*Biochimica et Biophysica Acta*, 1015 (1990) 141–149
Elsevier



BBABIO 43114

# Formation by NO of nitrosyl adducts of redox components of the Photosystem II reaction center. II. Evidence that $HCO_3^-/CO_2$ binds to the acceptor-side non-heme iron

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(Received 14 April 1989)
(Revised manuscript received 29 August 1989)

Key words: Photosystem II reaction center; Nitric oxide; Non-heme iron; ESR; Bicarbonate; Quinone-iron complex

**We have demonstrated (Petrouleas and Diner, accompanying paper) that NO binds to the non-heme iron of the PS II reaction center. We show here that, in spinach chloroplasts, NO ($K_d \approx 30$ μM), like formate, slows electron transfer between the primary and secondary quinone electron acceptors of PS II, $Q_A$ and $Q_B$, respectively. In a series of saturating flashes given to dark-adapted chloroplasts treated with NO, this electron transfer is slowed by at least a factor of 10 from the second saturating flash excitation onward as compared to untreated chloroplasts. This slowing is completely reversed by the addition of $NaHCO_3$ (10 mM), indicating that NO, like formate, displaces bicarbonate from the reaction center. The NO-enhanced dissociation of $HCO_3^-$ from the reaction center is pH-dependent, occurring much faster at pH 5.5 than at 7.4. In the reverse experiment, the $S = 3/2$ Fe(II)-NO EPR signal at $g = 4$ is diminished by the addition of $NaHCO_3$, indicating that $HCO_3^-$ dissociates the NO ligation to the iron. These data argue in favor of $HCO_3^-/CO_2$ as a ligand to the iron. Formate does not dissociate NO from the iron and it is possible that formate and NO displace $HCO_3^-/CO_2$ by different mechanisms.**

## Introduction

Electron transfer between the primary and secondary quinone electron acceptors, $Q_A$ and $Q_B$, respectively, of the Photosystem II (PS II) reaction center is strongly modulated by bound bicarbonate (for reviews see Refs. 1, 2). Depletion of this species by either exposure to pH 5 [3] or treatment with a number of anions, particularly formate [4], leads to a slowing of electron transfer between the quinones [5–9]. In dark-adapted chloroplasts, depletion of bicarbonate by formate results in a moderate slowing (approx. 4-fold) following the first saturating flash ($Q_A^-Q_B \rightarrow Q_AQ_B^-$) and considerably greater slowing (factor of 10–30) for the second and third saturating flashes and beyond ($Q_A^-Q_B^- \rightarrow Q_AQ_BH_2$) [8,9]. Readdition of bicarbonate results in a restoration of the rates observed prior to treatment [5–9]. Bound bicarbonate has been suggested to act as either an allosteric activator for the reduction of $Q_B$ [10] and release of $Q_BH_2$ [10] or as a participant in protonation reactions associated with reduction of $Q_B$ to the semiquinone anion [9] or of $Q_B^-$ to the quinol [11].

There is some disagreement as to the active species responsible for the above described modulation of electron transfer rates. Blubaugh and Govindjee [10] have argued that the active species is bound bicarbonate based on equilibrium measurements as a function of pH, while Stemler [12] has argued for $CO_2$ based on kinetics of binding of $^{14}C$. We prefer at the moment not to take sides in this discussion, and will refer to the active species as $HCO_3^-/CO_2$.

It has recently been suggested by a number of workers [8,9,13–18], that $HCO_3^-/CO_2$ is bound onto or in close proximity to the acceptor-side, non-heme iron of the reaction center. These arguments are indirect and are based: (a) on the effect of $HCO_3^-/CO_2$ on electron transfer rates between $Q_A$ and $Q_B$ described above [5–9]

Abbreviations: BBY, Berthold, Babcock, Yocum [20]; DMSO, dimethylsulfoxide; EPR, electron paramagnetic resonance; $F$, fluorescence yield following illumination; $F_0$, initial fluorescence yield preceding illumination; Mes, 4-morpholinethanesulfonic acid; PS II, Photosystem II; $Q_A$, primary quinone electron acceptor of PS II; $Q_B$, secondary quinone electron acceptor of PS II.

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and the location of the non-heme iron midway between the two [13]; (b) on the effects of low pH [14,15] and formate [16] on the EPR spectrum of $Q_A^-Fe(II)$, on the Mössbauer spectrum of Fe(II) [17] and on the midpoint potential of the Fe(III)/Fe(II) redox couple [18]; and (c) on comparison of the primary structure of the L and M polypeptides of the bacterial reaction centers as compared to the D1 and D2 polypeptides of PS II, with the suggestion that $HCO_3^-$ might replace glutamate as a ligand to the iron [13].

We have recently shown that NO binds to the non-heme PS II acceptor-side iron giving rise to a typical Fe(II)-NO, $S = 3/2$ EPR signal at $g = 4$ [19]. We show here that this is the site at which NO and $HCO_3^-/CO_2$ compete for binding to the reaction center. We consequently provide here direct evidence in favor of $HCO_3^-/CO_2$ binding to the non-heme iron.

## Materials and Methods

*Photosynthetic membranes*

Thylakoid membrane fragments (BBY membranes [20]) were prepared as described in Ref. 19. After the final centrifugation step, the membranes were resuspended at 10–15 mg Chl/ml in 5 mM Mes-NaOH (pH 6.5), 15 mM NaCl, 5 mM $MgCl_2$ and 0.4 M sucrose, frozen in liquid $N_2$ and stored at $-80\,^\circ C$ until use.

