The Interaction of Borate Ions with Cytochrome *c* Surface Sites: A Molecular Dynamics Study

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ABSTRACT Ionic interactions of cytochrome *c* play an important role in the electron transfer process. Molecular dynamics simulations of the binding of borate ion, which serves as a model ion, at three different cytochrome *c* surface sites are performed. This work is motivated by previous NMR studies of cytochrome *c* in borate solution, which indicate the existence of two types of binding sites, a slow exchange site and a fast exchange site. These two types of binding behavior were observed in the dynamic simulations, offering a molecular interpretation of "loose" and "tight" binding. At the "loose" binding sites (near Lys^{25}/Lys^{27} and Lys^{55}/Lys^{73}) the ion forms two to three hydrogen bonds to the nearest lysine residue. This binding is transient on the time scale of the simulation, demonstrating the feasibility of fast exchange. At the "tight" binding site (near Lys^{13}/Lys^{86}), on the other hand, the ion becomes integrated into the protein hydrogen bond network and remains there for the duration of the simulation (exemplifying slow exchange). Binding simulations of the ion at the "tight" site of H26Q mutant cytochrome *c* also showed integration of the ion into the protein's hydrogen bond network. However, this integration differs in details from the binding of the ion to the native protein, in agreement with previous NMR observations.

INTRODUCTION

Mitochondrial cytochrome *c*, a globular 12-kDa heme protein, is a part of the oxidative phosphorylation chain (Dickerson and Timkovich, 1975). Ionic interactions with the surface of the cytochrome play an important role in the electron transfer process. Cytochrome *c* has a positively charged surface resulting from many lysine residues, for example, 19 lysine residues in horse heart cytochrome *c* (Bushnell et al., 1990); (Margoliash et al., 1961), of which many are found in the specific binding sites for the mitochondria membranal enzymes, cytochrome reductase and oxidase (Pettigrew and Moore, 1987). The ionic interaction with the surface of the cytochrome is believed to have a significant role in the direction of the interaction between the cytochrome and the enzymatic aggregates, thus influencing the efficiency of electron transfer (Dickerson and Timkovich, 1975; Pettigrew and Moore, 1987).

Cytochrome *c* is known to bind small anions, including metabolites such as P_i, ATP, and various salts (Pettigrew and Moore, 1987; Pielak et al., 1996). This interaction is known to affect the mobility of this electron carrier protein and has been interpreted as an extra role for the protein as an ion carrier (Margalit and Schejter, 1973; Margoliash et al., 1970). NMR measurements (Taler et al., 1998) revealed that cytochrome *c* binds the tetrahedral borate anion, $B(OH)_4^-$, which structurally resembles P_i. This ion serves as a model compound for studying ion interaction with the surface of cytochromes, with the advantage that it can be studied by both ^1H and ^{11}B NMR. NMR measurements of horse cytochrome *c* in borate solutions revealed two types of ion binding sites: a slow exchange site (residence time of 40 ms) observable by 1D ^1H and ^{11}B NMR and a fast exchange site (shorter residence time of a few μs) observed only by ^{11}B multiple quantum filtered (MQF) NMR techniques (Taler et al., 1998; Taler and Navon, manuscript in preparation).

The binding of borate ions, which exchange very slowly with free borate ions, can be detected in the 1D ^1H NMR spectrum of cytochrome *c* in borate solutions, indicating a specific cytochrome binding site. The new set of peaks, observed upon binding in the proton NMR spectrum, reflects a perturbation in the heme environment, corresponding to protons of the heme methyls 3 and 8, the β proton of the iron ligand His^{18}, and the methyl protons of Leu^{68} (Taler et al., 1998). Although all of these protons are in the vicinity of the cytochrome heme (Bushnell et al., 1990), the borate binding site cannot be exactly identified, because these protons are located at different parts of the heme environment. The ionic nature of the interaction between the borate and the cytochrome binding site is demonstrated by the dependence of the binding on ionic strength (Taler et al., 1998).

A similar behavior of the proton NMR spectra is found for rat cytochrome *c*, which has 95% sequence identity with horse cytochrome *c* and shares with it a similar heme domain structure (Carlson et al., 1977), as well as for its H33F mutant. On the other hand, the rat cytochrome *c* mutant H26Q, which is a stable mutation (Qin, 1996) with no direct effect on the NMR spectrum of the heme vicinity protons, shows a marked difference upon borate binding. The proton NMR spectra of H26Q-borate solutions show a significant change in the heme methyl 3 protons, but leave the heme methyl 8 protons and the Leu^{68} methyl protons nearly unperturbed, implying a different binding character for the borate ion (Taler and Navon, unpublished results).

Using ^{11}B NMR multiple quantum filtered (MQF) NMR techniques, which allow detection of small ligands bound to

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rotating macromolecules (Eliav and Navon, 1991; Jaccard et al., 1986), we observed an NMR MQF peak originating from binding sites in fast exchange with the free borate ion (on the NMR time scale). These binding sites could not be detected as new sets of peaks by the 1D proton NMR, either because the borate ions bound to these sites may have little effect on the proton NMR spectrum or because the fast exchange of the borate ions averages the proton NMR peaks in both free and bound forms of the cytochrome (Taler and Navon, manuscript in preparation).

