Kinetic characteristics of formate/formic acid binding at the plastoquinone reductase site in spinach thylakoids

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Oxidation of the reduced primary electron acceptor, Q_A^-, of Photosystem II (PS II) in formate-treated spinach thylakoids, was inhibited more after the second than after the first actinic flash. This indicates a slowing of electron flow on the acceptor side of PS II from Q_A^- to Q_B^-, the semiquinone form of the secondary plastoquinone acceptor, formed by electron transfer after the first flash. A hypothesis of electron transfer on the acceptor side of PS II is proposed to accommodate the bicarbonate-reversible formate/formic acid inhibition of electron transfer after single turnover flashes. We suggest that the large inhibition in Q_A^- oxidation after the second flash reflects a blockage of the proton uptake that stabilizes Q_B^-. Kinetics of onset of inhibition following formate addition were followed by measuring the chlorophyll *a* fluorescence yield, reflecting the concentration of Q_A^-, 1 ms after the second actinic flash as a function of time after the addition of formate. The apparent rate constants for binding and unbinding, and the dissociation constant of formate were determined in the pH range from 5.5 to 7.5. The rate of onset of inhibition following formate addition, reflecting formate or formic acid binding, was highly dependent on the medium pH. Measurements on the initial binding rate, when one of the two (HCO_2^-/HCOOH) equilibrium species was kept constant and the other varied, suggested that formic acid is the binding species. This conclusion was consistent with the observed pH dependence of formate binding.

Introduction

Electrons are transferred from Photosystem II (PS II) to the plastoquinone pool through a two-electron gate mechanism involving bound primary (Q_A) and secondary (Q_B) plastoquinone (see reviews [1–3]). Bicarbonate (HCO_3^-) has been shown to stimulate electron transfer in PS II depleted of bicarbonate by treatment with formate or nitric oxide, and this effect has attracted increasing interest in recent years because of the possible role of bicarbonate in the mechanism of electron transfer in the quinone binding region, and in

ligation of the non-heme iron (see reviews [4–6]). The bicarbonate effect is observed in green plants [4–6] and cyanobacteria [7,8], but not in photosynthetic bacteria [9], which suggests that there are significant structural differences between the quinone-binding regions in the reaction centers (RCs) of oxygenic and anoxygenic photosynthetic organisms.

Several observations show that a major bicarbonate-reversible formate effect exists on the electron acceptor side of PS II in the D_1 and D_2 proteins of RC II:

(1) There is a dramatic inhibition, particularly after the second and subsequent flashes, of the reoxidation of the reduced primary quinone acceptor of PS II, Q_A^-, as measured by the chlorophyll (Chl) *a* fluorescence yield decay [10–13], or by the absorbance change at 320 nm [14] in the presence of formate.

(2) Light-induced EPR signals in the $g = 1.6$ to $g = 8$ region [15–17], attributed to magnetic interaction between semiquinone forms of Q_A and Q_B, and the non-heme iron of the acceptor complex ($Q_A^- Fe^{2+} Q_B^-$,

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Abbreviations: Chl, chlorophyll; PQ, plastoquinone; PS II, Photosystem II; Q_A, bound plastoquinone, one-electron acceptor of Photosystem II; Q_B, loosely bound plastoquinone, two-electron acceptor of Photosystem II; RC, reaction center.

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$Q_A^- Fe^{2+}$, $Q_B^- Fe^{2+}$ and Fe^{3+}), and the Mossbauer spectra of the non-haem Fe^{2+} [18-20], are altered by formate, NO and bicarbonate.

(3) The binding of several herbicides, known to interact with D1, is drastically affected by the presence or the absence of bicarbonate and vice versa [21,22].

(4) Various *Synechocystis* 6714 [23] and *Chlamydomonas reinhardtii* [24] herbicide-resistant mutants, altered in single amino acids on D1, are differentially sensitive to subsaturating concentrations of formate.

Several possible roles for bicarbonate in the function and structure of the acceptor side complex have been suggested. Bicarbonate may provide a bidentate ligand to Fe^{2+} in the Q_A-Fe-Q_B complex [25], and keep the D1-D2 proteins in their proper functional conformation, thereby facilitating electron transfer from Q_A^- to Q_B or Q_B^-. Experiments with nitric oxide (NO), that binds to Fe^{2+} [20], suggest [26] that bicarbonate binds to Fe^{2+} in PS II. Bicarbonate may promote protonation associated with PQ reduction at the Q_B site, explaining the larger bicarbonate-reversible formate effect on Q_A^- decay after the second and subsequent actinic flashes than after the first one (Refs. 12, 13; this paper). This latter concept is reinforced by observations on the effects of formate or bicarbonate on H^+ exchange related to PS II reactions [27,28].

