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Modulation of herbicide-binding by the redox state of Q_{400}, an endogenous component of Photosystem II

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Preincubation of isolated chloroplasts with ferricyanide, prior to addition of DCMU, unmasks a high-potential electron acceptor (Q_{400}) in Photosystem II that acts as an additional quencher and prolongs the fluorescence induction curve in the presence of DCMU (Ikegami, I. and Katoh, S. (1973) Plant Cell Physiol. 14, 829–836). This study confirms that Q_{400} is endogenous to Photosystem II and is not a bound ferricyanide, and several new characteristics of this high potential acceptor are established. (a) It is accessible to ferricyanide even in the presence of DCMU. The rate of oxidation, however, is very slow, consistent with access only via Q_A. Accessibility may be enhanced by magnesium, reminiscent of the oxidation of Q_A^- by ferricyanide. (b) Oxidation of Q_{400} drastically suppresses the binding of DCMU at neutral and alkaline pH. Below pH 6, however, DCMU binding is essentially normal. The pH dependence of DCMU binding is consistent with the known pH dependence of the redox midpoint potential of Q_{400}. (c) Binding of many other inhibitors of Q_A-to-Q_B electron transfer is much less affected or even completely unaffected. These results have implications for current notions of herbicide binding and may also bear on the origin of slow phases of fluorescence induction in the presence of DCMU.

Introduction

Although some progress has been made in understanding the mechanisms of Photosystem II, the complexity of the processes has yielded information slowly. In the case of oxygen-evolution the complexity is intrinsic to the high redox potential and charge storage chemistry involved. The electron acceptor reactions, on the other hand, are confounded by a bewildering heterogeneity of behaviors involving two-electron and one-electron mechanisms operating in several different redox-potential ranges.

Particularly perplexing among the acceptor activities is a high-potential component, first identified by Ikegami and Katoh [1]. In the presence of sufficient cations to maintain stacked grana membranes, the normal fluorescence induction kinetics, in the presence of saturating levels of DCMU, show a fast sigmoidal rise with a light-intensity-dependent half-time. The sigmoidicity reflects the transfer of excitation energy between Photosystem II units which gives rise to a non-linear relationship between the fluorescence yield and the level of reduction of the primary quinone, Q_A [2]. The area over the induction curve, on the other hand, is a linear measure of the number of turnovers of

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid.

the reaction center necessary to fully reduce Q_A [3,4]. At constant quantum yield this is proportional to the number of electron acceptors involved. In the presence of DCMU this amounts to one – Q_A itself. Ikegami and Katoh found that incubation of chloroplasts with ferricyanide, followed by addition of DCMU, resulted in an uncharacteristically slow fluorescence induction – the 'Ikegami-Katoh effect' [1]. The half-time of the DCMU-inhibited fluorescence rise was increased 50–100% by pretreatment with ferricyanide. When quantified in terms of the area over the induction curve, the ferricyanide incubation was found to cause an approximate doubling of quenching activity associated with electron acceptors prior to the DCMU block. Redox titrations showed this novel component to have a redox midpoint potential (E_m) of +360 mV at pH 7.8. It is distinct from cytochrome b-559_{HP} because addition of DCMU before the ferricyanide completely blocked the development of the Ikegami-Katoh effect, while oxidation of cyt b-559_{HP} was not affected.

A later study of Bowes et al. [5] confirmed the original observations and showed that, while the effect is evident even in the fluorescence rise during a microsecond actinic flash, with a 20 ns laser flash only the high-potential quencher (Q_{400}) * was reduced. Thus, Q_{400} appears to be active in series with Q_A and is connected to it on a time scale less than 5 μs. They also found that the E_m was pH-dependent, in the range pH 6–8, which further distinguishes it from cyt b-559_{HP} [9]. However, quantitation of the Ikegami-Katoh effect is not readily achieved with precision and the pH-dependence, although reasonably consistent with a slope of −60 mV/pH unit, could not resolve possible pK values.

Ferricyanide is also capable of oxidizing Q_A^- in the presence of DCMU in a slow reaction which is very sensitive to the surface charge of the membrane [10]. A more recent study concluded that this oxidation was direct rather than via an endogenous high-potential, Ikegami-Katoh component and it was suggested that no such component exists [11]. This raised the possibility that Q_{400} is, in fact, a bound ferricyanide. The potential for rapid reactivity between ferricyanide and the acceptor quinones has recently been demonstrated in reaction centers from *Rhodopseudomonas viridis*, a photosynthetic bacterium [12]. Such a direct role for ferricyanide in the Ikegami-Katoh effect might be considered to be ruled out by the pH dependence of the midpoint potential. However, it is easy to show that pH-dependent competitive binding of both ferri- and ferrocyanide can give rise to an apparently pH-dependent E_m for the bound species (see Appendix).

