The effects of quinone analogues on cytochrome b_6 reduction and oxidation in a reconstituted system

Eric Lam

Division of Molecular Plant Biology, 313 Hilgard Hall, University of California, Berkeley, CA 94720, USA

Received 7 May 1984

The reconstituted system containing Photosystem I, plastocyanin and the cytochrome b_6–f complex is used to study the effects of various quinone analogues on the redox behavior of cytochrome b_6. The effects of DBMIB, DNP-INT and HQNO are compared in an attempt to discern the modes of action of these quinone analogues. Both DBMIB and DNP-INT are potent inhibitors of the plastocyanin reductase activity of the isolated cytochrome complex. However, while DBMIB abolished the oxidant-induced reduction of cytochrome b_6, DNP-INT only inhibited about 25% of the net reduction. On the other hand, HQNO does not show any significant inhibition of plastocyanin reductase activity of the isolated cytochrome complex at concentrations up to 20 μM. An enhancement of the net amount of cytochrome b_6 reduced is observed in the presence of HQNO. Both DNP-INT and HQNO inhibited the dark oxidation rate of cytochrome b_6. The possible identity of the oxidant for cytochrome b_6 is discussed. Plastoquinone is concluded to be the most likely candidate. DNP-INT is concluded to have at least two sites of inhibition in the cytochrome complex. The implications of these findings on quinone functions in the cytochrome b_6–f complex are discussed.

Reconstitution *Cytochrome* b_6–f *complex* *Photosystem I* *Quinone analog* *Cytochrome* b_6

1. INTRODUCTION

Photosynthetic electron flow involves two membrane-bound cytochromes in higher plants, cytochrome *f* and b_6 [1]. Among them, the role of cytochrome *f* as the electron carrier between the Rieske Fe-S center and plastocyanin seems to be firmly established [2]. In contrast, the role of cytochrome b_6 remains to be elucidated. In particular, the nature of its reduction and oxidation pathways are important questions that bear on the functional role of this segment of the electron transport chain, since the oxidation of *b* cytochromes in analogous situations during mitochondrial and bacterial energy transduction has been implicated to be the electrogenic step leading to the generation of an electrochemical gradient [3,4].

The use of electron transport inhibitors, such as antimycin A and myxothiazol, has provided us with strong evidence for the existence of a 'Q-cycle' type of electron transfer pathways in the mitochondrial and bacterial cytochrome b_6–c_1 complexes [5]. On the other hand, our understanding of the cytochrome b_6–f complex in higher plants is comparatively meager. The potent inhibitors for the mitochondrial-type cytochrome complexes, such as antimycin A, are usually without effects on the isolated functional b_6–f complex at low concentrations. There exists, however, a set of inhibitors which are quite specific for the b_6–f complex. Most notably among these are the quinone analogues DBMIB and DNP-INT [6,7]. An inhibitor that is common between the mitochondrial and plant-type cytochrome complexes is UHDBT. This inhibitor, however, is effective only at lower pH values below the pH optimal for the cytochrome complexes (~pH 8). This is found in both the mitochondrial-type complexes (cf. [5]) and in the b_6–f complex from spinach (un-

Published by Elsevier Science Publishers B.V.
00145793/84/$3.00

published). The inhibitors DBMIB, DNP-INT and UHDBT have all been shown to interact with the Rieske Fe-S protein in the b_6–f complex [8]. There exists, however, evidence that they might interact with the cytochrome complex in different manners. DNP-INT and UHDBT were reported to stimulate ferredoxin-mediated reduction of cytochrome b_6 by NADPH while DBMIB was ineffective [9]. This observation is confirmed in [10] where it was proposed that DNP-INT inhibits at a different site in the cytochrome b_6–f complex from DBMIB. In [11] it was reported that DNP-INT is a better cyclic phosphorylation inhibitor than DBMIB while the opposite is true when non-cyclic phosphorylation was examined. These observations suggest that the modes of action for DBMIB and DNP-INT are different.