Since formate behaves as a competitive analogue of HCO_3^-, a study of its binding characteristics is expected to provide an understanding of the bicarbonate effect. In this work, we measured the time-course of formate binding in spinach thylakoids to gain an insight into the mechanism of formate-induced inhibition. Analysis of binding data showed that formic acid, but not formate, is the binding species. A hypothesis of electron transfer on the acceptor side of PS II is proposed to accommodate the bicarbonate-reversible formate/formic acid inhibition of electron transfer after single turn-over flashes.

In this paper and elsewhere [29,30], we have assumed that the binding of formate/formic acid displaces CO_2/HCO_3^- bound to PS II reaction centers, since formate/formic acid (1) behaves as a competitive inhibitor of HCO_3^- binding [21,29,30], and (2) releases CO_2 from thylakoid membranes [31]. Our work does not address the question of the nature of the species displaced. However, because the pH dependence of inhibitor binding is sufficiently explained in terms of the formic acid concentration, it is not necessary to invoke other changes in protonation state either of the binding site, or the displaced species, to explain our data.

Material and Methods

Spinach (*Spinacia oleracea*) thylakoids were isolated as previously described [12]. Thylakoids, suspended in 0.4 M sorbitol, 15 mM NaCl, 5 mM $MgCl_2$ and 20 mM Hepes (pH 7.8), were frozen rapidly, stored in liquid nitrogen, and thawed immediately prior to use. Chlorophyll concentration was spectrophotometrically determined in 80% acetone (v/v) extracts of thylakoids [32].

Mes, Hepes, and quinhydrone were purchased from Sigma. Sodium formate was purchased from J.C. Baker.

To monitor the redox state of Q_A, Chl *a* fluorescence yields after single-turnover saturating flashes (EG & G FX-124 flash lamp, 2.5 μs duration) were measured by an instrument described elsewhere (Ref. 12, see also Refs. 11, 33). Using weak measuring flashes, the 'O' (or F_o) level of Chl *a* fluorescence yield of 5 min dark-adapted thylakoids, and the decay of the variable (F_v) fluorescence after saturating flashes were measured at 685 nm, using a 10 nm bandwidth interference filter, by a photomultiplier (EMI 9558). The F_o measures the yield when all the Q_A is in the oxidized state (see, for example, Ref. 34), and the decay of F_v reflects the reoxidation of Q_A^- to Q_A. Assuming that the probability of the intersystem energy transfer is 0.5, the concentration of Q_A was calculated from the variable Chl *a* fluorescence yield, as outlined earlier [12,36,37].

Thylakoids, containing 10 μM Chl, were suspended in 0.4 M sorbitol, 50 mM NaCl and 2 mM $MgCl_2$ [12]. 80 μM quinhydrone was added to the sample to keep Q_A in its oxidized state. pH of the suspension was adjusted by using 20 mM Mes (from pH 5.5 to 6.5) or 20 mM Hepes (from pH 7.0 to 7.5).

The kinetics of formate binding in the dark-adapted thylakoid suspension were measured by monitoring Chl *a* fluorescence yield changes after an actinic flash at various times after injecting a known amount of formate (preadjusted to the pH of the suspension medium) with a syringe. The decay of Chl *a* fluorescence yield, reflecting oxidation of Q_A^-, was significantly slowed only after the second and subsequent actinic flashes. Those centers in which the Q_A^- oxidation was blocked were considered to be formate-bound. The concentration of formate bound PS II RC's was estimated from the fraction of centers showing inhibited Q_A^- oxidation (measured as above) after the addition of formate.

Theory

The two electron gate scheme, developed for anoxygenic photosynthetic bacteria and for oxygenic PS II [2], was modified to accommodate formate binding at PS II RC. Both oxidized and fully reduced forms of the secondary PQ acceptor (Q_B or $Q_B H_2$) bind loosely to the D1 protein of the RC II, whereas the semiquinone form, Q_B^-, binds tightly (for a review, see Ref. 2). Complete reduction of the bound quinone, Q_B, requires not only two electrons but transfer of two protons to Q_B^{2-}. The $Q_B H_2$ thus formed exchanges rapidly with PQ from the pool and completes the electron flow

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at this site, the so-called 'two electron gate'. Many inhibitors block the electron transport beyond Q_A by competing with Q_B at its binding site (see review in Ref. 2). We have assumed here that formate and Q_B binding are independent of each other.

