EVIDENCE FOR AEROBIC AND ANAEROBIC MECHANISMS FOR REDUCTION OF CYTOCHROME c BY XANTHINE OXIDASE

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There are several points to be clarified about the route of electrons from xanthine oxidase to various acceptors. The pathway of electron transfer appears to regulate the formation of uric acid and to play an important role in the metabolic control of the catabolism of purines. In this connection it is worthy of notice that cytochrome c acts as an electron acceptor for xanthine oxidase, but there has been considerable discussion about this reaction. Horecker and Heppel (1949) demonstrated that molecular oxygen is indispensable for the reduction of cytochrome c. This has subsequently been confirmed in several laboratories (Weber et al., 1956; Fridovich and Handler, 1962; Handler et al., 1964). On the other hand, in the observations on anaerobic reduction of cytochrome c by this enzyme, Morell (1952) reported that oxygen was not required and rather inhibitory for the reaction. The present paper describes our recent findings which explain both the aerobic and anaerobic mechanisms for the reduction of cytochrome c via xanthine oxidase.

Xanthine oxidase was prepared from fresh cream of cow's milk by the method of Kubo et al. (1962). The A_{280}/A_{450} ratio of the preparation was 5.7 - 5.9. Routinely, 1 μmole of hypoxanthine, 0.15 μmole of cytochrome c (Sigma's horse heart preparation, type III), 1 ml of 0.1 M potassium phosphate buffer (pH 7.4) and 100 μg of enzyme were present in the assay system in a final volume of 3 ml. The reduction of cyto-

chrome c was determined by the increase in absorbancy at 550 mμ.

It has been demonstrated that oxygen lack and a higher concentration of catalase inhibit the reduction of cytochrome c , and that H_2O_2 accelerate the reduction of cytochrome c under both aerobic and anaerobic conditions in the presence of hypoxanthine and 8-hydroxyquinoline. It has been suggested that the aerobic reaction proceeds through the free radical mechanism (Muraoka et al.,in preparation), as in the reduction of cytochrome c by plant peroxidases (Yamazaki, 1958; Yamazaki and Piette, 1963).

It is difficult to obtain the anaerobic reduction of cytochrome c without addition of any electron carriers, as stated in other reports. The electron carriers with which the anaerobic reduction of cytochrome c was obtained were *menadione*, *vitamin* K_1 , *coenzyme* Q_7 , 1,4-naphthoquinone, trinitrobenzene sulfonate, methylene blue, phenazine methosulfate and 2,6-dichlorophenolindophenol at concentration of 0.1 μmole/3 ml. Since 8-hydroxyquinoline (1 μmole/3 ml) and m-phenylenediamine (1 μmole/3 ml) promote a slow reduction under anaerobic conditions, they appear to act as weak electron carriers from the enzyme to cytochrome c . It is possible that loss of an endogenous electron carrier during purification of the enzyme might be responsible for the discrepant results and opinions on the anaerobic reduction of cytochrome c .

It has been postulated that the anaerobic reduction proceeds by a completely different mechanism from the aerobic one. In Table I, it is shown that 1 mole of hypoxanthine caused aerobic reduction of 0.81 - 0.92 mole of cytochrome c or anaerobic reduction of 3.21 - 4.08 moles of cytochrome c. This indicates that the stoichiometry of the two reaction systems is different. With menadione the anaerobic type of reaction was obtained under both aerobic and anaerobic conditions (Table I). Fig. 1 shows that the pH optimum of the aerobic reaction is approximately 10.0 and that of the anaerobic reaction is pH 8.0.

TABLE I

Effect of 8-hydroxyquinoline and menadione on the aerobic and anaerobic reductions of cytochrome c

Three ml of reaction mixture contained 0.15 μmole of cytochrome c, 1 ml of 0.1 M potassium phosphate buffer and 100 μg of enzyme. The cytochrome c reduced was calculated from ΔA at 550 mμ ($19.5 \times 10^3/M$ at pH 7.4). Anaerobic conditions were obtained evacuating and filling the vessel with purified nitrogen gas 6 times. Temperature 25°. 8-OHQ, 8-hydroxyquinoline; K_3, menadione; Cyt. c, cytochrome c.

Substrate (μmole)	Additions (μmole)	Gas Phase	Cyt. c Reduced (μmole)	Cyt. c/Substrate (mole/mole)
Xanthine, 0.01	None	Air	0.0046	0.46
" , 0.02	"	"	0.0077	0.38
" , 0.03	"	"	0.0131	0.44
Xanthine, 0.01	8-OHQ, 1.0	Air	0.0054	0.54
" , 0.02	" , 1.0	"	0.0115	0.58
" , 0.03	" , 1.0	"	0.0154	0.51
Xanthine, 0.01	K_3, 0.5	Air	0.0154	1.54
" , 0.02	" , 0.5	"	0.0334	1.67
" , 0.03	" , 0.5	"	0.0508	1.69
Xanthine, 0.01	8-OHQ, 1.0	N_2	0.0169	1.69
" , 0.02	" , 1.0	"	0.0338	1.69
" , 0.03	" , 1.0	"	0.0569	1.90
Xanthine, 0.01	K_3, 0.5	N_2	0.0169	1.69
" , 0.02	" , 0.5	"	0.0324	1.62
" , 0.03	" , 0.5	"	0.0453	1.51
Hypoxanthine, 0.01	None	Air	0.0092	0.92
" , 0.02	"	"	0.0162	0.81
" , 0.03	"	"	0.0246	0.82
Hypoxanthine, 0.01	8-OHQ, 1.0	Air	0.0077	0.77
" , 0.02	" , 1.0	"	0.0154	0.77
" , 0.03	" , 1.0	"	0.0208	0.69
Hypoxanthine, 0.01	K_3, 0.1	Air	0.0284	2.84
" , 0.02	" , 0.1	"	0.0545	2.72
" , 0.03	" , 0.1	"	0.0968	3.23
Hypoxanthine, 0.01	8-OHQ, 1.0	N_2	0.0400	4.00
" , 0.02	" , 1.0	"	0.0815	4.08
" , 0.03	" , 1.0	"	0.0963	3.21
Hypoxanthine, 0.01	K_3, 0.1	N_2	0.0362	3.62
" , 0.02	" , 0.1	"	0.0738	3.69
" , 0.03	" , 0.1	"	0.1015	3.38