The notion that a bound ferricyanide might function as the high-potential acceptor is not easily reconciled with the extreme order-of-addition requirement originaly reported [1,5]. In this study I show that the effect can be seen when ferricyanide is added after the DCMU. However, further observations on the reversibility of the phenomenon clearly show that the component is not ferricyanide but an endogenous species. A new manifestation of the component is also reported – a dramatic effect of its oxidation state on the binding of certain inhibitors of $Q_A^-Q_B$ to $Q_AQ_B^-$ electron transfer – and a model of close interaction between the primary quinone, the high-potential component and the binding site of some inhibitors is discussed.

Materials and Methods

Chloroplasts, from two-week old dwarf peas or from market spinach, were normally prepared by blending cut leaves in 0.4 M sorbitol/10 mM NaCl/5 mM $MgCl_2$/20 mM Tricine (pH 7.8), followed by centrifugation. The chloroplasts were washed in 10 mM NaCl, 5 mM $MgCl_2$ and finally resuspended in sorbitol-NaCl-$MgCl_2$-Tricine medium. Experimental measurements were generally done in 0.1 M sorbitol/10 mM NaCl/5 mM $MgCl_2$/20 mM buffer, pH range 5.4–8.3 plus 1 μM gramicidin. Minor variations in preparation procedure or suspension medium did not alter the observed behavior. Buffers used were: succinate (pH 5.4–5.6), Mes (pH 5.8–6.5), Mops (pH 6.8–7.5), Hepes (pH 7.5), Tricine (pH 7.8–8.3).

Fluorescence induction was measured in a con-

* The high-potential acceptor of Photosystem II has been referred to by many names: R (Ref. 1), Q_2 (Ref. 5), C_{400} (Ref. 6), A_H (Ref. 7), Q_{400} (Ref. 8). The latter is adopted here because it includes some useful information – the redox midpoint potential at pH 7.0 ($E_{m,7} = +400$).

ventional fluorimeter, with the detector, guarded by a Schott RG 665 filter, at right angles to the actinic light source, a 55W quartz-iodine bulb, filtered through a Corning 4-96 filter. The detector output was fed into a Biomation 805 transient recorder with a split time base, and stored in a Tracor Northern 575 signal averager. Traces were plotted out on an *X-Y* recorder.

The chlorophyll concentration for fluorescence measurements was 3.5 μg/ml, and potassium ferricyanide, when present, was added to 0.25 mM. For experiments involving inhibitor titrations, a large volume of sample was made up at twice these concentrations (7 μg Chl/ml and 0.5 mM potassium ferricyanide) and incubated at room temperature (25°C) for 10–15 min. This stock suspension was then placed on ice and 1 ml aliquots were diluted 1 : 1 with buffer for assay of the fluorescence induction kinetics. A similar protocol was followed for long incubation times except the stock suspension was kept at room temperature throughout. All sample manipulations were carried out under very low light.

DCMU binding determinations were performed as described by Tischer and Strottman [13], using DCMU with a specific activity of 4.2 μCi/μmol (a gift from Ms. C. Astier, CNRS, Gif-sur-Yvette). The chlorophyll concentration was 35 μg/ml. Potassium ferricyanide, when present, was 0.5 mM. Standards were run for all DCMU concentrations, with and without ferricyanide. The binding measurements were performed in total darkness except for a luminous clock. Even subdued room light, as normally used for such determinations, largely eliminated the effect of ferricyanide preincubation. Because of the time taken to perform the manipulations in the dark, the ferricyanide preincubation varied from 10–20 min and the incubation with DCMU varied from 5–15 min. The whole preparation time, for each set of eight samples, was 25 min.

Results

Fig. 1 shows the time-dependence of the appearance of the Ikegami-Katoh effect, measured as a slower rate for the fast phase of the fluorescence induction kinetics, during preincubation with 0.25 mM potassium ferricyanide. When incubated before the addition of DCMU, the effect was half maximal in 2–3 min, and essentially complete after 15 min. When DCMU was added before the ferricyanide the time required was much longer: in unstacked chloroplasts, the half time for appearance of the Ikegami-Katoh effect was in excess of 50 min. The presence of 5 mM magnesium ions, known to affect substantially the surface potential of the thylakoid membrane, and to induce restacking (appression) of the membranes, caused a significant acceleration of the development of the effect when DCMU was added first (half-time, approx. 20 min). The effect of magnesium was only reliably observed when added to previously unstacked chloroplasts. Chloroplasts that were prepared and maintained fully stacked, in the presence of magnesium, showed very little development of the Ikegami-Katoh effect when DCMU was added before ferricyanide (see, for example, Fig. 4). This may imply that the native, stacked structure is not readily regained, in vitro, after unstacking, leaving the appressed membrane regions more accessible to ferricyanide. Nevertheless, the experiment of Fig. 1 contradicts the strict order-of-addition requirement orginally reported [1,5]. Fig. 1 also indicates that prolonged (10–15 min) incubation with DCMU, added after the pretreatment with ferricyanide, did not cause any

