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Detection and Characterization of Boric Acid and Borate Ion Binding to Cytochrome *c* Using Multiple Quantum Filtered NMR

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The application of multiple quantum filtered (MQF) NMR to the identification and characterization of the binding of ligands containing quadrupolar nuclei to proteins is demonstrated. Using relaxation times measured by MQF NMR multiple binding of boric acid and borate ion to ferri and ferrocytochrome *c* was detected. Borate ion was found to have two different binding sites. One of them was in slow exchange, $k_{diss} = 20 \pm 3\ s^{-1}$ at 5°C and D_2O solution, in agreement with previous findings by ^1H NMR (G. Taler *et al.*, 1998, *Inorg. Chim. Acta* 273, 388–392). The triple quantum relaxation of the borate in this site was found to be governed by dipolar interaction corresponding to an average B–H distance of 2.06 ± 0.07 Å. Other, fast exchanging sites for borate and boric acid could be detected only by MQF NMR. The binding equilibrium constants at these sites at pH 9.7 were found to be 1800 ± 200 M^{-1} and 2.6 ± 1.5 M^{-1} for the borate ion and boric acid, respectively. Thus, detection of binding by MQF NMR proved to be sensitive to fast exchanging ligands as well as to very weak binding that could not be detected using conventional methods.

Key Words: **^{11}B-NMR; cytochrome *c*; multiple quantum filtered NMR; borate ion; boric acid.**

INTRODUCTION

The aim of this work was to exploit the multiple quantum filtered (MQF) NMR technique for the identification and characterization of the binding of small molecules containing quadrupolar nuclei to proteins. It has been previously shown that spin $\frac{3}{2}$ nuclei in systems with a slow reorientation correlation rate, τ_c^{-1}, relative to the Larmor frequency, ω_o, can give rise to double and triple quantum filtered NMR signals (*1*). Thus, this technique can be used to follow binding of ligands containing quadrupolar nuclei to macromolecules. We present here the first demonstration of such an application, applied to the system of boric acid and borate ion binding to cytochrome *c*.

Cytochrome *c* is a globular, 12 kDa, heme protein with a positively charged surface that serves as an electron carrier in the cell mitochondria (*2*). 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, cytochrome reductase and oxidase, thus influencing the efficiency of electron transfer (*2, 3*). Cytochrome *c* is known to bind small anions including metabolites such as phosphate ions and ATP (*3, 4*). This interaction is known to affect the mobility of the electron carrier protein and has been interpreted as an extra role for the protein as an ion carrier (*5, 6*). Recent ^1H and ^{11}B NMR measurements revealed that the tetrahedral borate anion, $B(OH)_4^-$, binds specifically to cytochrome *c* (*7*). Borate ion can serve as a model compound for studying the ion interaction with the cytochrome's surface and can be studied by both ^1H and ^{11}B NMR.

^{11}B NMR spectra of dilute boric/borate solutions consist of a single peak which is a weighted average of the signals of boric acid ($B(OH)_3$) and borate ion ($B(OH)_4^-$) which are in a fast equilibrium according to the reaction (*8–11*):

$$H_2O + B(OH)_3 \rightleftarrows B(OH)_4^- + H^+ \quad pK\ 9.2. \qquad [1]$$

The individual chemical shifts of $B(OH)_3$ and $B(OH)_4^-$ are 20 and 2 ppm, respectively, relative to the standard $Me_2O.BF_3$ (*9*). The tetrahedral symmetry of $B(OH)_4^-$, which results in a small electric field gradient and therefore a very small quadrupolar interaction, leads to a relative narrow linewidth of about 10 Hz while the trigonal structure of $B(OH)_3$ leads to a linewidth of about 100 Hz (*10*).

In the presence of cytochrome *c* the appearance of new peaks was detected in the ^{11}B NMR spectrum (*7*). These peaks were assigned to borate ion specifically bound to the protein and exchanging slowly with the free borate in solution (*7*).

In the present study additional binding sites for both borate ion and boric acid that are in a fast exchange with the free species in the bulk were revealed by MQF NMR spectroscopy. This technique enabled the characterization of the different binding sites in terms of binding constants, exchange rates, and relaxation parameters.

MATERIALS AND METHODS

Horse heart cytochrome *c*, di-sodium tetraborate (borax), and boric acid were purchased from Sigma. The cytochrome solutions were prepared in either aqueous or deuterated (~98% D_2O) solutions of sodium borax (which dissociates in the solution into four monovalent borate ions) or of boric acid. The

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pH was adjusted with DCl and NaOD or HCl and NaOH; the pH readings for the deuterated solutions are given without correction for the deuterium isotope effect and are designated as pH*. Small amounts of potassium ferricyanide, $K_3Fe(CN)_6$, were added to the ferricytochrome solutions to ensure full oxidization of the iron atom. Ferrocytochrome *c* was obtained by reduction of the cytochrome solution with dithionite in a strictly anaerobic atmosphere followed by sealing of the NMR test tube.

^{11}B NMR spectra were acquired using Bruker AMX360-WB and ARX500 NMR spectrometers with 10 mm broadband probes. The 90° pulse length in both spectrometers was about 30 μs. Chemical shifts for ^{11}B were measured using an external reference of sodium tetraphenylborate with $\delta(^{11}B) = -6.3$ ppm for this compound relative to $Me_2O.BF_3$ standard.

The ratio of borate ion to boric acid at 5°C and D_2O solutions was determined based on the pH* value and a pK* of 9.28 ± 0.05 that was determined from the fitting of a titration curve of the chemical shift of boric/borate solutions vs pH* at the same conditions. Similar work by How *et al.* (*9*) in aqueous solutions and a temperature of 33°C gave pK 8.7.

MQF NMR spectra were measured using the standard pulse sequence (*1, 12*)

$$90°–\tau/2–180°–\tau/2–90°–t_1–90°–\text{Acq}; \qquad [2]$$

double quantum (DQ) or triple quantum (TQ) coherences were selected using the appropriate phase cycling (*13*). T_{2s} and T_{2f} were determined by fitting of the dependence of the intensity of the MQF signal, I_{MQ}, on the creation times, τ, to the expression

$$I_{MQ} = A[\exp(-\tau/T_{2s}) - \exp(-\tau/T_{2f})]. \qquad [3]$$

The MQ relaxation times T_{TQ} or T_{DQ} were determined from the monoexponential decay of the MQ signal as a function of the evolution time, t_1. These measurements were performed using a modified sequence (Eq. [2]), where a 180° pulse was introduced in the middle of the t_1 evolution time. The τ value was the time (τ_{max}) that gave the maximum MQF signal.

The transverse relaxation time, T_2, for the protein-free solutions was measured using the spin echo sequence. Two methods were used for the measurements of the longitudinal relaxation time, T_1: (1) The inversion recovery method; (2) Owing to partial overlap between the broad, intense peak at 7.1 ppm and the narrow, weak peaks at 2 ppm, another pulse sequence, denoted as IR-TQF (Eq. [4]), was used to measure the T_1 of the 2 ppm peaks.

$$180°–t–63.1°–\tau_{max}/2–180°–\tau_{max}/2–90°–t_1–90°–\text{Acq}. \qquad [4]$$

In this pulse sequence the 90° pulse of the conventional inversion recovery experiment is replaced by a TQ filter. As a result, the 7.1 ppm peak with the smaller T_{2s}/T_{2f} ratio is more attenuated (see Fig. 1), thus reducing its influence in the T_1 measurement of the 2 ppm peaks. The IR-TQF pulse sequence (Eq. [4]) is different from the IR-DQF used previously for ^{2}H (*14*) in that the second pulse has a tip angle of 63.1° instead of 90°. This pulse transfers the tensor $T_{1,0}$ into $T_{1\pm1}$ tensors, but unlike a 90° pulse, eliminates the transfer of the undesired $T_{3,0}$ that was formed during the time interval, t, into $T_{3\pm1}$ and thus measures only the recovery of $T_{1,0}$.

