Hydrogen Bonds between Nitrogen Donors and the Semiquinone in the Qi-site of the bc1 Complex*

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The ubisemiquinone stabilized at the Qi-site of the bc1 complex of Rhodobacter sphaeroides forms a hydrogen bond with a nitrogen from the local protein environment, tentatively identified as ring N from His-217. The interactions of 14N and 15N have been studied by X-band (≈9.7 GHz) and S-band (3.4 GHz) pulsed EPR spectroscopy. The application of S-band spectroscopy has allowed us to determine the complete nuclear quadrupole tensor of the 14N involved in H-bond formation and to assign it unambiguously to the Nα of His-217. This tensor has distinct characteristics in comparison with H-bonds between semiquinones and Nε in other quinone-processing sites. The experiments with 15N showed that the Nα of His-217 was the only nitrogen carrying any considerable unpaired spin density in the ubiquinone environment, and allowed calculation of the isotropic and anisotropic couplings with the Nα of His-217. From these data, we could estimate the unpaired spin density transferred onto 2s and 2p orbitals of nitrogen and the distance from the nitrogen to the carbonyl oxygen of 2.38 ± 0.13 Å. The hyperfine coupling of other protein nitrogen with semiquinone is <0.1 MHz. This did not exclude the nitrogen of the Asn-221 as a possible hydrogen bond donor to the methoxy oxygen of the semiquinone. A mechanistic role for this residue is supported by kinetic experiments with mutant strains N221I, N221H, N221L, N221S, N221P, and N221D, all of which showed some inhibition but retained partial turnover.

The bc1 complex (ubihydroquinone:cytochrome c oxidoreductase, or complex III of the mitochondrial respiratory chain) functions to oxidize ubihydroquinone (QH2,4 quinol) in the membrane and reduce cytochrome c (or cytochrome c2 in bacteria) in the aqueous phase, using the redox work to transport 2H+ per electron across the membrane (1–4). In the photosynthetic bacterium Rhodobacter sphaeroides, the bc1 complex completes the photosynthetic chain containing the photochemical reaction center, which provides the substrates for the complex following photoactivation (5, 6). The Q-cycle mechanism is a well tested model to account for the function of the bc1 complex (7–9), but several details are still controversial. The Q-cycle involves separate catalytic sites on opposite sides of the membrane for oxidation of QH2 and reduction of ubisemiquone (Q, quinone). The work function is provided by a bifurcation of electron transfer at the quinol-oxidizing site (the Qα-site). The first electron is transferred to a high potential chain (the Rieske iron-sulfur protein, cytochrome c1, and cytochrome c), and the second to a low potential chain, consisting of heme b1 and heme b14 in cytochrome b, which delivers electrons to the quinone reduction site (Qβ-site). Some of the work from the first electron transfer is stored in the strong reducing potential of an unstable semiquinone intermediate formed at the Qα-site. This provides the driving force for reduction of Q, or of ubisemiquinone (SQ), in the Qβ-site, and for generation of the proton gradient. Controversial aspects of the Qα-site mechanism (10, 11) are not pertinent to this study, because it can be regarded simply as a device for delivery of electrons to the Qβ-site. At the Qβ-site, Q is thought to be reduced to QH2 in two sequential one-electron reactions, with a sequence that depends on the initial occupancy. With the pool initially oxidized (EmQ = ~200 mV) the quinone is reduced to SQ, which is then the acceptor for the second electron reaching the site after a subsequent turnover of the Qα-site. The SQ can also be formed as a stable intermediate on redox titration (12–15) or by reduction of the oxidized complex by added QH2 (16, 17). Because the latter reaction is insensitive to myxothiazol, it does not require the Qα-site.

The Q-cycle mechanism has been well supported by structures from crystallography (18–20). These include bc1 complexes from mitochondria from several vertebrate sources (beef, chicken, and rabbit), from yeast, and from two at which quinone is reduced; Qβ-site, site of the bc1 complex at which quinol is oxidized; SQ, ubisemiquinone; nQ, nuclear quadrupole interaction; MOPS, 4-morpholinopropenesulfonic acid; mT, millitesla; DFT, density functional theory.
Qi-site Semiquinone in the bc₇ Complex

Rhodobacter species (21, 22) (Rhodobacter capsulatus and R. sphaeroides). Structures have now been solved with many different inhibitors occupying the two postulated quinone-processing sites, which have allowed identification of binding domains with good resolution (better than 2.2 Å in some structures) (23–26). For the Qi-site, a natural quinone substrate has been modeled as the occupant in “native” structures and is presumed to be the oxidized (Q) form. However, the reaction cycle involves all three forms, Q, SQ, and QH₂, and an understanding of the mechanism requires identification of ligands for each. The structures suggest three potential side chains as ligands, His-201, Asp-228, and Ser-205 (in bovine numbering). Unfortunately, several different configurations have been observed in crystallographic structures, involving direct H-bonds and H-bonds mediated through H₂O molecules, in several different combinations (17, 25–28).

Alternative approaches to studying the Qi-site are through spectroscopy, and a substantial literature on characterization of the SQ form using continuous wave EPR or ENDOR has been reported (12, 28–31). More recently, applications of pulsed EPR have allowed a more detailed analysis of structure local to the SQ (17, 32). We have shown using X-band ESEEM that the SQ at the Qi-site of the bc₁ complex of R. sphaeroides interacts with a nitrogen atom identified as the N⁶ or N⁰ of a histidine side chain. This assignment could only be based on an estimate of the quadrupole coupling constant for the interacting ¹⁴N, because the X-band ESEEM spectra failed to provide the complete nuclear quadrupole tensor. In the structures from Berry and co-workers (17), a histidine serves as an H-bond donor through the N⁰ atom to one of the quinone carbonyls, leading us to suggest that the histidine identified in the R. sphaeroides enzyme by EPR is most probably His-217, equivalent to His-201 in the bovine mitochondrial complex. The nitrogen assigned to His-217 possesses unpaired electron spin density, which implies the existence of an atomic bridge through the H-bond for the transfer of spin density from the SQ to this protein nitrogen. We did not detect in the X-band ¹⁴N ESEEM spectra any major contributions with comparable unpaired spin density from other nitrogens in the SQ environment (17).