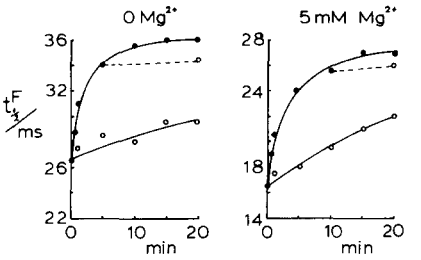


Fig. 1. Dependence of the half-rise time ($t^F_{1/2}$), for the DCMU-inhibited fast fluorescence induction kinetics, on ferricyanide pretreatment. Pea chloroplasts were prepared unstacked by omitting $MgCl_2$ from the wash and resuspension media. A portion were restacked by addition of 5 mM $MgCl_2$ to the stock suspension. The assay medium (pH 7.8) contained magnesium only where indicated (right panel). Incubation with ferricyanide (0.25 mM) occurred in the cuvette for the time indicated, either in the presence (○———○) or absence (●———●) of 10 μM DCMU. In the latter case, DCMU was added 1 min before illumination. (— — —), DCMU was added at the time indicated by the filled circle and the fluorescence recorded at the time indicated by the open circle.

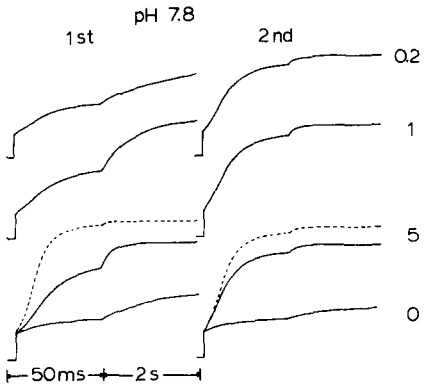


Fig. 2. DCMU titration of the fluorescence induction kinetics. Stacked spinach chloroplasts, in Mg^{2+}-containing medium (pH 7.8), were incubated with 0.5 mM potassium ferricyanide, at 7 μg Chl/ml and then diluted 2-fold 3 min in prior to assay. DCMU was added as indicated in the figure (μM), 2 min before the first illumination. Each illumination period was 5 s (only the first two seconds were recorded) and the dark time between illumination periods was 3 min. Induction kinetics with 5 μM DCMU, in the absence of, and without pretreatment by, ferricyanide (— — —).

significant reversal of the phenomenon.

It was noted during these studies that, in the presence of normally saturating levels of DCMU, ferricyanide-treated samples often manifested a large, abnormally slow phase of the fluorescence induction, with a half rise-time of about 1 s, compared to less than 50 ms for the fast phase (Fig. 2). When varying amounts of DCMU were used it became evident that this arose from incomplete inhibition by even quite high levels of DCMU (> 1 μM), indicating a much weaker binding of DCMU after the ferricyanide treatment. During illumination, the DCMU binding equilibrium shifted towards the normal position, giving a slower induction phase to a final level indistinguishable from that seen when ferricyanide was added after DCMU. The rate of the slow fluorescence rise was dependent on both light intensity and DCMU concentration (not shown). The effect of ferricyanide pretreatment is, therefore, evidently relieved by illumination, concomitant with the photoreduction of Q_{400}; DCMU can subsequently bind in a concentration dependent (second order) process. Essentially identical behavior has been described by Joliot and Joliot [14] for chloroplasts preilluminated with one flash to place the acceptor quinone complex in the $Q_AQ_B^-$ state. In this state, DCMU binds much more weakly and effective inhibition is delayed until a second turnover allows the reoxidation of the quinone complex by the plastoquinone pool, via the two-electron gate mechanism [15–18]. The fluorescence level reached at a fixed time, chosen to be close to when the fast rise phase was completed, is a convenient measure of the degree of inhibition of the sample. Fig. 2 shows the induction kinetics at several DCMU concentrations during the first illumination after ferricyanide pretreatment and again three minutes later, when Q_A^- was largely reoxidized, but the high potential component was not. The second illumination, therefore, provides a measure of DCMU binding under 'control' conditions, i.e., Q_{400} reduced. Separate experiments in the absence of ferricyanide confirmed that the second illumination is similar, but not identical, to a true control (Fig. 3).

Data from the experiment of Fig. 2 are plotted in Fig. 3, along with others at several different pH values. The data are represented as the ratio of the variable fluorescence (ΔF_t) at time t, the break in the dual time trace, to ΔF_m, the maximum variable fluorescence at time t seen at high DCMU levels. Because of the rather arbitrary choice of t and the variation in fast rise kinetics due to the fer-

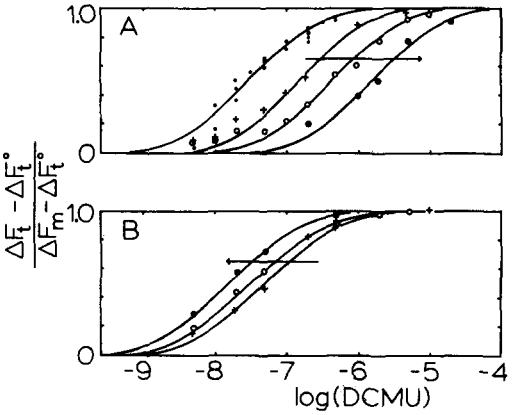


Fig. 3. pH-dependence of the DCMU-titration of fluorescence. The variable fluorescence at time t (ΔF_t) is normalized to the minimum level reached with no DCMU added (ΔF_t^0) and to the maximum level, also at time t, obtained with saturating levels of DCMU (ΔF_m). (A) Pretreatment with ferricyanide as for Fig. 2, except: +, pH 5.4; ○, pH 6.3; ●, pH 7.8; points (·), collected data for a second illumination at pH 5.8, 6.3, 7.8, and 8.3. (B) No ferricyanide: +, pH 5.8; ○, pH 6.9; ○, pH 8.3. Arrows indicate increasing pH.

