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INHIBITION OF CHEMICAL OXIDATION AND REDUCTION OF CYTOCHROMES *f* AND *b*-559 BY CARBONYLCYANIDE *p*-TRIFLUOROMETHOXY PHENYLHYDRAZONE

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SUMMARY

The presence of low (1–4 μM) concentrations of carbonylcyanide *p*-trifluoromethoxyphenylhydrazone during actinic illumination of chloroplasts generally inhibits the rate of subsequent dark chemical oxidation–reduction reactions of cytochrome *f* and *b*-559 Ferricyanide oxidation and ascorbate reduction of cytochromes *f* and *b*-559 are inhibited, as is hydroquinone reduction of cytochrome *b*-559 Inhibition by carbonylcyanide *p*-trifluoromethoxyphenylhydrazone of hydroquinone reduction of cytochrome *f*, the most rapid of these chemical oxidation–reduction reactions, cannot be detected The rate of the chemical redox reactions of the cytochromes in the presence of carbonylcyanide *p*-trifluoromethoxyphenylhydrazone are all markedly dependent upon the concentration of oxidant or reductant except the hydroquinone reduction of cytochrome *b*-559 photooxidized in the presence of carbonylcyanide *p*-trifluoromethoxyphenylhydrazone

The data is interpreted in terms of an effect of carbonylcyanide *p*-trifluoromethoxyphenylhydrazone on thylakoid membrane structure which generally inhibits accessibility to the hydrophobic interior of the membrane, possibly through an increase in membrane microviscosity The question of whether such an effect on membrane structure could be involved in uncoupling or inhibition effects of the carbonylcyanide-phenylhydrazone compounds is discussed, as is the special effect of these compounds on the cytochrome *b*-559 photoreactions at room temperature

INTRODUCTION

The ring-substituted derivatives of carbonylcyanide phenylhydrazone were found in 1962 to be very effective uncouplers of photosynthetic phosphorylation [1, 2] Since then it has been found that these compounds have other unusual effects on

Abbreviations FCCP carbonylcyanide *p*-trifluoromethoxyphenylhydrazone, CCP, carbonylcyanide phenylhydrazone

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electron transport In particular, the trifluoromethoxy derivative at low ($\leqslant$ 1 μM) concentrations increases the amplitude of light minus dark spectra of the photosynthetic cytochrome *b*-559 [3–9] At higher concentrations [10] or after somewhat extended incubation times (Cramer, W A , unpublished), FCCP results in inhibition of steady-state oxygen evolution FCCP is a member of a class of reagents which accelerate the deactivation reactions of photosystem II, as determined by measurements of average oxygen flash yields as a function of the time between flashes [11] Because other uncouplers like NH_4Cl and atebrin do not exert these effects in electron transport [4], one is led to consider the possibility of special structural effects of the CCP compounds on the membrane or electron transport system These effects might be associated with or independent of those relating to proton translocation [12–14] Interaction of CCP compounds or phenolic uncouplers with membrane protein [15,16] and albumin [17,18] has been observed Binding of these compounds to mitochondria [19, 20] and lipid-depleted mitochondria [19] has previously led to the hypothesis of special interactions of the phenolic uncouplers with membranes and membrane protein [21] Such interactions might relate to sulfhydryl reagent function [2, 4, 15, 22] or possibly the -NH group p*K* of compounds resembling CCP [23] Recent work in our laboratory has provided some evidence for membrane structural changes in the presence of FCCP FCCP (5 μM) causes an increase in the microviscosity of the *Escherichia coli* cell envelope [24] The experiments presented here were initiated in order to gain insight into the causes of the relatively slow hydroquinone reduction of cytochrome *b*-559 photooxidized in the presence of FCCP [6] We have previously attributed this relatively slow reduction to a transient formation of a low potential species of cytochrome *b*-559 which cannot be reduced by hydroquinone [6] The work discussed below indicates that the presence of FCCP during exposure of chloroplasts to actinic light causes a decrease in the accessibility of both cytochromes *b*-559 and *f* to chemical oxidant and reductant, making previous inference based on the hydroquinone reduction experiment of ref 6 more tenuous