In this paper, we will demonstrate (for earlier results, see Refs. 10, 12, 13) that formate bound at the PS II RC's greatly decreases the rate of electron flow after the second, but much less after the first actinic flash in the Q_A-Fe-Q_B complex. This is most economically explained by invoking an inhibition of protonation at the Q_B site.

Fig. 1 shows a simplified scheme that explains the formate effect on the two electron gate. When the plastoquinone pool is oxidized, the total (T) amount of the dark-adapted PS II RC's, to which formate (F) was added, are in one of the following states: Q_A (without Q_B bound), Q_AQ_B (with Q_B bound), FQ_A (Q_A with formate bound) and FQ_AQ_B (Q_A with both Q_B and formate bound). The formate-free and formate-bound RC's can be represented as:

$$[RC] = [Q_A] + [Q_AQ_B] = \{1 + K_o/[PQ]\}[Q_AQ_B] \qquad (1)$$

and

$$[FRC] = [FQ_A] + [FQ_AQ_B] = \{1 + K_o/[PQ]\}[FQ_AQ_B] \qquad (2)$$

Where [PQ] is the in vivo PQ concentration. [RC] and [FRC] represent the concentration of formate-free and formate-bound PS II RC's respectively. The total amount of RC, $[RC]_T$, is composed of [RC] and [FRC]. Eqns. 1 and 2 imply that the concentration of each form of RC (RC and FRC) can be directly determined by the concentration of the Q_B bound complexes (Q_AQ_B and FQ_AQ_B), K_o and [PQ]. In our scheme, we have two branches of electron flow in the Q_A-Fe-Q_B complex: (1) without formate bound (front of the scheme) and (2) with formate bound (back of the

scheme). Open arrows indicate reduction of Q_A following the first or second actinic flash. Solid arrows represent equilibria in dark reactions involving electron flow, the binding of quinone or formate and protonation. K_o is the dissociation constant for PQ at Q_B site and is assumed to be independent of formate binding. K_F and K_F' are dissociation constants for formate at the PS II RC when Q_B is absent and when Q_B is bound, respectively. $K_F' = [FQ_AQ_B]/[F][Q_AQ_B]$; $K_F = [FQ_A]/[F][Q_A]$. The estimation of kinetic parameters for formate binding at PS II RC's is based on the above scheme.

The following assumptions were made in the derivation of equations describing formate binding to PS II RC's:

(1) k_F and k_{-F} are defined as the on-rate and off-rate binding constant of formate at PS II RC's; their ratio defines the dissociation constant of formate binding. K_F. k_F is a second-order constant, whereas k_{-F} is a first-order constant. We assume that the dissociation constant of formate at PS II RC's with vacant Q_B site and that with bound Q_B site are equal, i.e., $K_F = K_F'$. This means that, unlike herbicides, the binding of formate does not compete with PQ. The two types of center are separate and formate does not exchange rapidly between them. For the same reason, K_o, the dissociation constant of PQ or PQH_2 for Q_B binding site, is assumed to remain unchanged in the formate binding process.

(2) Compared to the rates of the reduction of Q_A, the electron transfer from Q_A to Q_B or Q_B^-, the binding of Q_B at its binding site and the protonation of reduced Q_B, formate binding is assumed to be relatively slow. Therefore, the concentration of [FRC] is determined only by the formate-binding process, and other reactions mentioned above can be assumed to be at their equilibrium poise. Furthermore, the rate of formate binding is very slow even when compared with the dark interval (1 s) between two actinic flashes, so
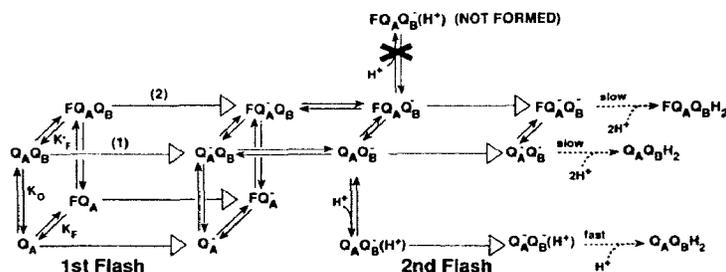
Fig. 1. A working scheme showing the formate/formic acid (F) binding mechanism which affects the two electron gate in PS II reaction centers. Open arrows indicate photoreaction of Q_A following the first or second actinic flash. Solid arrows represent equilibria for dark reactions involving (1) electron flow, (2) binding of quinone or formate/formic acid and (3) the protonation of reduced Q_B. K_o is the dissociation constant for plastoquinone. K_F' and K_F are dissociation constants for formate at the Q_B binding site or at the Q_B vacant site. The heavy cross implies the blockage of a protonation reaction.

that the fraction of formate bound centers does not change during the period between the last actinic flash and the assaying flash sequence.

