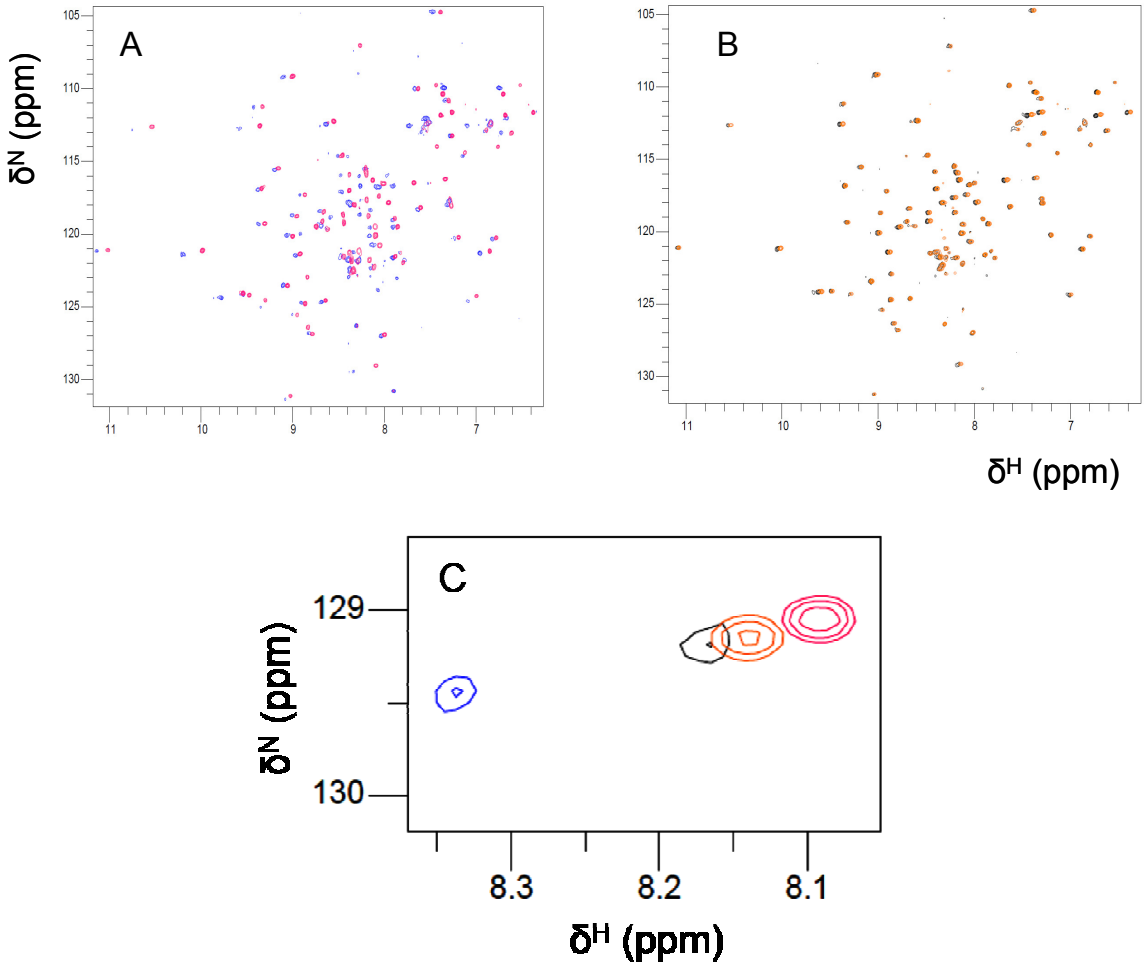


**Figure 6.2.** Overlaid HSQC spectra of free cyt $b_5$ (red in A, orange in B) and in the presence of 0.15 molar equivalent Mb(+8) (blue in A, black in B) in 10 mM KPi buffer, pH 6.0 (A) and 10 mM KPi, pH 6.0 and 100 mM NaCl (B). C) Zoom-in of an overlay of the spectra in A and B for Ala 54.