ricyanide treatment, ΔF_m is slightly different for the ferricyanide-treated and control curves, and serves as a normalizing factor. It is clear that after pretreatment with ferricyanide the inhibitory activity of DCMU was very pH-dependent, becoming much weaker at high pH (Fig. 3A). In the absence of ferricyanide, the pH-dependence of DCMU inhibition was slight, actually becoming stronger at high pH (Fig. 3B). Although a single curve has been drawn through each data set in Fig. 3, it should be noted that at the higher pH values, after ferricyanide treatment, there was some effect of DCMU even at concentrations much below the overall I_{50}. This may indicate a heterogeneity in PS II centers, with respect to their DCMU sensitivity after treatment. The fluorescence increase at such low DCMU levels was only 15% of the maximum variable fluorescence, but the relationship between fluorescence and the proportion of closed centers is non-linear, due to energy transfer between photosynthetic units [2]. If one assumes that all PS II centers are equally connected by an effective energy transfer probability of 0.5 [2,19], the observed 15% fluorescence increase would correspond to about 35% of the total PS II centers.

The correspondence between the effect of ferricyanide on DCMU inhibition, shown in Figs. 2 and 3, and the classic Ikegami-Katoh effect upon the rate of the fast fluorescence induction process is shown in Fig. 4. Incubation with ferricyanide, prior to addition of 2 μM DCMU, modified both the binding of DCMU and the half-time of the fast fluorescence rise in a similar manner. Addition of DCMU after 10 min did not reverse these effects during a subsequent incubation of nearly one hour, in contrast to the results of Fig. 1 where unstacked/restacked pea chloroplasts were used. However, if the initial incubation was performed with a mixture of ferri- and ferrocyanide (0.5 : 2.0 mM), addition of 2 μM DCMU caused a reversal of the effects with a half-time of 10–15 min. This is most readily explained by a DCMU-induced increase in the midpoint potential of an endogenous component allowing it to be rereduced by the ferrocyanide.

The effect of ferricyanide-treatment on the inhibitory potency of DCMU, shown in Figs. 2 and 3, is very pH dependent (see also Fig. 7). At pH 8.3, the difference in I_{50} values, between treated

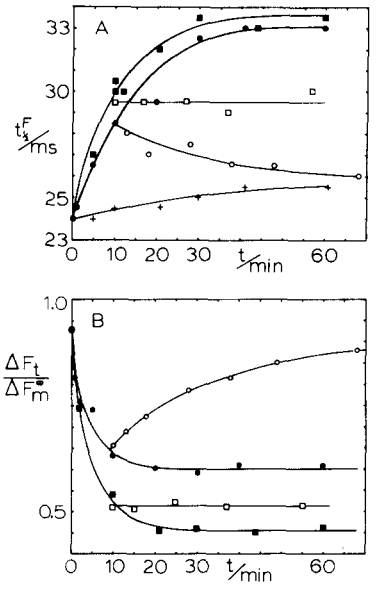


Fig. 4. Comparison of the effect of ferricyanide pretreatment on the rate and extent of the fast, DCMU-inhibited fluorescence induction phase. (A) Half-rise time of the fast fluorescence induction phase (classic Ikegami-Katoh effect). (B) Extent of the fast fluorescence induction phase (ΔF_t) normalized to the maximum variable fluorescence achieved after 2 s illumination in the presence of high levels of DCMU (ΔF_m^∞). Sample preparation as in Fig. 2, except with pea chloroplasts. ■, □, plus 0.5 mM ferricyanide; ●, ○, plus 0.5 mM ferricyanide/2 mM ferrocyanide. ■, ●, 2 μM DCMU was added at the times indicated, and the fluorescence induction assayed 1 min later. □, ○, 2 μM DCMU was added after 10 min preincubation and the fluorescence assayed after a total incubation time as indicated by the position on the abscissa. +, 2 μM DCMU was added 1 min before the ferricyanide; the points indicate total incubation time. The rate of the fast rise phase was quantitated by measuring the half-time to reach a level obtained by extrapolating the slow phase back to $t = 0$. Because of the great difference in rates of the fast and slow phases, the extrapolation was small and its accurary was not crucial.

and control samples, was almost three orders of magnitude. At low pH, the I_{50} values appeared to coincide. This behavior was confirmed by direct binding studies on pea chloroplasts using [^{14}C]DCMU (Fig. 5). The precision of the experiment was limited by the low-specific activity of the DCMU (4.2 μCi/μmol), and at low pH the two sets of data could be taken to indicate competitive behavior. However, at high pH, where the fluorescence data indicated a large difference between the treated and control K_D values, the binding data