After the first actinic flash, all the PS II RC's have transferred one electron to form Q_B in most centers. We suggest that the protonation of Q_B is prohibited in the formate-bound PS II RC's (see bold cross in Fig. 1). Therefore, although the second photoreaction can take place in the formate bound PS II RC's and $FQ_A^-Q_B^-$ can form, further reoxidation of Q_A^- will be hampered after the second flash since protonation is needed for fast plastoquinol (Q_BH_2) formation. This brings about the large increase of the Chl a fluorescence yield measured at 1 ms after the second actinic flash. We shall use the fluorescence yield at 1 ms after the second flash to calculate the fraction of the formate-bound PS II RC's. The protonation steps during Q_BH_2 formation have not yet been fully deciphered. However, Wraight [38] showed that in bacterial reaction centers, electron transfer from Q_A^- to $Q_B(H^+)$ was rapid compared with the transfer from Q_A^- to Q_B, and Crofts et al. [39] interpreted their results in chloroplasts as showing a similar effect. From our present results we cannot decide whether the first or the second or both protonation steps are hindered in formate-bound RC's.

On the basis of the assumptions above, we equate the formate-bound reaction centers detected after the second flash with the fraction of formate-bound reaction centers before the assaying flash sequence, so that [FRC] = [$FQ_A^-Q_B^-$]. Changes in the concentration of the formate-bound PS II RC's are given by Eqn. 3 and monitored by the Chl a fluorescence yield after the second actinic flash:

$$d[FRC]/dt = k_f[F][RC] - k_{-f}[FRC]$$

$$= k_f[F]([RC]_T - [FRC]) - k_{-f}[FRC] \qquad (3)$$

Under initial conditions, its normalized solution is:

$$[FRC]/[RC]_T = k_f[F](1 - exp\{-(k_f[F] + k_{-f})t\})/(k_f[F] + k_{-f}) \qquad (4)$$

The time course of formate binding in line with Eq. 4 is obtained by plotting [FRC]/[RC]$_T$ against time. [FRC]/[RC]$_T$ will reach a constant level when the incubation time is long enough. Its first derivative is:

$$d([FRC]/[RC]_T)/dt = k_f[F]exp\{-(k_f[F] + k_{-f})t\}) \qquad (5)$$

The initial on-rate binding constant of formate, $\{d([FRC]/[RC]_T)/dt\}_{t=0}$, is equal to $k_f[F]$ when t approaches 0 and is defined as C. C is dependent on the formate concentration in the initial condition and is directly measured from the initial slope of the time

course. After plotting C against formate concentration, k_f is thereby determined, as defined.

The dissociation constant of formate, K_f, was calculated by using the formate concentration and the ratio between formate bound or unbound RC's. This ratio was determined from the constant level reached during the time-course of formate binding. The off-rate binding constant, k_{-f}, was obtained from the ratio of k_f and K_f.

Results and Discussion

Flash number dependence of the PS II electron flow: basic binding property of formate