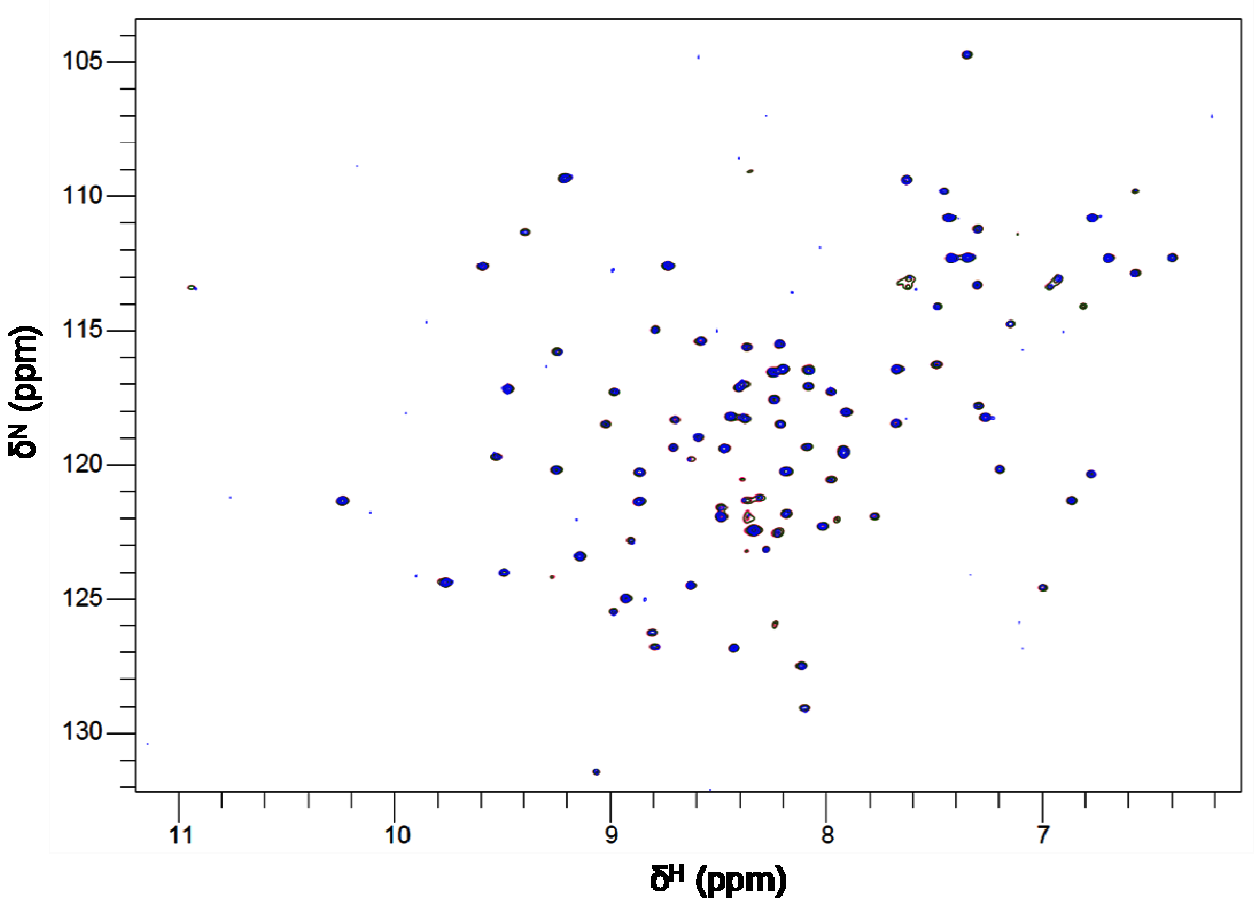


**Figure 6.3.** Overlaid HSQC spectra of free cyt $b_5$ (pink) and in the presence of 0.15 (blue) and 5 (green) molar equivalent Mb (+8) in 10 mM KPi, pH 6.0 and 250 mM NaCl.