We have also identified three exchangeable protons likely involved in H-bonds to the SQ, which were visualized by substitution of ¹H₂O by ²H₂O. Two of these showed large anisotropic hyperfine couplings consistent with out-of-plane H-bonds to carbonyl O atoms of the SQ. The third proton showed lower hyperfine coupling, which we considered to be compatible with either an involvement of Asn-221 (which is the R. sphaeroides equivalent to Ser-205) in an H-bond with a methoxy O atom (see model in Fig. 1) or an H-bond to H₂O (32).

Powder-type ESEEM spectra of native proteins in frozen solution are limited in their information content because of the nuclear quadrupole interactions (nqi) of ¹⁴N magnetically coupled with the SQs, which has a nuclear spin, I = 1 (33, 34). The nqi require special relationships between the nuclear Zeeman frequency and isotropic hyperfine constant for optimal resolution of the peaks from well defined transitions. These requirements limit interpretation because of the following two effects: (i) only partial information about hyperfine and quadrupole tensors of those ¹⁴N nuclei that generate resolved lines can be obtained, and (ii) contributions from anisotropic weakly coupled nuclei may be completely suppressed. These uncertainties can, in principle, be overcome through experiments at different microwave frequencies (leading to proportional variations of nuclear Zeeman frequency), or by using proteins labeled isotopically with ¹⁵N, which possesses I = ½.

Here we report the first studies of the SQ at the Qi-site of the bc₁ complex, using ¹⁴N S-band ESEEM with the native protein, and two-dimensional X-band ESEEM with the complex uniformly labeled with ¹⁵N atoms, with the specific aim of characterizing the interaction of the semiquinone with nitrogen atoms in the protein environment in greater detail.

Experimental Procedures

Preparation of Isolated bc₁ Complex—The bc₁ complex was isolated from chromatophores prepared from R. sphaeroides strain GBH6 and purified following protocols described previously (17, 35, 36), with minor modifications as follows. The concentration of chromatophores was adjusted to give an absorbance of ΔA₅₆₂–₅₇₇ nm = 1.2, and solubilization was performed for 1 h. Unless otherwise indicated, the bc₁ complex was used without added cryoprotectants, because Me₂SO or glycerol decreased the amplitude of the Qi-site semiquinone signal.

FIGURE 1. Structural model of the Qi-site in R. sphaeroides. The structure from the bovine mitochondrial bc₁ complex (Protein Data Bank code 1pp9) is shown, mutated in silico, and renumbered to conform to the R. sphaeroides sequence. Groups discussed in the text are shown as ball and stick models. The distances shown are in Å and represent the H-bond length between donor and acceptor atoms. Heme b₉ is colored yellow. Other critical residues involved as putative ligands to the quinone species at the site shown in CPK colors, and labeled by residue number. (Stereo pair for crossed-eye viewing.)
Protein isotopically labeled with $^{15}$N was prepared from *R. sphaeroides* grown in Sistrom media as described previously (36, 37).

**Construction of Mutations at Asn-221**—Conventional PCR-based protocols proved troublesome with the segment of DNA encoding the Asn-221 because of a very high GC ratio. A modification of the three-stage protocol previously developed (35) was used. Forward and reverse primers, which flanked the entire region, were used in conjunction with complementary mutagenic primers in two steps, PCR1 and PCR2, to generate intermediate sequences of similar length that overlapped and were complementary in the region of interest. These were then extended by a third step in which they acted both as primers and templates, and the product of this reaction was further amplified by a fourth step, PCR3. The flanking regions contained restriction sites previously engineered in the plasmid, through which the product could be clipped out and reincorporated into the mutagenic plasmid. A detailed protocol will be provided in the context of a more extensive paper dealing with the properties of these strains.

**Generation of Semiquinone at the Q$_i$-site**—Protocols used to generate semiquinone were as described previously (17, 32). In the experiments shown, the samples were reduced by ascorbate, and the concentrations of the bc$_1$ complex (monomer) in samples used for EPR were in the range 300–500 $\mu$M.

**Kinetic Measurements of Cytochrome Changes**—Changes in concentration of heme $b_{11}$ were measured in a single beam kinetic spectrophotometer designed and constructed in-house, as described previously (7), but with updated electronics interface and software. Experimental details are included in the figure legends.

**X-band EPR Measurements**—The EPR and ESEEM experiments were carried out using an X-band Bruker ELEXSYS E580 spectrometer with an Oxford CF 935 cryostat. Several types of ESEEM experiments with different pulse sequences were employed, with appropriate phase cycling schemes to eliminate unwanted features from experimental echo envelopes. Among them are two-, three-, and four-pulse sequences. In the two-pulse field-sweep experiment ($\pi/2$–$\pi/2$–$\pi$–$\pi$–echo), the intensity of the echo signal at fixed interval, $\tau$, between two microwave pulses with spin vector rotation angles $\pi/2$ and $\pi$ is measured as a function of magnetic field. In the one-dimensional three-pulse experiment ($\pi/2$–$\tau$–$\pi/2$–$\pi$–$\pi$–echo), the intensity of the stimulated echo signal after the third pulse is recorded as a function of time, $T$, at constant time, $\tau$. The Fourier transformation of this echo envelope gives the ESEEM spectrum with the frequency ordinate $v$ (as shown in Fig. 2).

In the two-dimensional four-pulse experiment ($\pi/2$–$\tau$–$\pi/2$–$\tau$–$\pi/2$–$\tau$–$\pi/2$–$\tau$–echo, also called HYSCORE), the intensity of the stimulated echo after the fourth pulse was measured with $t_2$ and $t_1$ varied and $\tau$ constant. Such a two-dimensional set of echo envelopes gives, after Fourier transformation, a spectrum with two frequency coordinates $v_1$ and $v_2$ (as in Fig. 3). The spectrum shows correlations between nuclear frequencies from two manifolds with opposite electron spin projections belonging to the same nucleus, with equal resolution in each frequency coordinate. The data are usually presented as contour plots to show the peak positions in the spectra arising from different nuclear spin interactions.

