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A MYXOTHIAZOL-SENSITIVE Q-BINDING PROTEIN ISOLATED FROM *CHROMATIUM VINOSUM*

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A quinol: ferricytochrome *c* oxidoreductase has been isolated from chromatophores of *Chromatium vinosum* by two procedures, involving extraction by bile salts and methanol, respectively. The steady-state kinetics indicate a random mechanism, with a K_m for 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinol of 1.1 μM and for the acceptor cytochrome *c* 1.75 μM. The enzyme is inhibited by myxothiazol, competitively with respect to quinol, with a K_i of about 2.3 μM. The protein reacts with ubiquinol produced by the succinate: Q oxidoreductase in submitochondrial particles or isolated succinate: cytochrome *c* reductase and can partially restore activity to myxothiazol-inhibited, antimycin-sensitive ubiquinol: cytochrome *c* oxidoreductase. The protein is considered to be analogous to the postulated myxothiazol-sensitive Q-binding protein in ubiquinol: cytochrome *c* oxidoreductase.

Introduction

The proposal that branching of the electron transfer takes place at the quinol-oxidation step in the respiratory chain [1,2] requires the stabilization of the intermediary semiquinone by binding to a specific site. The detection of relatively stable semiquinones in ubiquinol-cytochrome *c* oxidoreductase [3–6] has made it clear that these semiquinone-stabilizing sites exist, possibly located on specific Q-binding proteins, similar to the Q-binding protein isolated from succinate: ubiquinone oxidoreductase [7].

Two different semiquinone anions have been demonstrated in ubiquinol : cytochrome *c* oxidoreductase, one being sensitive to antimycin and the other requiring the presence of antimycin and an oxidant [8]. Additionally, we have shown the presence of two binding sites for the Q analogue HMHQQ with different affinities, one near the antimycin-binding site and the other near the Rieske Fe-S cluster and the site for myxothiazol [9]. Thus, the mitochondrial QH_2 : cytochrome *c* oxidoreductase contains two Q-binding proteins which could be the same as or different from the metal-containing subunits in the enzyme. Wang and King [10] have reported the isolation from this enzyme of a Q-binding protein with mol.wt. 15 000 which is significantly lower than that of the Fe-S protein or the cytochromes.

Nishi et al. [11] and Snozzi et al. [12] have isolated and purified a Q-binding protein from chromatophores of *Rhodospirillum rubrum* that catalyses the reduction of cytochrome c_2 by quinol [12]. The enzymic activity is not affected by antimycin or Q analogues and is slightly stimulated by HQNO. Snozzi et al. concluded that the protein is

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Abbreviations: DBH, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone; HMHQQ, 7-(*n*-heptadecyl)mercapto-6-hydroxy-5,8-quinolinequinone; HQNO, 2-(*n*-heptyl)-4-hydroxyquinoline *N*-oxide; Mops, 4-morpholinepropanesulphonic acid.

involved in electron transfer in the chromatophores, but that it loses its sensitivity to HQNO and antimycin during isolation. We have isolated a very similar enzyme from the chromatophores of the purple bacterium *Chromatium vinosum* and found it to be sensitive to myxothiazol, a specific inhibitor of QH_2 : cytochrome *c* oxidoreductase [13,14]. Moreover, the isolated enzyme is able to mediate electron transfer between succinate : ubiquinone oxidoreductase and ubiquinol : cytochrome *c* oxidoreductase and partially to restore activity to myxothiazol-inhibited succinate : cytochrome *c* oxidoreductase. It is suggested that this protein is a myxothiazol-sensitive ubiquinone-binding protein involved in the Q cycle at the ubiquinol-oxidizing site [9].

Materials and Methods

Cells of *C. vinosum* were cultured anaerobically under light, using Na_2S as reducing reagent according to the procedure of Hendley [15], as modified by Van Heerikhuizen et al. [16]. The cells were harvested by centrifugation and broken by grinding with glass beads. After the removal of glass beads and intact cells by centrifugation at 3000 rpm for 30 min, chromatophores were spun down at $315\,000 \times g$ for 1 h and suspended in 50 mM Mops-NaOH buffer (pH 7.0), containing 100 mM KCl. Residual nuclei and large fragments were removed by centrifugation at $17\,000 \times g$ for 20 min and the chromatophores were collected again by centrifugation for 1 h at $315\,000 \times g$. After homogenisation in the Mops-KCl buffer, the suspension was diluted to an $A_{800\,nm}$ of about 100.