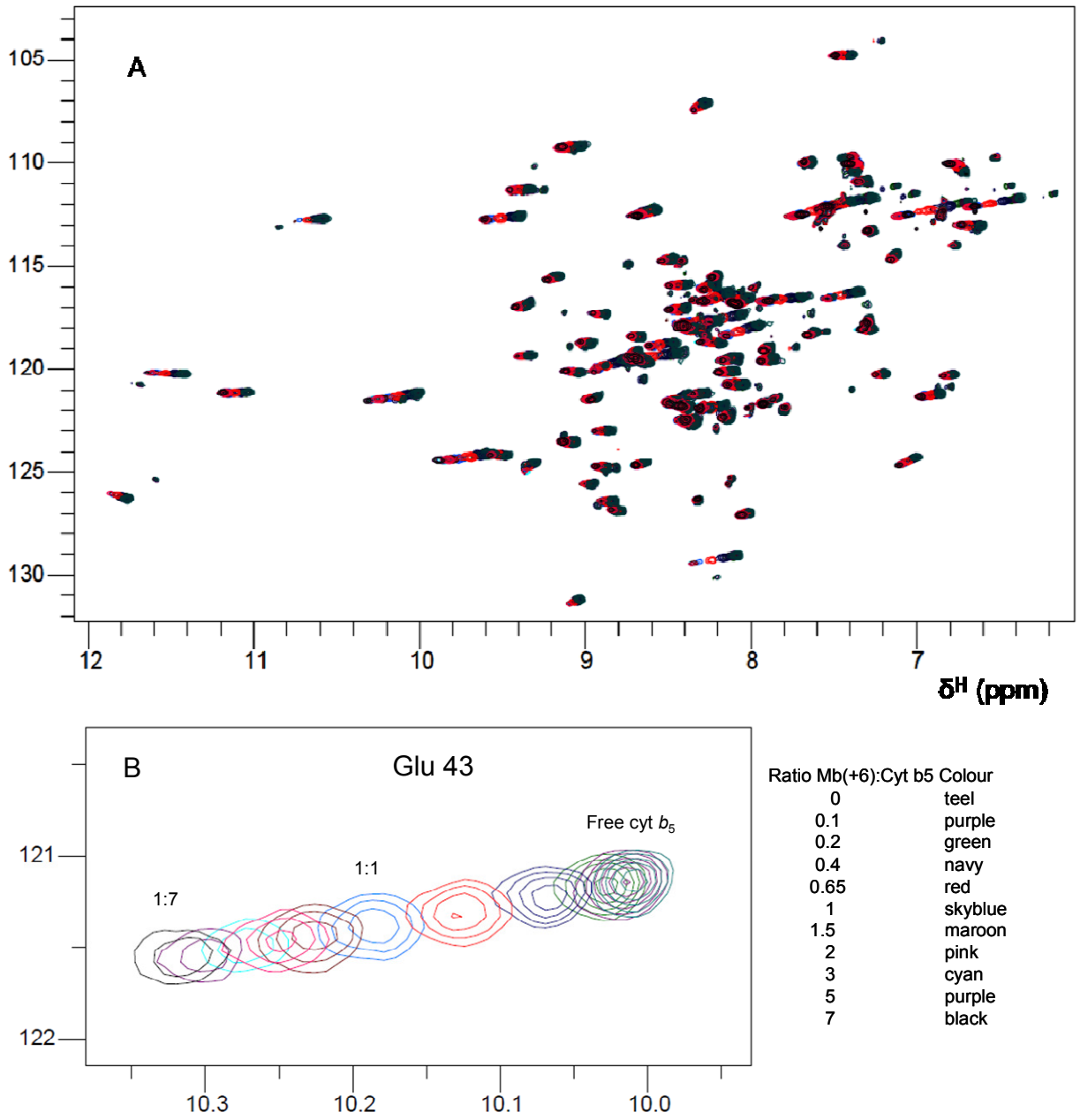


**Figure 6.4.** A) Overlaid HSQC spectra of free cyt $b_5$ and in the presence of Mb(+6) in a series of titration points shown in different colours. B) Chemical shift perturbations for Glu 43 are shown in detail.

Chemical shift perturbations of several $^1$H nuclei were plotted against the ratio of Mb (+6) and cyt $b_5$. The curves were fitted to a 1:1 binding model (eq. 4.1), yielding a $K_d$ value of 63 ± 8 μM (Fig. 6.5). On the basis of the $K_d$ the fraction of cyt $b_5$ that was bound at a 7:1 ratio of Mb(+6) over cyt $b_5$ was calculated to be 93%. The averaged

chemical shift perturbations observed at the 7:1 ratio were extrapolated to the 100% bound form of cyt $b_5$ for each residue ($\Delta\delta_{avg}$) and mapped on the surface of cyt $b_5$ (Fig. 6.6).