Formate-induced inhibition of the oxidation of Q_A^-, at pH 6.5, is shown in Fig. 2. In comparison to the earlier experiments [12,13], in which formate treatment lasted for hours, we measured Chl a fluorescence intensity after direct injection of formate into spinach thylakoid suspensions followed by a 15 min dark adaptation. A moderate slowing of electron flow was found following the first flash, but considerably greater slowing occurred after the second and subsequent flashes in formate-treated thylakoids at pH 6.5 (Fig. 2). Experiments at pH 6.0 and pH 7.0 confirmed this conclusion (cf. data in Refs. 12, 13). In all cases, addition of 20
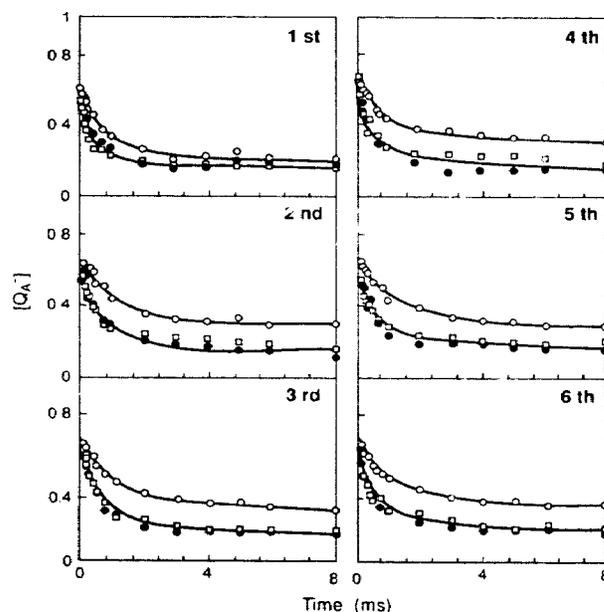
Fig. 2. The effect of formate/formic acid binding on the decay of [Q_A^-], calculated from Chl a fluorescence yield at pH 6.5. The number in each panel refers to the flash number of the actinic flash. The added formate concentration was 100 mM. The dark-adaptation time was 15 min. In the reaction medium, 0.4 M sorbitol, 50 mM NaCl, 2 mM MgCl$_2$ and 80 μM quinhydrone were included. Open circles, formate-treated; closed circles, control; open squares, formate-treated to which 20 mM bicarbonate was added.

mM bicarbonate totally reversed the above-mentioned inhibitory effects to match the data in the controls (data at pH 6.5 is shown in Fig. 2).

If the difference of Q_A^- concentration between formate-treated and control samples, $[Q_A^-]_{(formate)} - [Q_A^-]_{(control)}$, was plotted (data not shown), a maximal increase was found after the second and subsequent flashes. The largest increase of $[Q_A^-]$ was found at 1 ms after the actinic flash. Therefore, the change of $[Q_A^-]_{(formate)} - [Q_A^-]_{(control)}$ at 1 ms was chosen throughout our study. The fact that two actinic flashes are necessary to produce the biggest inhibitory effect on the electron flow can be explained by assuming that formate binding limits the availability of protons for the formation of Q_BH_2. Since H^+ binding has a relatively weak effect on the kinetics and equilibrium of electron transfer after the first flash (Robinson, H.H. and Crofts, A.R., unpublished data), this results in the greatest slowing of electron transfer after the second actinic flash, as indicated in Fig. 1 (see top horizontal line).

In order to control the effect on surface potential resulting from addition of sodium formate, 100 mM of NaCl was added to spinach thylakoid suspensions instead of sodium formate in experiments otherwise identical to those used in Fig. 2. No effect was observed on the decay of $[Q_A^-]$ after any of the six actinic flashes (data not shown). Therefore, the effect observed on addition of sodium formate cannot be attributed to changes in the surface potential of thylakoids.

The determination of the rate constants for binding and unbinding, and the dissociation constant of formate at the plastoquinone reductase site

The time-course of formate binding to spinach thylakoids at pH 6.5, as measured at different formate concentration, is shown in Fig. 3. At pH 6.5, the initial rate of formate binding increased as the concentration
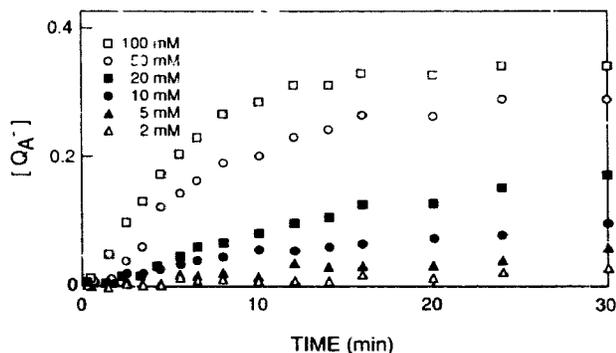
Fig 3. The concentration- and time-dependence of formate/formic acid binding at pH 6.5, as measured by $[Q_A^-]$ 1 ms after the second actinic flash. Different symbols indicate the different concentration of added formate (see key in the figure). The other experimental details were the same as in the legend of Fig. 2.
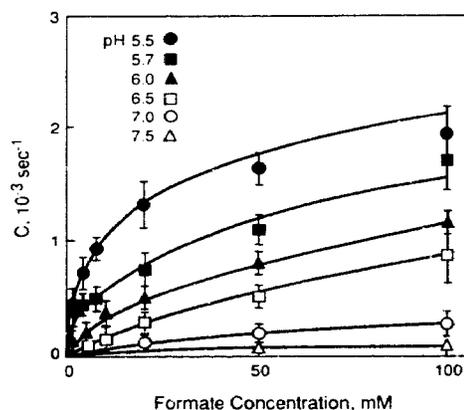
Fig. 4. Dependence of the initial rate constant, C, calculated from the slope of $[Q_A^-]$ versus mixing time, for formate/formic acid binding on formate concentration in spinach thylakoids after the second actinic flash. Different symbols indicate the medium pH (see key in the figure). The other experimental conditions were the same as in the legend of Fig. 2. Number of experiments to obtain error bar was 4 to 6.