Chloroplasts were prepared according to Avron [21] from spinach grown in growth chambers. Chloroplasts were resuspended in 50 mM Tris-HCl (pH 7.5) containing 10 mM NaCl, 0.4 M sucrose and 5% DMSO and stored at $-80\,^\circ C$.

*Electron spin resonance spectroscopy*

The electron spin resonance spectrometer, cryostat and illumination conditions were as described previously [22]. Measurement conditions are as described in the figure legends.

*Optical spectroscopy and fluorescence relaxation kinetics*

A flash-detection spectrophotometer similar to that designed by Joliot et al. [23] was used to measure rates of oxidation of $Q_A^-$ by $Q_B$ in *Rb. sphaeroides* reaction centers (a gift of Dr. Kathleen Giangiacomo), by detection of absorbance changes at 747 nm [24] following a saturating laser flash (Candela model SLL250 using

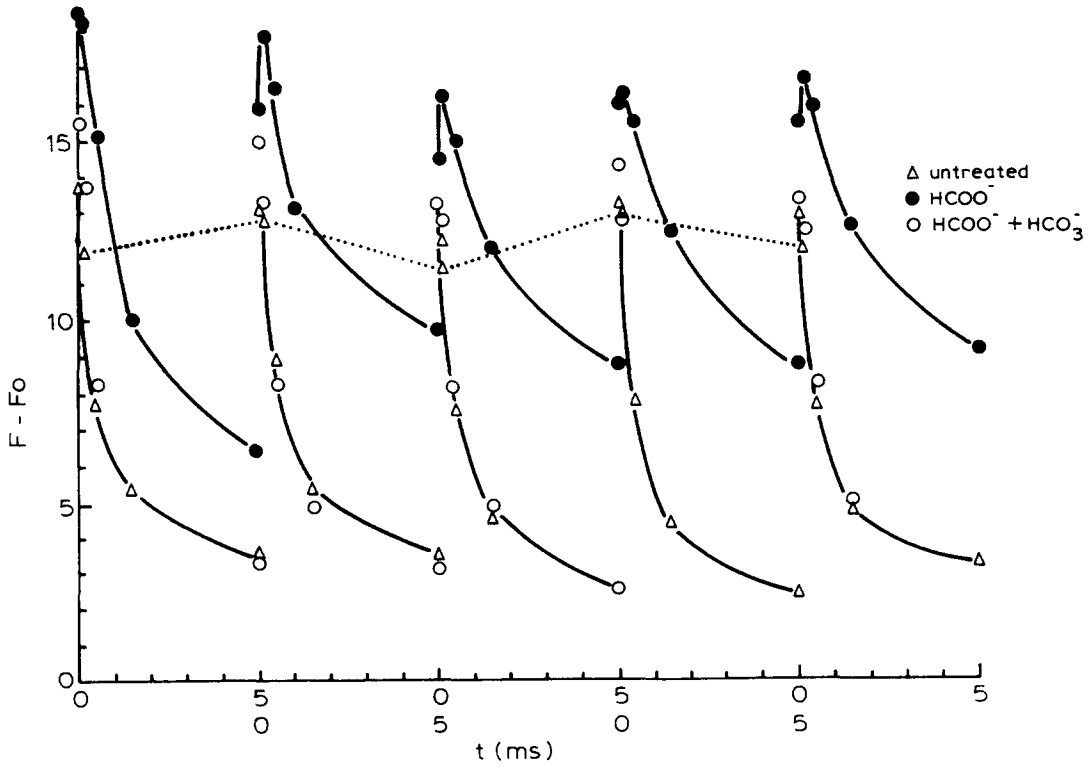


Fig. 1. Effect of formate incubation and bicarbonate addition on the kinetics of fluorescence yield relaxation, following each of a series of five saturating flashes (0.68 Hz). The fluorescence yield, $F$, following flash illumination, is measured relative to the initial fluorescence yield, $F_0$, preceding illumination. Spinach chloroplasts (250 μg Chl/ml) were suspended in 0.3 M sorbitol, 50 mM sodium phosphate (pH 6.1), 10 mM NaCl, 5 mM $MgCl_2$ containing 10 mM sodium formate. The buffer had been previously bubbled with a stream of $N_2$ for 5 min, to reduce the $CO_2$ concentration. Incubation with formate proceeded for 1 h at 25 °C under an $N_2$ atmosphere. Just prior to measurement, the chloroplasts were diluted to 10 μg Chl/ml in the same buffer adjusted to pH 6.3, previously bubbled with $N_2$. The kinetics of relaxation of the fluorescence yield were measured after each actinic flash in this material and after the addition of 10 mM $NaHCO_3$. Control chloroplasts were diluted directly to 10 μg Chl/ml in pH 6.3 buffer without formate and measured.

Sulforhodamine 640, $\lambda_{max} = 645$ nm, 600 ns duration). The sample photodetector (UV-444BQ, EG&G Co. – was protected by a Corning 7-96, 2-64 and a Corion 750 nm (10 nm bandpass interference filter). This instrument was also used in the fluorescence detection mode for the experiments involving spinach chloroplasts. A saturating actinic xenon flash was filtered by an infrared reflecting filter (MTO Athervex TA2) and a Corning 4–96. The fluorescence yield was detected using a probe flash at 422 nm, spaced at various times after the actinic xenon flash. The chlorophyll fluorescence was filtered through a set of blocking filters – Schott KV550, Ulano Rubylith, Corning 2-64 and a Kodak Wratten 70, together transmitting at 670 nm or above.