**S-band ESEEM**—The S-band EPR experiments were performed on a home-built pulsed S-band EPR spectrometer (38). The experiment was controlled with Xper software via an ELEXSYS console, including SpecJet and PatternJet (Bruker BioSpin, Rheinstetten, Germany). The probe was an ER 4118CF liquid helium flow cryostat with a Flexline (Bruker BioSpin, Rheinstetten, Germany) cavity holder and a home-built bridged-loop-gap resonator. The frequency of the microwave source was $\approx 3.4$ GHz, which corresponds to the resonance field $\approx 129$ mT for paramagnetic species with $g$-factor $\approx 2$. Spectral processing of one-dimensional and two-dimensional ESEEM patterns was performed using WIN-EPR software (Bruker).

**The Tensor of Nuclear Quadrupole Interaction**—Nuclei with spin $I > 1/2$ possess a nonspherical charge distribution characterized by a nuclear quadrupole moment, $Q$. The tensor of nqi is a traceless tensor describing the interaction of a nuclear quadrupole moment with the electric field gradient of the surrounding electrons. Its matrix elements (Equation 1) are defined as in Ref. 70,

$$Q_{ij} = eQV_{ij}/(2I(2I - 1)\hbar) \quad (\text{Eq. 1})$$

where $Q_{ij}$ are the components of the electric field gradient tensor, $e$, the electron charge, and $ij = x,y,z$. For the $^{14}$N nucleus with the nuclear spin $I = 1$ (Equation 2),

$$Q_{ij} = eQV_{ij}/2\hbar \quad (\text{Eq. 2})$$

To fully describe the nqi tensor in a principal axes system one needs only two parameters called the quadrupole coupling constant (Equation 3),

$$K = e^2Q/4\hbar \quad (\text{Eq. 3})$$

and the asymmetry parameter shown in Equation 4,

$$\eta = (V_{xx} - V_{yy})/V_{zz} \quad (\text{Eq. 4})$$

with $|V_{xx}| \geq |V_{yy}| \geq |V_{zz}|$, and $V_{zz} = eQ$. The $g$ is defined as the electric field gradient in the same units as $e$, and $\hbar$ is Plank’s constant. The value of $\eta$ lies between 0 and 1, and in the case of axial symmetry one obtains $|V_{xx}| = |V_{yy}|$ and $\eta = 0$.

With these definitions the principal values of the nqi tensor (in frequency units) are equal to that shown in Equation 5,

$$Q_{zz} = 2K, Q_{yy} = -K(1 + \eta), Q_{xx} = -K(1 - \eta) \quad (\text{Eq. 5})$$

The distribution of the electrons depended on the configuration of the bonds around the nitrogen and its occupancy, i.e. it is determined by the type of the chemical group containing the nitrogen. Therefore, the nqi tensors of the $^{15}$N atoms in the different chemical groups are strictly individual and can therefore be used for identification and characterization of the nitrogen. In this study the nqi tensor of the nitrogen involved in the interaction with the SQ was derived using S-band ESEEM.

**Factors Influencing $^{14}$N Powder ESEEM Spectra**—The $^{14}$N nucleus interacting with an unpaired electron spin $S = 1/2$ can produce up to six lines in an ESEEM spectrum. These lines are from transitions between three nuclear energy sublevels in each.
of the two electron spin manifolds with $m_s = +\frac{1}{2}$ or $-\frac{1}{2}$. Because of the different dependence on orientation, not all transitions contribute equally to the spectra in ESEEM measurements of amorphous samples (as in the case of the frozen suspensions of the $bc_1$ complex used in these experiments). The ESEEM spectrum expected from $^{14}$N with predominantly isotropic hyperfine coupling $A$ is governed by the ratio between the effective nuclear frequency in each manifold, $v_{\text{ef},z}$, given by $v_{\text{ef},z} = |v_I + A/2|$, and the quadrupole coupling constant, $K$ (33, 34).

If $v_{\text{ef},z}/K \approx 0$, i.e., $v_{\text{ef},z} \approx 0$ (the situation is called a cancellation condition, because $v_I \approx A/2$) then the three nuclear frequencies from a corresponding manifold will be close to three pure nuclear quadrupole resonance frequencies of $^{14}$N transitions between the energy levels defined by the principal values of the nqi tensor in Equation 5 (Equation 6).

$$v_+ = K(3 + \eta) \quad v_- = K(3 - \eta) \quad v_0 = 2K\eta \quad \text{(Eq. 6)}$$

In this case, three narrow peaks at the frequencies shown in Equation 6 will be present in the powder ESEEM spectra. These frequencies possess the property $v_+ = v_- + v_0$ and can appear in spectra up to a ratio of $v_{\text{ef},z}/K \approx 0.75 - 1$.

If $v_{\text{ef},z}/K > 1$, only a single line without pronounced orientation dependence from each corresponding manifold is expected. This line is produced by a transition at the maximum frequency, which is actually a double-quantum transition between the two nuclear outer states with $m_I = -1$ and $1$. The frequency of this transition is well described by Equation 7 (33),

$$v_{\text{dq},z} = 2(v_{\text{ef},z}^2 + \kappa)^{1/2} \quad \text{(Eq. 7)}$$

where $\kappa = K^2(3 + \eta^2)$. Two other single-quantum transitions, involving the central level with $m_I = 0$, usually do not show any resolved peaks because of significant orientation dependence from quadrupole interaction.

RESULTS

$^{14}$N ESEEM—The SQ in frozen solution of native (with the 99.63% of $^{14}$N natural abundance) and $^{15}$N-labeled $bc_1$ complex has the same line width ~0.8–0.85 mT in X-band continuous wave EPR and in field sweep ESE spectra. This is because the primary contribution to the line shape is given by g-tensor anisotropy, which largely masks the influence of $^{14}$N/$^{15}$N substitution at weakly coupled nitrogen nuclei.