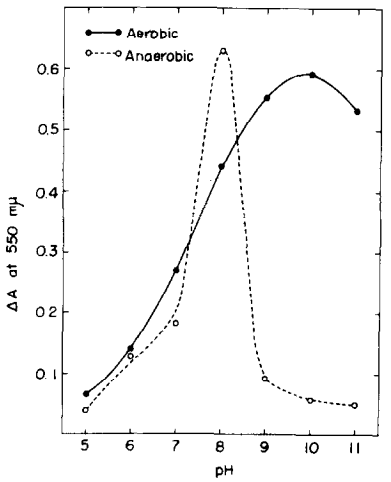

Fig. 1. Effect of pH on the aerobic and anaerobic reductions of cytochrome c in the presence of 8-hydroxyquinoline. The reaction mixture contained 0.1 μmole of hypoxanthine, 0.15 μmole of cytochrome c, 1 μmole of 8-hydroxyquinoline, 100 μg of enzyme and 1 ml of 0.1 M potassium phosphate buffer.

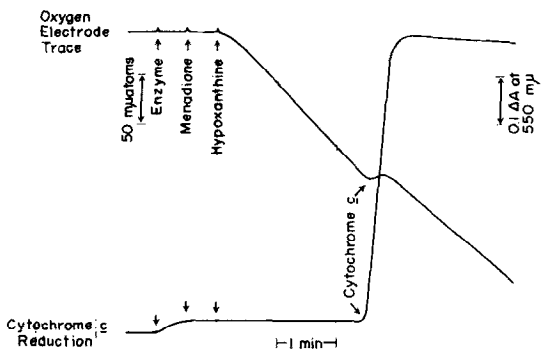

Fig. 2. Arrest of oxygen consumption during menadione-induced reduction of cytochrome c . The sample cuvette for polarographic and spectrophotometric analysis contained 0.6 ml of 0.1 M potassium phosphate buffer in a total volume of 2 ml. The other constituents were as follows: 1 mg of enzyme, 0.1 μmole of menadione, 1 μmole of hypoxanthine and 0.1 μmole of cytochrome c. Temperature 25°.

Since the extents of the aerobic and anaerobic reductions differ, the two reactions can readily be distinguished from one another. The reaction can be classified into three categories on the basis of the

electron carrier used: (a) In the presence of molecular oxygen as the hydrogen acceptor the aerobic type of reduction occurs, as already described. (b) In the presence of 8-hydroxyquinoline or m-phenylenediamine the aerobic type of reduction was observed under aerobic conditions while the anaerobic type of reduction proceeds under anaerobic conditions (Table I). The latter reaction is inhibited by traces of oxygen. An endogenous electron carrier similar to 8-hydroxyquinoline might have been present in Morell's enzyme preparation, since he reported that the reaction was inhibited by oxygen. (c) In the presence of menadione or other electron carriers the anaerobic type of reaction occurs under anaerobic conditions. As already mentioned, this type of the reduction is observed even under aerobic conditions. This is supported by the fact that menadione arrests oxygen consumption at the stage of cytochrome c reduction (Fig. 2).

From these results, it is suggested that the reduction of cytochrome c is achieved by hydrogen or electron from reduced enzyme via either aerobic or anaerobic pathway, and the pathway by which it occurs is determined by the electron carrier used.

REFERENCES

Fridovich, I., and Handler, P., J. Biol. Chem., 237 , 916 (1962).

Handler, P., Rajagopalan, K. V., and Aleman, V., Fed. Proc., 23 , 30 (1964).

Horecker, B. L., and Heppel, L. A., J. Biol. Chem., 178 , 683 (1949).

Kubo, H., Shiga, K., Isomoto, A., Uozumi, M., Kadota, K., and Kondo, Y., Proceedings of Symposia on Enzyme Chemistry (Japan), 1962, p.37.

Morell, D. B., Biochem. J., 51 , 666 (1952).

Muraoka, S., Sugiyama, M., and Yamasaki, H., in preparation.

Weber, M. M., Lenhoff, H. M., and Kaplan, N. O., J. Biol. Chem., 220 , 93 (1956).

Yamazaki, I., Proc. Sym. Enzyme Chem. (Tokyo-Kyoto), 1957, p.224.

Yamazaki, I., and Piette, L. H., Biochim. Biophys. Acta, 77 , 47 (1963).