METHODS

The method for chloroplast preparation is described in Methods (1) and the spectrophotometer parameters in Methods (2) of ref 25 The reaction medium contains 25 mM Tricine–KOH, pH 7 8, 5 mM K_2HPO_4, 5 mM NaCl, 2 mM $MgCl_2$, and 0 1 mM methyl viologen at 22–23 °C The chloroplast concentration is 80 μg/ml chlorophyll except where noted FCCP was kindly given to us by Dr P G Heytler

RESULTS

The kinetics of the ferricyanide oxidation of cytochrome *f* after prior illumination are monophasic with a half-time of approx 30 s (Fig 1A and ref 25) If FCCP (2 μM) is present during illumination, the subsequent ferricyanide oxidation is appreciably slower, as is the rate of ascorbate reduction of the oxidized cytochrome *f* (Fig 1B) The effect of adding FCCP in the dark after illumination is marginal A similar effect of FCCP present during prior illumination is seen on the rate of ascorbate reduction of chemically oxidized cytochrome *b*-559, with the half-time for reduction increasing from 9 s in the control (FCCP absent) to 17 and 24 s, respectively,

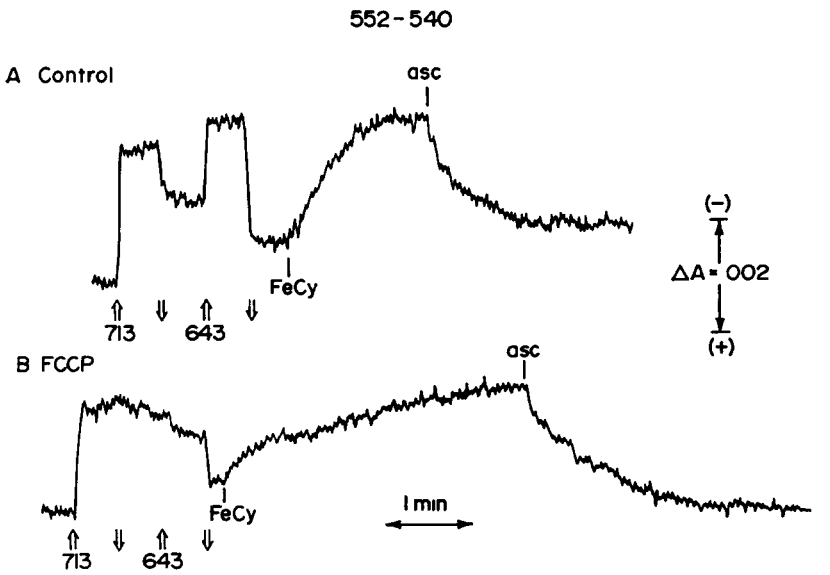

Fig 1 Comparison of the rates of chemical oxidation and reduction by ascorbate (asc) of cytochrome *f* in the absence (A) and presence (B) of FCCP during prior illumination Chloroplast concentration 80 μg/ml Concentration of FCCP, 2 μM, ferricyanide, 250 μM, ascorbate 1 mM Upward deflection in the traces corresponds to oxidation and absorbance decrease, upward arrows, actinic light on

with 2 and 4 μM FCCP present during a period of actinic illumination (Fig 2, Table I)

Some additional features of the light-induced absorbance changes in Figs 1 and 2 should be noted (a) FCCP causes an increase in the amplitude of the far-red light-induced oxidation of cytochrome *b*-559, and not cytochrome *f* (b) The uncou-

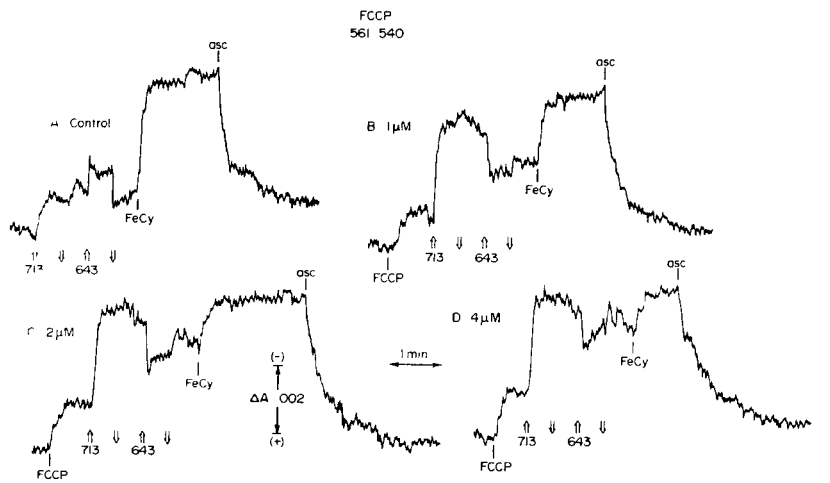

Fig 2 Comparison of chemical oxidation by ferricyanide and reduction by ascorbate of cytochrome *b*-559 in the absence (A) and presence (B–D) of FCCP during prior illumination Concentration of FCCP in (B) 1 μM, (C) 2 μM, and (D) 4 μM