of formate was raised. This result can be explained by the second-order binding process indicated in Eqn. 4. Furthermore, the higher plateau for $[Q_A^-]$ at higher formate concentration is also in agreement with Eqn. 4. Thus, our experimental results are consistent with the binding scheme in Fig. 1.

In Fig. 4, the initial on-rate binding constant of formate, C, calculated from the slope of $[Q_A^-]$ as a function of mixing time, and at different pH values, was plotted against formate concentration. The shape of the curves can be described by the Michaelis-Menten equation, implying a saturation effect. C increased with increasing formate concentration at all pH values (5.5–7.5) examined (Fig. 4; Eqn. 5). The initial slope of C, reflecting the initial binding rate of formate when the formate concentration approaches zero, was also dependent upon the suspension pH. Its value was relatively larger at lower than at the higher pH levels. Since the second-order on-rate constant for formate binding, k_F, is obtained from the initial slopes in Fig. 4, the pH dependence of the initial slope of C also demonstrates the pH dependence of k_F, the on-rate binding constant of formate.

The dissociation constant, K_F, was obtained under constant binding conditions. k_{-F}, the first-order rate constant for the release of formate from its binding site, was derived from the ratio between k_F and K_F in each case. The dissociation constant for formate was calculated to be 94 mM at pH 6.5. The rate constants for binding and unbinding, and the dissociation constant of formate are listed in Table I. The on-rate binding constant of formate, k_F, was highly pH dependent (top panel of Fig. 5): it is 18, 14, 5.6, 1.7 and $0.50 \cdot 10^{-2}$ s^{-1} M^{-1} at pH 5.5, 5.7, 6.0, 6.5, and 7.0, respectively (Table I): k_F decreased with decreasing

TABLE I

The on-rate (k_F), the off-rate binding constant (k_{-F}), and the dissociation constant (K_F) of the binding species (considered to be formate here) in spinach thylakoids

n = number of experiments; + indicates standard error.

pH	k_F $(10^{-2}\,s^{-1}\,M^{-1})$	k_{-F} $(10^{-2}\,s^{-1})$	K_F (mM)	n
5.5	18 ± 6	0.21 ± 0.04	12 ± 4	6
5.7	14 ± 5	0.18 ± 0.05	13 ± 5	4
6.0	5.6 ± 3.0	0.17 ± 0.03	30 ± 16	4
6.5	1.7 ± 0.93	0.16 ± 0.03	94 ± 51	4
7.0	0.50 ± 0.25	0.13 ± 0.01	260 ± 130	4

proton concentration. However, the off-rate constant, k_F, is independent of pH. K_F also shows a pH dependence. It changes from 12 to 260 mM in the pH range of 5.5 to 7.5.

The binding species: formate or formic acid?

It has been tacitly assumed thus far that formate is the binding species that produces bicarbonate-reversible inhibition of electron flow between Q_A and the PQ pool. However, the pH dependence of the on-rate
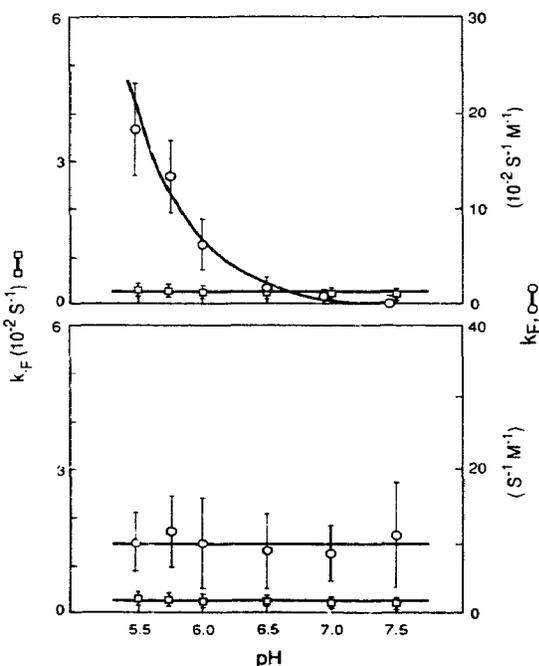
Fig. 5. Top: The pH dependence of the on-rate (k_F) and the off-rate (k_{-F}) binding constants after the second actinic flash when formate is considered to be the binding species. Bottom: The pH dependence of the on-rate (k_F) and the off-rate (k_{-F}) binding constant after the second actinic flash when formic acid is considered to be the binding species. Number of experiments to obtain error bar was 4 to 6. Open circles: k_F; open squares: k_{-F}.