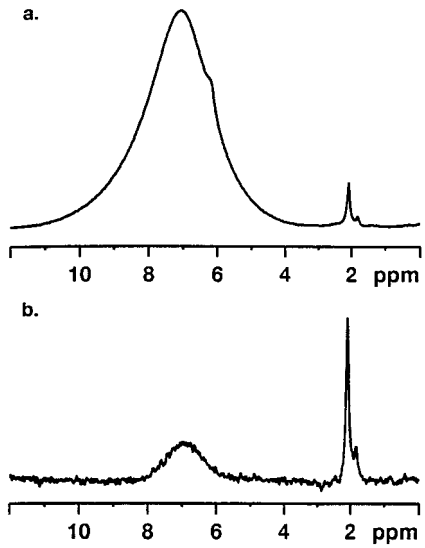

FIG. 1. The ^{11}B (a) SQ and (b) TQF (τ = 1.4 ms) NMR spectra of 100 mM borate solution with 4 mM ferricytochrome *c* at 5°C, 11.4 T, and pH* 9.7.

THEORETICAL BACKGROUND

It has been shown (*1, 15*) that for nuclei with spin of $I = \frac{3}{2}$ under the condition of $\omega_o\tau_c > 1$ one can measure four transverse relaxation times: two of the single (T_{2f}, T_{2s}), one of the double (T_{DQ}), and one of the triple quantum (T_{TQ}) coherences. A T_{2s}/T_{2f} ratio larger than one is essential for obtaining MQF signal (*1*). In recent publications (*15, 16*), it has been shown that T_{TQ} and T_{2s} are significantly affected by dipolar interactions. The decay of the longitudinal magnetization consists of two relaxation times, T_{1s} and T_{1f}, and is given by (*17*)

$$M_Z(t)–M_0 = (M_Z(0) - M_0)\{0.8\exp(-t/T_{1s}) + 0.2\exp(-t/T_{2f})\}. \qquad [5]$$

Since the ratio of T_{1s} and T_{1f} is always between a factor of 1 and 4, this decay curve cannot be resolved into two exponents and the result of the monoexponent decay, $T_1^{\text{effective}}$, was approximated as $1/T_1^{\text{effective}} = 0.8/T_{1s} + 0.2/T_{1f}$ (*18*).

The system of borate/boric acid and cytochrome *c* can be described by a fast chemical exchange between free borate and free boric acid in the bulk, a slowly exchanging bound species

(borate bound to the protein), and a fast exchanging bound species (either borate or boric acid bound to the protein).

The exchange rates of these processes obey the following conditions, respectively,

$$\text{condition (I) for the slow exchange process:} \quad k < p\Delta\omega$$

$$\text{condition (II) for the fast exchange processes:} \quad k \gg p\Delta\omega,$$

where $k = 1/\tau_a + 1/\tau_b$ and τ_a, τ_b are the exchange lifetimes of the two exchanging species in each case, p is the coherence order of the MQ transition (p equals 3 for TQ, 2 for DQ, and 1 for SQ), and $\Delta\omega$ is the frequency difference between the chemical shifts of the exchanging species. The bound species have a slow reorientation correlation rate so that $\omega_o\tau_c \gg 1$, whereas the free species in the bulk undergo a fast reorientation motion with $\omega_o\tau_c \ll 1$.

There are three mechanisms that contribute to the relaxation rate of the TQ, DQ, and SQ coherences: the modulation of the quadrupolar and dipolar interactions by reorientation motion and the modulation of the chemical shift difference by chemical exchange. The complete expressions for all the relaxation times (T_{1s}, T_{1f}, T_{2s}, T_{2f}, T_{DQ}, T_{TQ}) are given in the Appendix and the appropriate expressions for the various cases are given below.

For the bound species ($\omega_o\tau_c \gg 1$), assuming that the dipolar interaction is considerably smaller than the quadrupolar interaction, one can write for the different single and multiple quantum relaxation rates,

$$R_i^{\text{bound}} = R_i^{Q} + R_i^{D} + R_i^{EXC}, \qquad [6]$$

where i = 2s, 2f, DQ, TQ, 1s, or 1f. In this case, R_i^D, the dipolar contribution to the R_i^{bound} is given by

$$R_i^D = 1/5D^2\tau_c \quad (i = 2s, 2f) \qquad [7a]$$

$$R_{DQ}^D = 4/5D^2\tau_c \qquad [7b]$$

$$R_{TQ}^D = 9/5D^2\tau_c, \qquad [7c]$$

where $D = \hbar\gamma_H\gamma_B/r_{B-H}^3$ is the ^{11}B–^{1}H dipolar interaction, τ_c is the molecular reorientation correlation time, and the factors 4 and 9 in Eqs. [7b] and [7c] are the squares of the coherences of the DQ and TQ transitions, respectively. The dipolar interaction has negligible contribution to R_{1s} and R_{1f}. R_i^Q, the quadrupolar contribution to the R_i^{bound} is given by

$$R_i^Q = \chi^2/(16\omega_0^2\tau_c) \quad (i = 2s, TQ) \qquad [8a]$$

$$R_i^Q = \chi^2\tau_c/20 \quad (i = 2f, DQ) \qquad [8b]$$

$$R_{1s}^Q = \chi^2/(40\omega_0^2\tau_c) \qquad [8c]$$

$$R_{1f}^Q = \chi^2/(10\omega_0^2\tau_c), \qquad [8d]$$

where χ is the nuclear quadrupolar coupling constant and ω_0 is the ^{11}B Larmor frequency. For the slowly exchanging bound borate (condition I), R_i^{EXC}, can be simplified as

$$R_i^{EXC} = f_{\text{free}}k = 1/\tau_{\text{bound}} \quad (i = 2s, 2f, DQ, TQ, 1s, 1f), \qquad [9]$$

where f_{free} is the fraction of borate not bound to the cytochrome and τ_{bound} is the exchange lifetime of the bound borate.

For the case of the fast exchanging bound borate or boric acid (condition II), one should consider the contributions to the relaxation of the free species in the bulk where $\omega_o\tau_c \ll 1$. Under the assumption that the dipolar interaction is considerably smaller than the quadrupolar interaction, one can write for the different single and multiple quantum relaxation rates of the fast exchanging species:

$$R_i = R_i^Q + R_i^D + R_i^{EXC}. \qquad [10]$$

The dipolar and quadrupolar contributions to R_i are weighted averages of contributions from the bound and free species in the system and simplify to

$$R_i^D = f_a R_{i,a}^D + f_b R_{i,b}^D + f_c R_{i,c}^D + f_d R_{i,d}^D$$

$$(i = 2s, 2f, TQ, DQ, 1s, 1f) \qquad [11]$$

$$R_i^Q = f_a R_{i,a}^Q + f_b R_{i,b}^Q + f_c R_{i,c}^Q + f_d R_{i,d}^Q$$

$$(i = 2s, 2f, TQ, DQ, 1s, 1f), \qquad [12]$$

where f_a, f_b, f_c, f_d are the fractions of bound boric, bound borate, free boric and free borate, respectively. The expressions for the R_i^D and the R_i^Q of the bound borate or boric acid are given in Eqs. [7] and [8], respectively. R_i^D and the R_i^Q of the boric acid or borate ion in solution where $\omega_o\tau_c \ll 1$ are

$$R_i^D = D^2\tau_c \quad (i = 2s) \qquad [13a]$$

$$R_i^D = 6D^2\tau_c \quad (i = DQ, TQ, 2f). \qquad [13b]$$

The dipolar interaction has negligible contribution to R_{1s} and R_{1f}.

$$R_i^Q = \chi^2\tau_c/10 \quad (i = 2s, 2f, DQ, TQ, 1s, 1f) \qquad [14]$$

For the case where the R_i is governed by fast chemical exchange (condition II) the contribution of the fast exchange between free borate and boric acid to R_i^{EXC} can be simplified as

$$R_i^{EXC} = f_c f_d(\Delta\omega)^2/k \quad (i = 2s, 2f) \qquad [15a]$$

$$R_{DQ}^{EXC} = f_c f_d(2\Delta\omega)^2/k \qquad [15b]$$

$$R_{TQ}^{EXC} = f_c f_d(3\Delta\omega)^2/k, \qquad [15c]$$

where the factors 2 and 3 are the coherence of the DQ and TQ transitions, respectively. The exchange term has no contribu-

tion to R_{1s} or R_{1f}. The fast exchange between the free and bound borate or between the free and bound boric acid has a negligible contribution to R_i^{EXC}, since the fractions of the bound species are smaller than the free ones and the frequency differences between the free and bound species are considerably smaller than that between the borate and boric acid.