In an attempt to elucidate the mode of inhibition by DBMIB and DNP-INT, the effects of these inhibitors have been studied on the reactions of cytochrome b_6 in a system containing the isolated cytochrome b_6–f complex, plastocyanin and Photosystem I (PS I). As reported previously, the reduction of cytochrome b_6 upon light-activation of PS I is absolutely dependent on the addition of a low-potential donor to the cytochrome complex [12]. This system is attractive since the absence of ferredoxin precludes the reduction of cytochrome b_6 by the cyclic pathway while the limited numbers of components will facilitate our interpretation of data obtained. The results indicate that DNP-INT inhibits both the oxidation and reduction pathways of cytochrome b_6 while DBMIB only inhibited the latter process. HQNO, an inhibitor known to inhibit b_6 oxidation in chloroplasts [13] and heterocysts [14], also inhibits b_6 oxidation in our PS I coupled system. These results indicate that the endogenous plastoquinone molecule might be the oxidant for cytochrome b_6 and that DNP-INT has two sites of action on the cytochrome b_6–f complex.

2. MATERIALS AND METHODS

The cytochrome complex isolated in the absence of Triton [9] and the PS I preparation [15] are obtained from greenhouse spinach leaves by published methods. The reconstituted system containing PS I, plastocyanin and the b_6–f complex has been described [12]. The concentrations of the components are described in more detail in the figure legends.

Light-induced absorbance changes are followed by an Aminco DW-2 spectrophotometer with side illumination. The wavelength couple, 563–575 nm, is used to follow redox changes of cytochrome b_6 in the dual mode. Filter combination is described in [12]. All additions are done in darkness and experiments carried out at 22°C. Stock solutions of inhibitors are made with either absolute ethanol or dimethyl sulfoxide.

2-(*n*-Heptyl)-4-hydroxyquinoline *N*-oxide (HQNO), β-octylglucoside and Hepes are from Sigma. DNP-INT (2-iodo-6-isopropyl-3-methyl-2,4,4′-trinitrodiphenyl ether) and DBMIB (2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone) are gifts from Professor A. Trebst (Bochum, FRG). Duroquinol and duroquinone (tetramethylquinone) are obtained from K and K Chemical Co. All other reagents are of highest grade available.

3. RESULTS

The pH profile of the extent of cytochrome b_6 reduction in the PS I coupled system has been investigated. As the data in fig.1 show, the max-

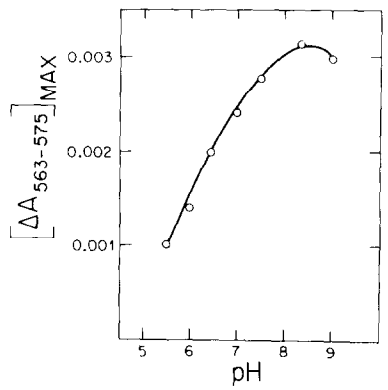

Fig.1. pH profile of the PS I-activated cytochrome b_6 reduction by duroquinol. The assay medium contains 20 mM buffer (Mes–NaOH for pH 5.5–6.5; Hepes for pH 7.0–7.5; Tricine–KOH for pH 8.0–9.0), 5 mM $MgCl_2$ and 15 mM NaCl. PS I complex (7 μg chl/ml), cytochrome b_6–f complex (32 μM), 0.15 μM plastocyanin and 0.5 mM sodium ascorbate were added prior to the addition of 60 μM duroquinol. All additions were made in the dark.

imum of the light-induced absorbance due to this cytochrome is about pH 8.5. The net amount of cytochrome *f* photooxidized is inversely proportional to this dependency, it decreases as the pH increases (not shown). These observations fit well with the observation that the cytochrome complex has an activity maximum at about 8–8.5 [16]. Thus, apparently as the rate of electron flow through the cytochrome complex increases, the amount of cytochrome b_6 undergoing reduction also increases. This might be due to an enhanced rate of generation of reductants for cytochrome b_6 during non-cyclic electron flow via a Q-cycle mechanism.