To better characterize the different ion binding sites at the cytochrome surface and to give a structural interpretation of the NMR findings, a set of molecular dynamics (MD) simulations (Brooks et al., 1988; McCammon and Harvey, 1987) were designed and performed in the present work. In particular, three cytochrome surface sites (near positively charged lysine residues) were selected to demonstrate possible binding behaviors of the negative borate ion at the protein's surface. We did not attempt an exhaustive search of all possible binding sites. Rather, the goal of this work is to analyze possible ion binding sites and obtain a molecular interpretation of the processes observed by NMR.

The molecular dynamics (MD) simulations performed in this study were designed to test several hypotheses regarding the nature of the interactions of ions (in particular, the borate ion) with the protein cytochrome *c* (native and the H26Q mutant), based on the above-described NMR studies. The computational study targeted four specific issues: 1) a comparison of native cytochrome *c* with its H26Q mutant in the absence of the ion; 2) the behavior of the borate ion at native cytochrome *c* surface sites suspected as a candidate "loose binding" sites (exhibiting fast exchange); 3) the behavior of the borate ion at a native cytochrome *c* site suspected as a candidate "tight binding" site (exhibiting slow exchange); and 4) the behavior of the borate ion at the candidate "tight binding" site in the H26Q cytochrome *c* mutant.

It should be stressed that the time scale of the molecular dynamics simulations, which is a few hundred picoseconds, is significantly shorter than the time scale of ion binding. Even at the "loose binding" site, the experimental residence time is a few μs, indicating that a direct molecular dynamics study of the binding process is not feasible. Therefore, the computer experiments described in this work were designed to analyze the energetic interactions of the ion with the protein after the ion is placed near selected surface sites (without actually perturbing the protein structure). We do not intend to reproduce the time evolution of the binding process. The nature of the binding to these sites is inferred from the energetics and dynamic fluctuations observed in the MD simulation.

EXPERIMENTAL

Molecular dynamics simulations of native horse heart cytochromes *c* and cytochromes *c* H26Q mutant were carried out using the CHARMM molecular dynamics program (version 23) (Brooks et al., 1983), the CHARMM22 all-atom force field (MacKerell et al., manuscript in preparation), the SHAKE algorithm, a dielectric constant of $\epsilon = 1$, and a 10-Å energy cutoff. The models were based on the native horse heart ferricytochrome *c* x-ray structure (Protein Data Bank entry 1hrc; Bushnell et al., 1990), including the six structurally preserved water molecules (Bushnell et al., 1990; Sanishvili et al., 1995). The protein was embedded in a 25-Å sphere of TIP3P water molecules (Jorgensen et al., 1983), using stochastic boundary conditions (Brooks and Karplus, 1983; Brünger et al., 1985). The water sphere was added in two steps, each of which involved overlaying a sphere of equilibrated water molecules at a random orientation followed by 20 ps of equilibration at 280 K. In the first step 1478 water molecules were added to the model, and 26 water molecules were added in the second step, giving a total of 1504 water molecules. The total number of atoms in the simulation (protein and water) was 6279 atoms.

The solvated native cytochrome *c* and its H26Q mutant were gradually heated to 280 K over a period of 5 ps and then equilibrated at 280 K for 50 ps. The systems were then simulated for 200 ps at 280 K, from which conformations were sampled every 0.5 ps. A total of 400 conformations were collected from the molecular dynamics trajectories of each system.

The tetrahedral borate ion, $B(OH)_4^-$, was modeled based on its known x-ray structure (Corti et al., 1980; Gupta and Tossel, 1981; Muetteties, 1967) and its calculated charge distribution (Laesson, 1974). The bond distances used were d(B-0) = 1.48 Å, d(O-H) = 0.96 Å; the charge was set at +0.70 on the boron atom and −0.74 on the oxygen atoms; bond angles were set according to the tetrahedral structure. The initial borate structure was minimized for 200 steps with the steepest descent algorithm, followed by 200 steps of the adopted basis Newton-Raphson algorithm (Brooks et al., 1983).

At each computer experiment a single borate ion was added to the protein (based on the fact that bound borates were detected by NMR even at dilute borate solutions (Taler and Navon, manuscript in preparation)). The borate ion was added to the equilibrated solvated protein models (cytochrome *c* and its H26Q mutant) at various locations near the protein surface, replacing seven to nine water molecules. In all cases, the borate ion was introduced near the protein surface without perturbing the initial protein structure. Each of the combined protein-water-borate systems was reequilibrated for 10 ps and then simulated for 250 ps at 280 K. Conformations were collected from the resulting molecular dynamics trajectories at 0.5-ps intervals (500 conformations per trajectory).