## Results

Fig. 1 shows the kinetics of relaxation of the quantum yield of fluorescence following each of a series of five saturating flashes at pH 6.3 in untreated dark-adapted spinach chloroplasts. These show half times of several hundred microseconds relaxation. To a first approximation these kinetics indicate the reoxidation of $Q_A^-$ by $Q_B$ and $Q_B^-$. While sufficient for the demonstration we wish to make here, an exact measurement of the rate of $Q_A^-$ reoxidation would require correction for energy transfer between PS II reaction centers [25]. At intermediate times (150 $\mu$s (...), Fig. (1) one observes an oscillation of period two of the fluorescence yield, consistent with the two-electron quinone gate functioning on the acceptor side of the PS II reaction center, faster on the odd-numbered flashes ($Q_A^-Q_B \rightarrow Q_AQ_B^-$) and slower on the even-numbered flashes ($Q_A^-Q_B^- \rightarrow Q_AQ_BH_2$) [26]. At longer times there is an oscillation of period four, which is dependent on the S states of the oxygen-evolving complex (fluorescence yield low in states $S_0$ and $S_1$, high in states $S_2$ and $S_3$, [27]). The chloroplasts were treated with formate to remove bound $HCO_3^-/CO_2$ as described in the figure legend (similar to Ref. 11) and the fluorescence relaxation was again measured following each saturating flash. As previously noted [8,9], the relaxation is slowed to a half-time of 2 ms following the first flash and to 5–10 ms on the second and subsequent flashes. The kinetics returned to the control rates following the addition of 10 mM $NaHCO_3$ to the formate-treated chloroplasts at the same pH (6.3, Fig. 1). Observations of this type have been interpreted as demonstrating the requirement of bound bicarbonate for rapid electron transfer between the quinones, with formate competing with bicarbonate for binding to the reaction center [1].

It has been proposed by a number of authors, based on indirect arguments, that the non-heme iron of the reaction center might be a potential binding site for bicarbonate (see Introduction and Refs. 9,13,15,17,29). We sought direct experimental evidence for such liga-

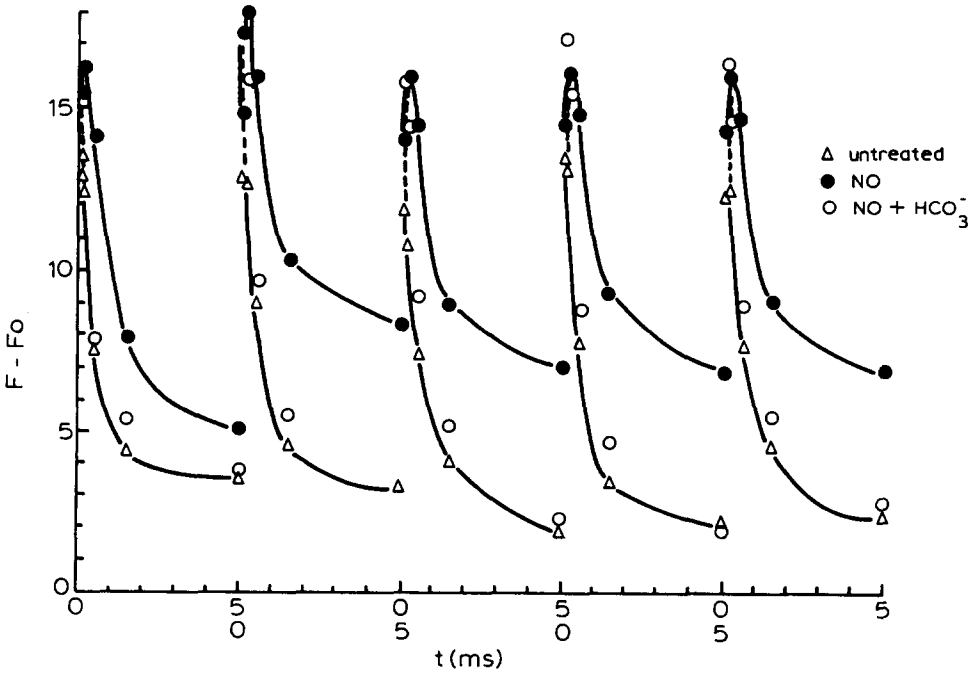


Fig. 2. Effect of NO incubation and bicarbonate addition on the kinetics of fluorescence yield relaxation, following each of five saturating actinic flashes (0.68 Hz). Spinach chloroplasts (10 $\mu$g Chl/ml) were suspended in 0.3 M sorbitol, 50 mM sodium phosphate (pH 6.3, 10 mM NaCl, 5 mM $MgCl_2$ previously bubbled with $N_2$. NO (30 $\mu$M) was added from a 3.3 mM stock solution (saturated solution in water at 0 °C). The chloroplasts were incubated for 45 min at 25 °C in a sealed vial free of $O_2$. The kinetics of relaxation of the fluorescence yield were measured after each actinic flash in this material and after the addition of 10 mM $NaHCO_3$ from a stock solution of 1 M $NaHCO_3$ previously made anaerobic by bubbling with $N_2$. The control chloroplasts were as in Fig. 1.