Two methods were used to isolate the protein described by Snozzi et al. [12]. The first method, using cholate plus deoxycholate for extraction, was essentially the same as reported by these authors [12], except that the chromatography on Sepharose 6B was omitted. On a Sephadex G-100 column, the yellow band of the protein moved much more slowly than the deep-red coloured front and was easily separated from the latter. The protein isolated by this method contains ubiquinone. It was used for experiments without further purification.

The second method yielded a quinone-free preparation. To 1 vol. of the suspension of chromatophores, 3 vol. of methanol were added. The suspension was centrifuged at 2000 rpm for 5 min, the slightly green-coloured supernatant discarded and a further 2 vol. of methanol were added to the precipitate. After dispersal and stirring for 5 min and centrifugation at 2000 rpm for 5 min, the deep-green coloured supernatant was applied to a Sephadex LH-20 column (1.2×50 cm) equilibrated with methanol. On elution with methanol, the deep-green coloured band was collected and after dilution with an equal volume of 10 mM Tris-HCl buffer (pH 8.0) the sample was applied to a 2-ml DEAE-cellulose column equilibrated with methanol/10 mM Tris-HCl buffer, pH 8.0 (1 : 1, v/v). The column was eluted with a methanol gradient (from 50 to 90%) in the same buffer. The blue-green bacterial chlorophyll was eluted at 70–80% methanol. When the eluate became colourless, the column was washed with 15–20 ml of the Tris-HCl buffer, after which the protein was eluted with about 10 ml of the same buffer containing 2% cholate and 2 M NaCl. An equal volume of 10 mM Tris-HCl buffer (pH 8.0) was added to the eluate, followed by solid $(NH_4)_2SO_4$ to 40% saturation. The sticky precipitate was collected by centrifugation, dissolved in a small volume of 50 mM Tris-HCl buffer (pH 8.0) and dialysed overnight against the same buffer without stirring. The protein isolated by this method was nearly free of bacterial chlorophyll and contained only a small amount of a pigment with an absorption peak at 690 nm. Due to the separation on Sephadex LH-20 in methanol, this preparation does not contain Q. Most of our experiments were done with the protein isolated by this method.

Submitochondrial particles and mitochondrial succinate : cytochrome *c* oxidoreductase were prepared according to Refs. 17 and 18, respectively. QH_2 : cytochrome *c* oxidoreductase activity and succinate : cytochrome *c* oxidoreductase activity were monitored at 550–540 nm with an Aminco DW-2 spectrophotometer, the cuvette holder being thermostatically controlled at 20°C and the reaction mixture in the cuvette stirred from the bottom by a magnetic stirrer. Duroquinone (from Aldrich Europe) was dissolved in methanol and reduced with $NaBH_4$. DBH, a Q-2 analogue with a saturated side chain (a gift from Dr. S.H. Speck), was dissolved in ethanol and also reduced with $NaBH_4$. Horse-heart cytochrome *c* (from Sigma)

was used as electron acceptor.

The oxidation of succinate and NADH by submitochondrial particles was measured at 30°C with an Oxygraph equipped with a Clark-type electrode. The treatment of submitochondrial particles with lipoxygenase (a gift from Dr. T. Schewe) was carried out according to the method described in Ref. 19. The absorbance spectra were measured with a Cary-19 spectrophotometer. The protein concentration of the preparations was measured by the method of Lowry et al. [20].

Antimycin, HQNO, myxothiazol (a gift from Dr. G. Thierbach), rotenone and 2-thenoyltrifluoroacetone were added from a stock solution in ethanol. HMHQQ (a gift from Dr. K. Folkers) was added from a stock solution in ethanol/2 M Tris-HCl buffer, pH 8.0 (9 : 1, v/v).

Results

The absorbance spectrum of the Q-binding protein isolated by methanol extraction (Fig. 1) shows a shoulder at 276 nm and a broad band around 400 nm. The origin of the latter is not clear. The spectrum is similar to that of the reduced form of the Q-binding protein of *R. rubrum* [12], but, since it does not contain Q, reduction with $NaBH_4$ causes no absorbance change at 276 nm. The high absorbance at wavelengths below 250 nm is due to the detergent in the preparation.