Clearly R_{DQ} and R_{TQ} cannot be measured unless some binding to the protein occurs so that $R_{2s} \neq R_{2f}$ (*1*); however, the expressions for all the relaxation times are given for completeness.

RESULTS

Figure 1a is the ^{11}B spectrum at 11.4 T of 100 mM borate solution with 4 mM ferricytochrome *c* at pH* 9.7 and 5°C. The large peak at 7.1 ppm is the same as that of the weighted average of 70% $B(OH)_4^-$ exchanging with 30% of $B(OH)_3$. The peaks at 2.1 and 1.8 ppm were assigned to $B(OH)_4^-$ specifically bound to two conformations of the ferricytochrome *c* that are present at the given pH value (*2, 7*). The assignment was based on the chemical shift which is similar to the chemical shift of free $B(OH)_4^-$, which appears at 2.0 ppm, and the narrow linewidth of 14 Hz which is consistent with the tetrahedral symmetry of this species (*7*). Only one peak, at about 2 ppm, appears in the spectrum of ferrocytochrome *c* and 100 mM borate solution under similar pH and temperature conditions (not shown). This is in agreement with the finding that ferrocytochrome appears in one conformational form in the pH range discussed above (*2*). The peak around 6 ppm in Fig. 1a is observable also in ferrocytochrome *c* borate solution and is presently unassigned. The chemical shifts of the peaks appearing in the presence of ferri and ferrocytochrome *c* (around 2 and 6 ppm) are independent of temperature and pH in the range of 9–10.

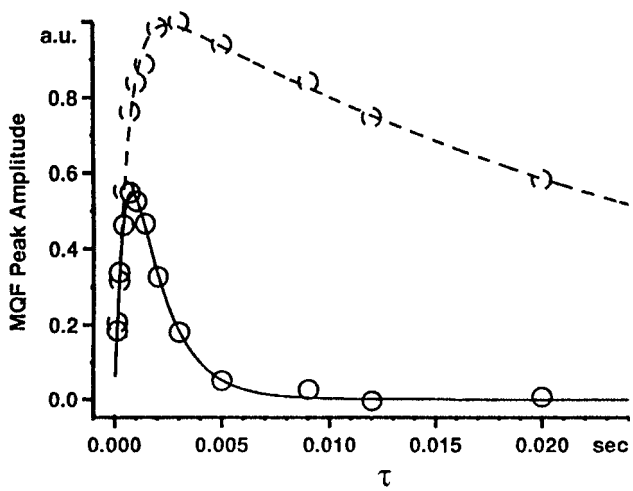

FIG. 2. Experimental results (circles) and fitted curves of the changes in the TQF peak intensities as a function of the creation time of the third rank tensor, τ, (Eq. [1]) for the peaks at 2 (dashed line) and 7.1 ppm (solid line). The lines were fitted according to Eq. [2] yielding the values of T_{2s} and T_{2f} for the two types of binding sites. The experiment was carried out on a sample of 100 mM borate solution with 4 mM ferricytochrome *c* at 5°C, 11.4 T, and pH* 9.7.

TABLE 1

Transverse and Longitudinal Relaxation Times at Different Magnetic Fields for the 2 ppm Peaks at 5°C, pH* 9.7 and H_2O Solutions

H_2O	Magnetic field	
Relaxation times[a] (ms)	8.45 T	11.74 T
T_1	22	26
T_{2s}	13	16
T_{2f}	0.65	0.73
T_{TQ}	8.4	9.0

[a] The experimental errors were ±10%.

A triple quantum filtered (TQF) NMR experiment yields a spectrum with three peaks (Fig. 1b), two at the specifically bound site around 2 ppm and one at the chemical shift of the borate/boric acid bulk peak at 7.1 ppm, suggesting two different types of binding sites designated as I and II, respectively. The 6 ppm shoulder and most of the bulk peak at 7.1 ppm are eliminated in the TQF spectrum. The TQF spectrum obtained for ferrocytochrome *c* (not shown) consists of two peaks at 2 and 7.1 ppm suggesting the same two types of binding sites as for the ferricytochtome.

Relaxation Studies of Binding Site Type I (the Peaks at 2 ppm)

The changes in the TQF peak intensities as a function of the creation time of the third rank tensor (τ in Eq. [2]) for both types of binding sites are given in Fig. 2. The curves were fitted to Eq. [3] yielding the values of T_{2s} and T_{2f} for the two binding sites. Tables 1 and 2 summarize the different transverse relaxation times (T_{2f}, T_{2s}, T_{TQ}) and the longitudinal relaxation time (T_1) at two different magnetic fields: 11.7 and 8.4 T, at 5°C, in H_2O solution at pH 9.3 and D_2O solution at pH* 9.7 so that pH = pD. Since the T_{2f}, T_{2s}, and T_{TQ} values for the two peaks

TABLE 2

Transverse and Longitudinal Relaxation Times at Different Magnetic Fields for the 2 ppm Peaks at 5°C, pH* 9.7 and D_2O Solutions

D_2O	Magnetic field	
Relaxation times[a] ± 10% (ms)	8.45 T	11.74 T
T_1	38	43
T_{2s}	25	32
T_{2f}	0.51	0.60
T_{TQ}	18	20

[a] The experimental errors were ±10%.

at 2.1 and 1.8 ppm were identical we will refer simultaneously to the two peaks.

As can be seen from Tables 1 and 2 the T_{2s} and the T_{TQ} values are appreciably different and the T_{TQ} value is independent of the magnetic field. This rules out a relaxation mechanism which results predominantly of a quadrupolar interaction because for such a mechanism T_{2s} is expected to be equal to T_{TQ} (Eq. [8a]). The fact that the T_{TQ} value remains practically the same at the two magnetic fields rules out fast modulation of chemical shift as a significant relaxation mechanism (Eq. [15c]). Also, it provides further evidence that the contribution of the quadrupolar interaction to T_{TQ} is not significant (Eq. [8a]). The only interaction that gives rise to field independent T_{TQ} which is shorter than T_{2s} is dipolar (Eqs. [7a] and [7c]). Although contribution of slow exchange to T_{TQ} is also field independent, it cannot give rise to different T_{TQ} and T_{2s} (Eq. [9]). Thus, the main interaction that governs the TQ signal relaxation time, T_{TQ}, is dipolar and if there is some exchange of the bound species with the bulk it must be very slow. The slight increase in the T_1 and T_{2s} values with the magnetic field may originate from a small contribution of quadrupolar interaction to these relaxation times.

Since T_1 of the 2 ppm peaks due to the bound species (38 ms in D_2O at 8.7 T, Table 2) is significantly longer than the T_1 value of the protein free borate/boric acid solution (6.2 ms), under the same conditions, the value of $1/T_1$ of the bound species provides an upper limit for their exchange rate (*18*). The excess relaxation of $1/T_{TQ}$ (55 s^{-1}) over that of $1/T_1$ (26 s^{-1}) is mainly due to dipolar interaction (see Eq. [6]).