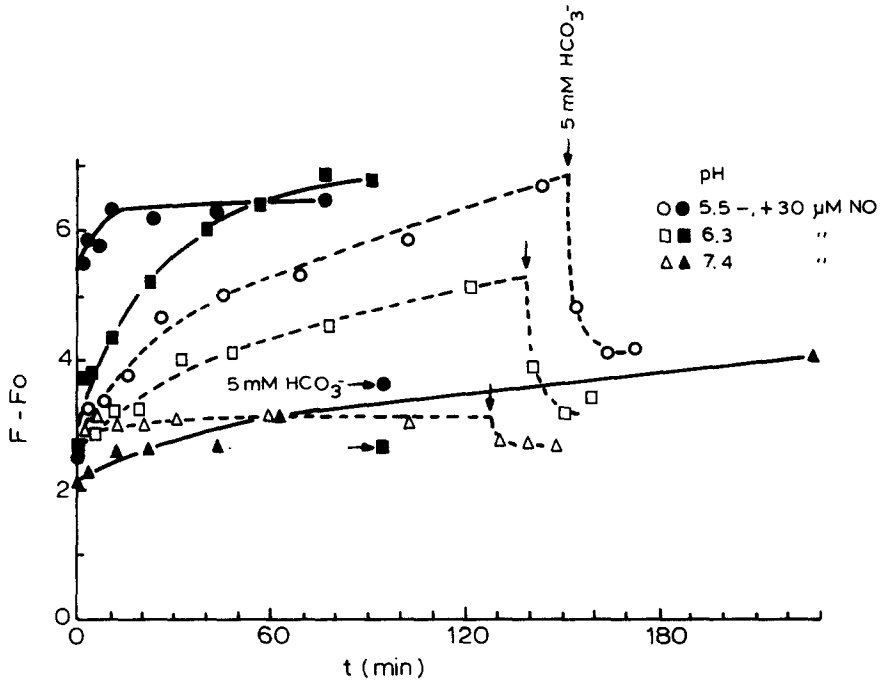


Fig. 3. Effect of pH and NO on the fluorescence yield at 5 ms following the second of two saturating flashes (0.68 Hz). At time zero, spinach chloroplasts (10 μg Chl/ml) were suspended in the same buffer as in Fig. 2 but at pH 5.5, 6.3 or 7.4 without NO or with 30 μM NO, added from a 3.3 mM anaerobic stock solution. The vertical arrows indicate the addition of 5 mM $NaHCO_3$. The points marked by the horizontal arrows were measured 5 min after the addition of 5 mM $NaHCO_3$.

tion and have used as a probe NO, which we showed in the accompanying paper [19] to ligate to the iron. Were $HCO_3^-/CO_2$ to compete with NO for binding, then one might expect NO binding to induce a formate-like effect on the electron transfer from $Q_A$ to $Q_B$. Fig. 2 shows that this is indeed the case. The addition of NO slows electron transfer between the quinones. This effect is considerably greater, as for formate, on the second and subsequent flashes than on the first. The concentration used in Fig. 2 is only 30 μM NO, consistent with a $K_d$ of 30 μM for binding of NO to the non-heme iron in spinach chloroplasts [19]. Higher concentrations of NO are not shown as they quench chlorophyll fluorescence. Addition of 10 mM $NaHCO_3$, even in the presence of 30 μM NO, results, as in the case of formate treatment, in a return of the electron transfer rate to control levels. Thus NO, like formate, dissociates bound $HCO_3^-/CO_2$ from the reaction center at low concentrations of bicarbonate. Upon raising its concentration, $HCO_3^-/CO_2$ is rebound.

The $K_d$ of $HCO_3^-/CO_2$ binding to the reaction center is known to increase as the pH is lowered [3]. Depending on the mechanism of dissociation of $HCO_3^-/CO_2$ from the reaction center in the presence of NO, the latter could either accelerate or have no effect on the rate of dissociation. Spinach chloroplasts were incubated in the dark at three different pH values (5.5, 6.3 and 7.4) in the presence and absence of 30 μM NO. At various time points following the dilution of the chloroplasts in buffers at these pH levels from a stock suspension at pH 7.5, an aliquot was taken and assayed for the rate of $Q_A$ to $Q_B$ electron transfer on the second of two saturating flashes. Fig. 3 shows the fluorescence intensity of chlorophyll at 5 ms after the second of two saturating flashes (1.48 s apart) during incubation in the presence and absence of 30 μM NO. In the absence of NO, the slowing of the fluorescence relaxation appears at faster rates the lower the pH between 7.4 and 5.5. It is not yet clear whether this dissociation at low pH is due to a protonation of $HCO_3^-$ directly, or of a site on the protein, the non-protonated form of which is necessary for $CO_2$ binding (e.g., lysine carbamate). NO greatly accelerates the rate of dissociation of $HCO_3^-/CO_2$ as the pH is lowered over the same range. The dissociation of $HCO_3^-/CO_2$ is accelerated from a $t_{1/2}$ at pH 5.5 of 40 min (−NO) to less than 1 min (+NO) and from a $t_{1/2}$ at pH 6.3 of 100 min (−NO) to 15 min (+NO). Both rates at pH 7.4 are extremely slow, with very little dissociation over a 3 h period. As in Fig. 2, rapid fluorescence relaxation rates were restored by the addition of 5 mM $NaHCO_3$. These data clearly indicate that NO has accelerated the dissociation of $HCO_3/CO_2$.