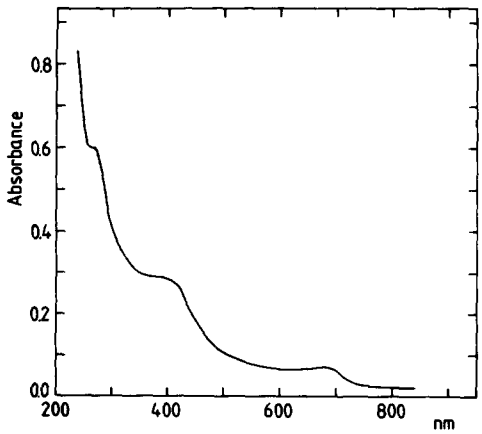

Fig. 1. The absorbance spectrum of the Q-binding protein (0.07 mg/ml) isolated by methanol extraction. The medium contained 50 mM Tris-HCl buffer (pH 8.0), 0.2% cholate and 0.1% deoxycholate. The high absorbance at wavelengths below 250 nm is due to the cholate in the preparation.

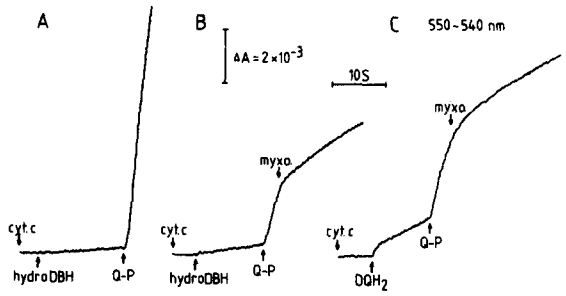

Fig. 2. The oxidation of reduced DBH and durohydroquinone (DQH_2) by horse-heart cytochrome *c* (cyt. c) catalysed by the Q-binding protein (Q-P) isolated by methanol extraction. The reaction mixture contained 50 mM Mops-NaOH buffer (pH 7.0) and horse-heart cytochrome *c* (20 μM). The non-enzymatic reactions were started by adding reduced DBH (10 μM in A and 5 μM in B) or durohydroquinone (75 μM, C) and the enzymatic reactions by a further addition of Q-binding protein (10 μg in A, 2.5 μg in B and C). The concentration of myxothiazol (myxo.), when added, was 25 μM.

The molecular weight of this protein as determined from its elution pattern on a Sephadex G-100 column was about 12000, similar to that reported for the *R. rubrum* preparation (11000). The molecular weight as determined by SDS gel electrophoresis was about 11000. The isolated protein still contains lipid.

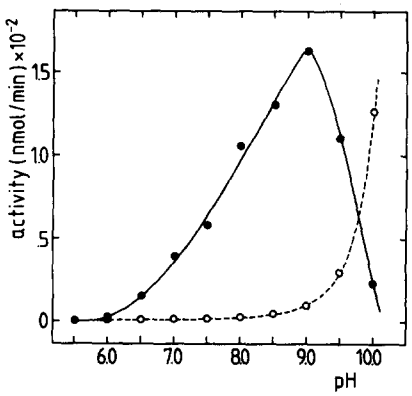

Fig. 3. The pH dependence of the reduced DBH-cytochrome *c* oxidoreductase activity of the Q-binding protein (●———●). The reaction mixture contained 20 mM acetic acid, 10 mM EDTA, 20 mM Mops, 20 mM Tris and 20 mM phosphate. NaOH or HCl were added to obtain the desired pH. After the addition of 20 μM cytochrome *c* and 2.5 μg Q-binding protein, the reaction was started by the addition of 10 μM reduced DBH. The measured rates were corrected for the non-enzymic reaction (○------○) measured under the same conditions but without the Q-binding protein.

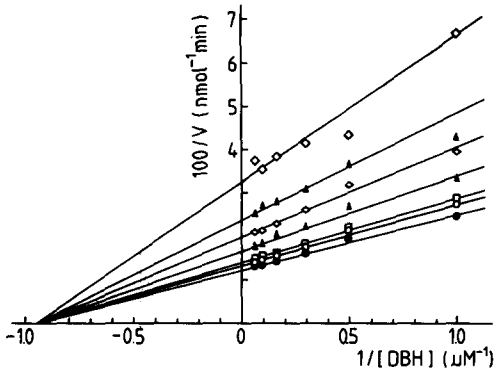

Fig. 4. The determination of the K_m for reduced DBH. The reaction mixture contained 50 mM Mops-NaOH buffer (pH 7.0) and 2.5 μg Q-binding protein. (◇———◇) 1 μM, (▲———▲) 2 μM, (◆———◆) 3 μM, (△———△) 5 μM, (□———□) 10 μM, (○———○) 20 μM, (●———●) infinite concentration of cytochrome *c*, obtained from Fig. 5. The K_m equals 1.1 μM.