In order to investigate the possible effect of ^1H–^{11}B dipolar interaction on the relaxation mechanism of this site one should compare the relaxation times for the H_2O and D_2O solutions (Tables 1 and 2, respectively). In the H_2O solution the T_{TQ}, T_1, and T_{2s} values are significantly decreased while the T_{2f} value is increased relative to the D_2O solution. These results are consistent with the expected effects of replacing deuterons with protons, i.e., increasing the dipolar interaction, D, and decreasing the viscosity. Increasing the dipolar interaction, D, is expected to shorten the T_{TQ} value (Eq. [7c]), as indeed was observed (Table 1). Lower viscosity results in shorter reorientation time, τ_c, and larger chemical exchange rate, k, which are expected to shorten T_1 and T_{2s} (Eqs. [8a], [8c], and [9]), again, consistent with the experimental results. On the other hand, T_{2f}, which is mainly governed by quadrupolar interaction, is expected to be affected mostly by $1/\tau_c$ (Eq. [8b]) and thus should be lengthened.

Analysis of Relaxation Times for Binding Site Type I

The measured parameters for this site at the different magnetic fields and types of solvents were fitted to a relaxation model that assumes exchange between free and bound states using Eqs. [A1]–[A5] in the Appendix. The fitted parameters that describe the borate at this binding site are summarized in Table 5. The quadrupolar coupling constant, $\chi/2\pi$, was found to be 260 ± 10 KHz. This relatively small value is consistent with a distorted tetrahedral nature of the bound borate, as it is comparable to solid tetrahydrate borates and is much smaller than trigonal borates (*19*).

The dipolar interactions for the bound borate in H_2O solution were found to be 8.8 ± 0.6 KHz. The relaxation in D_2O solution agreed with the ^{11}B–^2H dipolar interaction (which is smaller than the ^{11}B–^1H dipolar interaction by the ratio of $\gamma(^1H)/\gamma(^2H) = 6.5$) with some contribution from about 3% residual protons that were present in the D_2O solution. Thus, the dipolar interaction of the ^{11}B nucleus in H_2O solution results solely from exchangeable protons.

Other values that resulted from the fitting of the experimental results were the correlation times, τ_c, for the bound borate. These values were calculated separately for the H_2O and D_2O solutions and were 10 ± 1.5 ns and 15 ± 2.5 ns, respectively. These correlation times are in fact the correlation times of the protein in solution and since the viscosity of the D_2O solution is greater than that of the H_2O solution by a factor of 1.309 at 5°C (*20*), the correlation times in the two solvents are different.

The contribution of exchange to the equations resulted in a relative slow exchange of the bound borate with free borate with $k_{dissociation}$ of 20 ± 3 s^{-1} in D_2O solution in good agreement with the value of 24 ± 2 s^{-1} found independently using exchange measurements on the cytochrome *c* protons (*7*). A somewhat higher value of 30 ± 4 s^{-1} was found for the H_2O solution as expected for less viscous solvent. The fittings were not sensitive to either chemical shift differences or to the relaxation rates of the free borate, as expected for a system in slow chemical exchange with the bulk.

Relaxation Studies of Binding Site Type II (the Peak at 7.1 ppm)

The results of the various relaxation times, T_1, T_{2s}, T_{2f}, and T_{TQ}, in two magnetic field strengths (8.7 and 11.4 T) and two temperatures (5 and 25°C) at D_2O solution and pH* 9.7 for the signal at 7.1 ppm are summarized in Tables 3 and 4. The ratio of T_{2s}/T_{2f} of 4.3 obtained for site II (Table 3) is much smaller than that obtained for site I ($T_{2s}/T_{2f} = 53$, Table 2). This is because the relaxation rates of site II are weighted averages of free and bound species while those of site I are those of bound species only. The lower T_{2s}/T_{2f} ratio of site II is responsible for the fact that the TQF signal intensity is much weaker than that of the SQ one (*21*), although both TQF and SQ spectra represented the same species. All relaxation times were found to increase with temperature. This is an indication that all equilibria contributing to this signal are in fast exchange. The similarity of T_1 and T_{2s} in the protein solution (4.6 and 2.0 ms, respectively) to the T_1 and T_2 in the protein-free borate solution at the same conditions (6.2 and 1.8 ms, respectively) indicates that the relaxation times T_1 and T_{2s} are mainly determined by the relaxation times of the free species in the bulk with a small effect from the interaction with the protein.

TABLE 3

Transverse and Longitudinal Relaxation Times at Different Magnetic Fields for the 7.1 ppm Peak at 5°C, pH* 9.7 and D_2O Solutions

5°C	Magnetic field	
Relaxation times[a] ± 10% (ms)	8.45 T	11.74 T
T_1	4.6	6.0
T_{2s}	2.0	1.7
T_{2f}	0.65	0.39
T_{TQ}	0.35	0.15[b]

[a] The experimental errors were ±10%.

[b] This value is less precise than the other measurements since the relaxation time is comparable to the 180° pulse length of 60 μs.

The increase of T_1 in the protein solution with increased temperature is a result of the decrease of the reorientation correlation time, τ_c, of the free species, which fulfills the extreme narrowing condition, $\omega_0\tau_c \ll 1$. Since the T_1 of the free boric acid (2.5 ms) is much shorter than that of the tetrahedral borate ion (which is larger than 300 ms) and the measured $R_1 = 1/T_1$ is a weighted average of the relaxation rates of the two species (Eq. [12]), the effect of the temperature on the T_1 of their mixture is due to the variation of the τ_c of the boric acid (Eq. [14]).

T_{2s} of the protein solution and T_2 of the protein-free solution are shorter than the T_1 of the corresponding solutions. This is due to the contribution to the transverse relaxation stemming from chemical exchange between free borate and boric acid (Eq. [15a]). A rough estimate of k based on Eq. [15a], using the chemical shift difference between the borate and boric acid of 18 ppm (*9*) and Eq. [16],

$$R^{EXC} = R_2 - R_1, \qquad [16]$$

gives $k = (0.9 \pm 0.1) \times 10^5\ s^{-1}$ and $(1.0 \pm 0.1) \times 10^5\ s^{-1}$ based on the measurements at magnetic fields of 8.7 and 11.4 T, respectively, in protein-free D_2O solution at 5°C. The value of T_{2f} for the protein solution, 0.65 ms, is shorter than T_{2s}, 2.0 ms, indicating a significant contribution from the relaxation in the bound state. The increase of T_{2f} with temperature stems from the shortening of τ_c of the protein (Eq. [8b]).

T_{TQ} is much shorter than T_{2s} and was found to have a strong dependence on the strength of the magnetic field and to increase with temperature. As was discussed above for site type I, quadrupolar relaxation alone is not consistent with different T_{TQ} and T_{2s} values. The two relaxation mechanisms that can give rise to such a difference are dipolar interaction and fast chemical exchange. However, the strong dependence of T_{TQ} on the magnetic field points to the chemical exchange as the main contribution to the TQ relaxation rate. One may note that the contribution of the chemical exchange to T_{TQ} is greater by a factor of $p^2 = 9$ than the contribution of the exchange to T_{2s} (Eqs. [15a] and [15c]). The increase of T_{TQ} with the temperature is consistent with a TQ relaxation mechanism that is governed by chemical exchange, because the exchange rate increases with temperature.

In order to assess the contribution of the dipolar interaction to the relaxation, the measurements were repeated in H_2O solutions instead of D_2O. No significant changes were observed for the T_1, T_{2s}, T_{2f}, and T_{TQ} values. Thus, a major effect of the dipolar interaction on the relaxation of the ^{11}B nuclei at this site was ruled out.