A further prediction of competition between $HCO_3^-/CO_2$ and NO for binding to the reaction center is that at high enough concentration $HCO_3^-/CO_2$ should dissociate NO, previously ligated to the iron. Fig. 4 shows EPR dark-minus-light (200 K illumination) difference spectra for the Fe(II)-NO signal in the $g = 4$ region for BBY membranes at pH 6.3. We recall from the previous paper [19] that illumination under such

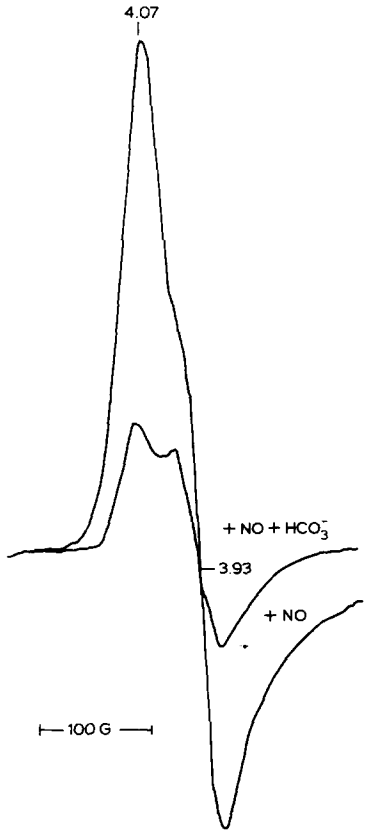


Fig. 4. Effect of bicarbonate addition on the amplitude of the $g = 4$Fe(II)-NO EPR signal. BBY membranes (3.1 mg Chl/ml, 50 mM Mes (pH 6.3)) were treated with 0.9 mM NO (labelled + NO) or with 0.9 mM NO, followed immediately afterwards with 5 mM $NaHCO_3$ (labelled + NO + $HCO^{-3}$). Both samples were subsequently incubated in darkness at 4° C for 40 min. EPR conditions: temperature 4.2 K, microwave frequency 9.42 GHz, microwave power 12.6 mW, modulation amplitude 16 G. These are dark-minus-light (200 K illumination) difference spectra.

conditions generates $Q_A^-$ which, through exchange coupling, largely eliminates the $g = 4$ Fe(II)-NO, $S = 3/2$ signal. Fig. 4 shows the $g = 4$ EPR signal at pH 6.3 in BBY membranes, treated in darkness at 4° C with 0.9 mM NO, followed by subsequent dark incubation with and without 5 mM $NaHCO_3$. A significant decrease in the Fe(II)-NO EPR signal size is observed following bicarbonate addition. The ability of a given concentration of $HCO_3/CO_2$ to dissociate NO is pH-dependent occurring more completely at higher than at lower pH. The $HCO_3^-/CO_2$-treated spectrum in Fig. 4 shows a larger decrease in the more axial component of the spectrum. This observation is consistent with the titration of the Fe(II)-NO adduct in the accompanying paper [19] in which the more axial component showed a somewhat higher $K_d$.

These results are consistent with NO and $HCO_3^-/CO_2$ competing for the same ligation site on the Fe(II). Considering that formate and $HCO_3^-/CO_2$ also compete for binding to the reaction center [3,28], it is worthwhile to see if indeed formate and NO compete for ligation to the iron, consistent with formate ligation to the iron. Fig. 5 shows an EPR dark minus light (200 K illumination) difference spectrum for the Fe(II)-NO signal in the $g = 4$ region for BBY membranes at pH 6.0. BBY membranes were incubated at pH 6.0 with either 75 mM NaCl or 75 mM sodium formate for 30 min at 4° C, and then for an additional 45 min with 300 $\mu$M NO. Unlike bicarbonate, formate causes a slight increase in the integrated area of the Fe(II)-NO EPR spectrum, arising from a widening of the spectrum. There also may have been some displacement of residual $HCO_3^-/CO_2$. Two sets of resonances are clearly distinguished; $g_x = 4.13$, $g_y = 3.87$ corresponding to an $E/D = 0.022$ and $g_x = 4.06$, $g_y = 3.95$, corresponding to an $E/D = 0.009$ (see the spin hamiltonian analysis in the preceding paper [19]). Formate clearly does not displace NO from the iron, as did $HCO_3^-/CO_2$, but rather binds simultaneously to the reaction center with NO, either on the iron or in its immediate vicinity.

We showed in the previous paper [19] that exchange coupling between either the paramagnetic $Q_A^-$ or $Q_B^-$ ($S = 1/2$) and the $S = 3/2$, Fe(II)-NO adduct gives an integral spin system resulting in the disappearance of the $g = 4$ EPR signal arising from the latter. Similarly, one would expect to see a decrease of the $Q_A^-$-Fe(II) EPR signal in the presence of bound NO. The latter EPR signal is normally rather weak, giving rise to two

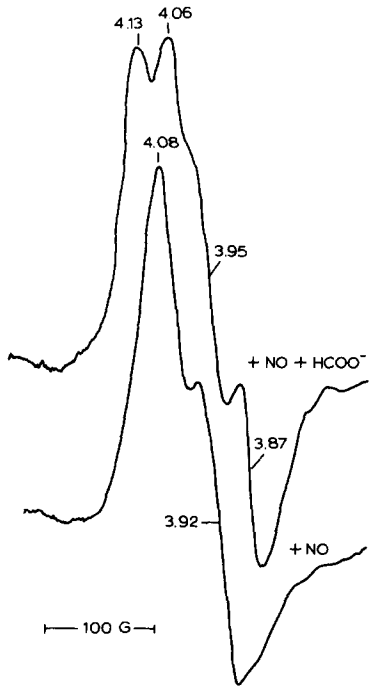


Fig. 5. Effect of formate on the $g = 4$ Fe(II)-NO EPR signal. BBY membranes (2.9 mg Chl/ml, 50 mM Mes (pH 6.0)) were incubated for 30 min in the presence of 75 mM NaCl (unlabelled) or 75 mM sodium formate (labelled + NO + $HCOO^-$). Each sample was subsequently incubated with 300 $\mu$M NO for 45 min. EPR conditions as in Fig. 5. These are dark-minus-light (200 K illumination) difference spectra.

