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KINETICS OF UBIQUINONE REDUCTION BY THE RESOLVED SUCCINATE: UBIQUINONE REDUCTASE

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A significant lag in the thenoyltrifluoroacetone (TTFA)-sensitive succinate: ubiquinone reductase activity was observed when a ubiquinone-deficient resolved preparation of the enzyme was assayed in the presence of exogenous ubiquinone-2 (Q_2) and 2,6-dichlorophenolindophenol. No such lag was seen when the free radical of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (Wurster's Blue) was used as the terminal electron acceptor, or when the reduction of Q_2 was directly measured. The apparent K_m value for exogenous Q_2 was determined in the Q_2-mediated TTFA-sensitive succinate: Wurster's Blue reductase reaction. When the enzyme activity was measured directly by monitoring Q_2 reduction without terminal acceptors, the time course of the reaction deviated from zero-order kinetics at Q_2 concentrations which were much higher than those expected from the $K_m^{Q_2}$ value determined in the presence of Wurster's Blue. The time course of Q_2 reduction fits a curve describing a competitive interrelationship between oxidized and reduced Q_2 at the specific binding site. The data obtained are in agreement with the Q-pool behavior of ubiquinone in mitochondrial membranes and suggest that the rate of ubiquinone reduction by succinate is dependent on the Q/QH_2 ratio.

The functioning of ubiquinone as an obligatory component of the proton-translocating respiratory chain is the subject of growing interest (see Refs. 1–5 and references cited therein). The mechanism of electron transfer between respiratory chain-linked dehydrogenases and membrane-bound ubiquinone is not known. The recent successful reconstitution of TTFA-sensitive succinate: ubiquinone reductase from soluble succinate dehydrogenase (EC 1.3.99.1) and a low molecular weight protein [6–9] suggests that the minimal structural unit of mitochondria capable of succinate: ubiquinone reductase activity is composed of at least three types of polypeptides. Limited information is available on the kinetics of ubiquinone reduction by intact, resolved or reconstituted preparations of succinate: ubiquinone reductase [1,10–13]. The difficulties of a simple steady-state kinetic analysis of the reductase-ubiquinone interaction are apparently due to extremely low solubility of the natural ubiquinone-10 (Q_{10}) in water [14] and also to the fact that most of the resolved preparations of succinate: ubiquinone reductase contain endogenous ubiquinone and show only partial dependence of the TTFA-sensitive activity on exogenous ubiquinone [15–17].

In this report we present evidence that an apparent competition between oxidized (Q_2) and reduced (Q_2H_2) ubiquinone-2 exists at the specific

Abbreviations; TTFA, thenoyltrifluoroacetone; carboxin, 5,6-dihydro-2-methyl-1,4-oxanthiin-3-carboxanilide; DCIP, 2,6-dichlorophenolindophenol; Wurster's Blue, a semiquinonediimine radical of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; Q_2, ubiquinone having two isoprenoid units in position 6 of the quinone ring.

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binding site in the succinate : ubiquinone reductase region of the respiratory chain.

Succinate : ubiquinone reductase was prepared by Triton X-100 extraction of either Keilin-Hartree bovine heart muscle preparation [18] or submitochondrial particles [19]. The purified enzyme shows five major bands in polyacrylamide gel electrophoresis in the presence of SDS [20]. The enzyme contains approx. 1.4 nmol acid nonextractable flavin per mg protein and catalyzes TTFA- and carboxin-sensitive oxidation of succinate by Q_2 at a maximal rate of 6–10 μmol succinate oxidized/min per mg protein at 23°C and pH 7.8. The preparation has no antimycin-sensitive succinate : cytochrome *c* reductase activity; it is inactive without added Q_2 in the assay systems containing DCIP or Wurster's Blue as the terminal electron acceptors. The complete procedure for preparation of the enzyme will be published elsewhere. The enzyme was assayed after 20 min preincubation with 20 mM succinate (to activate succinate dehydrogenase) in a mixture containing (final concentrations) 10 mM potassium succinate, 10 mM Tris-sulfate buffer (pH 7.8), 0.1 mM EDTA, 0.003% Triton X-100 and electron acceptors. Details are indicted in the legends to the figures. The protein and Triton X-100 contents were determined according to Refs. 21 and 22, respectively. The millimolar extinction coefficients for the acceptors used were 20 for DCIP (600 nm), 12 for Wurster's Blue (612 nm) and 12 for Q_2 (275 nm). Triton X-100 and DCIP were obtained from Sigma. Wurster's Blue was synthesized and recrystallized from methanol [23]. Q_2 was a kind gift from Professor T.E. King (New York State University at Albany, U.S.A.). TTFA was obtained from Fluka (Switzerland). Carboxin was a kind gift from Professor H. Lyr (Institute of Plant Protection Research, G.D.R.).