An important characteristic of the PS I-dependent in vitro system is that one can observe stable photo-induced reduction of cytochrome b_6 in the light. The reduced cytochrome b_6 then undergoes reoxidation in the dark [12]. These features are different from that reported in [17] using a different PS I preparation and PQ-1 as the electron donor. The authors in [17] observed a transient reduction of cytochrome b_6 which undergoes rapid reoxidation in the light. This might arise from the rapid oxidation of PQ-1 in their system as compared to duroquinol in ours. In any case, the duroquinol system [12] allows one to study the oxidation pathway of cytochrome b_6. The effects of DBMIB, DNP-INT and HQNO on the reduction and oxidation of cytochrome b_6 are compared in fig.2. DBMIB, as reported in [12], completely abolished the reduction of cytochrome b_6 observed in the light at low concentrations (~1 μM). On the other hand, DNP-INT at 4 μM did not abolish the observed reduction of cytochrome b_6 although the rate of its reduction is significantly slowed. The oxidation rate of cytochrome b_6 in subsequent darkness is also slowed by DNP-INT. HQNO, a well known electron transfer inhibitor of the mitochondrial complex III [18], is found to have little inhibitory effects on quinol-plastocyanin reductase activity of the isolated b_6–*f* complex (see table 1). In fig.2, it is shown that HQNO also shows an inhibition of the dark oxidation of cytochrome b_6. Concomitant with this inhibition, an increase in the net amount of cytochrome b_6 being reduced at steady state is observed.

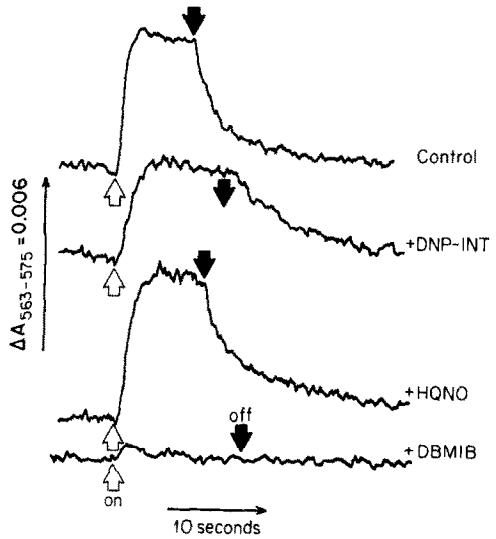

Fig.2. Effects of quinone analogues on reduction and oxidation kinetics of cytochrome b_6. The assay system contains 20 mM Hepes (pH 7.5), 5 mM $MgCl_2$, 15 mM NaCl, 0.9 μM cytochrome complex, 40 μg chl/ml PS I, and 0.6 μM plastocyanin. Duroquinol (100 μM) was added in the dark prior to the assay. DNP-INT (4 μM), HQNO (20 μM) and DBMIB (1 μM) were added prior to the assay.

To localize better the site of action for these inhibitors and the probable route of electron transfer from the reduced cytochrome b_6, the kinetics of cytochrome b_6 oxidation are examined under aerobic and anaerobic conditions by semi-logarithmic analysis. As fig.3 shows, the oxidation of cytochrome b_6 under aerobic conditions can be deconvoluted into two processes with about one

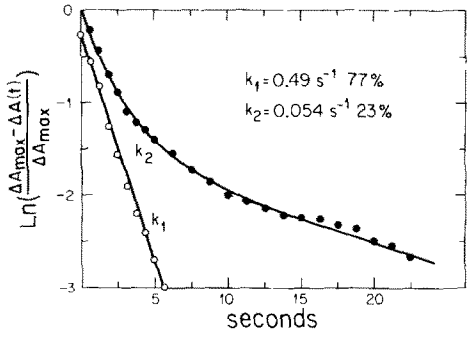

Fig.3. Kinetic analysis of the oxidation of cytochrome b_6. The trace in fig.2 (control) is analyzed by semi-logarithmic plot. ΔA_{max}, total absorbance change after termination of illumination. $\Delta A_{(t)}$, absorbance change at time *t* after illumination was stopped.

Table 1

Effects of inhibitors on quinol-plastocyanin oxidoreductase: kinetic parameters of the dark oxidation process of cytochrome b_6

	$DQH_2 \longrightarrow PC$	$DQH_2 \longrightarrow PC \longrightarrow$ PS I $\longrightarrow O_2$	Net b_6 photoreduction	b_6 Oxidation kinetics (s^{-1})	
				k_1	k_2
Control	100	100	100	0.5 (80%)	0.05 (20%)
+ 2 μM DBMIB	28	21	0	–	–
+ 4 μM DNP-INT	20	42	75	0.5 (45%)	0.06 (55%)
+ 20 μM HQNO	100	105	116	0.5 (56%)	0.06 (44%)
+ O_2 trap	–	–	100	0.6 (36%)	0.03 (64%)
+ O_2 trap + 4 μM DNP-INT	–	–	65	0.34 (30%)	0.04 (70%)
+ O_2 trap + 20 μM HQNO	–	–	119	0.13 (35%)	0.013 (50%)

