Characterization of Molecular Mechanism of Neuroglobin Binding to Cytochrome C: A Surface
Plasmon Resonance and Isothermal Titration Calorimetry Study

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ABSTRACT

Neuroglobin (Ngb) is a hexa-coordinate heme protein predominantly expressed in the brain tissue with a protective role against hypoxic/ischemic and oxidative stress-related insults. Several intracellular proteins including cytochrome c have been identified as potential neuroglobin binding partners. To understand the factors that control Ngb association to cytochrome c (Cyt c) the thermodynamic parameters for Cyt c association to Ngb variants were probed using surface plasmon resonance and isothermal titration calorimetry techniques. The dissociation constant for human Ngb binding to Cyt c was determined to be $\sim 10 \mu\text{M}$ using SPR and similar value was obtained by ITC confirming that the covalent attachment of Cyt c to the gold surface modified with mixed alkanethiols does not impair Cyt c interactions with Ngb. Modification of the heme iron coordination sphere by replacement of histidine 64 in the position of sixth axial ligand with cyanide effectively inhibits the inter-protein complex formation. On the other hand, the presence of the Cys 46 / Cys 55 disulfide bridge that restrain the CD loop flexibility and increases the distal histidine affinity for heme iron has no impact on the complex stability. ITC data also show that the inter-protein complex formation is entropy driven ($\Delta H = 3.3 \pm 0.2 \text{ kcal mol}^{-1}$ and $\Delta S = 32.3 \text{ cal mol}^{-1} \text{ K}^{-1}$) likely due to a reorganization of solvent molecules surrounding charged amino-acid residues. The experimental data are consistent with the results of a docking study in which the residues in the E- and F- helix of Ngb were identified to form a binding site for Cyt c. We propose that association of the exogenous ligand to the heme iron triggers a repositioning of the E-helix that prevents Ngb-Cyt c complex formation. These results indicate Ngb binds to Cyt c under hypoxic conditions. **(Online version of this Journal shows the figures in color).**

KEYWORDS

CD loop, SPR sensor, Electrochemical impedance spectroscopy, E-helix, Disulfide bridge

1. Introduction

Neuroglobin (Ngb) is a hexa-coordinate heme protein that is predominantly expressed in the neuronal tissue and some endocrine tissues [1]. In cultured neuronal cells Ngb over-expression is up-regulated under conditions of neuronal hypoxia and ischemia [2], whereas in transgenic animals Ngb over-expression reduces ischemic cerebral injury and its knockdown increases the infarct volume and worsens post-ischemic conditions [3]. In addition, Ngb was shown to block β -amyloid neurotoxicity *in vitro* and *in vivo* [4]. Decreased Ngb expression was associated with an increased risk of Alzheimer's dementia in humans [5]. Despite intensive efforts, the molecular mechanism of Ngb neuro-protective role remains unclear. The overall protein structure closely resembles to that of myoglobin (Mb) with a characteristic three over three fold of α -helices [6]. As other globins, Ngb reversibly binds small diatomic molecules including O₂, CO, and NO. The kinetics for diatomic ligand association are more complex compared to five coordinate globins due to the bis-histidine coordination of the heme iron [7] and are influenced by the presence of an internal disulfide bond connecting Cys 46 and Cys 55 in human Ngb [8]. Despite the high affinity for oxygen, the low micromolar intracellular Ngb concentration in neuronal tissue does not support an Ngb role as oxygen storage and/or transport protein [9]. Based on a rapid reaction of oxygen bound Ngb with nitric oxide forming ferric Ngb and nitrate anion, it was suggested that Ngb functions as a NO scavenger [10].

The unknown molecular mechanism of Ngb protective role stimulated a search for interacting partners. Although *in vivo* and *in vitro* studies have identified several intracellular proteins as hNgb interacting partners, only few protein-protein complexes were characterized in terms of affinity constant and identification of residues involved in the inter-protein complex formation. So far, prion [11], raft associated membrane protein flotilin-1 [12], α -subunit of heterotrimeric