Fig. 2 shows the $^{14}$N X-band three-pulse ESEEM spectrum of the SQ in the Q$_T$-site of the native $bc_1$ complex reported and analyzed in detail in our previous work (17). This spectrum exhibits two lines at frequencies 1.7 and 3.1 MHz. These two frequencies produce cross-peaks in two-dimensional ESEEM spectra and thus belong to opposite electron spin manifolds of a $^{14}$N nucleus. They were assigned to double-quantum transitions $v_{\text{dq},z}$ from two manifolds (see Equation 7). Formal application of Equation 7 to the observed frequencies using $v_I = 1.06$ MHz, corresponding to an external magnetic field 345 mT, gives $A = 0.8$ MHz and $\kappa = 0.43$ MHz$^2$. Assuming that $0 \leq \eta \leq 1$, these lead to $K = 0.35 \pm 0.3$ MHz, and give $v_{\text{ef},z}/K$ ratios ~2.2 and 1.9 (i.e. >1), respectively, providing support for the assignment of both frequencies to a single $^{14}$N, and for the analytic procedure used.

The estimate of $K$ with an accuracy of ~20% allows its assignment to the protonated nitrogen of an imidazole residue (17). Nevertheless, for a complete description of the nqi tensor, and its unambiguous assignment, direct determination of both quadrupole parameters, $K$ and $\eta$, would be desirable. These values could be determined directly from ESEEM experiments satisfying the cancellation condition $v_{\text{ef},z} \approx 0$ at one of the manifolds. For hyperfine coupling $A = 0.8$ MHz, the cancellation condition is reached at $^{14}$N Zeeman frequency $v_I \sim 0.4$ MHz, but this requires an experiment at S-band, with microwave frequency ~3 GHz.

Fig. 2 also shows the S-band three-pulse ESEEM spectrum of the SQ in the $bc_1$ complex obtained at magnetic field 121.9 mT, corresponding to $v_I = 0.375$ MHz. The spectrum contains three intense narrow peaks at 1.14, 1.03, and 0.09 MHz, and a broader line of lower intensity at 1.95 MHz. This spectrum has a shape typical of spectra recorded near cancellation conditions and allows for an immediate assignment of the narrow peaks to three nqi frequencies (see Equation 6) from the manifold with $v_{\text{ef},z} \approx 0$ and of the 1.9 MHz line to a double-quantum transi-
**Q-site Semiquinone in the bc1 Complex**

The intense diagonal peak 1 is a new feature in the $^{15}$N spectra, which is not seen in $^{14}$N spectra. This peak is contributed by all the other $^{15}$N nuclei around the SQ, which are involved only in weak interactions with the unpaired electron. One can note that all three peaks show an almost symmetrical line shape, with approximately rounded contours indicating very low anisotropy of the hyperfine interaction both for the nucleus possessing unpaired spin density and for weakly dipole-coupled nuclei.

The lack of extended anisotropic contours in the cross-peaks 2 along the line normal to the diagonal of the HYSCORE spectrum limits the accuracy of determination of the isotropic and anisotropic components of hyperfine tensor based on a contour presentation in quadratic frequency coordinates (39). Therefore, the isotropic constant $a$, and perpendicular anisotropic component $T$, of the axial hyperfine tensor ($a - T$, $a - T$, $a + 2T$), obtained from such an analysis, can only be used to provide initial values in the simulation of the HYSCORE spectra from the imidazole nucleus.

We were able to reproduce the location of cross-peak maxima and the length of the contour along the line normal to the diagonal with values for the isotropic constant, $a$, of $(-1.1)$ MHz, and for anisotropic constant, $T$, of $\pm (0.15-0.2)$ MHz couplings. The influence of the relative signs of $a$ and $T$ on the location of cross-peak maximum is negligible because of the small anisotropy. The absolute sign of $a$ and $T$ could not be determined from these experiments. The couplings found for the $^{15}$N nucleus correspond to $a = 0.7-0.78$ MHz and $T = \pm (0.1-0.14)$ MHz for a $^{14}$N nucleus. They will be used in the following.

The new intense peak 1 on the diagonal seen in the $^{15}$N spectra has a width at half-amplitude of $\sim 0.2$ MHz in one-dimensional projections on both axes. The peak arises from weakly coupled distant $^{15}$N atom(s), presumably involved only in anisotropic dipole-dipole interaction with the unpaired electron of the SQ. The width of this peak allows us to estimate an upper limit of $T \sim 0.2$ MHz (or $T \sim 0.14$ MHz for $^{14}$N) for any weakly coupled nuclei in the SQ environment contributing to this line.

**Role of Asn-221**—Our previous studies had shown three exchangeable protons, only two of which could be assigned to strong H-bonds to carbonyl O atoms of the SQ. The third, weakly coupled proton was tentatively assigned either to an H-bonded water or to a third putative H-bond seen in some structures of mitochondrial complexes as involving Ser-205, which was within H-bonding distance of a methoxy O atom (17, 32). Asn-221 has been identified as the homologue of Ser-205, and in silico modeling showed that it could also serve as a potential H-bond donor to a quinone bound in the R. sphaeroides Q-site (Fig. 1). The structures of Rhodobacter bc1 complexes now available do not contain any quinone species but show local structure similar to the mitochondrial complexes, from which a common architecture seems plausible, and a quinone can be modeled in these structures without undue distortion to give a structure similar to that of Fig. 1. To explore the possibility that an H-bonding role is essential, we have constructed mutants in which Asn-221 has been modified in strains N221T, N221H, N221I, N221S, N221P, and N221D. Kinetic properties pertinent to identification of the role in...

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**FIGURE 3.** The contour plots of $^{15}$N-HYSCORE spectrum of the SQ in the Q-site of the uniformly $^{15}$N-labeled bc1 complex. The spectrum was determined as described under "Experimental Procedures," with the time between first and second microwave pulses $\tau = 200$ ns; microwave frequency, 9.705 GHz, magnetic field, 345.8 mT; temperature, 90 K.
**Q<sub>i</sub>-site Semiquinone in the bc<sub>1</sub> Complex**