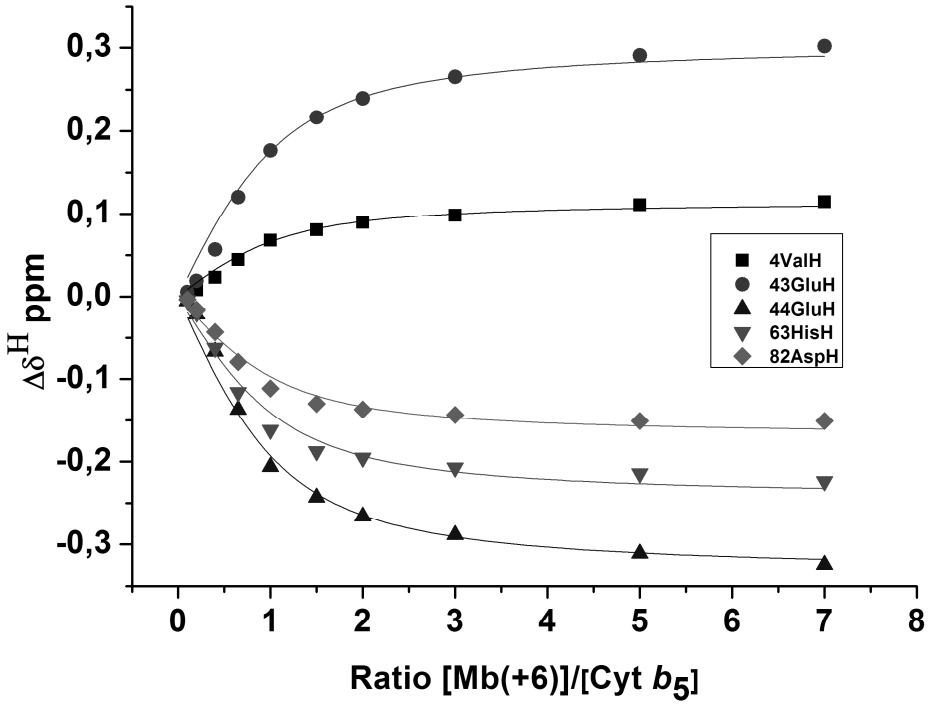


**Figure 6.5**. Plot of chemical shift perturbation titration curves of Mb(+6) to cyt $b_5$. The curves are fitted to a 1:1 binding model (eq. 4.1). using non-linear curve fitting. The $K_d$ of the complex is 63 ± 8 μM.

