Journal of Bioenergetics and Biomembranes, Vol. 17, No. 3, 1985

Antimycin-Resistant Alternate Electron Pathway to Plastocyanin in Bovine-Heart Complex III

Baishnab C. Tripathy[1,2] and John S. Rieske[1,3]

Received August 20, 1984; revised November 19, 1984

Abstract

Bovine-heart Complex III can catalyze the reduction of spinach plastocyanin by a decyl analog of ubiquinol-2 at a rate comparable with the rate of plastocyanin reduction by plastoquinol as catalyzed by the cytochrome b_6–f complex purified from spinach leaves. This plastocyanin reduction as catalyzed by Complex III was almost completely inhibited by myxothiazol at stoichiometric concentrations, partially inhibited by UHDBT (5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole) and funiculosin, and was relatively insensitive to antimycin and HQNO (2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide). Cytochrome *c* reduction as catalyzed by Complex III displayed a residual, inhibitor-insensitive rate of 5% of the uninhibited rate for each of the three inhibitors, antimycin, myxothiazol, and UHDBT. However, the residual rate that was insensitive to each of the inhibitors added singly was inhibited further by addition of the remaining two inhibitors. From these results it is concluded that plastocyanin reduction involves an electron-transfer pathway through Complex III that is distinct from the pathway utilized for reduction of cytochrome *c*.

Key Words: Bovine-heart Complex III; plastocyanin reduction; Complex III inhibitors; antimycin, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide; funiculosin; 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole; myxothiazol; inhibitor-insensitive reduction.

Introduction

Of current interest and attended with considerable controversy is the nature of the electron-transfer pathway from ubiquinol to cytochrome *c* through

[1]Department of Physiological Chemistry, College of Medicine, The Ohio State University, Columbus, Ohio 43210.
[2]Present Address: Department of Horticulture, University of Illinois, Urbana, Illinois 61801.
[3]To whom all correspondence should be addressed. A portion of the data in this report was presented at the IV International Symposium on Coenzyme Q_{10}, Munich, F.R.G., November, 6–9, 1983.

0145-479X/85/0600-0141$04.50/0

Complex III of the respiratory chain. Analogous systems are present in the photosynthetic apparatus of photosynthetic bacteria and plants. One such system present in chloroplasts of plants that functions in a manner analogous to Complex III is the cytochromes b_6–f complex. A differing aspect of this complex is its use of plastocyanin as an electron acceptor instead of cytochrome *c*. However, in systems *in vitro* cytochrome *c* can function as an electron acceptor (Hurt and Hauska, 1981; Lam and Malkin, 1983a; 1983b).

A commonly known but little understood phenomenon associated with the electron pathway through Complex III is the leakage of electrons through or around the antimycin block generally observed in mitochondria or submitochondrial particles. The importance of this phenomenon as a tool in the delineation of electron pathways through Complex III was magnified by the observation that this antimycin-insensitive electron flux was inhibited by certain other inhibitors of Complex III such as BAL[4] and UHDBT (Hatefi and Yagi, 1982).

In the present study we have examined this phenomenon further using both the reduction of plastocyanin and cytochrome *c* as catalyzed by bovine-heart Complex III. Antimycin, myxothiazol, and UHDBT appear to behave independently as essentially conformational inhibitors of Complex III. The insensitivity of plastocyanin reduction to antimycin can be explained by a residual leakage of electrons through the antimycin block. However, differences in inhibitory behavior of myxothiazol toward plastocyanin and cytochrome *c* reduction, respectively, as catalyzed by Complex III offer evidence that plastocyanin reduction in this system occurs by a different mechanism than cytochrome *c* reduction.