Fig. 1 demonstrates the time course of the reduction of the terminal electron acceptors (DCIP or Wurster's Blue) by succinate in the presence of Q_2. In agreement with other investigators [13,24], a significant lag in the initial rate was observed when DCIP was used as acceptor. At a fixed amount of the enzyme, the duration of the lag was dependent on both Q_2 and DCIP concentrations and it practically disappeared when high concentrations of Q_2 and DCIP were used. No lag

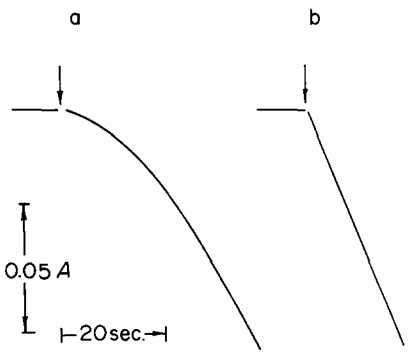

Fig. 1. Time course of Q_2 mediated reduction of DCIP (a) and Wurster's Blue (b) by succinate in the presence of succinate : ubiquinone reductase. 75 μM DCIP or 30 μM Wurster's Blue and 5 μM Q_2 were added to the assay mixture and the reaction was started by the enzyme (1.4 μg/ml; indicated by arrows).

was observed when Wurster's Blue was used as acceptor. Both DCIP and Wurster's Blue reduction was 90% sensitive to 100 μM TTFA or 30 μM carboxin. The constant rate of the reaction in the presence of 5 μM Q_2 measured as the reduction of Wurster's Blue (30 μM) was exactly the same as the initial rate of Q_2 (5 μM) reduction measured directly at 275 nm. In the presence of 75 μM DCIP, the constant rate of the reaction mediated by 5 μM Q_2 was 75% of that registered with Wurster's Blue as the terminal electron acceptor. The existence of a lag in the DCIP assay of succinate : ubiquinone reductase activity of succinate : cytochrome *c* reductase has been interpreted [24] as indicating that the exogenous quinone reacts with the endogenous ubiquinone and that time is required for the formation of a sufficient endogenous pool of $Q_{10}H_2$. Since no lag was seen with Wurster's Blue as acceptor or when reduction of Q_2 was monitored directly (see below), it seemed reasonable to propose that the lag is simply due to an insufficiently rapid reaction between Q_2H_2 and DCIP. We checked this possibility by direct measurement of DCIP reduction by Q_2H_2 prepared according to Ref. 25 under the conditions of the enzyme assay. A second-order reaction was observed with a rate constant equal to $2.5 \cdot 10^3$ $M^{-1} \cdot s^{-1}$, which gives a half-time equal to 10.3 s for 5 μM Q_2H_2 oxidation by 26.7 μM DCIP. Practically instant reduction of Wurster's Blue by

Q_2H_2 was observed under the same conditions. Thus, in the DCIP assay of succinate: ubiquinone reductase activity [15] the reoxidation of the reduced ubiquinone by DCIP might be rate limiting. The Wurster's Blue assay [26,27] does not seem to suffer from such a limitation.