### Table 1

Kinetic characteristics of strains with Asn-221 of cytochrome b modified

| Strain      | Photosynthetic growth | Reduction of heme b<sub>1</sub>t<sup>a</sup> | Fraction of b<sub>1</sub>t reduced<sup>b</sup> | Oxidation of heme b<sub>1</sub>t | Reoxidation (t<sub>1/2</sub>/ms)<sup>c</sup> |
|-------------|-----------------------|------------------------------------------|---------------------------------|-----------------|-----------------|
| Wild type   | + + +                 | 455                                      | 0.05                            | >353            | <1.6            |
| N221ID<sup>d</sup> | + + +             | Similar to wild type                   | 0.8                             | 15              | 12              |
| N221H       | + + +                 | 420                                      | 0.68                            | 75              | 12              |
| N221P       | + + +                 | 442                                      | 0.167                           | 342             | 2.0             |
| N221S       | + + +                 | Similar to wild type                   | 0.5                             | 10.0            |                 |
| N221T       | + + +                 | Similar to wild type                   | 0.37                            | 8.0             |                 |
| N221H       | + + +                 | Similar to wild type                   | 0.75                            |                 |                 |

<sup>a</sup> Rate of reduction of heme b<sub>1</sub>t measured following a 5-μs flash at E<sub>a</sub> ~ 120 mV in the presence of antimycin (units, mol of cytochrome b<sub>1</sub>/mol of bc<sub>1</sub> complex/s) (see Fig. 4 for experimental details).

<sup>b</sup> Fraction of heme b<sub>1</sub>t reduced at the maximal amplitude of the kinetic trace in the absence of antimycin, compared with the fraction reduced in the presence of antimycin.

<sup>c</sup> Rate of reoxidation of heme b<sub>1</sub>t, in the absence of antimycin, by subtraction of the traces in the presence and absence of antimycin (units, mol of cytochrome b<sub>1</sub>/mol of bc<sub>1</sub> complex/s). The rate in the wild-type reflects the rate-limiting reduction of the low potential chain through the Q<sub>i</sub>-site reaction. Since r ~ k(occupancy), and the fraction of heme b<sub>1</sub>t in the reduced form (column 4) is <1, the rate constant must be much greater than that for the limiting step.

<sup>d</sup> Half-times for reoxidation of heme b<sub>1</sub>t were measured from kinetic traces.

Rates of Q<sub>i</sub>-site turnover were similar to wild-type, as judged by kinetics measured over a longer time scale. In some strains the rates measured in the presence of antimycin were inhibited compared with the rates without inhibitor because of a dramatic lowering of the E<sub>a</sub> of heme b<sub>1</sub>t in the presence of antimycin. In these strains, the turnover of the Q<sub>i</sub>-site was estimated from the rate in the absence of antimycin, since the degree of inhibition allowed a significant reduction. The kinetic and thermodynamic properties of the mutant strains are described in detail elsewhere (29) (J. T. Holland, V. P. Shinkarev, and A. R. Crofts, manuscript in preparation).

H-bonding are summarized in Table 1, and example traces of flash-induced kinetics are shown in Fig. 4. The function of the Q<sub>i</sub>-site, as assayed by the rate of reduction of heme b<sub>1</sub>t in the presence or absence of antimycin, was unaffected. However, the kinetics at the Q<sub>i</sub>-site were inhibited in all strains. The inhibited kinetics for reoxidation of heme b<sub>1</sub>t in the absence of antimycin show that delivery of electrons to the Q<sub>i</sub>-site was impaired. Although comparison of half-times might suggest that the degree of inhibition was not great, it is likely that the effect on the rate constant was much greater, as discussed below. Nevertheless, all mutant strains were able to grow photosynthetically at close to normal doubling times, and the bc<sub>1</sub> complex turned over during the 20 ms between flashes, demonstrating that the specific properties of the asparagine side chain are not essential for turnover of the site.

**DISCUSSION**

**Transferred Spin Density and the Location of the H-bonded Nitrogen Atom**—The existence of a nonzero isotropic constant, a, for the interacting imidazole nitrogen indicates that unpaired electron spin density is transferred from the SQ onto this atom. The isotropic constant is determined mainly by the unpaired spin density in its 2p<sup>3</sup> orbital. It is proportional to the value of 1811 MHz for <sup>14</sup>N, which is computed for unit spin density in this orbital (40). Thus the isotropic hyperfine coupling ~0.7–0.78 MHz determined from HYSCORE spectra corresponds to the transfer of only a small fraction of this computed spin density, with a < 0.4 ± 0.03)·10<sup>-3</sup> on the 2s orbital of nitrogen.

The 2s<sup>2</sup>2p<sup>3</sup> valence shell of the nitrogen atom consists of four orbitals. One of them is a lone pair orbital. Usually the 2s orbital is involved in hybridization with p orbitals. The type of hybridization depends on the valence state and the bond geometry. The imine (N<sup>+</sup>) or amine (N<sup>−</sup>) nitrogens of imidazole residues are bonded with a hydrogen atom involved in H-bonds that possess sp<sup>2</sup> hybridization (41). The wave function of the hybridized orbital forming the N-H bond can be expressed as shown in Equation 8 (42),

\[
\psi = c_s|2s\rangle + c_p|2p\rangle \tag{Eq. 8}
\]

with \(c_s^2 + c_p^2 = 1\) and \(c_s = \cot \theta\). The \(c_s^2\) population of this orbital is determined by \(\cot^2 \theta\), where 29 is a CNC angle ~108–110°. This gives \(c_s^2 \sim 0.5\), suggesting that a similar spin density \(c_p^2\) must reside on the 2p orbital.

In contrast to the isotropic constant, the anisotropic hyperfine tensor is the result of two factors as follows: dipole-dipole coupling and spin transfer. The dipole-dipole contribution to the hyperfine tensor is determined chiefly by the O . . . N distance. The typical approach used for estimation of this contribution considers the dipole-dipole interaction between the nucleus and the unpaired spin density localized on the nearest carbonyl oxygen of the SQ (Equation 9),

\[
T = \rho_\sigma(g_\sigma\beta_\sigma\beta_h/h^2) = \rho_\sigma(8/r^3) \tag{Eq. 9}
\]

where \(\rho_\sigma\) is the \(\pi\) spin density at the quinone oxygen, and \(r\) is the O . . . N distance, \(g_\sigma\) and \(\beta_\sigma\) are the electronic and nuclear g-factors, and Bohr and nuclear magnetons, respectively. The value \(\rho_\sigma\) is estimated to be ~0.2 from experiments with <sup>17</sup>O labeled SQs both in vitro and in vivo (43, 44). Using Equation 9, one can estimate, for instance, that \(T_{dd} < 0.09–0.11\) MHz for O . . . N H-bonds, which typically have distances \(r > 2.6\) Å in well resolved x-ray crystallographic structures. The value of \(T_{dd}\) would increase for shorter O . . . N distances.