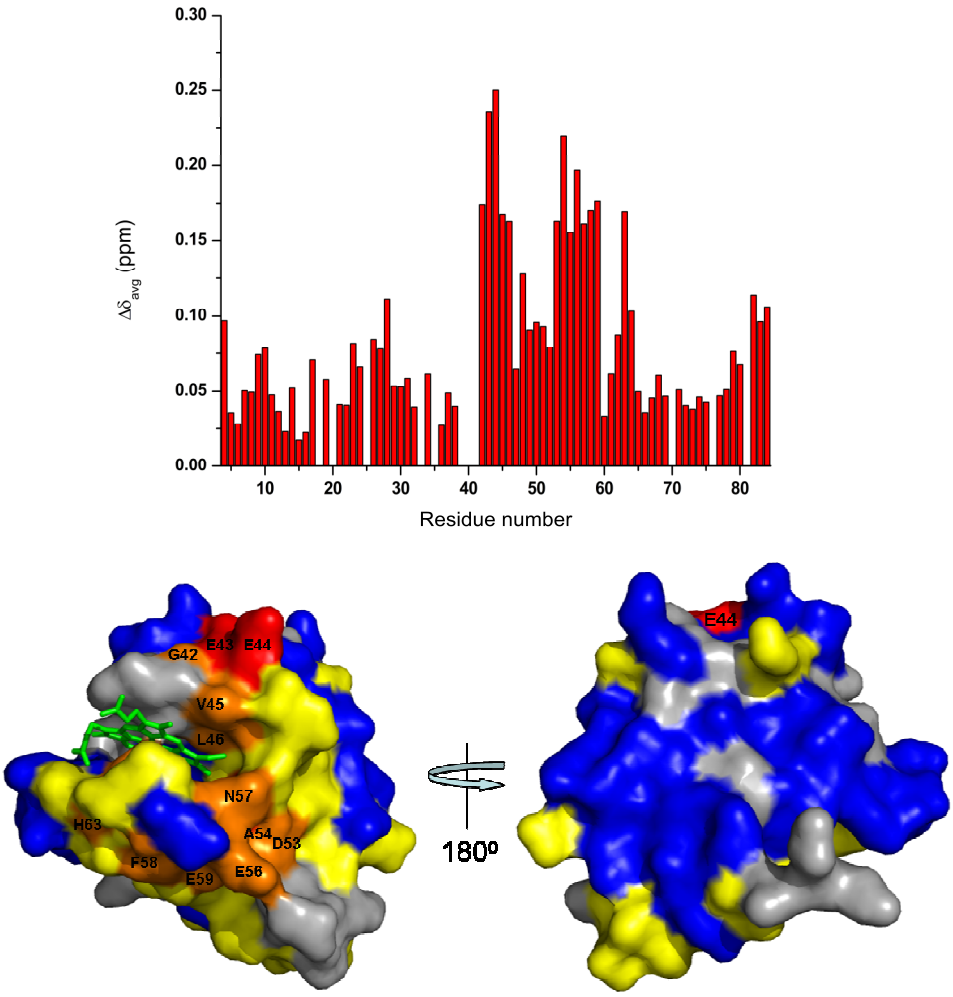


**Figure 6.6**. Binding map. Averaged amide chemical shift perturbations ($\Delta\delta_{avg}$, eq. 4.2) for ferric cyt $b_5$ extrapolated to the 100% bound form are plotted per residue (A) and on the surface of cyt $b_5$ (PDB entry 1CYO[231]) (B). The cyt $b_5$ residues are coloured according to the size of the perturbation, $\Delta\delta_{avg} \geq 0.2$ ppm in red, $\Delta\delta_{avg} \geq 0.15$ ppm in orange, $\Delta\delta_{avg} \geq 0.06$ ppm in yellow and $\Delta\delta_{avg} \leq 0.06$ ppm in blue. The unassigned residues are shown in grey. The haem is shown as green sticks.

**Discussion**

The complex between bovine cyt $b_5$ and horse Mb has been shown to be a highly dynamic and transient by various biophysical[207-210] and NMR studies.[211] Nocek *et al*.[225] showed on the basis of Brownian dynamics (BD) simulations that this protein complex can be strengthened electrostatically by optimizing the surface charges on the protein. In their study the BD hit profiles were generated based on two criteria. The first was the center-of-mass (COM), in which a hit was tallied when the distance between the COM of the partners comes roughly 39 Å within the sum of their radii. The second was the carboxylate (O-O) criterium, in which the hit was tallied if any distance between oxygen atoms of the cyt $b_5$ and Mb haem carboxylates was ≤ 4 Å. Based on these criteria the Mb mutant D44K/D60K/E85K, in which three acidic residues around the haem carboxylates were changed to lysine residues, came out as favourable. These mutations maximize the ratio of 'reactive (O-O)' hits over the total (COM) hits in the BD simulations. Based on the potential energies from the BD simulations, the ensemble of wt Mb configurations shows weak binding to cyt $b_5$, with the potential energy (V) varying from +1.6 to -4.6 kcal/mol. In contrast, the mutant Mb(+6) shows high affinity for cyt $b_5$, with V ranging from -6 to -8 kcal/mol. The results indicated that the complex between wt Mb and cyt $b_5$ involves mostly unreactive conformations, whereas the charge-reversal mutations preferentially enhance binding in configurations that are highly reactive, at least according to the O-O criterium. HyPARE computations[234]were used to calculate the association rate constants for the complex of Mb-cyt $b_5$. The predictions of BD simulations were tested experimentally by studying the photoinitiated ET between the Zn-substituted Mbs and cyt $b_5$. The mutant complex exhibited both a higher affinity and faster ET, indicating that the Mb(+6)-cyt $b_5$ complex is in the kinetic slow-exchange regime ($k_{off} << k_{et}$), whereas the wt Mb-cyt $b_5$ is in rapid exchange regime ($k_{off} >> k_{et}$).