TABLE I

Time for 50 % reduction of oxidized cytochrome *b*-559 by ascorbate (2 mM) as a function of FCCP concentration (calculated from Fig 2)

[FCCP] (μM)	$\tau_{\frac{1}{2}}$ (s)
0	9±2
1	11±2
2	17±2
4	24±2

pling effect of FCCP can be seen in the smaller amplitude of red light-induced oxidation of cytochrome *f* (c) The extent of the reduction by red light of cytochrome *b*-559 photooxidized in the presence of 1 and 2 μM FCCP is typical for a component located in the main electron transport chain joining photosystems I and II [4] With 4 μM (FCCP) (Fig 2D) it is apparent that reduction of cytochrome *b*-559 by red light is inhibited It should be noted that because the effects of FCCP at 552 nm and 561 nm are both so pronounced, no attempt was made to correct for mutual interference between cytochromes *f* and *b*-559 in these experiments

It has been shown that of the three oxidation–reduction compounds, ferricyanide, ascorbate, and hydroquinone, it is only the latter more lipophilic compound which affects the cytochrome *f* redox state more rapidly than that of cytochrome *b*-559 [25] In addition, there is no measurable effect of FCCP on the rate of reduction of chemically oxidized cytochrome *f* by 1 mM hydroquinone (Fig 3B) or by 0 25 mM

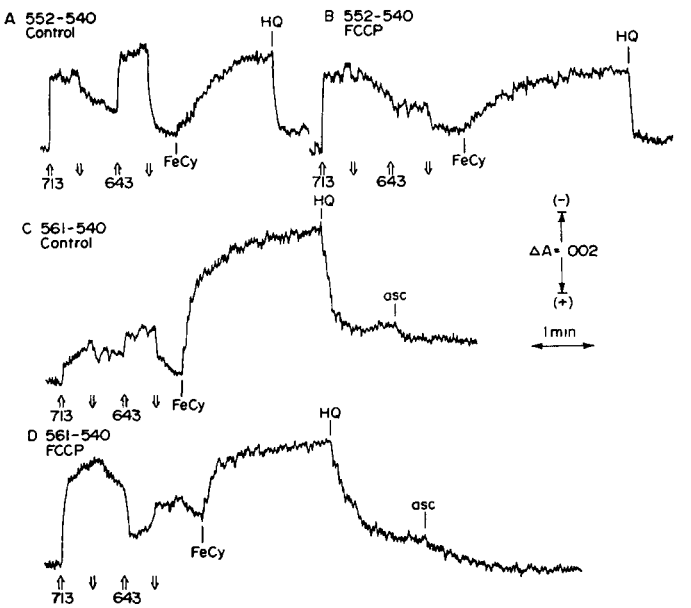

Fig 3 The effect of FCCP present during prior illumination on hydroquinone reduction of chemically oxidized cytochrome *f* (A, B) and *b*-559 (C, D) Concentration of FCCP, 2 μM, ferricyanide, 125 μM, hydroquinone and ascorbate 1 mM