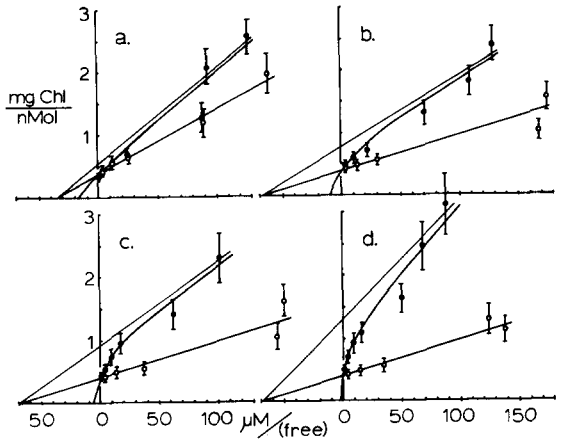


Fig. 5. Direct binding assay of [^{14}C]DCMU in pea chloroplasts. Preincubation with (●) or without (○) 0.5 mM ferricyanide; see Methods. (a) pH 5.40; (b) pH 5.85; (c) pH 6.75; (d) pH 8.05. Ordinate: reciprocal of the amount of inhibitor bound (nmol/mg Chl), I_b in the text. Abscissa: reciprocal of the concentration of free inhibitor (μM), I in the text. The error bars indicate the extreme values obtained from duplicate determinations of the stock activity. The upper line in each quadrant indicates a reasonable asymptote for the ferricyanide-treatment data (●) at low concentrations of free inhibitor. The curves show best fits to these data, according to Eqn. 2, using values for K_2, for the high affinity sites, obtained from the control data (○).

showed the separation of two binding populations in the treated samples – one with an affinity close to that of the control, and another with a much lower affinity. Thus, the binding assay reported a mixture of two binding functions, both of which might be assumed to be hyperbolic. The overall binding equation would then be:

$$I_b = \frac{B_1 I}{K_1 + I} + \frac{B_2 I}{K_2 + I} \tag{1}$$

or, in double reciprocal format:

$$\frac{1}{I_b} = \frac{K_1 K_2 + I(K_1 + K_2) + I^2}{(B_1 K_2 + B_2 K_1) I + (B_1 + B_2) I^2} \tag{2}$$

where I_b is the total amount of inhibitor bound, B_1 and B_2 and K_1 and K_2 are the concentrations and dissociation constants of the two different binding site populations, and I is free inhibitor. When $K_1 \gg K_2$, the high affinity behavior can be seen at low inhibitor levels ($I^2 \to 0$) and follows a straight line in the normal, double reciprocal representation. The negative intercept of this line on the abscissa provides an accurate measure of K_2, the dissociation constant for the tight-binding sites. However, the intercept of this linear construction on the ordinate (See Fig. 5) can overestimate the number of high-affinity sites (B_2):

$$I_b(I \to \infty) = B_2 + \frac{B_1 K_2}{K_1} \tag{3}$$

Eqn. 2, on the other hand, intercepts the ordinate at the total number of sites. Applying this analysis to the data in Fig. 5 revealed two classes of binding sites after preincubation with ferricyanide: one with a relatively weak, pH-dependent dissociation constant, the other with a high affinity that was almost pH-independent. As is most clearly seen at the highest pH the relative abundance of these sites was roughly 2.5 : 1. This is in very good agreement with the heterogeneity suggested by the fluorescence titrations of Fig. 3A. In all cases, the total number of binding sites was unchanged by the ferricyanide treatment. It is not clear, at this time, whether the coexistence of low-affinity and high-affinity sites indicates incomplete (approx. 65%) conversion by the ferricyanide treatment, or a manifestation of heterogeneity in DCMU binding sites. However, it is noteworthy that other manifestations of heterogeneity in PS II reveal similar proportions, e.g., PS II$_\alpha$ vs. PS II$_\beta$ [19,20] and B vs. non-B centers [21]. In either case, the observed relative abundance implies that the centers that show a dramatic effect of ferricyanide on their DCMU sensitivity, include active PS II centers.

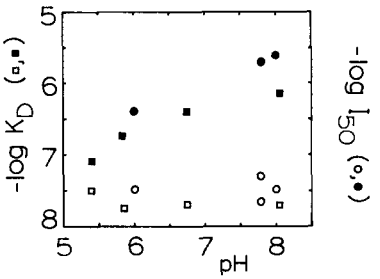


Fig. 6. Comparison of K_D and I_{50} for DCMU in pea chloroplasts. K_D values from Fig. 5; preincubation with (■) or without (□) 0.5 mM ferricyanide. I_{50} values obtained as in Figs. 2 and 3; first (●) and second (○) illumination.