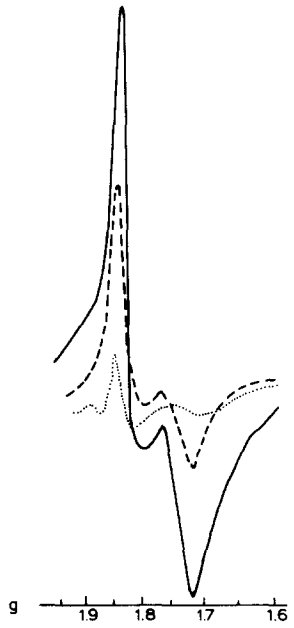


Fig. 6. Effect of NO on the $Q_A^-$Fe(II) $g = 1.82$ EPR signal amplitude in the presence of sodium formate. BBY membranes (2.9 mg Chl/ml, 50 mM Mes (pH 6.0)) were incubated for 30 min in the presence of 75 mM NaCl (·····), 75 mM sodium formate (———), or 75 mM sodium formate followed by 300 μM NO for 45 min (- - - - -). These are light- (200 K illumination) minus-dark difference spectra. The EPR conditions are as in Fig. 4.

alternative resonances at $g = 1.82$ and $g = 1.9$ [14,15], buried beneath the much stronger signal arising from free NO. Vermaas and Rutherford [16] have shown, however, that formate greatly stimulates a particular form of the $Q_A^-$Fe(II) EPR signal at $g = 1.82$. Fig. 6 shows the generation of this signal upon illumination at 200 K in the presence of 75 mM formate at pH 6.0 (———). In a similar sample treated with 300 μM NO, illumination at 200 K induces a much smaller signal (- - - - -). As NO and formate do not compete for binding to the reaction center, the decreased signal intensity is compatible with exchange coupling in centers which have NO as a ligand to the non-heme iron. The percent loss in the $Q_A^-$Fe(II) EPR signal is consistent with the $K_d = 250$ μM measured in the preceding paper [19] for NO binding to the non-heme iron in BBY membranes in the absence of formate. Higher concentrations of NO give further decreases in the amplitude of the $g = 1.82$ signal.

In light of the tight binding of NO to the non-heme iron in PS II and its competition with $HCO_3^-/CO_2$ for the same ligand position, we examined whether NO had any effect on $Q_A^- \rightarrow Q_B$ electron transfer in *Rb. sphaeroides* reaction centers. We found that 100 μM NO, at pH 5.9 and 6.5, had little effect on this electron transfer (no shown). While the effect of NO is greatest on the reduction of $Q_B^-$ to the quinol in PS II, there is still some slowing by NO observed for the formation of $Q_B^-$ (Fig. 2) in this photosystem. Attempts to look for a light-sensitive $g = 4$ Fe(II)-NO EPR signal in *R. rubrum* chromatophores and *Rb. sphaeroides* reaction centers were also unsuccessful and it would appear that NO does not bind to the non-heme iron in these reaction centers.

## Discussion

There are a number of experimental observations which have led some investigators in the past to propose binding of $HCO_3^-/CO_2$ on or in close proximity to the non-heme acceptor-side iron of PS II. These are: (a) the presence of rapid electron transfer between the primary and secondary quinone electron acceptors, $Q_A$ and $Q_B$, when $HCO_3^-/CO_2$ is present, which is slowed upon its removal by formate treatment or low pH [5–9]; (b) a heterogeneity in the g-values for the $Q_A^-$-Fe(II) EPR signal. There are at least two forms, a $g = 1.82$ favored at low pH [14,15,18] and in the presence of formate [16], and a $g = 1.9$ favored at high pH [14]; (c) the appearance, at low pH, of a high-potential form of the Fe(III)/Fe(II) redox couple [18]. A low potential form ($E_{m,7} \approx 400$ mV) which starts to disappear below pH 6.5, is observed in most but not all PS II centers; (d) a heterogeneity in the Mössbauer spectrum for Fe(II) with quadrupole splittings ranging from 2.1–2.9 mm/s [17]. A decrease in quadrupole splitting is observed with the addition of formate and a restoration of the initial signal on adding back $HCO_3^-$ [17]; (e) differences in the length of the connecting loop between transmembrane helices IV and V of D1/D2 compared to L/M, with the suggestion that a ligand other than a D2 carboxylic acid residue (e.g., $HCO_3^-$) might be involved in Fe coordination [13].

All of these characteristics distinguish PS II from the bacterial reaction centers, which do not show a bicarbonate effect [31]. It has been proposed [15,17,29] that the heterogeneity mentioned above in the spectroscopic signals and in the redox behavior of the non-heme iron might arise from a mixture of centers, those with bound $HCO_3^-/CO_2$ and those without.

We have shown [19] that NO is able to ligate to the PS II non-heme iron, producing an EPR resonance in the $g = 4$ region, characteristic of a Fe(II)–NO adduct, and sensitive, through exchange coupling, to the presence of the $s = 1/2$, $Q_A^-$ and $Q_B^-$ semiquinones. We demonstrate here that, associated with NO binding to the reaction center ($K_d \approx 30$ μM in spinach chloroplasts, see Ref. 19), there is a slowing of $Q_A$ to $Q_B$ electron transfer, similar to that observed upon formate treatment and characteristic of a dissociation of $HCO_3^-/CO_2$ from the reaction center. We also observe that NO is displaced from its binding site on the non-heme iron by $HCO_3^-/CO_2$.

While we do not present an absolute proof of binding of $HCO_3^-/CO_2$ to the PS II non-heme iron, there

are several arguments which separately favor this model and which together argue strongly that this is the case.