RESULTS

The experimental results, discussed above, suggest two different types of interaction sites for the borate ion on the surface of cytochrome *c*. One type of interaction site involves "tight" binding (experimental residence time of ~40 ms), whereas the other is characterized by much weaker interactions (experimental residence time of a few microseconds). To study the two types of interactions, given the limited length of available molecular dynamics simulations, the local dynamic stability of a borate ion placed at three different surface sites was examined. The ion-protein interactions were also compared for native and H26Q mutated cyrochromes.

Native versus H26Q cytochrome *c*

The H26Q mutant was modeled from the solvated rat cytochrome *c* model. Specifically, the glutamine side chain in the H26Q mutant was modeled from the coordinates of the His^{26} residue of native cytochrome *c*. The rms differences between the average structure of native cytochrome *c* and the average structure of its mutant (oriented by the heme

group) was 0.84 Å for all heavy atoms, 0.67 Å for backbone atoms, and 0.16 Å for the heme heavy atoms. Fig. 1 shows the overlaid C_α traces of the two structures of native and H26Q mutated cytochrome *c*, averaged over the 200-ps MD trajectories. As can be seen, all of the main structural features of the native cytochrome *c* are preserved in the H26Q mutant.

Moreover, in native cytochrome, His^{26} forms two hydrogen bonds: one to the backbone carbonyl of Pro^{44} and one to the backbone amid nitrogen of Asn^{31} (Bushnell et al., 1990; Qin et al., 1995). These hydrogen bonds bridge two different loops and have an important role in stabilizing the protein's tertiary structure, as was reported by Qin et al. (1995), based on denaturation and stability experiments. Fig. 2 shows that the hydrogen bonding pattern of native His^{26} is retained intact in the Gln^{26} mutant. The fact that both specific hydrogen bonds are formed when His is replaced by Gln indicates that the overall stability of the protein is not affected by this mutation. This observation is in agreement with experimental results with rat cytochrome *c* that indicate that the H26Q mutant is as stable as the native protein, unlike other mutants, such as H26V, which lose the described hydrogen bonds and become less stable (Qin et al., 1995).

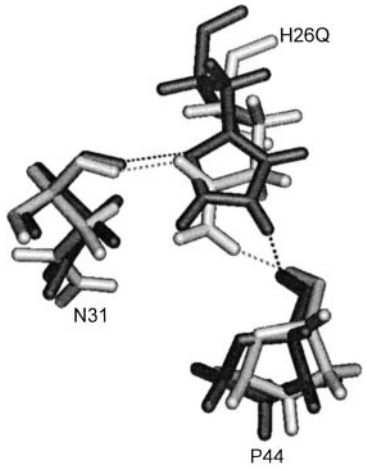

FIGURE 2 A section of the overlaid average structures of horse cytochrome *c* (*dark*) and its H26Q mutant (*light*). Shown are residues Asn^{31}, Pro^{44}, and 26 (His in native, Gln in mutant) and their hydrogen bond pattern. It is seen that the mutant retains the same hydrogen bonds pattern as the native.

Surface site no. 1: borate ion and native cytochrome *c*

The first candidate surface ion-binding site was selected between Lys^{25} and Lys^{27} of native cytochrome *c*. This site is highly exposed to water (the Lys^{25} side chain is almost completely solvated) and is thus suspected as a possible candidate for a fast exchange site. To simulate ion behavior at this site, a borate ion was placed near the protein surface midway between Lys^{25} and Lys^{27}, with the borate B atom at a distance of 4.5 Å from the amine nitrogen (NZ) of Lys^{25} and 5.1 Å from the amine nitrogen (NZ) of Lys^{27}. Fig. 3 presents the four distances between the four borate oxygen atoms and the Lys^{25} amine nitrogen during the 250 ps of the MD simulation. When shorter than 3.5 Å, these distances reflect possible H-bonds. During the simulation the borate ion did not form bonds with Lys^{27} and maintained an average distance of 6.2 ± 1.1 Å from the Lys^{27} amine nitrogen. Fig. 3 indicates that during the MD simulation the ion alternates between two states. In one state the borate ion is bound to the surface of the cytochrome (via Lys^{25}), whereas in the other state it is free of the protein and is completely solvated by the water molecules.

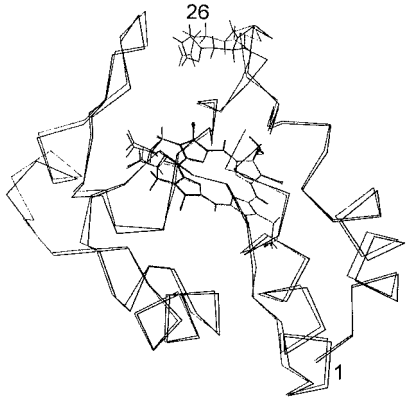

FIGURE 1 Average structures of horse cytochrome *c* (*dark*) and its H26Q mutant (*light*). Shown are the C_α traces, heme groups, and residues 26 of the two proteins (overlay orientation according to the heme group). Structures are averaged over 200 ps of molecular dynamics simulation (400 frames).