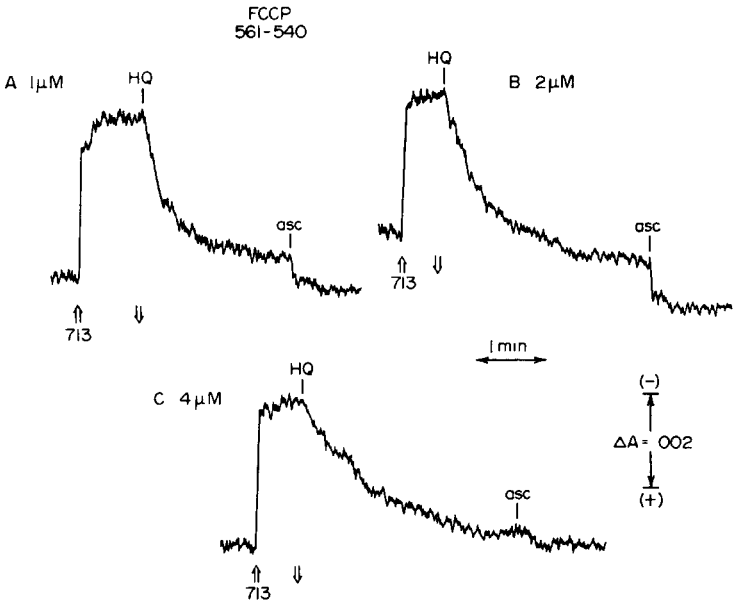

Fig 4 The dependence of the rate and extent of hydroquinone (HQ) reduction of cytochrome *b*-559 on the concentration of FCCP (A, 1 μM, B, 2 μM, C, 4 μM) present during prior illumination

hydroquinone (data not shown) The rate of hydroquinone reduction of cytochrome *b*-559 is inhibited by the presence of FCCP during prior illumination (Fig 3D), with the FCCP effect again markedly dependent on FCCP concentration above 1 μM (Fig 4, Table II) This result is similar to that obtained previously [6] when hydroquinone was added immediately after photooxidation by far-red light in the presence of FCCP

The rate of the chemical oxidation–reduction reaction is expected to be dependent on the concentration of oxidant and reductant in the case where the reactions are diffusion-limited The rate of ascorbate reduction of cytochromes *f* and *b*-559 photooxidized in the presence of FCCP is markedly dependent upon ascorbate concentration (Fig 5, Table IIIA) The rate of hydroquinone reduction of cytochrome *f* photooxidized in the presence of FCCP is also dependent upon hydroquinone concentration (Fig 6A, B, Table IIIB) The rate of hydroquinone reduction of cytochrome *b*-559 photooxidized in the presence of FCCP, however, is much less depen-

TABLE II

Time for 50 % reduction of oxidized cytochrome *b*-559 by hydroquinone (1 mM) as a function of FCCP concentration (calculated from Fig 4)

[FCCP] (μM)	$\tau_{\frac{1}{2}}$ (s)
1	13±2
2	24±2
4	42±3

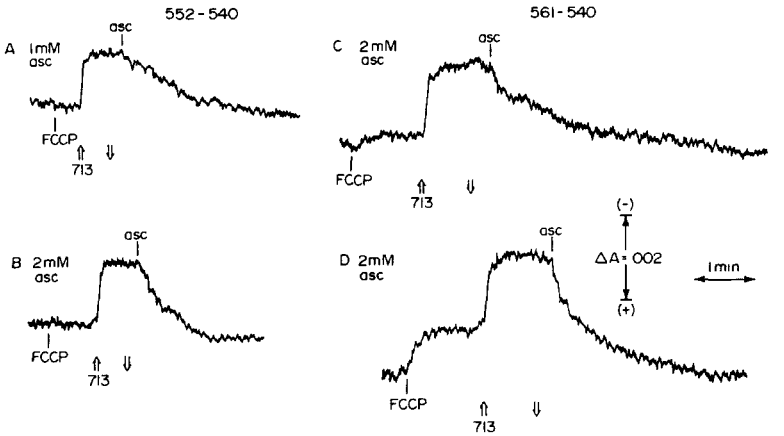

Fig 5 The dependence on ascorbate concentration of the rate of cytochrome *f* and *b*-559 reduction after oxidation by far-red light Ascorbate concentration in reduction of cytochrome *f* (A, 0 1 mM, B, 2 mM) and *b*-559 (C, 0 2 mM, B, 2 mM) FCCP concentration, 1 μM Chlorophyll concentration, 55 μg/ml

dent on hydroquinone concentration (Fig 6C, D, Table IIIB), suggesting the possibility that hydroquinone reduction of *b*-559 photooxidized in the presence of FCCP is limited by something other than diffusion

TABLE III

Time for 50 % reduction of oxidized cytochromes at different ascorbate and hydroquinone concentrations

Reductant	Concentration (mM)	Measured λ (nm)	$\tau_{\frac{1}{2}}$ (s)
Ascorbate	A (Calculated from Fig 5)		
	0 1	552	42±3
	2 0	552	18±2
	0 2	561	54±4
	2 0	561	18±2
Hydroquinone	B (From Fig 6)		
	0 25	552	11±2
	2 0	552	≦1
	0 25	561	10±2
	2 0	561	12±2