(1) NO is a small, diatomic molecule whose binding to the non-heme iron results in dissociation of $HCO_3^-/CO_2$. It is a neutral molecule which means that its competition with $HCO_3^-$ cannot be based simply on a coulombic effect. As NO binds to the iron, any steric overlap between the binding sites of NO and $HCO_3^-/CO_2$ would imply extremely close proximity or binding of the latter to the non-heme iron.

(2) NO does not appear to bind to the non-heme iron of bacterial reaction centers. This means that PS II has a replaceable ligand not present in the bacteria. While the four histidines of the bacterial reaction centers are probably conserved in PS II [13], the bidentate carboxylic acid residue would appear to be either absent or present as a weaker (monodentate?) ligand in the case of PS II. As the bacteria also do not show a bicarbonate effect [31], $HCO_3^-/CO_2$ is a likely candidate for the reversible ligand.

(3) The non-heme iron of PS II has a much lower $E_m$ (Fe(III)/Fe(II)) ($E_{m,7} = 400$ mV) than does the corresponding iron of the bacterial reaction centers ($E_{m,7} \geq 550$ mV). The presence of a basic or anionic moiety (e.g., $HCO_3^-$ or even $CO_3^{2-}$) as replaceable ligand would be a likely explanation for the lower $E_m$ in PS II.

(4) Formate decreases the quadrupole splitting by 0.7 mm/s in the Mössbauer spectrum of the PS II non-heme iron [17]. This effect is reversed by $HCO_3^-$. This effect is consistent with binding of $HCO_3^-/CO_2$ in the first coordination sphere of the iron. DCMU and *o*-phenanthroline, which bind in the second coordination sphere show little effect on the Mössbauer spectrum [17]. While we do not know whether the effect of formate treatment on the Mössbauer spectrum is due to the binding of formate or the displacement of $HCO_3^-/CO_2$ or both, it would appear that the responsible ligands are acting in the first coordination sphere. The effects of formate and NO on the kinetics of $Q_A/Q_B$ electron transfer are the same, however, despite their being very different molecules. The common denominator for their action would be displacement of the same molecule ($HCO_3^-/CO_2$) from its binding site.

A number of authors [3,28] have provided further evidence that formate displaces $HCO_3^-/CO_2$ from the PS II reaction center. While it is reasonable to expect that formate might ligate to the non-heme iron (e.g., $Fe(bimH_2)_2(HCO_2)_2$ [34]) in competition with $HCO_3^-/CO_2$, $HCOO^-$ does not appear able to displace bound NO at a concentration 250-times that of free NO. The $K_d$ for $HCO_3^-$ or $CO_2$ appears to be 40 $\mu$M [1,35], close to the 30 $\mu$M measured for NO [19]. Considering the inhibition constant, $K_i$, for formate of 2 mM [36], this anion should have been able to displace NO as well as $HCO_3^-/CO_2$.

We propose two possible models to interpret these observations. In both $HCO_3^-/CO_2$ would bind as a bidentate ligand (possibly as $CO_3^-$), or as a monodentate ligand with a second anionic ligand provided by the protein (e.g., glutamic acid).

(1) Formate displaces $HCO_3^-/CO_2$ by competing with a binding site outside the first coordination sphere of the iron, without itself being an iron ligand. This competition would dissociate $HCO_3^-/CO_2$ from the iron. Such a model would be consistent with an increase in the midpoint potential of Fe(III)/Fe(II) upon formate treatment, as it would result in the loss of an anionic ligand to the iron, but not prevent binding of NO;

(2) A more likely possibility is that one or both $HCO_3^-/CO_2$ ligation positions would be occupied by formate displacing $HCO_3^-/CO_2$. NO would occupy the other non-histidine ligation site or displace one of the two formates. Co-binding of NO and formate would explain the increased rhombicity of the Fe(II)-NO EPR spectrum in the presence of formate (Fig. 5). The increased $E_m$ of Fe(III)/Fe(II) upon formate addition in the absence of NO would be explained by a decreased anionic or basic character of the first coordination sphere i.e., a decrease in the number of anionic ligands or a lower p$K$ of formate (3.75) vs. (H)$HCO_3^-$ (6.37) or (H)$CO_3^{2-}$ (10.25) or glutamic acid (4.25).

We cannot, at the moment, choose between these models. The observation of broadening of the Fe(II)-NO EPR signal in the presence of $^{17}$O-labelled $HCOO^-$ would provide a clear-cut argument for the second proposal. Likewise, broadening of the axial Fe(III) EPR signal at $g = 6$ [17] in the presence of $^{17}$O-labelled $HCO_3/CO_2$, would provide further proof of direct ligation of this species to the iron.

NO (100 $\mu$M, not shown) has no effect on the rate of electron transfer from $Q_A^-$ to $Q_B$ in reaction centers of *Rb. sphaeroides*. Attempts to observe an EPR signal arising from an Fe(II)-NO adduct in *R. rubrum* chromatophores and *Rb. sphaeroides* reaction centers, sensitive to light, as in PS II, were also unsuccessful. Why NO is able to coordinate the iron in PS II and not in bacterial reaction centers is not immediately clear. Either the NO ligating the iron also interacts with a site external to the iron and absent in the bacteria or, in the latter case, the glutamic acid ligand binds too tightly to be displaced by NO.

