ENZYMIC ACTIVITY OF A CYTOCHROME a PREPARATION FREE OF CYTOCHROME a_3 FROM CARDIAC MUSCLE

Tsoo E. King and Morio Kuboyama
Oregon State University, Corvallis

Received July 13, 1964

The definitive work of Keilin and Hartree (1938) has conclusively placed cytochrome oxidase at the terminal position of the respiratory chain. Their further resolution (1939) of cytochrome oxidase spectrally into cytochromes a and a_3 has settled the controversy with Warburg regarding "das Atmungsferment" (see, 1949). But perhaps due to the unusal characteristics of cytochromes a and a_3 as hemoproteins, the dispute on their identity or non-identity, functional as well as structural, has prevailed in the last quarter of a century (see for example, Slater, 1958). The interesting preparation of cytochrome a free from cytochrome a_3 by Horie and Morrison (1964) leads toward the most convincing direction for settling the question. The criterion used by Horie and Morrison (1964) is, however, based on the spectral behavior. This communication reports the enzymic activity of the isolated cytochrome a in the oxidation of succinate through the respiratory chain.

Methods and Material -- The Keilin-Hartree preparation was prepared from beef heart by their method (1940) as adopted in this laboratory (King, 1961). Cytochrome oxidase was isolated from the heart muscle preparation according to Kuboyama, Takemori and King (to be published). The ratio of copper to heme or to heme

iron of the oxidase was 1.0 and the activity was found to be approximately 60 electron equivalents x sec^{-1} x mole heme $\underline{a}^{-1}$. Cytochrome $\underline{a}$ free of cytochrome $\underline{a}_3$ was prepared by the method of Horie and Morrison (1964). The enzymic activity of cytochrome $\underline{a}$ was determined at 600 mμ with a reference wave length of 630 mμ in an Aminco-Chance double-beam spectrophotometer. The change of absorbance was converted to the concentration of cytochrome $\underline{a}$ reduced by means of an absorbance index of 9.3 mM^{-1} cm^{-1} (see, Horie and Morrison, 1964).

Results and Discussion -- We confirmed the report of Horie and Morrison (1964); the isolated cytochrome $\underline{a}$ was found to be spectrally different from the oxidase. The shift of absorption maxima in both oxidized and reduced forms and insensitivity to carbon monoxide in comparison with cytochrome oxidase were observed. Catalytically cytochrome $\underline{a}$ was free of the cytochrome oxidase activity examined polarographically in the presence of ascorbate and cytochrome $\underline{c}$. In confirming the findings of Horie and Morrison (1964), the cytochrome $\underline{a}$ isolated by their method was mainly in the reduced form which was very slowly "autoxidizable." However, we found that the rate of the oxidation of reduced cytochrome $\underline{a}$ by oxygen was increased in the presence of the heart muscle preparation. The latter evidently supplied cytochrome $\underline{a}_3$ which reacts more efficiently with molecular oxygen.

It is interesting to note that our starting material, cytochrome oxidase, which was not subjected to a step of tryptic digestion gave the same result; Horie and Morrison (1964) used the cytochrome oxidase that was prepared by tryptic digestion (Horie and Morrison,

1963). However, the yield was low, less than 20% on the protein basis by either of the original method or the method with our modifications. Horie and Morrison (1964) did not report the yield. A large amount of precipitate of an apparently denatured hemoprotein was found in a discarded fraction from ammonium sulfate fractionation after the borohydride action on the cyanide-treated oxidase.