To complement the above studies we have performed a structural characterization of the mutant Mb(+6) and cyt $b_5$ by using NMR spectroscopy. Earlier results by Worrall *et al*. showed that the complex of wt Mb and cyt $b_5$ is highly dynamic[196]. This was concluded on the basis of chemical shift mapping, $^{15}N$ relaxation data and protein docking

simulations. NMR results of the mutant Mb(+6) and cyt $b_5$ are reported here. In contrast to wt Mb, Mb(+6) seems to bind cyt $b_5$ in a specific, well-defined state. This conclusion is based on the chemical shift perturbation mapping. Mb(+6) causes large chemical shift perturbations of cyt $b_5$ residues upon binding. The chemical shift perturbations were 15 to 20 times larger as compared to those for the wt Mb-cyt $b_5$ complex. The $K_d$ for the Mb(+6)-cyt $b_5$ complex was 63 ± 8 μM (free energy of binding, $\Delta G_B$ = -5.8 kcal/mol), three times less than the wt Mb-cyt $b_5$ complex under identical conditions (200 μM, $\Delta G_B$ = -5.09 kcal/mol). A comparison of the average chemical shift plots and the binding maps of cyt $b_5$ upon binding the wt Mb and Mb(+6) shows that most of the cyt $b_5$ residues involved in the binding are common in both complexes. However, the more specific complex leads to much larger shifts and, thus, significant shifts are observed for more residues, such as Gly42, Val45, Leu46, Asp 53, Thr55, Asn57 and Phe58. In contrast, residues Leu9 and Asp60 are involved in binding of Mb(WT) but show no significant chemical shift perturbation in the complex with Mb(+6).

The BD and ET study suggested that the wt Mb-cyt $b_5$ complex exists only as encounter complex, while the introduction of positive charges around the Mb haem leads progressively to a better defined ensemble of structures, with greater average reactivity. The cyt $b_5$ residues involved in binding the mutant Mb (Fig. 6.6) match the residues reported in the BD study[225]. Most of the affected residues of cyt $b_5$ are located around the haem (Fig. 6.6), but that is also true in complex with the wt Mb. Cyt $b_5$ appears to use the same face for binding wt and mutant Mb, but binding leads to much larger perturbations, suggesting better defined interactions and less averaging. It is concluded that in the complex with Mb(+6) cyt $b_5$ assumes many fewer orientations. The BD calculations and ET experiments also indicate that the mutations reduce the Mb surface that is sampled by cyt $b_5$. NMR experiments on labelled Mb will be necessary to confirm this finding.

It is clear that the interaction between Mb(+6) and cyt $b_5$ decreases rapidly in strength with increasing ionic strength of the buffer. It was observed that the chemical shift changes of cyt $b_5$ upon addition of Mb(+6) are quite large at low ionic strength but the size of the perturbations reduces with 100 mM and at 250 mM NaCl binding appears to

be abolished. Though the chemical shift perturbations of cyt $b_5$ are large with Mb(+6) compared to with wt Mb, they are not as large as those observed for specific complexes, like the Src SH3-peptide complex (peptide RALPSIPKLA, Fig. 2.3). The $K_d$ for the Src SH3-peptide is 63 μM, which happens to be the same as for the Mb(+6)-cyt $b_5$ complex, yet the averaged chemical shifts for the SH3-peptide complex, even in the presence of 100 mM NaCl, are more than two times bigger than the low ionic strength perturbations of the Mb(+6)-cyt $b_5$ complex. These results raise the question whether the artificially created complexes like Mb(+6)-cyt $b_5$ and Mb(-6)-cyt *c* have properties similar to naturally occurring well-defined protein complexes. Though the interface mutations make the artificial complexes more stable, the complex seems only to be stabilized by the electrostatic interactions. Short range interactions like H-bonds and the hydrophobic effects are not optimized. In the BD calculations only charge interactions are evaluated, so the finding that the experimental binding energy matches the (lower end) of the interaction energy range found in the BD calculations supports the view that only charge interactions contribute to the binding.