Both the Q-containing and the Q-free preparations catalyse the oxidation of duroquinol or reduced DBH by horse-heart cytochrome *c* (Fig. 2). With reduced DBH as substrate, the optimal pH is about 9.0 (Fig. 3). The steady-state kinetics (Figs. 4 and 5) indicate a rapid equilibrium, random Bi Bi mechanism [21], with separate binding sites for DBH and cytochrome *c*, with dissociation constants of 1.1 and 1.75 μM, respectively. The maximum specific activity of protein prepared by

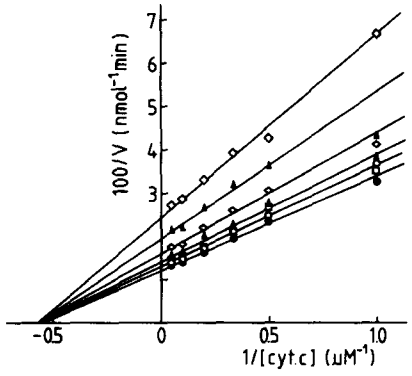

Fig. 5. The determination of K_m for horse-heart cytochrome *c*. The experimental conditions were the same as in Fig. 4. (◇———◇) 1 μM; (▲———▲) 2 μM; (◆———◆) 4 μM; (△———△) 6 μM; (□———□) 10 μM; (○———○) 15 μM; (●———●) infinite concentration of reduced DBH, obtained from Fig. 4. The K_m equals 1.75 μM.

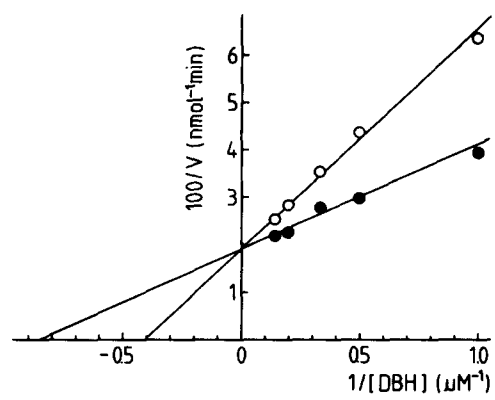

Fig. 6. Competitive inhibition by myxothiazol. The reactions were carried out in 50 mM Mops-NaOH buffer (pH 7.0) with 20 μM cytochrome *c* and 1.5 μg Q-binding protein added. After incubating the Q-binding protein with the mixture with (○) or without (●) 2.5 μM myxothiazol for 2 min, the reactions were started by the addition of reduced DBH. In this experiment a crude methanol extract was used. The K_m for reduced DBH equals 1.2 μM and the inhibition by myxothiazol is competitive with a K_i of 2.3 μM.

methanol extraction was 32.6 μmol/min per mg protein at 20°C. Assuming a molecular weight of 11 000 and a single catalytic site, the turnover number is 6.0 s^{-1}.

This enzymic activity was not inhibited by anti-

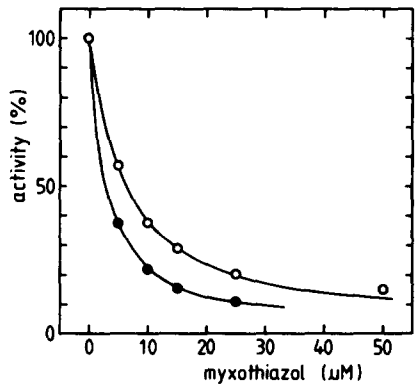

Fig. 7. The inhibition by myxothiazol of the reduced DBH : cytochrome *c* oxidoreductase activity at different concentrations of substrates. After incubating the Q-binding protein (2.5 μg) with different concentrations of myxothiazol for 2 min in 50 mM Mops-NaOH buffer (pH 7.0) containing 20 μM cytochrome *c*, reduced DBH (10 μM, ○———○; 2.5 μM, ●———●) was added to start the reaction. The curves in the figure are theoretical inhibition curves with apparent K_i of 6.6 and 2.9 μM, respectively. The real K_i (from a plot according to Hunter and Downs [22]) equals 2 μM.

mycin (50 μM), HQNO (50 μM), 2-thenoyltrifluoroacetone (380 μM), rotenone (15 μM), CN^- (2 mM), EDTA (10 mM) or 1,10-phenanthroline (1 mM). HMHQQ (25 μM) increased the activity by a factor of 2–5, the absolute amount of the activation becoming larger at higher pH. It was, however, inhibited by myxothiazol (Fig. 2). The inhibition is competitive with respect to quinol (Fig. 6) with a K_i of 2.3 μM. Also, from the data of the experiment of Fig. 7 in which a purified preparation was used, a K_i for myxothiazol of 2 μM was obtained using the plotting method of Hunter and Downs [22].