When bound, the ion usually forms two or three hydrogen bonds with Lys^{25} amine nitrogen. In some instances a rotational motion of the borate ion, while still bound, is observed. In these cases, for example, at $t = 200$ ps, one H-bond is broken and the oxygen that was previously oriented toward the water replaces it with a newly formed hydrogen bond. During the 250 ps of the simulation, the bound species was formed three times for periods of varying lengths: 15 ps, 30 ps, and 90 ps (5–20 ps, 120–150 ps, and 180–250 ps, respectively). The average electrostatic interaction energy between the borate ion and the protein during these "bound" intervals was −91 ± 12 kcal/mol (the van der Waals contribution to the interaction energy was negligible, ~1 kcal/mol). The interaction of the bound species with the solvent was −80 ± 15 kcal/mol.

The rest of the time the borate ion is surrounded by water, without direct contact with the protein. The electrostatic interaction of the unbound ion with the solvent was about −130 ± 15 kcal/mol. After being detached from the protein surface site, the ion rebinds either spontaneously or after it bounces against the simulation solvent boundary (at $t = 95$ ps). The observed behavior of the borate ion, which alternates between a bound state and a free state, supports the hypothesis that this type of surface site may correspond to

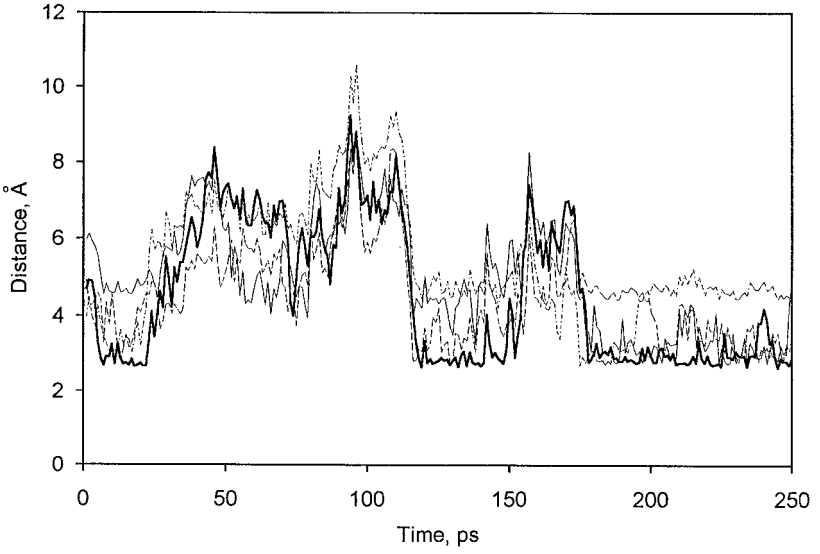

FIGURE 3 The distances between the four borate oxygen atoms and the Lys^{25} amine nitrogen during a 250-ps molecular dynamics simulation of the ion near surface site no. 1 (Lys^{25}/Lys^{27}) of native horse cytochrome *c*. Each distance is designated by a different line style.

the experimentally observed "loosely" bound ion. Depending on actual concentrations, the alternating behavior observed in this simulation can also represent sequential binding of different borate ions to a single binding site, and not necessarily geminate recombination of the same ion.

Surface site no. 2: borate ion and native cytochrome *c*

The second candidate surface ion-binding site was selected between Lys^{55} and Lys^{73} of native cytochrome *c*. Similar to the "surface site no. 1," this site is also exposed to water, with similar averaged distances between the NZ atoms of the two lysines. Again the borate ion was placed near the surface of the native protein midway between Lys^{55} and Lys^{73}, with the borate B atom at a distance of 5.0 Å from the NZ atom of Lys^{55} and 4.8 Å from the NZ atom of Lys^{73}. The binding pattern exhibited in the MD simulation of this site was similar to the pattern observed in the first site. Fig. 4 depicts the four H-bonding distances between the borate oxygen atoms and the amine nitrogens of Lys^{73} (Fig. 4 *a*) and Lys^{55} (Fig. 4 *b*). The two figures show a complementary pattern, indicating that after 175 ps the borate ion alternated from binding to Lys^{55} to binding to Lys^{73}. When bound it forms the same H-bond pattern observed in the first site, where three borate hydroxyl oxygen atoms point toward the lysine amine nitrogen. Some of the distances between the oxygen atoms of the bound borate and the lysine residues were longer compared with site no. 1, resulting in a lower electrostatic interaction energy of -71 ± 16 kcal/mol (compared with -91 ± 12 kcal/mol for the former site) and a higher interaction with the solvent of about -100 ± 15 kcal/mol (compared with -80 ± 15 kcal/mol for the former site). The hopping of the ion between the two lysines in site no. 2 (Lys^{55} and Lys^{73}) is analogous to the borate leaving Lys^{25} for the bulk solvent in site no. 1. Each of the two lysines (Lys^{55} and Lys^{73}) serves as an independent binding site during part of the simulation.