Fig. 6 shows the binding constants obtained from the data of Fig. 5 together with I_{50} values from fluorescence studies on identical pea chloroplast preparations. The correspondence is strong over the whole pH range 5.5–8.0. In Fig. 7 more extensive I_{50} data are shown for spinach chloroplasts. It is apparent that the marked effect on binding seen for DCMU is not evident with other inhibitors tested so far. *O*-Phenanthroline, a relatively weak inhibitor of Q_A^- reoxidation, was chosen here because it has been shown to bind and unbind relatively quickly [22]. *O*-Phenanthroline binding showed only a small effect of the oxidation state of the high potential acceptor. Inhibition by atrazine, a representative of the s-triazine class of PS II-inhibitors, was completely unaffected by ferricyanide treatment. Similarly, ioxynil, a potent hydroxybenzonitrile, and dinoseb, a nitrophenol, showed less than 4-fold change in I_{50} upon oxidation of the high-potential component (not shown). All these inhibitors, however, reveal the Ikegami-Katoh effect on the time course of the fast phase of the fluorescence induction (not shown).

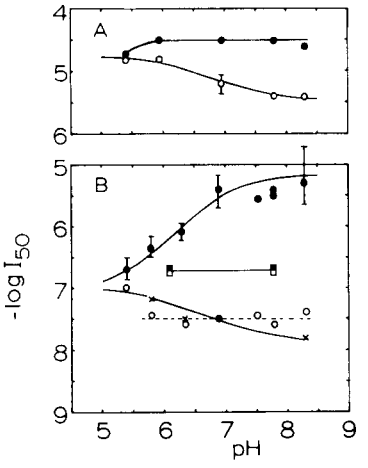


Fig. 7. pH dependence of I_{50} for various PS II inhibitors in spinach chloroplasts. I_{50} values obtained as in Figs. 2 and 3. Filled symbols, first illumination; open symbols, second illumination. (A) ●, ○, *o*-phenanthroline; (B) ●, ○, DCMU; ■, □, atrazine; ×, DCMU, with no ferricyanide treatment. The dotted line indicates that the pH-dependence for the second illumination after ferricyanide treatment (○) may be slightly different from that for the first illumination without ferricyanide treatment (×).

Discussion

The dramatic effect of ferricyanide treatment on the I_{50} for DCMU, at high pH, clearly indicates some sort of causative interaction between the two reagents. However, the strong order-of-addition requirement usually observed argues strongly against the identity of Q_{400} as a bound ferricyanide that is displaceable by DCMU, e.g., in the Q_B-site. On the other hand, the experiment of Fig. 4 shows that the effect of ferricyanide can be reversed by the addition of DCMU, providing ferrocyanide is also present. This raises the possibility that Q_{400} might be a bound ferricyanide, displaceable by ferrocyanide, but the interaction with DCMU is then still unaccounted for. Furthermore, the pH-dependence of the apparent midpoint potential of Q_{400} would require an unlikely degree of divergence between the binding properties of ferri- and ferrocyanide (See Appendix).

A much simpler and more plausible explanation is that Q_{400} is an endogenous component that is oxidized by ferricyanide and can be rereduced by ferrocyanide. The pH-dependence of the E_m of Q_{400} and of the I_{50} for DCMU can then be interpreted in terms of redox state-dependent interactions between Q_{400} and DCMU (Fig. 8).

When Q_{400} is reduced, the pH-dependence of DCMU binding is weak or non-existent . For spinach (Fig. 7), the I_{50} data in the absence of ferricyanide show a weak pH-dependence. There is currently much controversy over photosynthetic unit sizes [23,24]. We have consistently observed values of about 320 for the number of Chl per

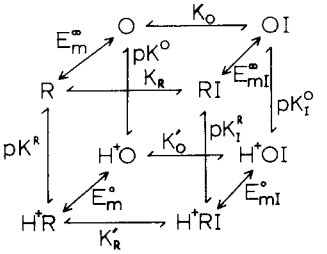


Fig. 8. Redox and ligand-binding states associated with Q_{400}. O, oxidized Q_{400}; R, reduced Q_{400}; I, inhibitor (DCMU); H^+, hydrogen ion. All K's are dissociation constants. See text for further discussion.

inhibitor binding site, using DCMU, terbutryn and ioxynil, and in either pea or spinach chloroplasts (Paterson, D.R. and Wraight, C.A., unpublished data). Similar values may be found throughout the literature [13,25,26], although higher ones are also common [30,31]. On the basis of this experimental value, the concentration of binding sites in the fluorescence experiments would be about 12 nM (3.5 μg Chl/ml). At low pH (less than 6), the I_{50} for DCMU in the absence of ferricyanide tends towards 10^{-7} M. This should be a reasonable estimate of K'_R, since it is significantly greater than the binding site concentration, and non-specific binding is negligible in this concentration range [13]. At high pH, however, the I_{50} tends towards 10^{-8} M and must be increasingly dominated by the concentration of binding sites. For a simple, monotonic binding-functional assay, $I_{50} = K + \frac{1}{2}$ B, so $K_R < 10^{-8}$ M. It seems likely, therefore, that DCMU binding when Q_{400} is reduced is more strongly pH-dependent at high pH than is indicated by the fluorescence assay. The I_{50} data are consistent with a value for $pK_I^R \geq 7.5$ and $pK^R \geqq 8$, but direct binding assays, with higher specific activity DCMU, are needed to establish these numbers.