The advantage of the preparation used in these studies is that at least functionally it contains practically no endogenous ubiquinone as indicated by the absence of TTFA-sensitive reduction of Wurster's Blue (or DCIP) without added Q_2. Thus, an apparent $K_m^{Q_2}$ value for the ubiquinone-binding site of the enzyme can be determined taking into account the very rapid reaction between Q_2H_2 and Wurster's Blue. Such a determination shown in Fig. 2 (curve 1) yields a hyperbolic dependence of the reaction on Q_2 with the $K_m^{Q_2}$ value equal to 0.3 μM. Since Q_2 is limitedly soluble in aqueous solutions and the apparent $K_m^{Q_2}$ value for the enzyme is very low, it was possible to compare the date shown in Fig. 2 (curve 1) with the kinetics of Q_2 reduction followed directly without the use of a reoxidizing dye. A representative experiment is

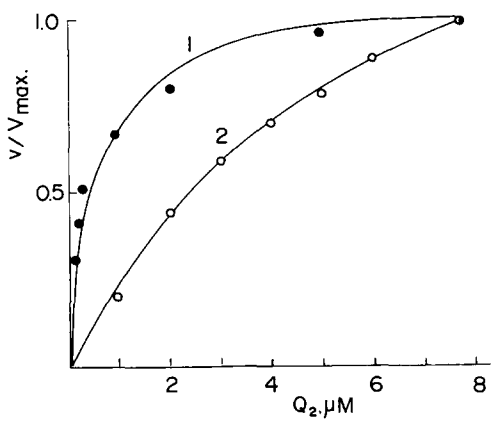

Fig. 2. The dependence of succinate: ubiquinone reductase activity on the concentration of Q_2. (1) 30 μM Wurster's Blue was used as the terminal electron acceptor (●). (2) The reduction of 8 μM Q_2 was monitored directly at 275 nm as in Fig. 3; the continuous line is the theoretical curve describing the dependence of v/V_{max} on the residual Q_2 concentration for the case where QH_2 formed during the reaction is inhibitor competitive with respect to Q_2; the following parameters were used for the construction of this theoretical curve: $K_m^{Q_2} = 0.3$ μM (see curve 1) and $K_i^{Q_2H_2} = 0.8$ μM. The points on the curve (○) represent the experimentally observed values of v/V_{max} corresponding to the residual concentrations of Q_2 during its reduction to Q_2H_2.

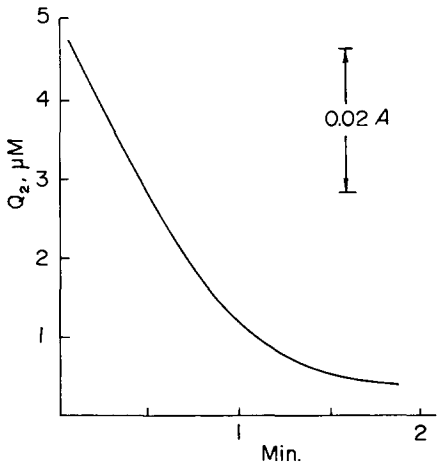

Fig. 3. The time course of Q_2 reduction by succinate in the presence of succinate: uniquinone reductase. The reaction was started at zero time by the addition of the enzyme (1 μg/ml) to the assay mixture containing 5 μM Q_2.

shown in Fig. 3. In agreement with the results on Q_2-mediated Wurster's Blue reduction (Fig. 1b), no lag in the initial rate of Q_2 reduction was observed. When the initial rates of the reaction were plotted as a function of Q_2 concentration, the $K_m^{Q_2}$ value calculated was found to be close to that determined in the experiments where Q_2 was used as a mediator of Wurster's Blue reduction, although the precision of such a determination is not high due to its very low absolute value (0.3 μM). The important feature of the curve shown in Fig. 3 is that the rate of the reaction deviates from the zero-order pattern much faster than expected from the apparent $K_m^{Q_2}$ value. It was found in particular experiments (not shown) that this deviation is not due to an irreversible inactivation of the enzyme or to accumulation of a small amount of fumarate formed during the reaction; nor does it depend on the amount of the enzyme: the normalized curves were found to be coincident when 0.4, 1.2, 2.4 and 4.8 mg/l of the enzyme were added to the assay mixture. Thus, the most conceivable reason for rapid deviation of the curve shown in Fig. 3 from zero-order pattern is that the Q_2H_2 accumulating during the reaction inhibits the reduction of Q_2. In order to quantitate the inhibiting effect of Q_2H_2, the instant rate values during the time course of Q_2 reduction were calculated using curves obtained as in Fig. 3 and which were plotted as a