Surface site no. 3: borate ion and native cytochrome *c*

The third site was selected to be near Lys^{13} and Lys^{86} of the native cytochrome *c*. Although this site is also exposed to water (the ion replaced seven water molecules in the simulation model), it was postulated as a possible "tight" binding site based on its relative proximity to the heme, allowing us to study the effect of binding on the heme surroundings. Moreover, it is known that this site is one of the binding sites of P_i (Pettigrew and Moore, 1987). The initial distance was 3.8 Å between the borate B atom and the NZ atom of Lys^{13} and 4.4 Å between the borate B atom and the NZ atom of Lys^{86}. The 250 ps of MD simulation of this system revealed a completely different dynamic behavior of the ion, in comparison with its behavior at the previous two sites. In particular, after 35 ps of dynamics, the borate ion moved into a stable position near Lys^{13}, where it remained for the duration of the simulation. Throughout the boron atom stayed at a fairly constant distance of 3.5 ± 0.2 Å from the Lys^{13} amine nitrogen. The very small fluctuations reflect the stability of this binding, indicating a possible "slow exchange" site.

Fig. 5 shows the averaged structures of residues Lys^{13}, Lys^{86}, and Glu^{90} in native cytochrome *c* superimposed over their average structures after the borate ion moves into its bound position between Lys^{13} and Lys^{86}. It is seen that in its "tightly bound" position the borate ion disrupts the Glu^{90}/Lys^{13} salt bridge that exists in the unperturbed cytochrome (Bushnell et al., 1990). This salt bridge is now replaced by a new hydrogen bond network connecting Lys^{13} to Glu^{90}, which essentially integrates the borate ion with an additional water molecule into the protein structure. The break-

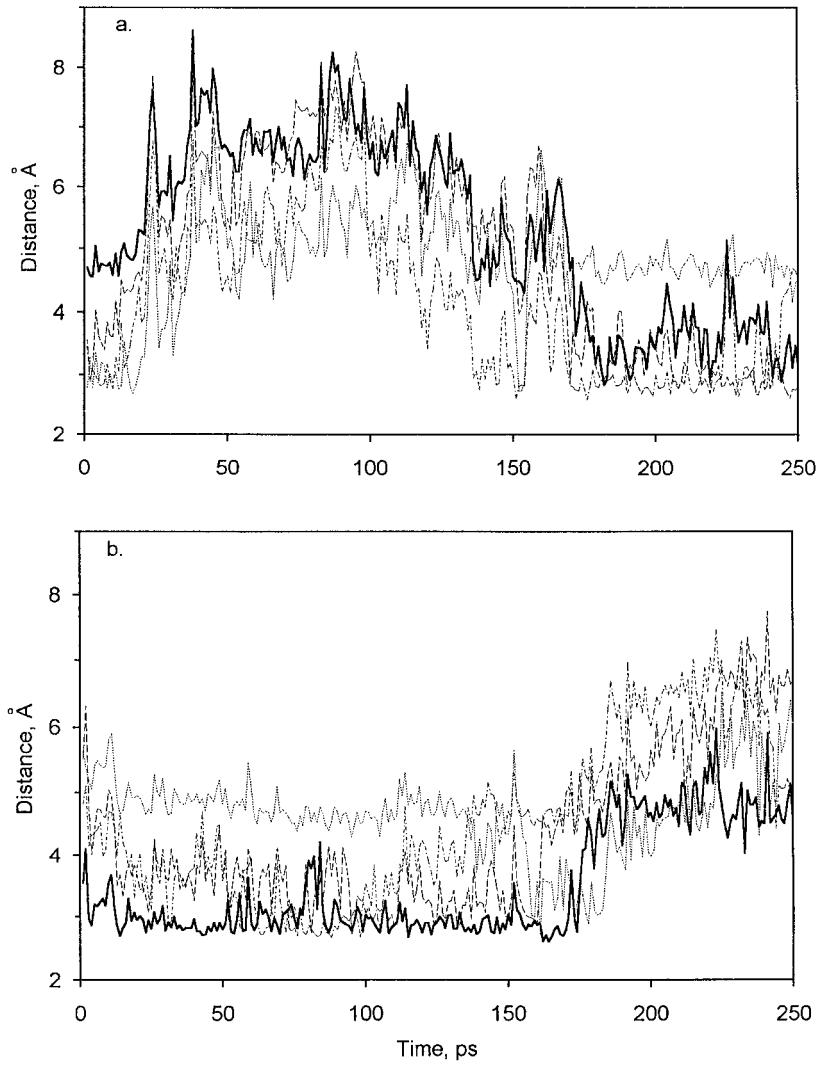

FIGURE 4 The distances between the four borate oxygen atoms and the amine nitrogen atoms of (*a*) Lys^{73} and (*b*) Lys^{55} during a 250-ps molecular dynamics simulation of the ion near surface site no. 2 of native horse cytochrome *c*. Each distance is designated by a different line style.

ing of the Glu^{90}/Lys^{13} salt bridge, both structurally and energetically, is demonstrated in Fig. 6. At $t = 35$ ps, the time when the borate ion moves into its "tightly bound" position, there is a sharp change in the electrostatic interaction between the components of the salt bridge, Lys^{13} and Glu^{90}. Simultaneously, the distance between these two residues suddenly increases and the direct salt bridge ceases to exist. The average electrostatic interaction of the borate ion with the protein at this binding site is -131 ± 21 kcal/mol, $\sim 40-60$ kcal/mol stronger than the interaction of the ion with the protein at the two "loosely bound" surface sites. This interaction energy reflects the fixed structure created when the borate ion is integrated into the salt bridge. The interaction with the solvent is about -50 ± 25 kcal/mol, reflecting the fact that in this site the borate is buried deeper in the protein and is less exposed to the solvent (compared to the previous two sites).