Materials and Methods

Complex III was purified according to the procedure of Rieske (1967). Plastocyanin, isolated from spinach leaves according to Davis and San Pietro (1979), was furnished by Dr. Elizabeth L. Gross of this institution. Cytochrome *c*, type VI and HQNO were purchased from Sigma Chemical Co. Antimycin was obtained as a gift from Dr. Claude Vezina (Ayerst Research Laboratories, Montreal, Canada). Funiculosin was obtained from C.S.K. Research Laboratories. UHDBT was a gift from Dr. Bernard L. Trumpower (Dartmouth Medical School, Hanover, New Hampshire). Myxothiazol was a gift from Dr. Wolfram Trowitzsch (G.B.F., Braunschweig-Stockheim,

[4]Abbreviations: HQNO, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide; UHDBT, 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole; BAL, British Anti-Lewisite (2,3-dimercapto-1-propanol); MES, 2(*N*-morpholino)ethane sulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; TAPS, 3-([tris-(hydroxymethyl)methyl]amino)propanesulfonic acid; decyl QH_2, reduced decyl analog of ubiquinol-2.

FRG). The decyl analog of ubiquinone-2 (decyl Q) was obtained as gifts from Dr. Karl Folkers (The University of Texas at Austin) and Dr. Bernard L. Trumpower and also was synthesized according to a procedure described by Margolis (1976).

Decyl QH_2-cytochrome *c* reductase activities were measured as the rate of reduction of cytochrome *c* recorded spectrophotometrically as the rate of increase in the absorbance difference between 550 and 540 nm using the coefficient 18 mM^{-1} cm^{-1}. For this assay, Complex III was diluted to 4 mg ml^{-1} in 20 mM MOPS buffer (pH 7.0) containing 0.1% potassium deoxycholate. The assay mixture contained 300 nmol decyl QH_2, 0.2% ferricytochrome *c*, and 10 μl of the diluted enzyme (0.133 nmol) in 1.0 ml of 20 mM MOPS buffer, pH 7.0. Plastocyanin reduction was measured spectrophotometrically using the wavelength pair 597 minus 500 nm and a coefficient of 4.9 mM^{-1} cm^{-1}. The initial assay mixture contained 5 μl of diluted stock enzyme (0.067 nmol), 70 nmol decyl QH_2, and the desired concentrations of plastocyanin in 1.0 ml of 20 mM MOPS buffer, pH 7.0. In both assays the rate of nonenzymic reduction of cytochrome *c* or plastocyanin was measured before addition of enzyme to correct the assay for nonenzymic reduction. All reductase assays were run at 25°C.

Results

Reduction of Plastocyanin by Decyl QH_2 Catalyzed by Bovine Heart Complex III

Complex III catalyzed the reduction of spinach plastocyanin by decyl QH_2 as measured by a decrease in absorbance at 597 nm. The catalyzed rate was much higher than the uncatalyzed rate; therefore, correction of the overall rate for the uncatalyzed rate was minimal (cf. Fig. 2). The K_m and V_m of this reaction were measured as 5 μM and 16.6 μmol plastocyanin reduced per milligram Complex III protein per hour, respectively (data not shown). This V_m corresponds to a value of 5.5 μmol plastocyanin reduced per nanomole cytochrome c_1 per hour. These values of K_m and V_m are in good agreement with the values of 4.6 and 4.2 μmol plastocyanin reduced per nanomole cytochrome *f* per hour, respectively, reported for the photochemical reduction of plastocyanin with a reconstituted cytochromes b_6–*f* system (Lam and Malkin, 1983b), although the activity observed in this study was somewhat lower than the activities (9.1–25.2) reported for the reduction of plastocyanin by plastoquinol-1 as reported by Hurt and Hauska (1981).

The pH profile of plastocyanin reduction is shown in Fig. 1. The optimal pH is 7.5. The uncatalyzed reduction of a plastocyanin by decyl QH_2 is completely different, showing an increasing rate at pH's above 7.5. This pH

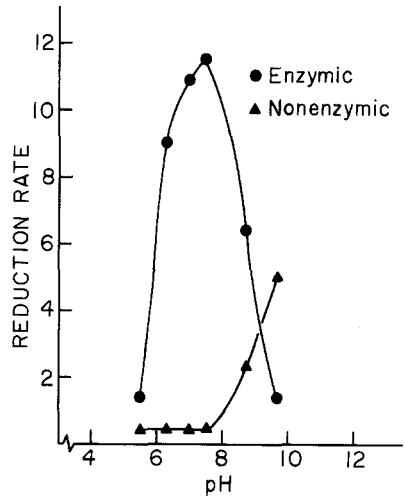

Fig. 1. pH profile of plastocyanin reduction as catalyzed by Complex III. ●, Enzymic; ▲, Nonenzymic rate. The 1.0-ml reaction mixture contained 67 nM complex III, 70 μM decyl QH_2, and 8 μM oxidized plastocyanin. Buffers were 20 μM and consisted of MES (pH 5.5–6.7), MOPS (pH 6.8–7.9), and TAPS (pH 8.0–9.0). Rate measurements were made spectrophometrically at 25°C according to the procedure described in Methods and are expressed as micromoles plastocyanin reduced per milligram Complex III protein per hour.