All activities were measured at pH 7.5. The activities are expressed as % of control. Conditions in all assays are identical to that of fig.2. The O_2 trap (glucose–glucose oxidase–catalase) system was described in [9]. In the oxygen electrode, the O_2 trap consumes at least 98% of the oxygen in less than 1 min

order of magnitude difference in the rate constants. Similar analysis can be applied to the oxidation process under anaerobic conditions also. Table 1 compares the effects of DNP-INT and HQNO on the decay kinetics under aerobic and anaerobic conditions. In aerobic conditions, both inhibitors decreased the contribution from the fast process of oxidation. Interestingly, the value of the rate constants themselves are not affected significantly. When the assay sample is rendered anaerobic by inclusion of the oxygen trap (glucose-glucose oxidase-catalase), different behaviors of the inhibitors are observed. The percentages of the two processes are relatively unchanged while the value of the fast process is decreased by both inhibitors. It is interesting to point out that depletion of oxygen decreased the percentage of the fast phase of oxidation while depressing the value of the slower rate constant. The net amount of cytochrome b_6 reduced under illumination remains unchanged by the exclusion of oxygen from the system.

4. DISCUSSION

To analyze the above results, we should first consider the mode of electron transfer in our in vitro system and the effects of the inhibitors on the associated activities of the cytochrome complex. In the presence of oxygen, both DBMIB and DNP-INT are potent inhibitors of the b_6–f complex catalyzed reduction of plastocyanin by duroquinol (table 1). HQNO has no detectable inhibitory action at 20 μM. In the absence of the cytochrome complex, relatively little light-dependent oxygen uptake can be observed when PS I is incubated with duroquinol and low concentrations of plastocyanin (~0.5 μM). Increasing plastocyanin concentration (to >5 μM) or inclusion of the b_6–f complex in the system will then provide a rapid rate of light-dependent oxygen uptake (not shown). These observations demonstrate that direct reduction of the PS I reaction center by duroquinol is relatively slow as compared to reduced plastocyanin. As expected from their inhibitory actions, both DBMIB and DNP-INT inhibited the light-dependent oxygen uptake while HQNO did not. Interestingly, however, DBMIB inhibited completely the light-induced cytochrome b_6 reduction while DNP-INT only inhibited about 25%. HQNO shows a 16% stimulation instead. The effects of DNP-INT might be rationalized in terms of a two-site model where this inhibitor acts on the oxidant-induced reduction pathway of cytochrome b_6 and also on the oxidation pathway of this cytochrome. On the other hand, HQNO only works on the oxidation pathway of cytochrome b_6 while DBMIB only inhibits the oxidant-dependent pathway of

cytochrome b_6 reduction. This model is consistent with the observation that DNP-INT inhibits severely the plastocyanin reduction process while only mildly affecting the net reduction of cytochrome b_6. Thus, with the inhibition of the reoxidation process, a much decreased rate of electron transfer through the cytochrome complex did not severely inhibit the observed reduction of cytochrome b_6, as is the case with DBMIB. This stabilization of reduced cytochrome b_6 by DNP-INT also offers a possible explanation for the difference in the stimulation of ferredoxin-mediated reduction of cytochrome b_6 by DNP-INT and DBMIB [9].