The I_{50} values obtained from a second illumination in the presence of ferricyanide are similar to those obtained in the absence of ferricyanide but are more or less constant throughout the pH range, yielding no information on pK values. The value of $3 \cdot 10^{-8}$ M is small enough to be somewhat influenced by the binding site concentrations. The reason for the small, but qualitative, difference between these data and those obtained in the absence of ferricyanide is not known.

After ferricyanide preincubation, the I_{50} for DCMU becomes markedly pH dependent. The binding is sufficiently weak ($K \geqq 10^{-7}$ M) that the I_{50} values should provide a good indication of the affinity; thus $K'_0 \approx 1 \cdot 10^{-7}$ M and $K_0 \approx 6 \cdot 10^{-6}$ M, with probable pK values of 5.3 (pK^0) and 7.2 (pK_I^0).

The parameters described so far define the front and back faces of the binding cube of Fig. 8. The other faces require data on the redox behavior of Q_{400}, some of which is available from the work of Bowes et al. [5]. In that study, as in all other studies of Q_{400} to date, the redox state of the high-potential acceptor was established prior to the addition of DCMU and the fluorescence induction assay carried out shortly thereafter. Thus, the E_m values reported by Bowes et al. correspond to the left-hand face of Fig. 8, and the pH dependence ought to reflect pK^R and pK^0. The value of pK^0 obtained in the present work would be too low to be readily observable in the E_m/pH data of Ref. 5, which did not extend below pH 6. Hoever, if we take $pK^0 = 5.3$, we may deduce that $E_m^0 \approx 500$ mV. pK^R is uncertain in the present work, but probably lies outside the range covered here. The data of Bowes et al. also indicate that pK^R cannot be less than 8, as there is no strong indication of a tailing-off of the pH-dependence of E_m. Thus, $E_m^\infty \leq 320$ mV. Furthermore, if $K'_R \approx K'_0 \approx 1 \cdot 10^{-7}$ M (Fig. 7), then $E_{mI}^0 \approx E_m^0 \approx 500$ mV. Finally, since $K_0 \geqq 600\ K_R$, $E_{mI}^\infty \geqq E_m^\infty + 170$ mV and, at high pH, the addition of DCMU causes a large increase in the E_m of Q_{400}. Thus, when Q_{400} is oxidized by preincubation with a mixture of ferri- and ferrocyanide, the addition of DCMU induces its rereduction by the ferrocyanide (Fig. 4). The assigned values to the parameters of Fig. 8 are summarized in Table I.

Another point for discussion concerns the differing responses of other PS II-inhibitors to the redox state of Q_{400}. Clearly, the interaction between Q_{400} and DCMU is at least moderately strong; a change in binding affinity of 600, seen at high pH, is equivalent to a change in interaction energy of 16 kJ $\cdot$ mol^{-1}. In contrast, the change for *o*-phenanthroline is less than 6 kJ $\cdot$ mol^{-1}, and for atrazine it is essentially zero. A substantial amount of work has shown that these, and many other, PS II-inhibitors display competitive binding behavior [13]. Furthermore, Velthuys [17] and Wraight [18] have previously suggested that the mode of action

TABLE I

APPROXIMATE AND ESTIMATED VALUES FOR PARAMETERS OF THE BINDING SCHEME (FIG. 8)

$K_0 \approx 6 \cdot 10^{-6}$ M	$K'_0 \approx 1 \cdot 10^{-7}$ M
$K_R < 1 \cdot 10^{-8}$ M	$K'_R \approx 1 \cdot 10^{-7}$ M
$pK^0 \approx 5.3$	$pK_I^0 \approx 7.2$
$pK^R \geqq 8$	$pK_I^R \geqq 7.5$
$E_m^\infty \leqq 320$ mV	$E_{mI}^\infty \geqq E_m^\infty + 170$ mV
$E_m^0 \approx 500$ mV	$E_{mI}^0 \approx 500$ mV

of these inhibitors is to displace competitively the secondary quinone, Q_B, from its binding site. The most parsimonious hypothesis would invoke a single site, or focus, for both quinone and inhibitors, but a number of observations require substantial modification of this simple view. These include the chemical diversity of PS-II-inhibitors, the existence of at least two classes of inhibitors, identifiable by which protein subunit is most readily labelled through covalent linkage of azido-inhibitors [26–29], and the appearance of mutant biotypes that are resistant to triazines, but are still sensitive to DCMU and are hypersensitive to nitrophenols [30–32]. Such mutants usually also show altered Q_B-activity [30,31,33], but there is one report of a herbicide-resistant cyanobacterial mutant with essentially normal Q_B-behavior [34].