Analysis of the Bound Species at Binding Site Type II

The fact that the peak at 7.1 ppm passes the TQ filter indicates that at least one of the species contributing to this peak binds to the protein. At pH* 9.7 the solution contains both borate and boric acid at a ratio of 7:3. In order to find out whether boric acid binds to cytochrome *c* we prepared a boric acid–cytochrome *c* sample at a pH* value of 5.4. At this pH* value the cytochrome preserves its native structure (*2*) and, based on the boric/borate equilibrium constant of pK* 9.28, the borate concentration is negligible. Both the SQ and TQF spectra gave a single peak at 20 ppm—the chemical shift of the pure boric acid. As can be seen in Fig. 3, the signal of the boric acid in the presence of the protein passes the TQ filter. This is a clear sign that the boric acid binds to the protein. The ^{11}B relaxation times T_1, T_{2s}, T_{2f}, and T_{TQ} at 5°C and 8.7 T were found to be 1.8, 1.9, 0.5, and 1.8 ms, respectively. The short T_{2f}, relative to the other relaxation times, is a result of the binding to the protein. The similarity between T_1, T_{2s}, and T_{TQ} indicates an extreme narrowing condition ($\omega_0\tau_c \ll 1$) and thus rules out an effect of the binding to the protein on these relaxation times. In fact these values are quite similar to $T_1 = 2.5$ ms measured for the protein-free solution of boric acid under the same conditions. Thus, the values of T_1, T_{2s}, and T_{TQ} of the protein solution are determined by the relaxation of boric acid in the bulk. The somewhat shorter values of these relaxation times, compared to the T_1 of the protein-free solution, is

TABLE 4

Transverse and Longitudinal Relaxation Times at Different Magnetic Fields for the 7.1 ppm Peak in D_2O Solutions pH* 9.7 and 25°C

25°C	Magnetic field	
Relaxation times[a,b] (ms)	8.45 T	11.74 T
T_1	7.3	7.2
T_{2s}[a]	2.7	unresolved
T_{2f}[a]	1.3	unresolved
T_{TQ}	0.50	0.24[c]

[a] The experimental errors were ±10%.

[b] For T_{2s}/T_{2f} less than 3, the bi-exponential separation is uncertain.

[c] This value is less precise than the other measurements since the relaxation time is comparable to the 180° pulse length of 60 μs.

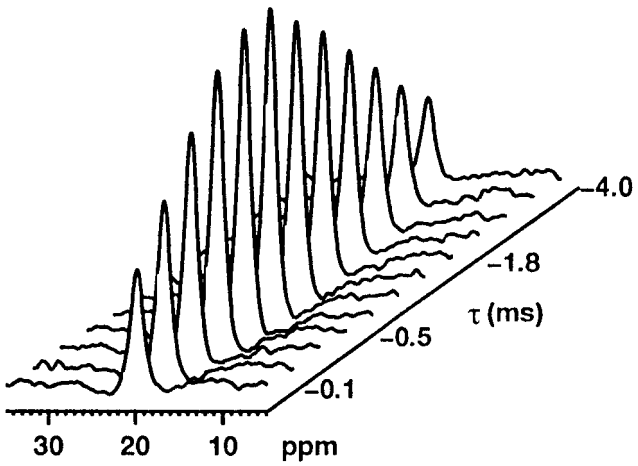

FIG. 3. The changes in the TQF peak intensities as a function of the creation time of the third rank tensor, τ (Eq. [1]), for a sample of 100 mM boric acid and 4 mM ferricytochrome *c* at 5°C, 8.7 T, and pH* 5.4.

consistent with the increased viscosity in the protein solution. The short relaxation times are the result of the strong quadrupolar interaction in the boric acid which does not have cubic symmetry.

The fact that the T_{2s}/T_{TQ} ratio at pH* 5.4 is close to unity rules out any contribution from modulation of a chemical shift by chemical exchange and from dipolar interaction (see Eqs. [13] and [15]). The T_{2s} and T_{TQ} relaxation times at both pH* 9.7 and pH* 5.4 are determined by the relaxation in the bulk. However since at pH* 5.4 the solution consists only of boric acid there is no contribution of chemical exchange to these relaxation times and therefore their ratio is unity, unlike the T_{2s}/T_{TQ} ratio at pH* 9.7.

The nature of the bound species in the mixture of borate and boric acid was studied by comparison of the relaxation times and the chemical shifts at different concentrations of boric acid or boric/borate mixture. The two relaxation times that are most sensitive to the effect of binding are T_{2f} and the double quantum relaxation time, T_{DQ} (Eq. [8b]). Since the T_{2s}/T_{2f} ratio obtained from conventional TQF experiment for the boric/borate mixture (pH* 9.7) was found to be in the range of 2–3, the sensitivity and accuracy of the fitted T_{2f} values was not sufficient to follow small changes in the amount of binding. As T_{DQ} is obtained by a fitting of the DQF decay to a single exponent its determination is more reliable than that of T_{2f} and therefore it was used for the evaluation of the extent of binding.

Figure 4a gives the measured values for the chemical shift, T_1 and T_{DQ}, for a sample of ferricytochrome *c* at different concentrations of boric acid, $[B]_0$. All measurements were carried out at pH* 5.4 and 5°C with a constant protein concentration of $[Cyt]_0 = 4.3$ mM. Assuming a single binding site for boric acid the equilibrium process is given by

$$\mathrm{Cyt} + \mathrm{B(OH)_3} \overset{K_{\mathrm{boric}}}{\rightleftharpoons} \mathrm{Cyt} - \mathrm{B(OH)_3} \qquad [17]$$

The three measured parameters, chemical shift, T_1, and T_{DQ}, remained practically constant with the change in the total boric acid concentration indicating weak binding. Thus, the conditions $[B]_0 \gg [\mathrm{Cyt} - \mathrm{B(OH)_3}]$ and $[\mathrm{Cyt} - \mathrm{B(OH)_3}] < [Cyt]_0$ apply to the present system. In this case the fraction of bound boric acid is given by

$$f_b^{\mathrm{boric}} = [Cyt]_0/(K_{\mathrm{boric}}^{-1} + [B]_0). \qquad [18]$$

The fact that the T_{DQ}, and thus also f_b^{boric} (Eq. [12]), remained constant with the increase in the total boric acid concentration up to 80 mM, gives an upper limit to the binding constant of $K_{\mathrm{boric}} \ll 12.5\ \mathrm{M}^{-1}$. An estimation of K_{boric} can be obtained by assuming that the value of the quadrupolar coupling constant of bound boric acid is the same as that of solid boric acid, i.e., $\chi_{\mathrm{boric}}/2\pi = 2.56$ MHz (*19*). This assumption is reasonable since the χ value is practically the same for a variety of trigonal borates (*19*). Subtracting the contribution of the free boric acid (given by R_{2s}) from R_{2f} and using Eqs. [8b] and [12], one obtains the net effect of binding given by $R_{2f} - R_{2s} = f_b^{\mathrm{boric}}\chi^2\tau_c/20$. Using a τ_c of 15 ns (Table 5, site type I) one obtains $K_{\mathrm{boric}} = 1.9\ \mathrm{M}^{-1}$.