The exact mode of inhibition of cytochrome b_6 oxidation by DNP-INT and HQNO is more difficult to ascertain. Under aerobic conditions, these two quinone analogues inhibited the process of b_6 oxidation by decreasing the percentage of the fast phase. Under anaerobic conditions, the actual decay rate constants are affected rather than the percentage of contribution by the two processes. In our system, the electron acceptor for cytochrome b_6 is unlikely to be the Rieske Fe-S center since cytochrome f is observed to undergo re-reduction in instrument-limited time scale (<0.3 s) when the light is turned off and it has been demonstrated that the Rieske Fe-S center is in rapid redox equilibrium with cytochrome f [19]. The fact that anaerobiosis did not abolish the dark oxidation of cytochrome b_6 also suggests that oxygen is not the immediate acceptor. It is noted, however, that oxygen depletion inhibited the kinetics of oxidation. Thus, oxygen might serve as a secondary electron acceptor pool. The only remaining probable candidate in our system as the direct electron acceptor for cytochrome b_6 is then duroquinone or the endogenous plastoquinone in the cytochrome complex. We have tested the first possibility by the addition of duroquinone prior to the activation of PS I. If duroquinone can indeed serve as the electron acceptor of cytochrome b_6, we would expect an inhibition of the net reduction of the cytochrome due to a stimulation of its reoxidation rate. No significant effect was observed however, even when duroquinone was added to 60–80 μM. Thus, the endogenous plastoquinone, in either the oxidized or stabilized semiquinone form, in the cytochrome complex is concluded to be the most likely candidate as the direct electron acceptor for cytochrome b_6 under the present conditions. This conclusion also suggests that the quinone analogues, DNP-INT and HQNO, might be able to interfere with this process by insertion into the vicinity of the tightly bound plastoquinone molecule. Although the conclusion that plastoquinone serves as the acceptor for cytochrome b_6 might explain the inhibition of its oxidation by the quinone analogues, the existence of two distinct phases in the oxidation process remains unresolved. At present, the heterogeneity in the plastoquinone [20] and cytochrome b_6 [21] populations reported to be present in the isolated b_6–f complex might be the cause for this phenomenon. Future experiments are required to definitively establish the identity of the two processes. Finally, the observation that HQNO can inhibit the oxidation process of cytochrome b_6 in the isolated b_6–f complex without significantly affecting quinol-plastocyanin reductase activity supports the idea that cytochrome b_6 turnover might not be obligatory during non-cyclic electron flow [22].

ACKNOWLEDGEMENTS

This work was supported in part by a grant from the National Institutes of Health. The support and encouragement from Dr Richard Malkin are gratefully acknowledged.

REFERENCES

[1] Cramer, W.A. and Whitmarsh, J. (1977) Annu. Rev. Plant Physiol. 28, 133–172.
[2] Malkin, R. (1982) in: Electron Transport and Photophosphorylation (Barber, J. ed.) pp.1–47, Elsevier, Amsterdam, New York.
[3] Mitchell, P. (1976) J. Theor. Biol. 62, 327–367.
[4] Wikström, M. and Krab, K. (1980) Curr. Top. Bioenerg. 10, 52–103.
[5] Hauska, G., Hurt, E., Gabellini, N. and Lockau, W. (1983) Biochim. Biophys. Acta 726, 97–133.
[6] Trebst, A., Harth, E. and Draber, W. (1970) Z. Naturforsch. 25b, 1157–1159.
[7] Trebst, A., Wietoska, H., Draber, W. and Knops, H.J. (1978) Z. Naturforsch. 33c, 919–927.
[8] Malkin, R. (1982) Biochemistry 21, 2945–2950.
[9] Lam, E. and Malkin, R. (1982) FEBS Lett. 141, 98–101.
[10] O'Keefe, D.P. (1983) FEBS Lett. 162, 349–354.

[11] Barton, J.R., Macpeek, W.A. and Cohen, W.S. (1981) in: Energy Coupling in Photosynthesis (Selman, B.R. and Selman-Reimer, S. eds) pp.73–77, Elsevier, Amsterdam, New York.
[12] Lam, E. and Malkin, R. (1982) Biochim. Biophys. Acta 682, 378–386.
[13] Selak, M.A. and Whitmarsh, J. (1982) FEBS Lett. 150, 286–292.
[14] Houchins, J.P. and Hind, G. (1983) Biochim. Biophys. Acta 725, 138–145.
[15] Mullet, J., Burke, J.J. and Arntzen, C.J. (1980) Plant Physiol. 65, 814–822.
[16] Hurt, E. and Hauska, G. (1981) Eur. J. Biochem. 117, 591–599.
[17] Hurt, E. and Hauska, G. (1982) Photobiochem. Photobiophys. 4, 9–15.
[18] Van Ark, G. and Berden, J.A. (1977) Biochim. Biophys. Acta 459, 119–137.
[19] Whitmarsh, J., Bowyer, J.R. and Crofts, A.R. (1982) Biochim. Biophys. Acta 682, 404–412.
[20] Hurt, E. and Hauska, G. (1982) Biochim. Biophys. Acta 682, 466–473.
[21] Hurt, E. and Hauska, G. (1983) FEBS Lett. 153, 413–419.
[22] Bendall, D.A. (1982) Biochim. Biophys. Acta 683, 119–152.