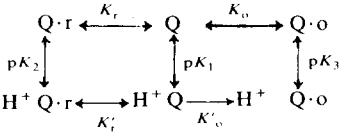
These properties can generally be accommodated by the model of 'overlapping' binding domains [30,35], where only a small crucial element is common to all compounds. However, they can, of course, also be explained by longer range, allosteric interactions. The results presented here show a marked distinction between two inhibitors, DCMU and atrazine, previously considered to be quite closely related in action [13,30,35]. The modulation of DCMU binding by the redox state of Q_{400} is likely to arise largely from electrostatic forces. X-ray studies of many proteins show that probably all charged species internal to proteins or membranes are extensively compensated [36]. Thus, the net charge change involved in oxidizing Q_{400} may be expected to induce a substantial conformational response within the protein. The widely differing responses of DCMU and atrazine to the redox state of Q_{400} leads one to speculate that the sites of action for these classes of inhibitor are well separated from other other. One the other, hand, the very rapid oxidation of Q_A^- by oxidized Q_{400} ($t_{1/2} < 5$ μs) suggests that these two acceptors approach closely to one another.

The pH-dependence of DCMU binding (Fig. 7) indicates that the effect of oxidized Q_{400} (more positive relative to the reduced form) can be counteracted by protonation (also adding a positive charge). How this arises is unknown, but it could arise from charge pairing of the oxidized Q_{400} with an ionizable counter-ion group in the protein. When Q_{400} is reduced, such a group would be protonated and neutral ($pK^R > 8$). Upon oxidation of Q_{400} the pK of the group is lowered ($pK^0 \approx 5.3$) and, at pH values above this pK, it becomes deprotonated (negative). Charge pairing may then occur with oxidized Q_{400}, inducing a significant structural rearrangement in the protein. At low pH, the charge on Q_{400} (ox) is insufficient to induce deprotonation of the counter ion species which, therefore, remains neutral and does not charge pair with Q_{400} (ox). Thus, no large conformational change occurs.

Finally, it was generally observed, at high pH ($\geqq 7.5$), that even in the absence of ferricyanide a small component of the fluorescence rise could be resolved that titrated at high levels of DCMU, similar to the behavior of chloroplasts with oxidized Q_{400}, i.e., $I_{50} > 1$ μM. There is a great deal of literature and confusion concerning the phenomena of slow phases of the fluorescence rise in the presence of DCMU. One recent study has, in fact, demonstrated the sensitivity of some aspects of the slow phase to very high levels of DCMU [37], and low affinity DCMU-binding sites have been noted in previous work [38,39]. The present study raises the possibility that partially oxidized Q_{400} could contribute to the slow phase phenomenology. Since most chloroplast fluorescent studies are performed aerobically ($E_h = 815$ mV) and the plastoquinone pool of dark adapted thylakoids is fully oxidized, the high potential of Q_{400} is not a strong argument against such a contribution.

Appendix

Assume that ferricyanide (o) and ferrocyanide (r) bind mutually exclusively to a single site (Q), in a pH-dependent fashion:



where the K's are dissociation constants. If bound ferricyanide were active as a rapid electron acceptor from Q, then Q_{400}-like activity would exhibit the following dependence on concentrations of ferricyanide, ferrocyanide and pH:

$$\frac{Q\cdot o + H^+ Q\cdot o}{\sum \text{ all species}} = \frac{\frac{[o]}{K_o}\left(1+\frac{[H^+]}{K_3}\right)}{1+\frac{[o]}{K_o}+\frac{[r]}{K_r}+\frac{[H^+]}{K_1}\left(1+\frac{[o]}{K'_o}+\frac{[r]}{K'_r}\right)} \qquad (A\text{-}1)$$

At half-maximal activity the ratio of ferri-/ferrocyanide in the solution, i.e., the redox potential, would be:

$$\left\{\frac{[o]}{[r]}\right\}_{1/2} = \frac{1+\frac{[H^+]}{K_2}}{1+\frac{K_o}{K'_o}\frac{[H^+]}{K_1}-\frac{K_o}{[o]}\left(1+\frac{[H^+]}{K_1}\right)}\cdot\frac{K_o}{K_r} \qquad (A\text{-}2)$$

If the concentration of ferricyanide is sufficiently high ($[o] \gg K_o$), this reduces to:

$$\left\{\frac{[o]}{[r]}\right\}_{1/2} = \frac{1+\frac{[H^+]}{K_2}}{1+\frac{[H^+]}{K_3}}\cdot\frac{K_o}{K_r} \qquad (A\text{-}3)$$

Since $E_m^{app} = E_m(\text{ferri/ ferrocyanide}) + 60\ \log\{[o]/[r]\}_{1/2}$, the effect of bound ferricyanide acting as an electron acceptor species could exhibit a fairly normal redox potential-dependent behavior with a pH dependence determined by the values of pK_2 and pK_3. Numerical solutions of these equations showed that redox potential-like behavior could be observed for a wide range of ferricyanide concentrations and that the requirement for $[o] \gg K_o$ was not stringent. However, for approximate simulation of the pH-dependence of Q_{400} reported in ref. 5, the difference between pK_2 and pK_3 had to be more than two pH units. It is not easy to see how such similar species as ferri- and ferrocyanide, differing in charge by only one unit, could have such divergent effects on the pK of a nearby group.

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