As seen in Fig. 5, the borate ion is also close to Lys^{86}. However, its interactions with this second residue are of a fluctuating nature. It was found that the motion of the Lys^{86} side chain controls the ion's exposure to the solvent. In particular, the interaction energy of the ion with Lys^{86} showed a pattern complementary to its interaction with the water molecules (Fig. 7). When the ion interaction with Lys^{86} is strong, the ion's interaction with the solvent weakens (i.e., it is less exposed to the solvent), and when the ion interaction with Lys^{86} weakens, its interaction with the solvent increases, indicating partial solvation. This alternation shows a period of ~ 100 ps (of course, from a 250-ps simulation we cannot determine whether this fluctuation pattern continues at longer times).

Surface site no. 3: borate ion and H26Q mutant cytochrome *c*

The proton NMR spectra described above, which reflect the "tightly bound" species, indicated that the interaction of the borate ion with the cytochrome *c* H26Q mutant is different from its interaction with native cytochrome. To see whether this difference can be reproduced in the molecular dynamics simulations, we repeated the simulation of borate binding at

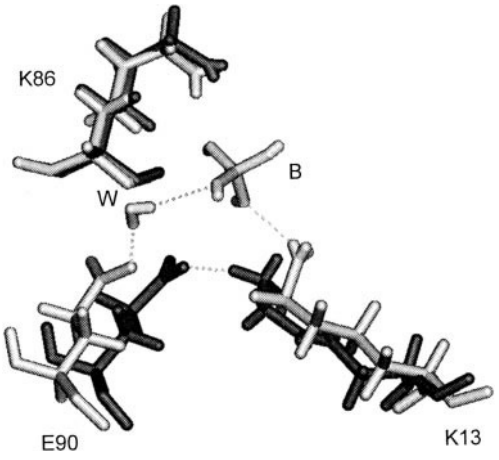

FIGURE 5 The average structures at surface site no. 3 (Lys^{86}/Lys^{13}) before (*dark*) and after (*light*) integration of the borate ion into the horse cytochrome *c* hydrogen bond network. The salt bridge Lys^{13}/Glu^{90} that exists in the absence of the ion (*dark*) is replaced by a new hydrogen bond pattern involving the borate ion, B, and a water molecule, W (*light*). (The structures are oriented according to the heme group.)

site no. 3 with the H26Q mutant instead of the native cytochrome *c*. The borate ion was introduced into the H26Q mutant at the same initial location near Lys^{13} and Lys^{86} (initial distance between the B atom and the NZ atom of Lys^{13} was 3.9 Å, and its distance to the NZ atom of Lys^{86} was 4.4 Å). After a short adjustment time of ~35 ps, in which the ion detached itself from Lys^{86}, the borate ion moved into a stable location equidistant between Lys^{86} and Lys^{13} (Fig. 8). At this position its electrostatic interaction energy with the protein is high, -141 ± 15 kcal/mol, similar to its interaction in the native protein. The electrostatic interaction energy of the ion with the solvent when bound in this site is only -30 ± 20 kcal/mol.

Structurally, however, there is a significant difference between the ion's binding pattern in native cytochrome and its binding pattern in the H26Q mutant. In particular, binding of the ion to the H26Q mutant does not disrupt the Glu^{90}/Lys^{13} salt bridge. Fig. 9 is an overlay of the averaged structures and hydrogen bond pattern of the H26Q mutant

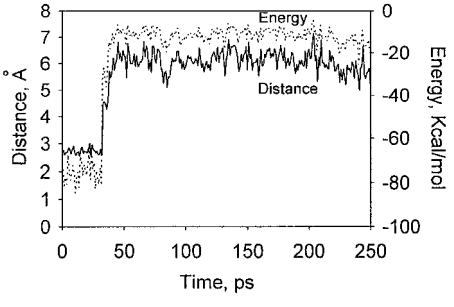

FIGURE 6 The rupture of the salt bridge Lys^{13}/Glu^{90} during the 250 ps of the simulation of the horse cytochrome *c* with a borate ion placed near surface site no. 3. Shown are the distance (in Å) and the electrostatic interaction energy (in kcal/mol) between Lys^{13} and Glu^{90}. The breaking of the salt bridge at $t = 35$ ps is clearly seen.