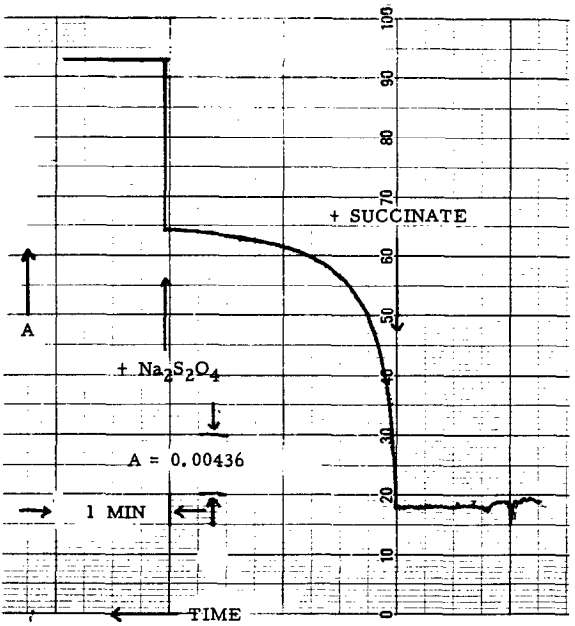

Fig. 1. The enzymic reduction of cytochrome a. The system contained phosphate buffer, 50 mM, pH 7.4; crystalline cytochrome c (beef), 0.7 μM; cytochrome a, approximately 5 μM; EDTA, 2.7 mM; and the heart muscle preparation, 11 μg protein. At proper time, 17 mM succinate and 2-3 mg sodium dithionite were added. The heart muscle preparation at 20 mg per ml was mixed with cholate up to 2%, pH 7.4. The mixture was incubated at 23° for 5 minutes, then appropriately diluted and kept at 0-4° for use. The measurement was made in a double-beam spectrophotometer with λ_1 at 600 mμ and λ_2 at 630 mμ. The full chart width was 10% T.

The enzymic activity of cytochrome a was demonstrated in a very simple and straight-forward manner. Cytochrome a was

used as a reactant in accepting electrons in succinate oxidation by succinate oxidase which was supplied from the heart muscle preparation. A protocol is depicted in Fig. 1. In the absence of the heart muscle preparation, cytochrome a was completely devoid of succinate oxidase activity. The cytochrome a in the heart muscle preparation present in the system in Fig. 1 was less than 0.01 μM (cf. King et al., 1964) in contrast to 5 μM added. The reaction was very much dependent on the concentration of cytochrome a. The initial, rapid reduction (Fig. 1) slowed down during the course of the reaction and the reaction approximately followed the characteristics of the first order kinetics.

Cyanide (0.5-1.0 mM) showed a weak inhibition in the initial rapid phase and stimulated the reaction at low concentrations of cytochrome a. The stimulation was likely due to the competition for the electrons between exogenous cytochrome a and oxygen (through cytochrome a_3 of succinate oxidase) and cyanide inhibited the reaction between cytochrome a_3 and oxygen. On the other hand, the flow of electrons from "exogenous" cytochrome a to "endogenous" cytochrome a_3 was slow. However, the weak inhibition of cyanide might involve more complicated reactions (cf. Keilin and King, 1960) and will be discussed with other experimental evidence elsewhere.

Under the conditions as shown in Fig. 1 the addition of dithionite further immediately reduced cytochrome a as expected. This further reduction was due to the fact that the enzymic reaction was still proceeding but at a slow rate because of the decrease of cytochrome a concentration. In a separate experiment,

prior to the addition of dithionite, addition of ferricyanide immediately re-oxidized the reduced cytochrome a as witnessed by a sudden drop of the absorbance (not shown in the figure) at 600 mμ.

Antimycin A at about 0.5 μg per ml of the assay system completely inhibited the reaction.

The prior treatment of the heart muscle preparation with cholate increased the reduction rate of cytochrome a. Analogous observations have been reported in the succinate oxidase system using cytochrome c as an electron acceptor (King and Takemori, 1964). Calculation from results similar to data presented in Fig. 1 gave a reaction rate of approximately 0.55 μmole cytochrome a reduced per minute per mg of the heart muscle preparation at 23°. This is equivalent to a Q_{O_2} value of nearly 200 μl. These values should be considered minimal because the experimental conditions might not be optimal. Moreover, the concentrations of cytochrome a used were low. It recalls that the K_m values for cytochrome c for both succinate-cytochrome c reductase and succinate oxidase (in the presence of cyanide) are approximately 4 μM (King and Takemori, 1964). Even an equivalent Q_{O_2} value of 200 μl is only slightly lower than that when oxygen or cytochrome c is used as an acceptor (see King, 1961; King and Takemori, 1964).

These results have clearly shown that the isolated cytochrome a is enzymically active. The demonstration of enzymic activity of the cytochrome a preparation, which is free of cytochrome oxidase action, coupled with the spectral evidence so richly documented in the literature, seems to leave no alternatives except in the recogni-

tion of the existence of the separate entities of cytochromes a and a_3 defined both structurally and functionally.

Acknowledgment -- This work was supported by grants from the National Science Foundation, the Public Health Service, the American Heart Association, and the Life Insurance Medical Research Fund. We are grateful to Dr. M. Morrison who made his paper with Dr. Horie available to us before publication (April 20, 1964).

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