DISCUSSION

Ferricyanide and ascorbate should not be able to penetrate very deeply into a continuous hydrophobic membrane [25] Direct oxidation and reduction of the electron transport components or of intermediates in the chemical oxidation–reduction reactions presumably takes place near the membrane surface The oxidation–reduction of electron transport components which tend to exist in the membrane interior

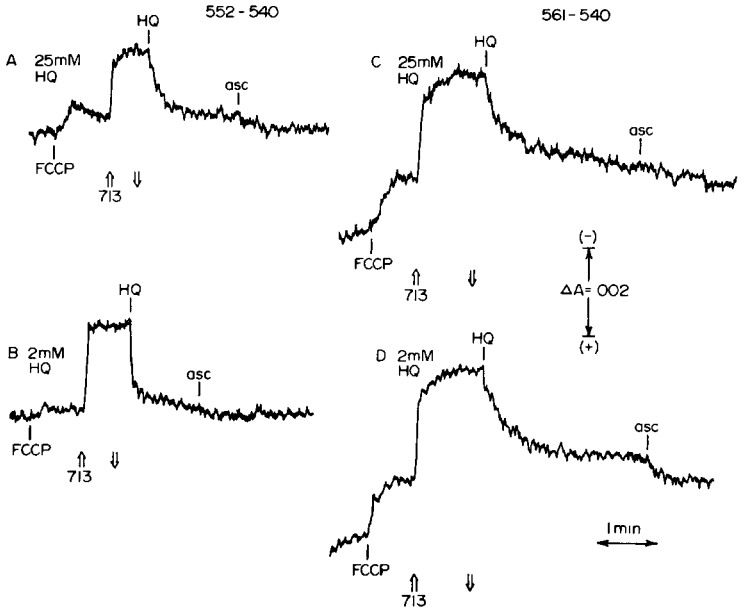

Fig 6 The dependence on hydroquinone concentration of the rate of cytochrome *f* and *b*-559 reduction after oxidation by far-red light Hydroquinone concentration in the reduction of cytochrome *f* (A, 0 25 mM, B, 2 mM) and cytochrome *b*-559 (C, 0 25 mM, D, 2 mM) Ascorbate and FCCP concentrations, 2 mM and 1 μM, respectively Chlorophyll concentration, 60 μg/ml

would involve either their occasional movement to allow exposure of a reactive side to the membrane surface, or a shuttling of endogenous redox mediators from fixed components inside the membrane to the surface This motion would occur in a direction normal to the membrane surface Inhibition of ferricyanide and ascorbate accessibility would imply a decrease in this trans-membrane motion A decrease in motion of intramembrane electron transport components could be caused by an increase in membrane microviscosity It has been documented by fluorescence probe techniques that FCCP causes a general increase in the microviscosity of *E coli* cell envelope [24] An additional effect of FCCP on the chloroplast electron transport components is a negatively directed shift in midpoint potential, which has been directly demonstrated for cytochrome *b*-563 under conditions very similar to those used in this work [26], and for cytochrome *b*-559 in the dark with higher FCCP concentrations after a 5 min incubation [6] It has been inferred that, in the presence of low concentrations of FCCP, light causes a transient shift of the cytochrome *b*-559 to a low potential form which can be oxidized by plastoquinone [6, 7, 27] It was tentatively proposed that the effect of FCCP on the cytochrome *b*-563 midpoint potential might be explained in terms of an increase in solvent accessibility [26] Although the experimental systems are very different, it is of interest to note that cholesterol, which is known to cause an increase in microviscosity of model lipid membranes (e g , ref 28), also has been shown to cause an increase in water penetration into a liposome preparation [29]

It is not clear whether the effect of FCCP described here on inhibition of

dark chemical oxidation–reduction reactions of cytochrome *f* and *b*-559 should be associated with uncoupling or inhibitory effects of FCCP on photosynthetic electron transport The inhibitory effects of FCCP on the dark chemical reactions can be observed at FCCP concentrations (1–2 μM) which do not measureably affect steady-state rates of oxygen evolution [7, 9] or red-light reduction of cytochrome *f* (Figs 1B, 3B) or *b*-559 (Figs 2B, C, 3D) However, FCCP is known to uncouple at lower concentrations, where FCCP inhibition of chemical oxidation-reduction reactions is not observable, suggesting that the effects of FCCP described here are rather associated with inhibition than with uncoupling A correlation between inhibitory effects of lipid soluble uncouplers in mitochondria and inhibition of substrate uptake has been observed [30]