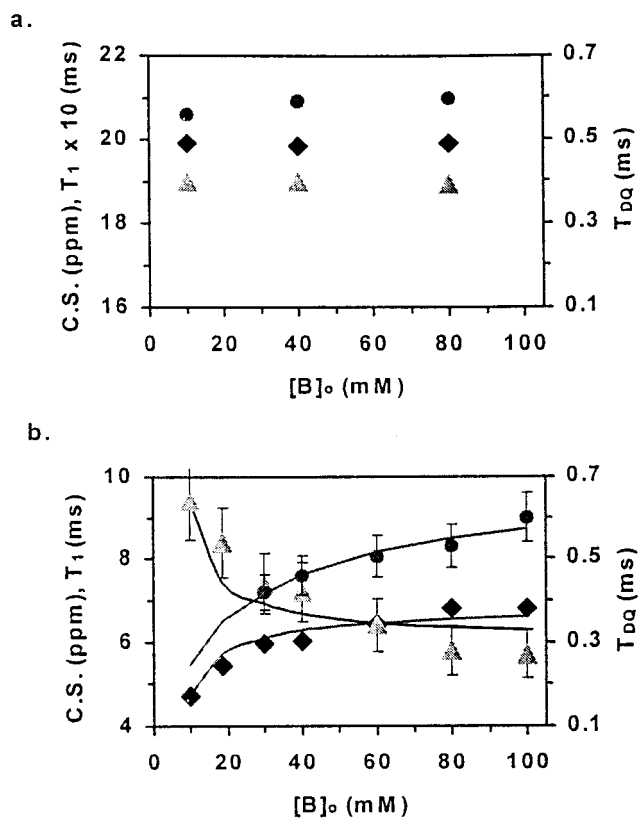

FIG. 4. The experimental values at 5°C and 8.7 T of T_1 (▲), T_{DQ} (●), and the chemical shift (◆) as a function of $[B]_0$ in solutions containing 4.3 mM ferricytochrome *c* at two pH values. (a) pH* 5.4. In this case only boric acid is present. (b) pH* 9.7. In this case $[B]_0$ = [borate] + [boric acid]. The continuous lines are computed using the fitted parameters given in Table 5 for site type II.

In Fig. 4b values for the chemical shift T_1 and T_{DQ} are given for a sample of 4.3 mM ferricytochrome *c* as a function of total borate/boric concentrations, $[B]_0$, at a constant pH* of 9.7 and 5°C. One may note that all three parameters exhibit marked change at low values of $[B]_0$, in contrast to the above case of pH* 5.4 where only boric acid is present. Two additional equilibrium processes should be considered for this sample in addition to the boric acid–cytochrome equilibrium (Eq. [17]). The equilibrium between the free boric acid and borate ion in solution (Eq. [1]) and the binding of the borate ion to the cytochrome (Eq. [19]) is

$$\mathrm{Cyt} + \mathrm{B(OH)_4^-} \underset{}{\overset{K_{\mathrm{borate}}}{\rightleftharpoons}} \mathrm{Cyt - B(OH)_4^-}. \qquad [19]$$

A model based on the three equilibrium processes given in Eqs. [A9]–[A16] in the Appendix was successfully used to account of the experimental measurements of the chemical shift, T_1 and T_{DQ}. The experimental results were fitted to Eq. [A16] with the resulting equilibrium constants and microscopic parameters given in Table 5. Curves calculated on the basis of the fitted parameters are shown in Fig. 4b. The value of $pK_{boric/borate}$ 9.28 ± 0.07 obtained in the fitting procedure agrees with the value of 9.28 ± 0.05 found independently for protein free borate/boric acid solution as explained in the experimental section. K_{boric} and K_{borate} were found to be 2.6 ± 1.5 and 1800 ± 200 M^{-1}, respectively. The value of the binding constant of the boric acid is consistent with the slightly lower value estimated for the acidic solution of pH* 5.4.

TABLE 5

The Parameters Obtained for Binding Sites Type I and II in D_2O Solutions at 5°C Using the Relaxation Analysis of Two and Four Sites Models, Respectively[a]

	Borate(I)[b]	Borate(II)[c]	Boric acid(II)[d]
$k_{dissociation}$, s^{-1}	30 ± 4 20 ± 3[d]	$>8 \times 10^4$	$>5 \times 10^3$
$K_{binding}$, M^{-1}	(13 ± 2)[e]	1800 ± 200	2.6 ± 1.5
$D/2\pi$, KHz	8.8 ± 0.6[d]	—	—
τ_c, ns	15.0 ± 2.5 10.0 ± 1.5[d]	15.0 ± 1.5	—
$\chi_{bound}/2\pi$, MHz	0.26 ± 0.01	0.48 ± 0.04	(2.56)[f]
$(boric/borate)_{free}$[c]	pK 9.28 ± 0.07	$k = (1.2 \pm 0.14) \times 10^5$ s^{-1}[g]	

[a] The error in the parameters was calculated by systematic variation of each of the parameters in the table to the extent that the residual of the least mean squares is not larger than its maximum value as can be determined by the lower and upper limits of the relaxation times given in Tables 1 and 2.

[b] Two site analysis using the relaxation measurements in Tables 1 and 2.

[c] Four site analysis using the T_1, T_{DQ}, and chemical shift measurements at different boric/borate concentrations at 8.7 T.

[d] Values obtained for H_2O solution.

[e] This value was obtained by Taler *et al.* using ^{1}H NMR exchange measurements on the cytochrome *c* protons in borate solutions (*7*).

[f] A value taken from Bray *et al.* (*19*).

[g] Using the relaxation times in Table 3, at 8.7 T and $R^{EXC} = R_{TQ} - R_1$ and Eq. [15c] or $R^{EXC} = R_{2s} - R_1$ and Eq. [15a], the same value of $k = (1.20 \pm 0.12) \times 10^5$ s^{-1} was obtained.

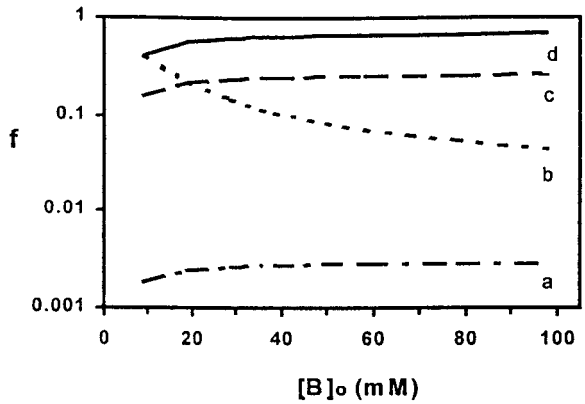

FIG. 5. The calculated fractions, (a) f_b^{boric}, (b)f_b^{borate}, (c) f_f^{boric}, and (d) f_f^{borate}, shown on a logarithmic scale, as a function of the total boron concentration, $[B]_0$, for a sample containing 4.3 mM ferricytochrome *c* at 5°C and pH* 9.7. The fractions were calculated according to the equilibrium constants K_1, K_2, and K_3 (Table 5) obtained in the fitting of T_1, T_{DQ}, and the chemical shift values (Fig. 4b) by the four site model give in the Appendix.

An insight into the various contributions to the measured chemical shifts and the T_1 and T_{DQ} relaxation times can be obtained from the fraction of the various species that can be calculated from the fitted values of the equilibrium constants (see Fig. 5). The trends of the chemical shift and T_1 values are the result of the increase of the combined fractions of the free and bound borate ($f_f^{borate} + f_b^{borate}$) at lower $[B]_0$. This is because both species are expected to have low chemical shift and long T_1 values. The double quantum relaxation rate, $1/T_{DQ}$, is governed by a combination of contributions from bound boric acid and borate ion and by the chemical exchange of the free species (Eqs. [10], [12], and [15b]). The relative proportions of these contributions vary with $[B]_0$.