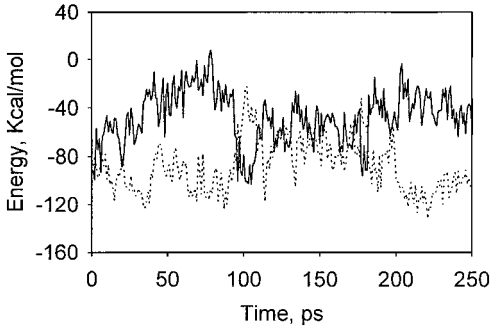

FIGURE 7 The complementary pattern of the electrostatic interaction energy between the borate ion and Lys^{86} (*dashed*) and between the borate ion and the solvent (*solid*) during the 250 ps of the molecular dynamics simulation.

(Lys^{13}, Lys^{86}, and Glu^{90}) with and without the borate ion. This picture clearly shows that in the H26Q mutant the borate ion does not perturb the Glu^{90}/Lys^{13} salt bridge, leaving the direct hydrogen bond between these two residues unchanged. Instead, the borate ion forms a stable hydrogen bond with the backbone nitrogen of Lys^{86} and with the amine nitrogen (NZ) of Lys^{13}, bridging between the two residues. A second interaction between the borate and Lys^{86}, which causes the side chain of Lys^{86} to tilt toward the borate, can also be seen in the averaged structure, presented in Fig. 9.

Of special interest in interpreting the NMR findings, is whether the simulation results can indicate the difference in the heme environment between native and mutant borate bound cytochromes. To check this, we measured the average distance between the borate ion and the heme iron atom in the native and mutant cytochrome *c*. It was found that in the H26Q mutant the borate ion is located a little closer to the heme group than in native cytochrome *c*; i.e., in the mutant it penetrates a bit deeper into the protein core. The average Fe-B distances were 15 Å in the native protein and 14 Å in the H26Q mutant.

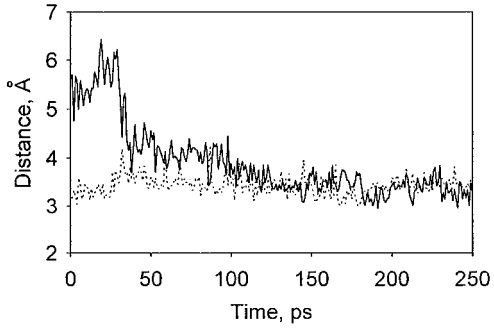

FIGURE 8 The distances between the borate B atom and amine nitrogen atoms of Lys^{13} (*dashed*) and Lys^{86} (*solid*) of the H26Q mutant during the 250 ps of the simulation, when the ion is initially placed near surface site no. 3.

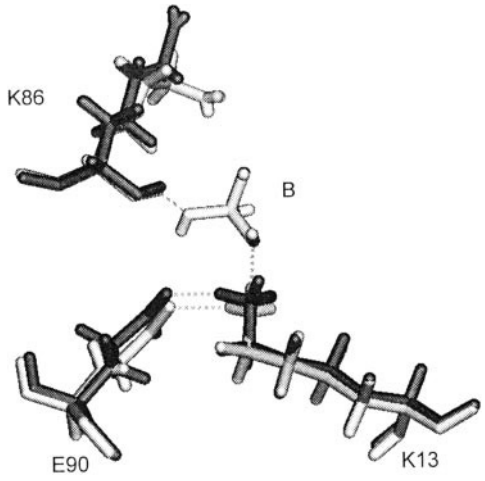

FIGURE 9 The average structures at surface site no. 3 (Lys^{86}/Lys^{13}) without the ion (*dark*) and after the borate ion stabilizes at this site (*light*) in the H26Q mutant cytochrome *c*. In this case, the Lys^{13}/Glu^{90} salt bridge is not affected by the ion, which forms stable hydrogen bonds connecting residues Lys^{86} and Lys^{13}. (The structures are oriented according to the heme group.)

DISCUSSION

The MD simulations presented in this paper of ion binding to three cytochrome surface sites (Fig. 10) clearly exemplify two types of distinct binding sites.

"Loose binding" sites

The binding patterns of the ion in the first two sites (Lys^{25}/Lys^{27} and Lys^{55}/Lys^{73}) are similar. In these sites, the borate ion binds to a single lysine residue, with three hydroxyl groups pointing toward the amine nitrogen (NZ) of the lysine, forming two or three hydrogen bonds. This type of binding appears to be transient on the time scale of the simulation, demonstrating the feasibility of fast transfer of the ion from one such site to another (e.g., Lys^{55} to Lys^{73}) or fast exchange of such "loosely" bound ions with free ions in the solution. That is, ion-binding sites that exhibit nonspecific fast exchanges in the simulation, such as at surface lysines 25, 55, and 73, offer a possible molecular interpretation of the MQ NMR experimental results. They exemplify the kind of protein sites that can contribute to the experimentally observed rapid exchange of borate ions among many sites.

"Tight binding" site

The third site (Lys^{13}/Lys^{86}) shows a completely different binding pattern, for both the native and the H26Q mutant cytochromes. The borate ion placed near the protein surface, as in the first two sites, rapidly becomes integrated into the protein hydrogen bonding network, bridging two protein residues: Glu^{90}/Lys^{13} in the native cytochrome (instead of the native salt bridge that is disrupted) and Lys^{13}/Lys^{86} in the H26Q mutant. After the initial motion (less than 40 ps) associated with the structural integration of the ion, the borate ion remains bound with very little positional fluctuations. This structural integration hinders the ion's motion, implying slow exchange with the solvent. Such binding characteristics have the potential of affecting the protons in the surrounding heme, causing the observed changes in the protein's proton NMR spectra. It is interesting to note that this surface site, which binds the borate ion "tightly," is also a known binding site of the P_i ion.