G-protein ($G\alpha_{i/o}$) [12, 13], and Cyt c [14] were found to bind Ngb with high affinity. Lechauve et al. [11] have reported that mixing of Ngb and cellular prion protein at sub-micromolar concentrations leads to a fast formation of protein aggregates due to the ionic interactions between the N-terminal domain of cellular prion protein and Ngb suggesting the potential role of Ngb as a scavenger protein. It was proposed that the prion peptide specifically binds into a narrow cleft on Ngb surface formed by acidic (Glu 51, Asp 52, and Glu 58) and polar residues (Asn 43, Ser 48, Ser 55, and Gln 46). Wakasugi et al. [12, 13] have intensively studied interactions between Ngb and $G\alpha_{i/o}$. They have shown that Ngb binds to the $G\alpha_{i/o}$ subunit in the ferric state and inhibits guanidine nucleotide dissociation, whereas the ferrous CO bound form of Ngb has negligible affinity for $G\alpha_{i/o}$ subunit. In addition, the authors have demonstrated that human Ngb (hNgb) mutants having charged residues Glu 53, Arg 97, Glu 118, and Glu 151 replaced by non-charged residues decreased the guanidine nucleotide inhibition activity [15]. Based on these results, the authors have proposed that hNgb serves as oxidative stress-responsive sensor for neuro-protection [12]. Fago et al. [14] have demonstrated a fast electron transfer between the heme iron centers in ferrous hNgb and ferric Cyt c proposing a fast association of hNgb to pro-apoptic Cyt c [16].

To better understand the molecular basis of interactions between Cyt c and hNgb, the affinity constants and thermodynamic parameters for formation of Cyt c-Ngb complexes were determined using surface plasmon resonance (SPR) and isothermal titration calorimetry (ITC) techniques. Since SPR measurements requires one binding partner to be immobilized on the sensor chip surface which may impact the inter-protein complex formation, the K_d values determined by SPR are compared to those observed in solution using ITC. To covalently attach Cyt c to the gold surface, the surface was modified via mixed alkanethiols. The formation of self-

assembled monolayer (SAM) on the gold surface, and subsequent Cyt c immobilization, and Cyt c-hNgb complex formation were confirmed by electrochemical impedance spectroscopy (EIS) technique. Since previous studies have shown that the interactions between Ngb and $G\alpha_{i/o}$ subunit are altered by changes in the heme iron coordination [13, 17], we have characterized association of Cyt c to hNgb with distal His 64 residue replaced by glutamine, as a model of a five-coordinate heme iron hNgb (His64GlnhNgb), as well as hNgb having distal His 64 replaced by cyanide ion to mimic the structure of the Ngb in complex with exogenous diatomic ligands. In addition, the impact of the internal disulfide bond between Cys 46 and Cys 55 and CD loop flexibility on the affinity between Cyt c and Ngb was probed by characterizing the association between Cyt c and the rat Ngb (rNgb) that is missing the Cys residue at position 46. Our data show that the transition between the six-coordinate bis-histidine heme iron and a six-coordinate cyanide bound heme iron in hNgb diminished the affinity for Cyt c, whereas hNgb association to Cyt c is not controlled by the presence of the internal disulfide bridge within the CD loop.

2. Materials and methods

2.1. Reagents and Materials

Horse heart cytochrome c, dithiothreitol (DTT), potassium hexacyanoferrate (III), 16-mercaptohexadecanoic acid (MHA), 11-mercapto-1-undecanol (MUO), and 5-aminolevulinic acid (ALA) were purchased from Sigma-Aldrich, ethanol (200 proof) from Decon Laboratories LLC and potassium ferrocyanide trihydrate from Acros Organics. N-hydroxysuccinimide (NHS) and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) were received from Thermo-Scientific. All other reagents were purchased from Fisher Scientific and used as received. Solutions were prepared using deionized (DI) water ($\sim 18\text{ M}\Omega$) (Ultra Purelab system, ELGA/Siemens). The polycrystalline gold chips (50 nm gold over 2.5 nm titanium adhesion

layers, coated on 18mm x 18mm cover slip glass slides) were purchased from Platypus Technologies, LLC, Madison, WI and each one was cut into two pieces before processing.