function of Q_2 concentration (Fig. 2, points on curve 2). The resulting curve fits perfectly to the simple Michaelis equation for the case where the product of the reaction (Q_2H_2) is a competitive inhibitor of the substrate (Q_2) binding with the following parameters $K_m^{Q_2} = 0.3$ μM and $K_i^{Q_2H_2} = 0.8$ μM.

Several reports in the literature suggest that TTFA, a specific inhibitor of succinate: ubiquinone reductase [28], competes with ubiquinone for its binding sites [24,29,30]. Thus, it was of interest to determine whether TTFA influences the kinetics of Q_2 reduction in a manner affecting $K_m^{Q_2}$ and $K_i^{Q_2H_2}$. In the presence of 4 μM TTFA, the following parameters determined as shown in Fig. 2 were found: $K_m^{Q_2} = 0.6$ μM; $K_i^{Q_2H_2} = 2.3$ μM. Thus, both K_m and K_i values for oxidized and reduced Q_2 are significantly altered by TTFA.

Several observations reported in this paper may related to the mechanism by which succinate dehydrogenase reduces ubiquinone. The simple hyperbolic dependence of ubiquinone-deficient succinate : ubiquinone reductase on the concentration of Q_2 (Fig. 2) and the absence of a lag in the initial rate, when properly fast-reacting Wurster's Blue is used as the terminal oxidant, fits the reaction mechanism where oxidized ubiquinone (not semiquinone) is an immediate electron acceptor for the enzyme. This would certainly not contradict the existence of a succinate dehydrogenase-associated QH· pair in the respiratory chain [31,32], since the semiquinone must be formed as an intermediate [33] of the reaction between a one-electron donor (succinate dehydrogenase center S-3 [34,35]) and a two-electron acceptor (ubiquinone). The fact that TTFA alters the interaction of both Q_2 and Q_2H_2 with the specific binding site is in accordance with the idea that this inhibitor competes with ubiquinone. It should be mentioned, however, that a more complicated behavior than that of the simple competitive type is observed in the kinetics of TTFA inhibition of succinate : ubiquinone reductase (unpublished results from this laboratory) and none of the proposed mechanisms of TTFA and carboxin inhibition [24,30,36,38,40] have been conclusively established. The central finding of this report is that the reduced form of Q_2 is a competitive inhibitor of succinate : ubiquinone reductase. It appears necessary to emphasize that the natural phospholipid environment of succinate : ubiquinone reductase in Triton X-100-extracted preparation is certainly altered and that there may be significant difference in the behavior of a less lipid-soluble Q_2 as compared to natural Q_{10}. Thus, quantitatively, K_m and K_i values for natural ubiquinone may differ from those found in this report. However, the existence of a competitive relationship between oxidized and reduced ubiquinone at the donor site of succinate : ubiquinone reductase presents an interesting possibility for the control of electron flow through this region of the respiratory chain. The first-order nature of the redox reactions of ubiquinone in the respiratory chain [10] and the interesting regulatory properties of the enzymes with the competitive substrate/product binding [39] are certainly relevant to the findings of this report.

The development of the procedure for isolation of succinate : ubiquinone reductase used in this paper was started by one of us (A.D.V.) in The Laboratory of Bioenergetics, Department of Chemistry, New York State University at Albany (U.S.A.). The author is grateful to Professor Tsoo E. King for his hospitality and continued encouragement in the initial stages of this work.

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