We show, in Fig. 3, that the rate of dissociation of $HCO_3^-/CO_2$ from the iron is pH-dependent. This dissociation is greatly accelerated by NO as the pH is lowered. The observed kinetics would appear to rule out a replacement mechanism in which the binding of NO occurs as soon as $HCO_3^-/CO_2$ dissociates from the reaction center (the rate-limiting step), otherwise no acceleration of $HCO_3^-/CO_2$ dissociation would be observed in the presence of NO. Two possibilities remain: either the NO drives the $HCO_3^-/CO_2$ off the iron with

both bound in a transition state; or the $HCO_3^-/CO_2$ can dissociate and reassociate many times with the iron in a restricted space, in the absence of NO, before being lost from the reaction center. Diffusion out of the restricted space would constitute the rate-limiting step for $HCO_3^-/CO_2$ dissociation. NO, when present, would block the reassociation in the binding pocket and accelerate the loss of $HCO_3^-/CO_2$.

Neither NO nor formate appear to have any intrinsic effect on rates of electron transfer, outside of that caused by the dissociation of $HCO_3^-/CO_2$. The effect of NO on the electron transfer rate is pretty much identical to that of $HCOO^-$, despite these being very different molecules. In both cases the slowing of electron transfer is considerably more marked on the second and subsequent flashes than on the first (Figs. 1 and 2, and Refs. 8, 9). $HCO_3^-/CO_2$ has been suggested to be involved in protonation steps linked to the reduction of $Q_B$ [9,11]. Those species, previously identified as producing a bicarbonate effect are all anions and include formate, acetate [3] and nitrite [37]. We have also found that 30 mM $CN^-$ will induce slowing of $Q_A/Q_B$ electron transfer similar to that observed for these anions and NO. Preliminary EPR results, in collaboration with Dionysus Koulougliotis, indicate that $CN^-$ competes with NO. These findings will be presented in a forthcoming publication. The observations presented here are, therefore, to our knowledge, the first evidence of a non-anionic inhibitor producing a bicarbonate effect.

In bacterial reaction centers, the rate of reaction $Q_A^-Q_B \rightarrow Q_AQ_B^-$ is slightly pH-dependent, while that of $Q_A^-Q_B^- \rightarrow Q_AQ_BH_2$ is strongly pH-dependent with a protonation step being rate-limiting [38]. In PS II, both reactions are only weakly pH-dependent [39–41]. Perhaps, in PS II, the bound $HCO_3^-/CO_2$ provides what would be the rate-limiting proton in the bacterial system for $Q_A^-Q_B^- \rightarrow Q_AQ_BH_2$. This explanation does not, however, explain why in formate, or NO-treated chloroplasts in the absence of $HCO_3^-/CO_2$, the rate remains slow on the third flash instead of returning to the first flash rate. One would have to argue that, in the absence of $HCO_3^-/CO_2$, no further proton exchange can occur coupled to $Q_B$ reduction.

There are reports that illumination in the presence of formate [35] or reductant [11] raise the $K_d$ of $HCO_3^-/CO_2$ binding, implying a dependence of the $K_d$ on the redox state of the acceptor-side quinones. While it is possible that there are reaction centers which still retain $HCO_3^-/CO_2$ in the presence of NO or $HCOO^-$, but lose it following single flash excitation, our own attempts (not shown) to observe an accelerated dissociation of $HCO_3^-/CO_2$ at pH 5.5 following one or two light flashes indicated no increase in dissociation rate.

Other questions that remain are the form in which $HCO_3^-/CO_2$ is bound to the reaction center, the mechanism by which this species participates, if it does at all, in protonation-deprotonation reactions coupled to $Q_B$ reduction, and finally the structural puzzle as to why the PS II reaction centers show a bicarbonate effect and NO ligation, while the bacterial reaction centers do not.

Also to be resolved is whether the $HCO_3^-/CO_2$ binding site is indeed regulating, under physiological conditions, electron flow in the photosynthetic electron transport chain. Stemler and Murphy [35] have shown the $K_d$ for $HCO_3^-/CO_2$ to be 80 $\mu$M (40 $\mu$M if corrected for the $HCO_3^-/CO_2$ equilibrium [1]). If $HCO_3^-$ is the active binding species, then at physiological pH approx. 8, and a stromal $[CO_2]$ of 10 $\mu$M [42], the $[HCO_3^-]$ should be approx. 400 $\mu$M, well above the $K_d$, and all centers should be saturated. If $CO_2$ is the active species, then considering the $K_d$ for this species (also 40 $\mu$M, using the data of Ref. 35), then few centers have $CO_2$ bound. Possible complicating factors are competing ligands to the iron, a pH at the negatively charged thylakoid surface [43] below that of the bulk phase, and a higher $K_d$ in the light than in the dark for $HCO_3^-/CO_2$ [35,44]. The question of physiological regulation of the PS II electron transfer by $HCO_3^-/CO_2$ is still an open one, though a recent paper by Ireland et al. [45] has provided evidence for such regulation.

Possible advantages to regulation by $HCO_3^-/CO_2$ in PS II would be to slow reaction center turnover under conditions of physiological $CO_2$ depletion (e.g., hot, dry weather or high light intensity). Under such conditions oxygenase activity of ribulose bisphosphate carboxylase/oxygenase would be increasingly favored relative to carboxylase function. Slowing of PS II would tend to lower intracellular $[O_2]$ and would also prevent the plastoquinone pool from becoming overreduced, maintaining the pool poised for efficient cyclic phosphorylation [46–48], both aspects would enhance carbon fixation. Photosynthetic bacteria, with only one photosystem and unable to evolve oxygen, would not need such regulation.

**Acknowledgements**

The authors would like to thank Dr. Kathleen Giangiacomo for her gift of *Rb. sphaeroides* R-26 reaction centers and Drs. Steven Gutteridge, George Lorimer, Daniel O'Keefe, Vincent Pecoraro and Jean-Pierre Tuchagues for helpful discussion.

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