2.2. Protein isolation and purification

Ngb was isolated and purified as described previously [18]. Cyt c was dissolved in deionized water, oxidized with potassium ferricyanide and passed through a Sephadex G-25 column to remove the excess of potassium ferricyanide. Subsequently, ferric Cyt c was dialyzed overnight against 10 mM Tris buffer (pH 7.0) or 10 mM phosphate buffer (pH 7.0) containing 10 mM KCN. The cyanide bound hNgb (CN-hNgb) was prepared by addition of KCN to Ngb to a final concentration of 10 mM and the CN-hNgb was dialyzed overnight against 10 mM Tris buffer or phosphate buffer containing 10 mM KCN (pH 7.0). Formation of CN-hNgb adduct was verified using UV-VIS spectroscopy (Cary 50, VARIAN).

2.3. Surface plasmon resonance

SPR experiments were performed using a home-built SPR instrument. The standard NHS-EDC coupling chemistry (25 mM NHS and 50 mM EDC for 8-10 minutes) was used to covalently immobilize Cyt c on the gold surface. The chip preparation and SPR instrumentation set-up are described in the supplementary information, sections S1 and S2, respectively. Cyt c was immobilized on the activated SAM via covalent amide bond and then different concentrations of Ngb were flowed over the Cyt c bound surface. All solutions were flowed through the channel and over the chip surface with a constant flow rate of 50 μ L/min at 22°C. 10 mM Tris or phosphate buffer (pH 7.0) was sequentially flowed through the channel until a stable SPR signal was achieved. Equilibrium data analysis method was used to obtain dissociation constants for Cyt c-Ngb complexes formation.

2.4. ITC measurements

The ITC experiments were performed on VP-ITC titration calorimeter (MicroCal, Northampton, MA). Samples of Cyt c and Ngb were prepared with the same batch of buffer (10 mM phosphate, pH 7.0) to minimize artifacts due to minor differences in buffer composition. Injections were started after equilibration to the baseline stability. The sample cell was loaded with 20 μ M Ngb solution and the concentration of Cyt c in the syringe (290 μ L) was 1.5 mM. The titration experiments consisted of 28 injections of 7 μ L aliquots of Cyt c with 2 min intervals between injections. The temperature was kept at 25 °C. The data were subsequently corrected by subtracting a small heat of diffusion determined in control experiments from the binding heat. The isotherm curves were analyzed using a single set of identical sites model.

3. Results and discussion

Protein – protein recognition and complex formation is a dynamic process that involves structural reorganization of interacting partners and concomitant rearrangement of hydration ions and shells. Mapping of binding sites and clarification of the binding modes required determination of dissociation constant and additional thermodynamic parameters such as enthalpy and entropy change for native proteins as well as proteins with an altered primary structure. Here we compare results of SPR and ITC measurements with the aim to probe molecular mechanism of Cyt c-Ngb complex formation by probing the K_d values for Cyt c association to hNgb, rNgb, His64GlnhNgb and a model of six-coordinate exogenous ligand bound hNgb (cyanide bound hNgb, CN-hNgb). His64GlnhNgb is a good model for a five – coordinate hNgb since the glutamine side-chain has a comparable van der Waals volume as a histidine side chain, but does not form a coordination bond with ferric heme iron in hNgb as evident from the UV-vis spectra (Supplementary information, Figure S1), as reported previously

[18]. Despite the fact that cyanide is not a physiological ligand for Ngb, cyanide bound heme proteins are often used as a model for low-spin six-coordinate globins due to a straightforward sample preparation and a high stability of cyanide bound heme protein complexes. Figure 2a shows the typical SPR response for Cyt c immobilization to activated SAM surface. The Cyt c immobilization was also confirmed by EIS. As shown in Table S1 (Supplementary information), the charge transfer resistance increases from 920 k Ω to 1390 k Ω when Cyt c is immobilized onto activated SAM. A detailed explanation of EIS data is given in the Supplementary information (section S4). Figure 2b and 2c show the association of CN-hNgb to Cyt c and hNgb to Cyt c as a function of hNgb concentration, respectively in the presence of phosphate buffer, pH 7.0. The Cyt c-hNgb complex formation was also confirmed by EIS. The charge transfer resistance increased from 1390 k Ω to 2420 k Ω (Table S1) when 30 μ M hNgb was allowed to bind with Cyt c, which indicates the Cyt c-hNgb complex formation. A control experiment was also performed by passing buffered solutions of myoglobin (Mb) in the concentration range between 1 μ M and 50 μ M through Au/SAM/Cyt c surface. Mb is a globular protein of similar tertiary structure as Ngb that does not bind Cyt c. Only negligible increase in the SPR signal with both Tris buffer and phosphate buffer were detected for Mb association to Cyt c that corresponds typically to non-specific interactions. The equilibrium SPR response (equilibrium pixel values, in Figure 2c) for each Ngb was then plotted as a function of Ngb concentration, as shown in Figure 3. The K_d values were determined from a simple non-linear hyperbolic (SNLH) fitting of the plot of equilibrium SPR response as a function of Ngb concentration according to equation 1 [19],