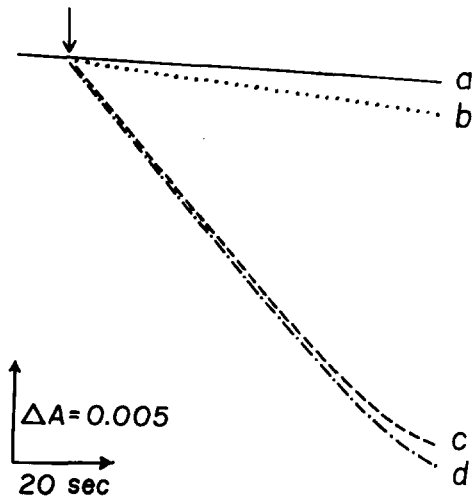

Fig. 2. Spectrophotometric traces of the time course of reduction of plastocyanin by decyl QH_2. The arrow indicates addition of Complex III. (a) Uncatalyzed reduction; (b) Complex III treated with 67 nM myxothiazol; (c) Complex III treated with 67 nM antimycin; (d) untreated Complex III. The reactions were run at 25°C and pH 7.0. Other experimental details are as described for Fig. 1 and in Methods.

dependency of the catalyzed reduction is very similar to that observed for the reduction of cytochrome *c-552* (*Euglena gracilis*) as catalyzed by purified cytochromes b_6–f complex from spinach (Hurt and Hauska, 1981).

Effect of Inhibitors on Plastocyanin Reduction

Figure 2 shows the time course of plastocyanin reduction by decyl QH_2 as measured by decrease of absorbance difference between 597 and 500 nm for the uncatalyzed reduction, the reduction catalyzed by Complex III, and the catalyzed reduction in the presence of antimycin and myxothiazol, respectively. The rate of plastocyanin reduction was strongly inhibited by myxothiazol but essentially unaffected by antimycin. The uncatalyzed reduction rate was essentially insignificant at pH 7.0 compared with the uninhibited, catalyzed rate. Figure 3 displays the inhibition of plastocyanin reduction as a function of the molar ratio of inhibitor to Complex III for several inhibitors. Antimycin and HQNO were ineffective as inhibitors of plastocyanin reduction even at 4-fold stoichiometric concentrations, whereas, myxothiazol inhibited this reaction almost completely at a concentration stoichiometric with that of the complex. Funiculosin and UHDBT were intermediate between antimycin and myxothiazol in effectiveness as inhibitors. It is interesting that UHDBT, which inhibits cytochrome *c* reduction almost completely at concentrations stoichiometric with Complex III (Trumpower and Haggerty, 1980), was considerably less effective as an inhibitor of plastocyanin reduction, inhibiting only 50% at a stoichiometric

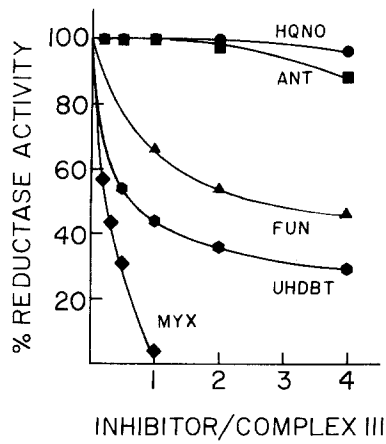

Fig. 3. Effect of inhibitors of Complex III on plastocyanin reduction, ●, HQNO; ■, antimycin; ▲, funiculosin; ●, UHDBT; ♦, myxothiazol. Reduction rates were measured at pH 7.0 and 25°C. Other experimental details are as described for Fig. 1 and in Methods.

concentration with a maximal inhibition approaching 80%. However, it may be significant that this inhibitory potency is similar to that reported for the cytochromes b_6–f-catalyzed reduction of plastocyanin (Hurt and Hauska, 1981).