DISCUSSION

In the present work three distinct binding sites of borate ion and boric acid to cytochrome *c* were characterized using MQF NMR measurements: One for slowly exchanging borate ion (type I) and two for fast exchanging (type II) borate ion and boric acid. The presence of the slowly exchanging borate ion is consistent with previous rate measurements using proton NMR of cytochrome *c* (*7*). The relatively small value of $\chi/2\pi$ for the bound borate at site type I (0.26 MHz) suggests a tetrahedral symmetry that is probably somewhat distorted within the binding site, so that the measured $\chi/2\pi$ value is higher than the 0.09 MHz found for solid state NMR borate ion in $Na_2B(OH)_4Cl$ but it is smaller than that for distorted borates with $\chi/2\pi \approx 0.4$ MHz and much smaller than for trigonal borates with $\chi/2\pi \approx$ 2.5 MHz (*19*). As was reported in our previous publications (*15, 16*) the TQ relaxation time of spin-$\frac{3}{2}$ nuclei is sensitive to

dipolar interaction. This was found to be the case for the borate ion bound to the slow exchanging site. Assuming, based on distance consideration, that the dipolar interaction found for H_2O solutions ($D/2\pi$ = 8.8 KHz) resulted mainly from the interaction of the boron atom with the hydroxyl protons of the borate itself, the distance between them calculated using the expression $r_{B-H} = \sqrt{2\hbar\gamma_H\gamma_B/D}$ is 2.06 ± 0.07 Å. This value is in agreement with the value of 2.09 Å calculated on the basis of the O–H and B–O distances of 0.96 and 1.48 Å, respectively (*22*) and the B–O–H angle of 116° found for boric acid at 130°K (*23*). The reorientation correlation time, τ_c = 10 ± 1.5 ns, that was found for the bound borate by fitting the results to the two site model may be compared with the τ_c of the cytochrome. A rough approximation of τ_c for the cytochrome was obtained using the Stokes–Einstein equation with a protein radius of 16 Å and the viscosity of pure water at 5°C (η = 1.519 cp) and yielded a value of 7 ns. The two values agree considering the higher viscosity of the cytochrome solution compared to pure water. Thus, the τ_c of the bound borate is the same as that of the cytochrome indicating restricted motion of the borate in its binding site.

Other binding sites for borate and boric acid were discovered by the MQF NMR measurements. The single MQ signal obtained for these sites resulted from borate and boric acid that bind separately to the cytochrome and undergo a fast exchange with free borate and boric acid in solution. The fast exchange made these sites undetectable by ^{1}H or ^{11}B SQ spectra. The binding of boric acid to cytochrome *c* was found to be rather weak with a binding constant, K^{II}_{boric}, of 2.6 ± 1.5 M^{-1} at pH* 9.7. An independent confirmation of this binding was obtained by MQF experiment at pH* 5.4. The binding constant at this pH was estimated to be 1.9 M^{-1}. The similarity between these values in spite of the fact that the cytochromes surface at pH* 9.7 is less charged (because some lysine residues on its surface become neutral) agrees with binding that is based on the formation of hydrogen bonds.

K^{II}_{borate}, the binding constant of the negatively charged borate ion in the fast exchange site is much higher than K^{II}_{boric}, indicating additional electrostatic interaction. It may be noted that the high value of K^{II}_{borate} is on the same order of magnitude as one of the binding constant of phosphate ions at pH 7 to cytochrome *c* (*3, 4*). Other, much smaller binding constants of phosphate ions to different cytochrome sites are also known (*3*), resembling the multiple binding pattern of the borate ion. Owing to the structural similarity between the $B(OH)_4^-$ and the dihydrogen phosphate ion, $H_2PO_4^-$, it is likely that the latter ion is that bound to the cytochrome in phosphate solutions.

The difference between the two binding sites of the borate ion is very striking. The slow exchange site (type I), which is reflected clearly in the NMR spectrum of the heme surrounding protons, has low binding constant (K^{I}_{borate} = 13 M^{-1}) (*7*) while the fast exchanging site (type II) has much higher binding constant (K^{II}_{borate} = 1800 M^{-1}). One may speculate that structural considerations dictate the binding mechanism, thus affecting the exchange rate. A suggested mechanism can be that the borate ion in the slow exchange site is buried and hindered while the fast exchange site results merely of electrostatic interaction of the borate ion with exposed parts of the surface of the cytochrome. This mechanism is supported by a recent molecular dynamics study (*24*) which indicates the presence of a "tight" binding site and two "loose" binding sites, in cytochrome *c*. In that work the difference between the tight and loose binding sites was not in the interaction but a kinetic one. The tightly bound borate is first placed near the protein surface as in the loose binding site, but it becomes integrated into the protein hydrogen bonding network and remains bound with little positional fluctuations, and therefore exchanges with the solvent very slowly.

CONCLUSION

MQF NMR measurements were demonstrated to be an effective tool in the identification and characterization of the binding of ligands containing quadrupolar nuclei to proteins. The MQF method made it possible to reveal the existence of binding sites that could not be detected by conventional SQ methods. Another benefit of the MQF method was the possibility to measure for the quadrupolar nucleus dipolar interaction with the consequent geometrical information.

APPENDIX

The full expressions for the single quantum transverse relaxation rate:

$$\frac{1}{T_{2i}} = \frac{\chi^2}{40}\left[J_Q(0) + 2J_Q(\omega_I) + J_Q(2\omega_I)\right] + \frac{1}{2}\left(\frac{1}{T^D_{1,1}} + \frac{1}{T^D_{3,1}}\right) \pm \frac{1}{2}\,\mathrm{SQRT2} \qquad [A1]$$

with $i = f$ for the plus sign and $i = s$ for the minus sign, where

$$\frac{1}{T^D_{1,1}} = \frac{D^2}{15}S(S+1)[4J(0) + J(\omega_I - \omega_S) + 6J(\omega_S) + 3J(\omega_I) + 6J(\omega_I + \omega_S)]$$

$$\frac{1}{T^D_{3,1}} = \frac{D^2}{15}S(S+1)[4J(0) + 11J(\omega_I - \omega_S) + 6J(\omega_S) + 33J(\omega_I) + 66J(\omega_I + \omega_S)]$$

$$\mathrm{SQRT2} = \sqrt{\left\{\frac{\chi^2}{20}\left[J_Q(0) - J_Q(2\omega_I)\right]\right\}^2 + \frac{\chi^2}{50}\left[J_Q(0) - J_Q(2\omega_I)\right] \times \left(\frac{1}{T^D_{3,1}} - \frac{1}{T^D_{1,1}}\right) + \left(\frac{1}{T^D_{3,1}} - \frac{1}{T^D_{1,1}}\right)^2}$$

and

$$D = \hbar \gamma_S \gamma_I / r^3.$$

The double quantum transverse relaxation rate:

$$\frac{1}{T_{DQ}} = \frac{\chi^2}{20}[J_Q(0) + J_Q(2\omega)] + \frac{8D^2}{15} S(S+1) \times [2J(0) + J(\omega_I - \omega_S) + 3J(\omega_S) + 3J(\omega_I) + 6J(\omega_I + \omega_S)]. \quad [A2]$$

The triple quantum transverse relaxation rate:

$$\frac{1}{T_{TQ}} = \frac{\chi^2}{20}[J_Q(\omega_I) + J_Q(2\omega_I)] + \frac{D^2}{5} S(S+1) \times [12J(0) + J(\omega_I - \omega_S) + 18J(\omega_S) + 3J(\omega_I) + 6J(\omega_I + \omega_S)]. \quad [A3]$$

The single quantum longitudinal relaxation rate:

$$\frac{1}{T_{1i}} = \frac{\chi^2}{20}[J_Q(\omega_I) + J_Q(2\omega_I)] + \frac{7}{2T_{1,0}^D} \pm \frac{1}{2} \text{SQRT1} \quad [A4]$$

$i = f$ for the plus sign and $i = s$ for the minus sign, where

$$\text{SQRT1} = \sqrt{\left\{\frac{\chi^2}{10}[J_Q(\omega_I) - J_Q(2\omega_I)] + \frac{3}{T_{1,0}^D}\right\}^2 + \left\{\frac{4}{T_{1,0}^D}\right\}^2}$$

$$\frac{1}{T_{1,0}^D} = \frac{2D^2}{15} S(S+1)[J(\omega_I - \omega_S) + 3J(\omega_I) + 6J(\omega_I + \omega_S)].$$

$J_Q(\omega)$ and $J(\omega)$ in the above expressions are the spectral densities due to quadrupolar and dipolar interactions, respectively.