$$R_{eq} = \frac{R_{max} [Ngb]}{K_d + [Ngb]} \quad (1)$$

where R_{eq} is the equilibrium response, R_{max} is the fitting parameter representing maximum response, and $[Ngb]$ is the Ngb concentration. The determined K_d values for formation of Cyt c-Ngb complexes in Tris and phosphate buffers are listed in Table 1. The K_d for hNgb, His64Gln hNgb and rNgb binding to Cyt c was found to be nearly identical, $\sim 30 \pm 10 \mu\text{M}$ in 10 mM Tris buffer (pH 7.0). Here the errors represent the standard error of the fitting. The observed values match well with the equilibrium constant ($K_d \sim 45 \mu\text{M}$) reported previously for DTT reduced ferric Ngb binding to ferric Cyt c using SPR [20]. Somewhat smaller K_d values of $\sim 10 \pm 1 \mu\text{M}$ were detected for hNgb binding to Cyt c in 10 mM phosphate buffer (pH = 7.0). On the other hand, binding of cyanide to heme iron effectively prevents the inter-protein complex formation.

Previous studies have shown that Cyt c association to negatively charged lipid membranes leads to a partial protein unfolding and alteration of the heme iron coordination [21]. To verify that the attachment of Cyt c to the negatively charged Au/SAM surface does not impair the complex formation, K_d values were determined independently using ITC. The representative ITC data for Cyt c binding to Ngb are shown in Figure 4. The data were analyzed using a single set of identical binding sites model and the thermodynamic parameters are listed in Table 2. Two binding sites of similar affinity were resolved for Ngb binding to Cyt c with a K_d value comparable to that determined in SPR measurements. As observed in SPR experiments, Cyt c does not bind to CN-hNgb. The hNgb, rNgb and His64GlnhNgb exhibit comparable positive reaction enthalpy ($\Delta H \sim 3 \text{ kcal mol}^{-1}$) and entropy ($\Delta S \sim 30 \text{ cal mol}^{-1} \text{ K}^{-1}$) for the association to Cyt c suggesting that the Cyt c-Ngb complex formation is predominantly entropy driven. We attribute the observed entropy increase to a release of water molecules from the binding area and/or water molecules surrounding charged amino-acid residues that are involved in the inter-protein complex formation (see below).

Human and rat Ngb proteins exhibit a high sequence homology (94 %), though rNgb structure lacks the disulfide bridge connecting Cys 46 to Cys 55 due to the presence of a Gly residue at position 46 [8]. The similar K_d values determined for hNgb and rNgb association with Cyt c indicate that the formation of the Cyt c-Ngb complex is not perturbed by the conformational flexibility of the CD loop, which is restricted in the human protein [22]. To investigate the impact of the changes in the heme-binding pocket on the hNgb association to Cyt c we have determined the dissociation constant for binding of ferric Cyt c to His64GlnhNgb and CN-hNgb. The five-coordinate His64GlnhNgb mutant binds Cyt c with a similar affinity ($K_d = 26 \pm 11 \mu\text{M}$) as wild type hNgb. On the other hand, when the distal histidine is replaced by CN^- , no formation of the inter-protein complex was observed, suggesting that the reorganization of the heme distal pocket due to the structural changes associated with the transition from the six-coordinate bis-histidine heme iron to six-coordinate cyanide bound heme iron inhibits the Cyt c association to hNgb.