The ineffectiveness of antimycin as an inhibitor apparently was not due to a lessened binding to Complex III. A fluorescence-quench analysis indicated that antimycin was bound stoichiometrically to Complex III in the presence of plastocyanin (data not shown).

Inhibitor-Insensitive Reduction of Cytochrome c –Inhibitory Relationships Among Antimycin, Myxothiazol, and UHDBT

Because of the low electron flux associated with plastocyanin reduction as compared with cytochrome *c* reduction under otherwise identical conditions, it became evident that plastocyanin reduction in the presence of antimycin may involve electron leakage around or through the antimycin site as observed during normal electron transfer through Complex III. To test this concept, experiments were performed to measure the extent of insensitivity of decyl QH_2-cytochrome *c* reductase to each of the three most potent inhibitors: antimycin, myxothiazol, and UHDBT. These compounds are representative of the three general classes of inhibitors of Complex III: antimycin-like, MOA (inhibitors containing a methoxyacrylate reactive group), and quinone (or ubiquinone antagonists), respectively.

The results of these experiments are recorded in Fig. 4. They show that with sufficiently concentrated enzyme a residual electron flux insensitive to the inhibitor can be demonstrated with all three inhibitors. This residual activity was near 5% of the uninhibited electron flux with all three inhibitors individually and was inhibited additionally by the remaining two inhibitors. With a stoichiometric excess of all three inhibitors present simultaneously, a residual activity between 1 and 2% of the uninhibited activity was observed. These results when compared with the effects of these inhibitors on plastocyanin reduction offer significant evidence that different electron-transfer pathways are involved in reduction of cytochrome *c* and plastocyanin, respectively. Myxothiazol inhibited completely the residual electron flux measured by plastocyanin reduction, yet inhibited only 60% of the electron flux leaking through the antimycin block in cytochrome *c* reduction. Furthermore, the myxothiazol-insensitive and the antimycin-insensitive electron fluxes involved in reduction of cytochrome *c* were similar in magnitude. Therefore, if both cytochrome *c* and plastocyanin accepted electrons from the same immediate donor, then plastocyanin reduction would be expected to be insensitive to myxothiazol as well as to antimycin, provided that both inhibitors blocked the electron pathway at points preceding the postulated common donor to cytochrome *c* and plastocyanin.

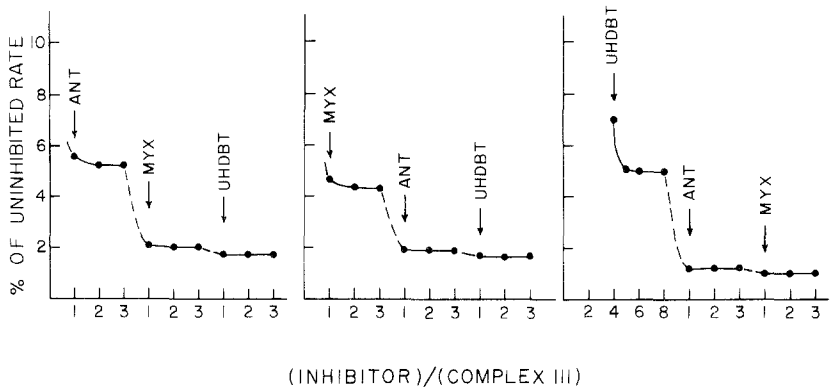

Fig. 4. Inhibitor-insensitive reduction of cytochrome *c* as catalyzed by Complex III. The 1.0-ml assay mixture contained 10 μl of a stock solution of enzyme containing 4 mg/ml Complex III in 20 mM MOPS buffer, pH 7.0, 0.1% potassium deoxycholate, 300 μM decyl QH_2, and 20 mM MOPS buffer, pH 7.0. The inhibitors antimycin, myxothiazol, and UHDBT were added as solutions in ethanol. Rate measurements were made as described in Methods.