Unpaired spin density transferred onto the 2p orbital determines the second contribution to the anisotropic tensor. The computed value of \(T_{pp}\) for a 2p electron is 55.5 MHz, which is about 32 times less than the value for isotropic coupling obtained for a 2s electron (40). For a spin density ~0.4 × 10<sup>−3</sup> on the 2p orbital one can find the \(T_{pp} \sim 0.02\) MHz. Thus, the dipole-dipole interaction provides the major contribution to the anisotropic hyperfine tensor of the imidazole nucleus. The contribution from 2p spin density falls within the accuracy of the experimental determination of \(T \sim 0.1–0.14\) MHz, which can be used for estimation of the O . . . N distance using Equation 9. Substitution then gives 2.38 ± 0.13 Å for the H-bond distance, within the range shown for the H-bond in the mitochondrial structure (2.40 Å to N<sup>−</sup> in Protein Data Bank code 1pp9).

The Characteristics of H-bonded Imidazole Nitrogen—The nqi characteristics for imidazole N<sup>6</sup> and N<sup>−</sup> have been extensively studied in different systems, including model compounds, and in several series of similar compounds with specif-
ically varying characteristics. The well pronounced linear correlation between reciprocal \( h/e^2 qQ \) and \( \eta \) has been found, for instance, for the imine \( ^{15}N \) coordinating the metal in zinc and cadmium imidazole complexes, and for the amine \( ^{15}N \) in these complexes and in a copper dien-substituted imidazole model and copper proteins. The slope of these correlations was \(~0.3 \pm 0.05\) MHz\(^{-1} \) (39, 45).

The formation of an H-bond between SQ and a histidine nitrogen, specifically \(^{15}N \), has been previously proposed for the several quinone sites on the basis of EPR spectroscopy, and correlated with structural evidence from x-ray crystallography and Fourier transformation IR spectroscopy. The nqi parameters and hyperfine coupling for these nitrogens are listed in Table 2. They show values for the quadrupole coupling constant \( K \) within 0.35–0.43 MHz and \( \eta \sim 0.6–0.8 \). Our S-band measurements have provided us with nqi parameters \( K = 0.36 \) MHz, and \( \eta = 0.17 \) for the imidazole nitrogen involved in hydrogen bond formation with the SQ. The value of \( K \) is in good agreement with previously reported values, but the asymmetry parameter is significantly lower than in all other sites. Assuming that the linear dependence between \( 1/K \) and \( \eta \) exist for the \(^{15}N \) nitrogens H-bonded with the SQs, one can estimate that a higher value for \( K \), in the range \(~0.55\) MHz, would correspond to an asymmetry parameter of this low value.

An explanation of the linear dependence of \( h/e^2 qQ \) versus \( \eta \) was developed in the context of the Townes-Dailey model (46), based on consideration of the electron population of the bonding orbitals of the nitrogen by \( \beta \) electrons. This analysis assumes that in comparison between different systems, the geometry of the nitrogen environment does not change, and this is likely true within the same class of compounds. However, significant deviations from linearity have been reported for some compounds, and these have been assigned to external influences on the \(^{15}N \) nitrogen (45).

The role of such external influence can be illustrated using the nqi data for the \(^{15}N \) and \(^{14}N \) atoms in \( \tau \)-histidine monochloride monohydrate where the crystallographic structure is also available (41). The quadrupole parameters reported for imine \(^{15}N \) and amine \(^{14}N \) nitrogens in noncoordinated imidazole and histidine are equal to \( K = 0.81–0.84 \) MHz, \( \eta = 0.13 \) (\( \kappa = 1.98–2.12\) MHz\(^{-2} \)), and \( K = 0.35 \) MHz, \( \eta = 0.915–0.996 \) (\( \kappa = 0.47–0.49\) MHz\(^{-2} \)), respectively (47–49). Both imidazole nitrogens are protonated in \( \tau \)-histidine monochloride monohydrate. Similar local molecular environments of the \(^{14}N \) and \(^{15}N \) nuclei lead to the expectation of similar electronic structures, and similar \(^{15}N \) nqi tensors. However, the quadrupole parameters become \( K = 0.32\) MHz, \( \eta = 0.946–0.974 \) (\( \kappa = 0.40\) MHz\(^{-2} \)) for \(^{14}N \), and \( K = 0.366\) MHz, \( \eta = 0.268–0.3 \) (\( \kappa = 0.41\) MHz\(^{-2} \)) for \(^{15}N \), i.e. the \(^{15}N \) has characteristics similar to the amine nitrogen in solid imidazole, whereas those of the \(^{14}N \) deviate from them considerably. The nqi diversity observed was explained by differences in intermolecular hydrogen bonding at each nitrogen. For the \(^{14}N \)-H . . . O hydrogen bond, the \(^{14}N \) and H . . . O distances are 2.64 and 1.58 A, respectively, and the bond angle is 171°, whereas for the \(^{15}N \)-H . . . O hydrogen bond, the \(^{15}N \) and H . . . O distances are 2.83 and 1.94 A and the bond angle 143° (50). The analysis of the nqi tensors using the Townes-Dailey model revealed that shorter hydrogen bonding at the \(^{15}N \) nitrogen site caused a greater electron population in the N-H orbital than that in the \( \pi_{a} \) orbital, whereas for the \(^{14}N \) those populations are essentially equal (49).