In agreement with Snozzi et al., the protein is very resistant to heat. In dilute solutions, heating to 100°C for 10 min resulted in about 10% loss of the activity. The activity is also insensitive to treatment with trypsin or chymotrypsin.

The interaction with mitochondrial QH_2: cytochrome c oxidoreductase

Mitochondrial QH_2: cytochrome *c* oxidoreductase has a very high affinity towards myxothiazol, the amount needed for a maximum inhibition of electron transfer being virtually stoicheiometric with the concentration of cytochrome c_1. Fig. 8 shows that, after inhibition with a 5-fold excess of myxothiazol, addition of the isolated protein brings about a restoration of the succinate : cytochrome *c* reductase activity. The activity increases over a period of half a minute, suggesting that the protein is being incorporated into the succinate : cytochrome *c* reductase. This suggestion is supported by the finding that the concomitant addition of phospholipid stimulates the recovery. Interestingly, the phospholipids have to be added as a solution (in methanol). Vesicles are not effective. The addition of Q-10 had little effect. The recovery of activity in the presence of added phospholipid can be as large as 25% of that of the original uninhibited succinate : cytochrome *c* oxidoreductase activity. The recovered activity is sensitive to high (25 μM) concentrations of myxothiazol, enough to inhibit the added quinol : cytochrome *c* reductase, and to 2-thenoyltrifluoroacetone, which inhibits the production of QH_2 (Fig. 9). In addition, the recovered activity is partly sensitive to antimycin (20–40%) (Fig. 9).

The interaction of the isolated protein with

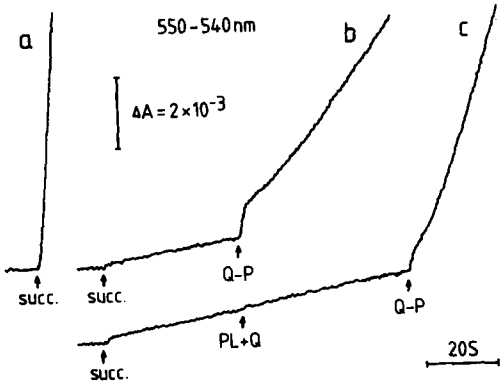

Fig. 8. The stimulation of the myxothiazol-inhibited succinate : cytochrome *c* oxidoreductase activity of mitochondrial succinate : cytochrome *c* oxidoreductase. The reaction mixture contained 50 mM Mops-NaOH buffer (pH 7.0), 50 μM cytochrome *c*, 20 μg succinate : cytochrome *c* oxidoreductase and 2 mM CN^- with (b and c) or without (a) 1 μM myxothiazol. The reactions were started by the addition of succinate (succ.) (2.5 mM). In b and c, 5 μg crude Q-binding protein (Q-P) in methanol was added after succinate (b) or after succinate followed by phospholipid (PL) (asolectin, 50 μg) and Q-10 (0.5 μM) in methanol (c).

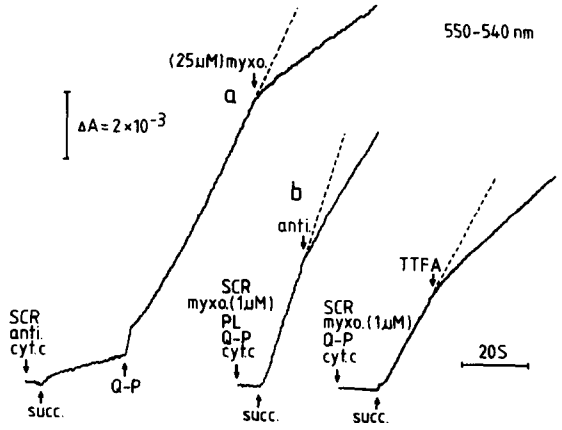

Fig. 9. The sensitivity to inhibitors of Q-binding protein-restored succinate : cytochrome *c* oxidoreductase in the presence of 1 μM myxothiazol (myxo.). The reactions were carried out in 50 mM Mops-NaOH buffer (pH 7.0) containing 2 mM CN^-. The additions are indicated in the figure. Concentrations or amounts: succinate : cytochrome *c* oxidoreductase (SCR), 20 μg; cytochrome *c* (cyt. c), 50 μM; succinate, (succ.), 2.5 mM; Q-binding protein (Q-P) (crude extract), 5 μg; antimycin (anti.) 1.5 μM; asolectin (PL); 50 μg; 2-thenoyltrifluoroacetone (TTFA) 0.25 mM.