The interaction energies, summarized in Table 1, between the borate ions and the protein, quantify the different binding characteristics of the two sites (mainly electrostatic interactions; the contribution of van der Waals interactions is small in comparison). Whereas the interactions of a "loosely bound" ion with the protein are in the -70 to -90 kcal/mol range, a "tightly bound" ion interacts with the protein much more strongly, in the -130 to -140 kcal/mol range (in both cases most of the interaction is electrostatic). A complementary picture is obtained when considering the interaction of the ion with the water molecules. "Loosely bound" ions are clearly more exposed to the solvent than "tightly bound" ions. Their interaction with the solvent is about -100 to -80 kcal/mol, whereas the interaction of the ion in the "tight" site with water is only -30 to -50 kcal/mol. We wish to emphasize that in all cases, the borate ion was introduced near the protein surface without perturbing the protein initial structure.

It is interesting to note that the overall interaction energies of the ion with its surroundings, protein and solvent together, at both sites are quite similar (Table 1). In all four cases the total ion interaction energy is about -170 to -180 kcal/mol. This means that energetically there is little difference between binding at one site or another, especially as the interaction energy of the ion with pure solvent is also of the same magnitude (total electrostatic interaction of -165 ± 15 kcal/mol). In addition, in terms of entropy one expects relatively small differences between the two types

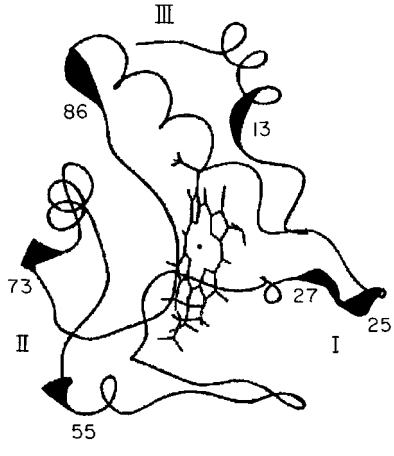

FIGURE 10 The three ion binding sites at the surface of horse cytochrome *c* studied in this work. The sites are indicated as pairs of residues on C_α traces of the protein and its heme group.

TABLE 1 Electrostatic interaction energies of the borate ion with the protein (native and H26Q mutant cytochrome *c*) and with the solvent at three surface binding sites

Surface site*/protein	Ion-protein interaction (kcal/mol)	Ion-solvent interaction (kcal/mol)
Site no. 1/native cytochrome	−91 ± 12	−80 ± 15
Site no. 2/native cytochrome	−71 ± 16	−100 ± 15
Site no. 3/native cytochrome	−131 ± 21	−50 ± 25
Site no. 3/H26Q mutant cytochrome	−141 ± 15	−30 ± 20

*Site no. 1 = (Lys^{25}/Lys^{27}). Site no. 2 = (Lys^{55}/Lys^{73}). Site no. 3 = (Lys^{13}/Lys^{86}).

binding sites, because at either site the bound ion forms multiple stable hydrogen bonds, which significantly restrict its conformational freedom. Moreover, even when solvated in pure water, because of its four tetrahedral OH^- groups, the borate ion forms ordered structures (ice-like) (Corti et al., 1980). Thus, upon binding to the protein, no great entropy loss is expected. We can therefore conclude that the difference in observed exchange rates is, to a large extent, of a kinetic origin and is not driven by thermodynamics. After integration into the protein hydrogen bond network and becoming further removed from the solvent, the ion faces steric hindrance that slows its escape from the "tight" binding site (hence we name it "tight" and not "strong").

The H26Q mutant simulations, which conserve the hydrogen bonding pattern of residue 26 (in agreement with known structural information), offer additional support for our "tight" binding site postulate. The reproduction in the H26Q mutant simulations of "tight" binding through integration into the protein's hydrogen bond network corroborates our suggested choice of this site as a "slow exchange" site. The changes in the location and in the hydrogen-bond pattern of the borate relative to the heme group indicate that ion binding to this mutant is expected to have a slightly different proton NMR spectrum compared with binding to native cytochrome, as was indeed observed (Taler and Navon, unpublished results).

To conclude: we have demonstrated that molecular dynamics simulations are a useful tool for adding structural and dynamical interpretations to experimental NMR measurements. The simulations allow us to study selected test cases specifically designed to check specific hypotheses. In particular, we were able to demonstrate, at a molecular level, the existence of the two distinct binding behaviors observed in the experiments. Moreover, from the simulation dynamics we can conclude that the observed behavior is not necessarily unique to the borate ion. Patterns of binding to cytochrome, similar to those found for the borate ion, are possible for other ions as well (e.g., for P_i).

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