In terms of the question of the pathway of cytochrome *b*-559 oxidation at room temperature, we note that the light-induced absorbance changes of cytochrome *b*-559 in the presence of 1–2 μM FCCP are very similar to those of cytochrome *f* (Figs 2B, C, 3D) in that far-red light oxidizes the cytochrome and red light reduces it This basic observation on cytochrome *b*-559 has been demonstrated previously [4] and as well by others (e g , Fig 3A of ref 8, Fig 1 of ref 31) If the chloroplasts are incubated for a prolonged period ($\sim$ 5 min) with 1–2 μM FCCP, or if higher concentrations of FCCP are used (Fig 2D), red-light reduction of oxidized cytochrome *b*-559 is inhibited

Since with equal incident intensities of illumination the rate of absorption of far-red quanta by the chloroplasts is smaller than that of red quanta, Figs 2B, C and 3D show that under these conditions cytochrome *b*-559 is oxidized by photosystem I and reduced by photosystem II The cytochrome *b*-559 is mostly in the high potential ($E_m \approx +0\,35$ V) state in the dark [32–35], and is located in close proximity to photosystem II as judged by preferential photosystem II photooxidation at 77 °K (e g , ref 36) If photosystem I is to oxidize high-potential *b*-559, the pathway for oxidation would have to bypass the plastoquinone pool with a midpoint of about +100 mV [37]

We have proposed instead that FCCP stimulates observable far-red oxidation of cytochrome *b*-559 because it facilitates conversion of this cytochrome to a lower potential form which can donate electrons to plastoquinone This hypothesis is based on (1) the ability of relatively high concentrations of FCCP to convert cytochrome *b*-559 to a lower potential form in the dark [6], and the expectation that in the presence of light lower concentrations would suffice, (2) the inhibition of far-red oxidation of cytochrome *b*-559 by the quinone analog 2,5-dibromo-3-methyl-6-isopropylbenzoquinone (DBMIB) [27, 9] and (3) the relatively slow reduction by hydroquinone of *b*-559 photooxidized in the presence of FCCP compared with hydroquinone reduction (a) of *b*-559 oxidized in the dark by ferricyanide, and (b) of cytochrome *f* photooxidized in the presence of FCCP [6] The relatively slow hydroquinone reduction in the dark of the photooxidized cytochrome *b*-559 was considered to be a measure of the dark conversion of low-potential *b*-559 back to high-potential *b*-559 [6] The hydroquinone experiment of ref 6 has been repeated [8] and again shows that concentrations of FCCP which allow stimulation of the oxidation of cytochrome *b*-559 by far-red light do not permanently convert the cytochrome to a low potential form This does not answer the question as to whether there is a transient change to a low potential form in the light The other alternative offered in ref 6 to explain the slow

reduction of cytochrome *b*-559 photooxidized in the presence of FCCP was a selective decrease in accessibility of hydroquinone to cytochrome *b*-559 caused by light and FCCP This possibility was considered relatively unlikely in ref 6 The general effect of FCCP on accessibility of oxidant and reductant documented above, combined with one exception of hydroquinone reduction of cytochrome *f* now make the analysis of the hydroquinone experiment of ref 6 much more complex It does appear that the lack of dependence on hydroquinone concentration of the rate of hydroquinone reduction of *b*-559 oxidized in the presence of light and FCCP (Fig 6C, D) is unique, requires an additional explanation beyond that of hindered accessibility, and is consistent with the formation of a transient low potential species of cytochrome *b*-559 Reversion to a high potential form in the dark would then be the limiting step in the hydroquinone reaction However, it must be admitted that because of the general effects of FCCP on rates of chemical oxidation and reduction the analysis of the hydroquinone experiment in terms of a low potential *b*-559 is now more complicated The argument for the formation of transient photoconversion of cytochrome *b*-559 to a low potential form is probably best supported by the need to explain the red-light reversible far-red oxidation of *b*-559 (Figs 2B, C, 3D, refs 4, 8, 31) which is inhibited by DBMIB [27, 9]

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