The Effect of Chemical Exchange

Two sites. Assuming isotropic chemical exchange between two sites, a and b, one can replace the expressions for the relaxation rates given in Eqs. [A1]–[A4] by

$$R_\pm = \frac{1}{2}\{-ip\Delta\omega + R_j^a + R_j^b + k_a + k_b \pm \sqrt{(-ip\Delta\omega + R_j^a - R_j^b + k_a - k_b)^2 + 4k_a k_b}\}, \quad [A5]$$

where j can be 1s, 1f, 2s, 2f, DQ, or TQ; R_j^a and R_j^b are the relaxation rates for sites a and b, respectively; $k_a = 1/\tau_a$ and $k_b = 1/\tau_b$ where τ_a, τ_b are the exchange lifetimes of the two sites; p is the coherence and equals 0, 1, 2, or 3 for the longitudinal, transverse, DQ, or TQ relaxation rates, respectively; and $\Delta\omega$ is the frequency difference between the chemical shifts of the exchanging species.

Four sites. The following equilibria are considered:

$$\text{Cyt} + \text{B(OH)}_3 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{Cyt} - \text{B(OH)}_3 \quad [A6]$$

$$\text{B(OH)}_3 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{B(OH)}_4^- \quad \text{(at a constant pH)} \quad [A7]$$

$$\text{Cyt} + \text{B(OH)}_4^- \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \text{Cyt} - \text{B(OH)}_4^- \quad [A8]$$

Yielding the simple relations (Eqs. [A9]–[A14]):

$$K_1 = \frac{f_a}{f_c P_1} \quad [A9]$$

$$K_2 = \frac{f_d}{f_c} \quad [A10]$$

$$K_3 = \frac{f_b}{f_d P_2} \quad [A11]$$

$$f_a + f_b + f_c + f_d = 1 \quad [A12]$$

$$P_1 = [\text{Cyt}]_0 - f_a[\text{B}]_0 \quad [A13]$$

$$P_2 = [\text{Cyt}]_0 - f_b[\text{B}]_0, \quad [A14]$$

where f_a, f_b, f_c, f_d are the fractions of bound boric acid, bound borate, free boric acid, and free borate, respectively; P_1 and P_2 are the concentrations of the sites on the protein available for binding boric and borate, respectively; and $[\text{Cyt}]_0$ and $[\text{B}]_0$ are the total concentrations of the cytochrome and the boric/borate, respectively.

Based on these equations a cubic equation for the fraction of bound boric acid in terms of the equilibrium constants $[\text{B}]_0$ and $[\text{Cyt}]_0$ is given as

$$a_3 f_a^3 + a_2 f_a^2 + a_1 f_a + a_0 = 0$$

$$a_0 = -c_3^2$$

$$a_1 = c_3 c_1 + c_3 c_2 + c_3^2 + c_4 c_3 + c_3 - c_5 c_3$$

$$a_2 = c_5 c_1 + c_5 c_2 - c_3 + c_5 c_3 - c_4 + c_5$$

$$a_3 = -c_5$$

$$c_1 = \frac{c_2}{K_2}$$

$$c_2 = \frac{K_2}{K_1[\mathrm{B}]_0}$$

$$c_3 = \frac{[\mathrm{Cyt}]_0}{[\mathrm{B}]_0}$$

$$c_4 = K_3[\mathrm{Cyt}]_0 c_2$$

$$c_5 = K_3[\mathrm{B}]_0 c_2 - 1. \qquad \text{[A15]}$$

On the basis of the expression for f_a and Eqs. [A9]–[A14] one can express all other fractions (f_b–f_d) using the same terms as for f_a. Having expressed f_a–f_d, P_1, and P_2 as a function of the equilibrium constants $[\mathrm{B}]_0$ and $[\mathrm{Cyt}]_0$, one can write the following relaxation matrix,

$$\begin{pmatrix} -k_{-1} - R_a + i\Delta\omega & k_1 P_1 & & \\ k_{-1} & -k_1 P_1 - k_2 - R_c + i\Delta\omega & k_{-2} & \\ & k_2 & -k_3 P_2 - k_{-2} - R_d & k_{-3} \\ & & k_3 P_2 & -k_{-3} - R_b \end{pmatrix}, \qquad \text{[A16]}$$

where $\Delta\omega$ is the chemical shift difference between the boric and the borate; R_b and R_d are the various relaxation rates of the borate in the bound and free states, respectively; and R_a and R_c are the corresponding relaxation rates of the boric acid.

The relaxation rates are the eigenvalues of the matrix in Eq. [A16]. Since we expect all four types of sites to be in very fast exchange the relevant eigenvalue is the one with the smallest real part. This eigenvalue is fitted to the experimental result by a nonlinear least mean square routine, yielding equilibrium constants and microscopic parameters such as quadrupole coupling and protein reorientation time.

REFERENCES

1. G. Jaccard, S. Wimperis, and G. Bodenhausen, *J. Chem. Phys.* **85,** 6282–6293 (1986).
2. R. E. Dickerson and R. Timkovich, *in* "The Enzymes" (P. D. Boyer, Ed.), Vol. 11, pp. 397–429, Academic Press, New York (1975).
3. G. W. Pettigrew and G. R. Moore, "Cytochromes c Biological Aspects," pp. 67–84, Springer-Verlag, Berlin (1987).
4. G. J. Pielak, D. S. Auld, S. F. Betz, S. E. Hilgen-Willis, and L. L. Garcia, *in* "Cytochrome c a Multidisciplinary Approach" (R. A. Scott and A. G. Mauk, Eds.), pp. 255–257, University Science Books, Sausalito, CA (1996).
5. R. Margalit and A. Schejter, *Eur. J. Biochem.* **32,** 500–505 (1973).
6. E. Margoliash, G. H. Barlow, and V. Byers, *Nature* **228,** 723–726 (1970).
7. G. Taler, A. Schejter, and G. Navon, *Inorg. Chim. Acta* **273,** 388–392 (1998).
8. F. A. Cotton and G. Wilkinson, "Advanced Inorganic Chemistry," Wiley, New York (1988).
9. M. J. How, G. R. Kennedy, and E. F. Mooney, *Chem. Com.* **1,** 267–268 (1969).
10. J. M. Coddington and M. J. Taylor, *J. Coord. Chem.* **20,** 27–38 (1989).
11. C. G. Salentine, *Inorg. Chem.* **22,** 3920–3924 (1983).
12. A. Bax, S. Freeman, and P. Kempsell, *J. Am. Chem. Soc.* **102,** 4849–4851 (1980).
13. G. Bodenhausen, H. Kogler, and R. R. Ernst, *J. Magn. Reson.* **58,** 370–388 (1984).
14. Y. Sharf, U. Eliav, H. Shinar, and G. Navon, *J. Magn. Reson. B* **107,** 60–67 (1995).
15. U. Eliav and G. Navon, *J. Magn. Reson. A* **123,** 32–48 (1996).
16. U. Eliav and G. Navon, *J. Magn. Reson.* **130,** 63–67 (1998).
17. P. S. Hubbard, *J. Chem. Phys.* **53,** 985–987 (1970).
18. T. E. Bull, *J. Magn. Reson.* **8,** 344–353 (1972).
19. P. J. Bray, J. O. Edwards, J. G. O'Keefe, V. Ross, and I. Tatsuzaki, *J. Chem. Phys.* **35,** 435–442 (1961).
20. G. N. Lewis and R. T. MacDonald, *J. Am. Chem. Soc.* **55,** 4730–4731 (1933).
21. G. Navon, *Magn. Reson. Med.* **30,** 503–506 (1993).
22. E. L. Muetterties, "The Chemistry of Boron and Its Compounds," pp. 196–198 Wiley, New York (1967).
23. E. Zobetz and A. Preisinger, *Monatsh. Chem.* **120,** 291 (1989).
24. G. Taler, G. Navon, and O. M. Becker, *Biophys. J.* **75,** 2461–2468 (1998).