The hydrogen bond with histidine nitrogen in the \( Q_{\gamma} \)-site possesses unique characteristics. The unusually small value of the \(^{14}N \) asymmetry parameter is accompanied by the smallest value of the hyperfine coupling among all available data for Q-sites determined from ESEEM spectra (Table 2). The \(^{15}N \) data provide the relatively short O . . . N distance \(~2.4\) Å for this H-bond consistent with the O . . . N \(^{14}N \) distance of \(~2.40\) Å in Protein Data Bank code 1pp9. One can therefore propose that the unique characteristics of the H-bonded nitrogen are associated with the \(^{15}N \) of His-217 with the short O . . . N \(^{15}N \) distance. The presence of an exchangeable proton with anisotropic coupling \( |T_{s}| = 5.5\) MHz (32), corresponding to the estimated O . . . H distance \(~1.4\) Å (51) for the in-plane H-bond geometry, supports this assignment. This estimate suggests a close to linear geometry for this H-bond, like that for \(^{14}N \) in \( \tau \)-histidine monochloride monohydrate, which is the property of a strong hydrogen bond (52).

In the light of these results one can compare available structural information for the SQ in the \( Q_{\gamma} \)-site with those at the \( Q_{\lambda} \) and \( Q_{\beta} \) sites in the reaction center, which have been most extensively studied so far, and where the H-bonds with histidine \(^{15}N \) possess typical characteristics. The x-ray structure of the reaction center shows the O . . . N \(^{15}N \) distances 2.9 and 2.8 Å (\(~0.3\) Å) for \( Q_{\lambda} \) and \( Q_{\beta} \)-sites, respectively (53), with modeled O . . . H distance \(~2.0\) Å and O . . . H . . . N angle 143°. However, a recent \(^{1}H \) and \(^{2}H \) ENDOR study (54) has reported the hyperfine and nqi tensor for the proton or deuteron in the O . . . H . . . N \(^{15}N \) (His M219). This proton is characterized by an axial hyperfine tensor with \( T = -5.2\) MHz. The O . . . H distance \(~1.60±0.04\) Å was determined from the nqi tensor. The estimate from the hyperfine coupling using a point-dipole model gives an even shorter distance \(~1.30±0.04\) Å. The O . . . H . . . N angle is \(~160±9°\). This comparison shows that despite a very close value of the hyperfine couplings for the proton involved in H-bond with histidine, the nitrogens in the \( Q_{\lambda} \) and \( Q_{\beta} \) sites possess significantly different nqi tensors.

In addition, the effect of hydrogen bonding to the primary quinone \( Q_{\lambda} \) in bacterial reaction centers was studied by Sinnecker et al. (55) and Fritscher et al. (56) through DFT calculations. Optimization of the hydrogen atom positions of model systems extracted from 15 different x-ray structures derived mean values of the H-bond lengths and directions for the charge neutral \( Q_{\lambda} \) state. It was found that His M219 forms an H-bond to \( Q_{\lambda} \) with O . . . H distance 1.79 Å and \(^{15}N \)-H . . . O angle N–H . . . O angle of 170°. Two geometry optimizations with a different number of flexible atoms were performed for the anion radical \( Q_{\lambda}^{-} \). The calculations revealed that the length of this bond is \(~1.65\) Å with similar angle. In addition the change in \(^{15}N \) . . . O distance was reported from 2.8 to 2.69 Å.

The different structural models of the quinone-binding site consist of \( Q_{\lambda} \) and several of the surrounding amino acid residues with coordinates taken from the crystal structure of Stowell et al. (53) were used in Ref. 56. This work reports \(^{15}N \)–O and H . . . O distances 2.66 and 1.71 Å and reasonable agreement with ENDOR-derived (60) hyperfine tensor for H-bonded pro-
ton of His M219 for the model of $Q_{\alpha}^-$ state, including maximal number of surrounding amino acid residues.

DFT calculations of the influence of hydrogen bond geometry on $^{14}N$ nqi tensor for the systems imidazole-water and methylimidazole-benzosemiquinone have been reported by Fritscher (57) and reveal a strong dependence of the nqi tensor on intermolecular arrangement, especially for the asymmetry parameter $\eta$. The largest influence was found for a variation of
the O . . . N length, whereas the effects of an in- or out-of-plane distortion of the bond geometry were found to be less pronounced. However, results for the N\(^{4}\) and N\(^{6}\) of L-histidine hydrochloride monohydrate, for Q\(_{\gamma}\), and for the Q\(_{\alpha}\) and Q\(_{\delta}\) sites discussed above, are in contradiction with the calculations that predict a decrease in the parameter \(\eta\) with an increase of O . . . N distance (57). On this basis, one can therefore suggest that the results of calculations performed for imidazole in vitro with only one protonated nitrogen might not reproduce correctly the nqi characteristics for a group with both N\(^{4}\) and N\(^{6}\) protonated, and thus may not be directly applicable to an explanation of the differences obtained for N\(^{4}\) and N\(^{6}\) in situ. A model with both nitrogens protonated would be more appropriate in this case. This suggestion finds support in the calculations of nqi tensors for the N\(^{4}\) and N\(^{6}\) of His M219 in Q\(_{\alpha}\) state by Fritscher et al. (56), which demonstrated the best agreement with experiment when the model included interaction of the N\(^{4}\) with Zn\(^{2+}\).

In particular, the disagreement between our observation and the theoretical predictions for the N\(^{4}\) of His-217 in the Q\(_{\gamma}\) site allow us to propose that the N\(^{4}\) of His-217 in the Q\(_{\gamma}\)-site might also be protonated. There is no protein group to serve as an alternative partner. However, the schematic model for the reaction sequence we discussed previously (17) suggested an involvement of this histidine in protonation on formation of the SQ. Because the SQ has the characteristics of the anionic state (Q\(^{+}\)), protonation of the SQ state following electron transfer would be expected to involve a site other than the SQ itself, and this histidine represents an attractive possibility.

Another potentially informative characteristic of the interacting N in the Q\(_{\gamma}\)-site is the observed value of the isotropic hyperfine coupling, which is smallest among all reported values, despite the evidently short hydrogen bond O . . . N distance. A nonzero isotropic coupling, \(a_{\text{i}}\), for the interacting 14N nucleus indicates that nonzero unpaired electron spin density is transferred from the SQ onto this nucleus. Unfortunately, the work by Fritscher (57) did not report the variations of 1H and 14N hyperfine couplings accompanying the changes of the nqi tensor. Hybrid density functional calculations for an imidazole-H-bonded to a phenol radical suggest that spin polarization through the H-bonded proton transfers spin density to the interacting nitrogen nucleus from the \(\sigma\) spin density present on the O atom (58). The recent calculations report the isotropic hyperfine couplings 2.15 MHz (55) and ~2.5 MHz (56) for N\(^{6}\) of His M219 of the Q\(_{\alpha}\) state but do not provide any discussion of the values or of the mechanism of spin density transfer. In addition, the results by Fritscher et al. (56) have excluded possible assignment of the double-quantum peak in the three-pulse 14N ESEEM spectra to N\(^{6}\) of His M219 (59). However, this double-quantum peak was used for estimation of the hyperfine coupling of 1.8 MHz with the His M219 nitrogen. Thus, the real value of the hyperfine coupling with this histidine N\(^{6}\) remains questionable.