The structure of the transient Cyt c-hNgb complex was modeled by Bønding et al [20]. The authors proposed that the inter-protein complex is stabilized through ionic interactions between Glu 87, Glu 60 and Arg 66 in hNgb and Lys 25, Lys 72 and Asp 50 in Cyt c and the dissociation constant for hNgb binding to Cyt c decreases in the presence of 100 mM NaCl [20, 23]. We have used a ZDOCK protein docking algorithm [24] to model orientation of Cyt c and Ngb in the inter-protein complex. Among the five complexes identified in ZDOCK simulations, two complexes with the distance between the heme irons of less than 18 Å were used for further analysis since the proximity of the heme groups is necessary to satisfy a fast electron transfer rate between reduced hNgb and oxidized Cyt c ($k = 2 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$) [14]. The Ngb binding surface identified in ZDOCK simulation is similar to that described previously by Bønding et al [20] and

involves mostly residues from the E and F helices, whereas the binding region on Cyt c surface consists of residues of a highly conserved loop D (residues 70-87) and residues in a short 70s helix. In one model, the Cyt c-Ngb complex is stabilized through a hydrogen bond between Ser 84 side-chain in Ngb and Pro 76 carbonyl oxygen in Cyt c, and two salt bridges between Asp 73 and Lys 73 and heme propionate group, and Lys 72 in Ngb and Cyt c, respectively (Figure 5). In the second model, a hydrogen bond between Arg 66 and Gln 16 and a salt bridge between the heme propionate group and Lys 79 contributes to the inter-protein interactions. Interestingly, the Ngb residues identified in docking studies are not conserved in the sequence of Mb supporting the involvement of these residues in the Ngb/Cyt c complex formation. Analogous residues in Mb sequence and corresponding Ngb residues (shown in parenthesis) are: Gly 74 (Asp73), Glu 84 (Ser84), Asn 67 (for Arg 66), and Lys 64 (for Asp63). The involvement of the D loop Lys residues from Cyt c in the inter-protein complex formation is consistent with the increased affinity of Cyt c for hNgb in the phosphate buffer compared to the Tris buffer. Phosphate anions were shown to interact with the side-chain of Lys 72, 73, 86, and 87 that are exposed on the Cyt c surface [25] and thus may serve as a hydrogen bond donor/acceptor bridge between Ngb and Cyt c. In addition, association of the phosphate anion to the D-loop in Cyt c may reduce the loop flexibility promoting the Ngb-Cyt c complex formation.

The crystal structure of the six-coordinate bis-histidine mouse Ngb (mNgb) and a six-coordinate CO bound mouse Ngb shows that the binding of the exogenous ligand leads to a reshaping of the internal hydrophobic cavities, sliding of the heme group into protein interior, and an alteration of the EF-loop flexibility [6]. Surprisingly, no significant changes in the heme distal pocket including the orientation of the distal His 64 side chain and a repositioning of the E-helix were reported. On the other hand, based on the computational studies, it was proposed that

the heme sliding mechanism may be unique for mouse Ngb and the transition between the six-coordinate and the five-coordinate heme iron in hNgb may trigger analogous structural changes previously observed for Mb including displacement of the E-helix that moves away from the heme iron and rotation of the distal His 64 side-chain [22]. Since the helix E residues were identified as part of the binding interface between Ngb and Cyt c in docking studies, we propose that reorientation of the distal histidine side chain and displacement of the E-helix as a consequence of the cyanide binding to the heme iron contribute to the decreased affinity of cyanide bound hNgb for Cyt c. In addition, cyanide binding to the Ngb triggers an alteration of the hydrogen bonding network between the heme propionate groups and nearby amino acid residues. In the ligand free protein, Lys 67 form a hydrogen bond with the heme propionate group 7, but the hydrogen bond between heme propionate group and Lys 67 is destabilized in cyanide bound Ngb [26]. Indeed, the position of Lys 67 seems to be more flexible; for example Lys 67 forms a hydrogen bond with heme 7- propionate in the structure of Fe³⁺mNgb (PDB entry 1Q1F) whereas and in the structure of CO bound and Fe²⁺mNgb (PBD entry 1W92 and 2VRY, respectively) the side-chain is positioned within hydrogen bond distance to both heme 6- and 7- propionate groups. Since the Ngb heme propionate group is part of the hydrogen bonding network with Cyt c, alteration of the hydrogen bonding network involving the heme propionate groups likely contributes to the CN-hNgb decreased affinity for Cyt c.