constant (top panel of Fig. 5) and of the initial binding rate (Fig. 4) of formate leads to a new insight into the binding species problem. In the medium, formate equilibrates with formic acid. The pK of formate is 3.75. In the 5.5 to 7.5 pH range, used in this study, the change of formic acid concentration with the pH change is far greater than that of formate. At pH 6, 99.4% of the total amount of the two equilibrium species is formate, changing only to 99.94% at pH 7.0. It is obvious that the change in formate concentration is negligible when the medium pH changes from 5.5 to 7.5. In contrast, the change in formic acid concentration is highly dependent on the medium pH in the pH range of this study. Results (Fig. 4 and the top panel of Fig. 5) indicating the high pH dependence of the initial on-rate binding constant, C, and the second order rate constant, k_F, imply that formic acid, rather than formate, may be the binding species in PS II.

Since formic acid, not formate, appears to be the binding species, all the data in Table I were recalculated using the concentration of formic acid. Using the pK value of formate (3.75) and the medium pH, the ratio between concentrations of formate and formic acid was derived. At pH 5.5, 6.0, 6.5 and 7.0, the concentration of formate is 56-, 178, 563- and 1778-times the concentration of formic acid, respectively. The actual concentration of formic acid was calculated and replaced with that of formate in previous [F] in all calculations. New results listed in Table II are based upon the assumption that formic acid is the binding species. A remarkable result is observed (bottom panel of Fig. 5, also see Table II): the on-rate constant, k_F (around 10 s^{-1} M^{-1}) becomes pH-independent in the pH range 5.5 to 7.5. The off-rate constant of formate, k_{-F}, however, still remains pH independent as before. The dissociation constant, K_F, that was larger than 10 mM when formate was thought to be the binding species, becomes 200 μM and relatively independent of pH. It is satisfying that the concentration of formic acid required to inhibit Q_A-to-Q_B reaction is in the micromolar range, not in the 10 millimolar range as would be the case if formate were the inhibitory species.

TABLE II

The on-rate (k_F), the off-rate binding constant (k_{-F}), and the dissociation constant (K_F) of the binding species (considered to be formic acid here) in spinach thylakoids

n = number of experiments; ± indicates standard error.

pH	k_F $(s^{-1}\,M^{-1})$	k_{-F} $(10^{-2}\,s^{-1})$	K_F (μM)	n
5.5	10 ± 3	0.21 ± 0.04	210 ± 60	6
5.7	12 ± 4	0.17 ± 0.05	140 ± 50	4
6.0	10 ± 6	0.17 ± 0.03	170 ± 100	4
6.5	9.6 ± 5	0.16 ± 0.03	170 ± 90	4
7.0	8.9 ± 4	0.13 ± 0.01	150 ± 70	4
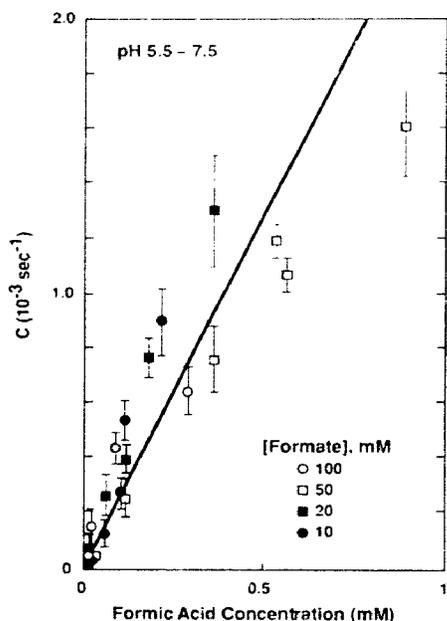
Fig. 6. The formic acid concentration dependence of the initial on-rate binding constant of formate/formic acid binding in spinach thylakoids. The data were obtained from Fig. 4 at different suspension pH and formate concentration. Different symbols indicate the equilibrium formate concentration at several different medium pH. The drawn line was obtained by the least-squares method. One point at 0.9 mM formic acid (50 mM formate) was not included in the analysis.