mitochondrial NADH : Q oxidoreductase and succinate : Q oxidoreductase was also investigated. Both in intact submitochondrial particles and in lipoxygenase-treated particles in which the interaction of the NADH and succinate dehydrogenase with ubiquinone is largely destroyed [19], the effects of the protein were marginal, activating slightly in one case (succinate oxidation) and inhibiting in the other (NADH oxidation), indicating specificity of this Q-binding protein for the QH_2 oxidase reaction.

Discussion

From the similarity of the preparations isolated by extraction with methanol or detergents (spectrum, molecular weight, electron-transfer activity, sensitivity to inhibitors, heat resistance) we may conclude that the two preparations are identical except for the presence of bound quinone in the one isolated with detergents. The presence of quinone in the latter preparation and the reactivity of both types of proteins with quinols show that it is a quinone-binding protein.

Since its activity is sensitive to myxothiazol, a specific inhibitor of the oxidation of ubiquinol at the center o of both mitochondrial and chromatophore ubiquinol : cytochrome *c* (c_2) oxidoreductase [13,14,23], we propose that this protein acts as the Q-binding and QH_2-activating protein proposed to function in the Q cycle. It has previously been suggested that the oxidation of QH_2 by the Fe-S protein and cytochrome *b* is sensitive to myxothiazol [24]. This idea is supported by the effects of ubiquinone and ubiquinone analogues on the EPR spectrum of the Rieske Fe-S cluster in the mitochondrial QH_2 : cytochrome *c* oxidoreductase [25–27] and the effect of myxothiazol on the spectrum of cytochrome *b*-566 [13].

Two properties of the isolated protein remain to be discussed: its activity towards cytochrome *c* and the relatively low affinity for myxothiazol.

The activity of the bacterial enzyme towards mammalian cytochrome *c* is certainly not physiological. We may suppose that the binding site for cytochrome *c* is exposed only after isolation, being buried within the enzyme in the intact system. Indeed, after reincorporation into succinate : cytochrome *c* reductase, cytochrome *c* reduction becomes partly antimycin sensitive, showing that some of the reincorporated protein is no longer accessible to cytochrome *c*. Also, the activity with reduced DBH as substrate is lowered after incorporation.

The low affinity for myxothiazol (relative to the intact system) of the isolated protein may be a consequence of the isolation procedure. Although we cannot exclude that the QH_2 : cytochrome c_2 oxidoreductase of *C. vinosum* has a low intrinsic affinity for myxothiazol, results with other bacterial systems [23] argue against this possibility.

It is possible that the protein described in this paper is the counterpart in *C. vinosum* of the 15-kDa subunit found by Wang and King [10] to be required for activity of the mitochondrial QH_2 : cytochrome *c* oxidoreductase. However, data on the activity of the protein obtained by Wang and King [10] towards ubiquinol and its sensitivity to myxothiazol and/or antimycin are required before this possibility can be further examined.

Our data on the restoration of succinate : cytochrome *c* reductase activity in myxothiazol-inhibited systems (with a low concentration of myxothiazol) show that the isolated Q-binding protein can react with the ubiquinol produced by the succinate : Q oxidoreductase moiety of the succinate : cytochrome *c* oxidoreductase. This conclusion is supported by the inhibitory effect of 2-thenoyltrifluoroacetone on the restored activity. The partial sensitivity to antimycin of the induced reduction of cytochrome *c*, although the isolated Q-binding protein is not inhibited by antimycin, suggests that some of the incorporated protein functions as in an intact system, delivering electrons to the intrinsic redox pathway in the enzyme.

Although we have not been able to isolate by similar procedures a Q-binding protein from submitochondrial particles, we propose that the isolated protein described in this paper is part of the intact QH_2 : cytochrome c_2 oxidoreductase of the bacteria and has an analogous counterpart in the mitochondrial system. The data on the inhibitory effect of myxothiazol clearly indicate that myxothiazol competes with the quinol substrate, which agrees with the finding of Meinhardt and Crofts [23] that myxothiazol displaces inhibitory Q analogues.

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