Discussion

Although the data presented in this report are consistent only with separate sites of interaction with Complex III for cytochrome *c* and plastocyanin, this concept presents some difficulty with respect to the present models of electron-transfer pathways within the complex. The differences in the sensitivity of plastocyanin reduction to antimycin and myxothiazol, both of essentially equal potency as inhibitors of cytochrome *c* reduction, support a nonlinear pathway of electron transfer through the complex. However, the stoichiometric inhibition by myxothiazol of plastocyanin reduction, the low rate of which would be insensitive to the inhibitor in the pathway to cytochrome *c*, indicates that the electron-transfer pathway from quinol to plastocyanin passes directly through the myxothiazol-sensitive component with virtually no bypass via a cyclic pathway such as the Q cycle.

Possible inhibitor-insensitive pathways of electron flow to cytochrome *c* and plastocyanin utilizing the hypothesized Q cycle are shown in Fig. 5. One pathway would involve a slow electron exchange between the cytochrome *b*'s and cytochrome c_1. This pathway would appear significant as a shunt that short-circuits the Q cycle only under conditions where the cycle is blocked on one side only of the bridging cytochromes *b*, i.e., at center "i" by antimycin or center "o" by myxothiazol or UHDBT. Simultaneous inhibition of electron access to cytochrome *b* from both centers "i" and "o" would be necessary to inhibit electron flow to cytochrome c_1 via the shunt as observed

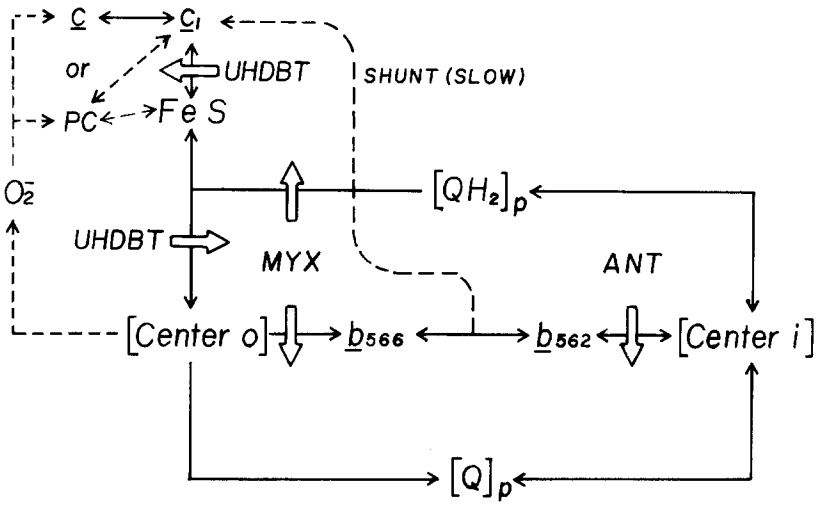

Fig. 5. Hypothesized mechanisms of inhibitor-insensitive reduction of cytochrome *c* and plastocyanin as applied to the Q-cycle hypothesis. Dashed lines indicate possible shunt pathways of electron transport to plastocyanin and cytochrome *c*. Plastocyanin is abbreviated PC. A discussion of these mechanisms is given in the text.

with simultaneous inhibition by antimycin and myxothiazol or antimycin and UHDBT. However, if plastocyanin accepts electrons directly from cytochrome c_1 as does cytochrome *c*, then inhibition of plastocyanin reduction by myxothiazol or UHDBT alone would not be expected if the shunt pathway was predominant.

A second possibility could involve direct electron transfer through the sequence: cytochromes *b*, cytochrome c_1, iron–sulfur center, plastocyanin via the antimycin-insensitive shunt. This route could only be sensitive to myxothiazol if myxothiazol inhibited the reduction of the iron–sulfur center by ferrocytochrome c_1. Although a recent paper (Von Jagow *et al.*, 1984) reports that myxothiazol inhibits the reduction of the iron–sulfur center by ubiquinol and displaces the quinone from the iron–sulfur protein, there is as yet no evidence that the inhibitor blocks electron transfer between cytochrome c_1 and the iron–sulfur center.