By taking advantage of the pH dependence of the equilibrium ratio of formate to formic acid to effectively hold one species constant while varying the other, we further probed the chemical nature of the active species of the inhibitor. Measurements of the initial on-rate binding constant. C, over a pH range from 5.5 to 7.5 indicated that the initial binding rate is proportional to the concentration of formic acid, but independent of formate concentration at constant formic acid concentration (Figs. 6 and 7). These results demonstrate that formic acid is the active species involved in the inhibitory process.

In order to test further the nature of the inhibitor binding site, we attempted to match our k_+ data with theoretical acid-base titration curves using The Henderson-Hasselbalch equation. If a fit were to be obtained, we could explain it by simply supposing the existence of a binding site in the RC protein containing a residue that undergoes protonation. In the anionic form, this residue would impede the binding, but in the neutral form it would facilitate its binding. The fitting could indicate that formate is the more logical binding species because of its negative charge. However, our k_+ data did not fit any of the theoretical curves with pK values of 5, 5.5, 6, 6.5 or 7 (data not shown). Thus,
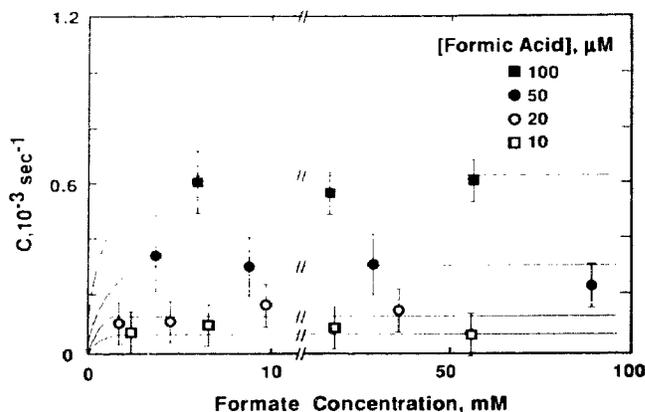
Fig. 7. The formate concentration dependence of the initial on-rate binding constant of formate/formic acid binding in spinach thylakoids. The data were obtained from Fig. 4. Different symbols indicate four different equilibrium formic acid concentrations at several different medium pH.

we take this information as opposing formate as the binding species.

It has been suggested that bicarbonate and formate bind to Fe^{2+}, to D1-R269 (Ref. 5; see also Ref. 40) and to D2 arginine [41]. If this is the case, formic acid would have to diffuse a certain distance (10–50 Å from stroma) before reaching the binding site. It is therefore necessary to consider the influence of diffusion when analyzing the binding kinetics. First, the dependence of the initial binding rate constant. C, on formate concentration (Fig. 4), does not show a linear characteristic at low concentration, as is expected for diffusion, but saturation. This is best seen from the set of straight lines obtained after the data in Fig. 4 were replotted as a double-reciprocal (Lineweaver-Burk) plot (Fig. 8). This implies that the effect of formate/formic acid follows saturation characteristic of binding at a specific
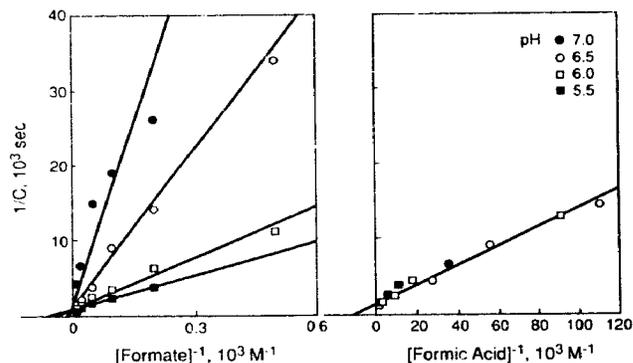
Fig. 8. A double-reciprocal (Lineweaver-Burk) plot for the initial binding constant, C, when formate (left panel) or formic acid (right panel) is considered to be the binding species. The data was calculated from that in Fig. 4. Different symbols represent different pH values (see key in the right panel).

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