Further calculations are also needed for a quantitative understanding of the mechanism of spin density transfer, and its relation to the geometry and strength of the H-bond. Particularly, it will be necessary to analyze the transfer of spin density over the H-bond for different O . . . N distances and O . . . H . . . N configurations. This is important in light of the role of H-bonds and the distribution of unpaired spin density on rates of electron and proton transfer (60).

Other Nitrogens—In accounting for the other 15N interaction observed (the diagonal peak I), we note that the structures show a local environment relatively poor in N. In the bovine structure (Protein Data Bank 1pp9), only the histidine N\(^{4}\) (at 2.4 Å) is within 4 Å of the SQ; other close N atoms are the histidine N\(^{6}\) (at 4.49 Å) and peptide N atoms from Ser-35 (4.24 Å) and Ser-205 (5.71 Å). In the Rhodobacter structures, no ubiquinone is modeled, so distances cannot be assigned. However, the only additional close N atom is that from the side chain of Asn-221 (which in the conformation modeled in Fig. 1 is 2.93 Å), which “replaces” Ser-205, as already discussed. It seems unlikely that dipole coupled N atoms at greater distance, for example those of the heme (nearest N at 7.18 Å), would contribute significantly to this diagonal peak, because the ESEEM amplitude caused by dipole-dipole interactions fall off rapidly, with 1/r\(^{6}\) distance dependence. The failure to detect additional isotropic hyperfine couplings from N atoms other than the histidine N\(^{6}\) ligand might suggest that no other side chain N atom H-bonds with an SQ carbonyl oxygen. However, one cannot exclude the possibility that Asn-221 forms an H-bond with the methoxy oxygen on the basis of the 15N-ESEEM. The DFT calculations of
spin density distribution in the anion radical of the ubiquinone give a value of $p_n \sim 0.01-0.02$ for the methoxy oxygen, depending on the orientation of the methoxy group, which would give an estimate of the anisotropic hyperfine component $T < 0.005$ MHz. On the one hand, the width of the central peak is not located at a distance of 2.4 Å from the nearest carbonyl oxygen. However, an H-bond from a bridging H$_2$O to the carbonyl involved in the H-bond to the N$^\alpha$ of histidine, seen in some structures (26, 61), is not consistent with the distances measured. To show the same interaction, a proton would need to be at a distance $\sim$1 Å from a methoxy oxygen, but this is too close for an H-bond between Asn-221 and this oxygen. We therefore tentatively assign this proton to a weak H-bond from H$_2$O to the SQ-carbonyl that H-bonds to Asp-252, which is seen as a crystallographic water in Protein Data Bank code 1pp9, but we cannot exclude a nonbridging H$_2$O H-bonded to the other carbonyl.

**Mechanistic Implications**—Although the ESEEM data and characteristics derived from them do not allow us to resolve the question of the involvement of Asn-221 in H-bonding to the SQ, our studies of mutations at Asn-221 have shown that, although some strains were quite strongly inhibited, this residue is not essential. Because mutants with substitution by proline or isoleucine were also active, we can say that if any H-bonding from the side chain occurs, it does not contribute a major binding term. Because the kinetics seen in the N221P strain appeared to be the least affected, we can also conclude that no H-bonding potential from the backbone –NH is needed. Neither does the polarity of the side chain appear to be an essential feature. Rather, the site can accommodate a wide range of side chain characteristics, because all mutant strains were able to grow photosynthetically, and the mutant strains, even though the occupancy of reduced heme $b_{14}$, as indicated by the maximal amplitude, approached 1. From these considerations, the rate constant is probably slowed by factors between 4 and 100 in the different strains.

As discussed previously (17, 32), the equivalent serine side chain in the bovine structure is involved in H-bonding to the heme propionate through a water bridge, and the asparagine chain in the bovine structure is involved in H-bonding to the heme propionate. The kinetic implications of these H-bonding considerations, the rate limit is not expected from the values in Table 1. The rate of heme $b_{14}$ oxidation in wild type is rapid compared with the rate of reduction. The kinetics observed reflect competing reduction and oxidation rates, in which the latter is determined by the rate constant for heme $b_{14}$ oxidation and the occupancy, $\nu = k_{cat}(\text{occupancy})$, where occupancy refers to the reaction complex. A maximal value for this can be obtained from the extent of the transient reduction of heme $b_{14}$, which is $<5%$ in wild type (Table 1 and Fig. 3). This occupancy indicates a rate constant $>10^4$ s$^{-1}$, 20-fold higher than the value of $\sim 500$ s$^{-1}$ needed to match the rate-limiting reaction at the Q$_o$-site that determines the rate of reduction of heme $b_{14}$. On the other hand, in the mutant strains, the reoxidation time was slower than the rate-limiting step, markedly so in the more inhibited strains, even though the occupancy of reduced heme $b_{14}$, as indicated by the maximal amplitude, approached 1. From these considerations, the rate constant is probably slowed by factors between 4 and 100 in the different strains.

**Acknowledgments**—We thank Dr. A. A. Shubin for providing us with unpublished results from DFT calculations on semiquinones. National Institutes of Health NCRR Grant S10-RR15878 was provided for instrumentation.

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Hydrogen Bonds between Nitrogen Donors and the Semiquinone in the Q$_1$-site of the bc$_1$ Complex
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J. Biol. Chem. 2007, 282:25831-25841.
doi: 10.1074/jbc.M702333200 originally published online July 5, 2007

Access the most updated version of this article at doi: 10.1074/jbc.M702333200

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