A mechanism that should receive serious consideration involves the reduction of plastocyanin by superoxide anions generated from the reaction of oxygen with ubisemiquinone formed at center "o" of the proposed Q cycle. Superoxide was reported as a reductant of cytochrome *c* when antimycin-inhibited submitochondrial particles were treated with succinate and ferricytochrome *c* (Boveris and Cadenas, 1975). Furthermore, Ksenzenko *et al.* (1983) reported that the generation of superoxide anions was stimulated by

antimycin, HQNO, and funiculosin and inhibited by cyanide, mucidin, myxothiazol, or 2,3-dimercaptopropanol (BAL). The sensitivity of plastocyanin reduction to myxothiazol and the insensitivity of the reduction to antimycin or HQNO is superficially in agreement with a mechanism involving superoxide; however, several other of our observations are in conflict with this mechanism. Plastocyanin reduction was insensitive to antimycin or HQNO but was not stimulated by these compounds. The reduction was inhibited rather than stimulated by funiculosin. The pH profile of generation of superoxide anions by submitochondrial particles (Boveris and Cadenas, 1975) is quite different from the pH profile of plastocyanin reduction as found in this investigation. Finally, in contradiction to our observations, cytochrome *c* reduction should show no residual insensitivity to myxothiazol or UHDBT if superoxide anion is the principal reductant of cytochrome *c* at low levels of electron flux through Complex III.

Direct evidence that the antimycin-insensitive reduction of cytochrome *c* or plastocyanin is due to superoxide generation would tend to minimize the significance of this study with respect to new information on electron-transfer pathways within Complex III. However, in any case, the close similarities in kinetic properties between cytochromes b_6f and cytochromes bc_1-catalyzed reduction of plastocyanin, respectively, raises the possibility that plastocyanin-reductase activities observed with current preparations of purified cytochromes b_6f may reflect abnormal pathways of electron transfer to plastocyanin not directly involving cytochrome *f*.

Unfortunately, because of logistic problems, we were unable at this time to test for superoxide contribution to plastocyanin reduction by addition of superoxide dismutase or by measurement of plastocyanin reduction in an anaerobic system. Planned experiments to test the effects of superoxide dismutase or anaerobiosis on the antimycin-insensitive plastocyanin reduction together with direct comparisons of kinetic parameters and inhibitor effects on plastocyanin reduction catalyzed by cytochromes b_6f and Complex III, respectively, should clarify the questions raised by this initial study.

Acknowledgements

This work was supported by a grant (HL-265298) from the National Institutes of Health, United States Public Health Service. We would like to thank Dr. Clinton D. Stoner for helpful discussions and for the use of his equipment. We also appreciate constructive suggestions made by the reviewers of the manuscript.

References

Boveris, A., and Cadenas, E. (1975). *FEBS Lett.* **54**, 311–314.
Davis, D. J., and San Pietro, A. (1979). *Anal. Biochem.* **95**, 254–259.
Hatefi, Y., and Yagi, T. (1982). *Biochemistry* **24**, 6614–6618.
Hurt, E., and Hauska, G. (1981). *Eur. J. Biochem.* **117**, 591–599.
Ksenzenko, M., Konstantinov, A. A., Khomutov, G. B., Tikhonov, A. N., and Ruuge, E. K. (1983). *FEBS Lett.* **155**, 19–24.
Lam, E., and Malkin, R. (1983a). *FEBS Lett.* **152**, 89–93.
Lam, E., and Malkin, R. (1983b). *Arch. Biochem. Biophys.* **224**, 456–463.
Margolis, P. A. (1976). Senior Honors Thesis, Dept. of Biol., Dartmouth College, Hanover, New Hampshire.
Rieske, J. S. (1967). In *Methods in Enzymology* (Estabrook, R. W., and Pullman, M. E., eds.), Vol. X, Academic Press, New York, pp. 239–245.
Trumpower, B. L., and Haggerty, J. G. (1980). *J. Bioenerg. Biomembr.* **12**, 151–164.
Von Jagow, G., Ljungdahl, P. O., Graf, P., Ohnishi, T., and Trumpower, B. L. (1984). *J. Biol. Chem.* **259**, 6318–6326.