It is interesting to compare the mechanism of hNgb binding to other intracellular proteins. The association of hNgb to G $\alpha_{i/o}$ subunit is inhibited by exogenous ligand since the affinity of CO bound hNgb for Cyt c decreases more than 1000 times compared to the ferric protein [13]. On the other hand, cystatin C binds to ferric and CO bound hNgb with comparable affinity, $K_d \sim 2.4$ μ M and 5.5 μ M, respectively [27], that is similar to the hNgb affinity for Cyt c in phosphate

buffer (pH 7.0). Furthermore, residues identified to be involved in hNgb interactions with intracellular targets are clustered in adjacent locations on the Ngb surface and relatively close to the heme group (Figure 5). For example, residues within the CD loop are involved in hNgb prion peptides binding, the CD loop and D-helix region form a binding site for the $G\alpha_{i/o}$ subunit [28], and the E-helix represents a binding interface for Cyt c. In case of Cyt c and $G\alpha_{i/o}$ subunit, the Ngb affinity for intracellular partners is inhibited upon exogenous ligand binding to the heme iron suggesting that the formation of Ngb/Cyt c and Ngb/ $G\alpha_{i/o}$ subunit complexes is promoted under hypoxic conditions. All together, these results suggest that depending on the intracellular conditions, Ngb may interact with distinct intracellular targets with comparable affinity allowing Ngb to carry multifunctional roles under hypoxic/ischemic conditions, oxidative stress, and neurogenetic disorders.

4. Conclusions

We have studied the interactions between various types of Ngb (hNgb, rNgb, His64GlnhNgb, and CN-hNgb) and Cyt c using Surface Plasmon Resonance and Isothermal Titration Calorimetry. The immobilization of Cyt c on the MHA/MUO modified gold substrate was evaluated by EIS. Based on the derived dissociation constant K_d from SPR signal and ITC measurements, we have found no significant impact of the internal disulfide bridge between Cys 46 and Cys 55 on the Ngb binding to Cyt c. On the other hand, replacement of the distal His 64 by cyanide ligand inhibits hNgb binding to Cyt c suggesting that the residues located within the E-helix, including Arg 66 and Asp 73 represent the binding sites for Cyt c on Ngb.

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Supplementary information

Supplementary information to this paper can be found online: <http://.....>

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Tables

Table 1. K_d values for Ngb binding to Cyt c as determined using SPR.

	K_d (μ M)	K_d (μ M)
	10 mM Tris buffer, pH 7.0	10 mM phosphate, pH 7.0
hNgb	30 ± 10	10 ± 1
His64Gln hNgb	26 ± 11	4 ± 1
rNgb	36 ± 12	9 ± 2
CN-hNgb	n.d.	n.d.

Table 2. Thermodynamic parameters for formation of Cyt c-Ngb complexes as determined in ITC measurements.

	n	K_d (μ M)	ΔH (kcal mol ⁻¹)	ΔS (cal K ⁻¹ mol ⁻¹)
hNgb	1.8 ± 0.1	22.0 ± 1.7	3.3 ± 0.2	32.3
His64Gln hNgb	1.9 ± 0.1	25.6 ± 2.2	4.0 ± 0.3	34.5
rNgb	2.3 ± 0.1	10.5 ± 1.6	1.5 ± 0.1	27.9
CN-hNgb	n.d.	n.d.	n.d.	n.d.

FIGURE CAPTIONS

Figure 1: Cartoon scheme of gold surface modification. The carboxyl groups of MHA were activated by standard NHS/EDC coupling for protein immobilization.

Figure 2: SPR sensorgrams: a) 20 μ M Cyt c immobilized onto Au/SAM surface, b) CN-hNgb binding to Cyt c, and c) hNgb binding to Cyt c. All traces were recorded in the presence of phosphate buffer pH 7.0.

Figure 3: Titration curves for Ngb binding to Cyt c. Symbols represent equilibrium pixel values and the continuous lines represent SNLH fitting using equation 1.

Figure 4: Binding isotherm for Cyt c-Ngb complex formation. The top panel represents the raw power titration curve and the bottom panel shows the integration data to a single set of identical binding sites model. The recovered thermodynamic parameters are listed in Table 2.

Figure 5: The model of Cyt c-hNgb complex. The residues found in the inter-protein interface are shown in orange and the side chain or residues participating in the hydrogen bond and salt bridges stabilizing the complex are shown as sticks.

FIGURES:

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5