OXALOACETATE INHIBITION OF SUCCINATE OXIDATION IN TIGHTLY COUPLED LIVER MITOCHONDRIA WITH FERRICYANIDE AS AN ELECTRON ACCEPTOR

G. Lynis Dohm and Edward B. Tapscott

Department of Biochemistry, School of Medicine
East Carolina University, Greenville, North Carolina

Received March 15,1973

SUMMARY

When ferricyanide is used as an artificial electron acceptor, succinate oxidation by tightly coupled liver mitochondria becomes inhibited after 1-3 min. No inhibition occurs in the presence of rotenone or glutamate establishing that oxaloacetate causes the inhibtion. Oxygen consumption by mitochondria oxidizing succinate does not become inhibited in the absence of rotenone suggesting that oxaloacetate accumulates to a greater extent when ferricyanide is added than when oxygen is the terminal acceptor. Higher levels of oxaloacetate in the ferricyanide reaction are apparently due to an increased rate of synthesis rather than a decreased rate of removal. Thus it appears that when succinate is the substrate and oxygen the terminal acceptor a control mechanism exists which blocks oxidation of malate. When ferricyanide is added as an artificial electron acceptor this control is lost and oxaloacetate accumulates to inhibit succinate oxidation.

INTRODUCTION

Although oxaloacetate is known to inhibit succinate dehydrogenase (1), inhibition of oxygen consumption by tightly coupled liver mitochondria oxidizing succinate is not observed because the rate of formation of oxaloacetate is matched by the rate of removal and inhibitory levels are not reached. However, when oxidative phosphorylation is uncoupled the rate of removal of oxaloacetate is decreased and succinate oxidation becomes inhibited (2,3,4).

We have observed inhibition of succinate oxidation by oxaloacetate in tightly coupled liver mitochondria when ferricyanide was used as an artificial electron acceptor. The inhibition seems to be due to an increased rate of synthesis of oxaloacetate rather than a decreased rate of removal.

METHODS

Rat liver mitochondria were isolated as described by Johnson and Lardy (5). Isolated mitochondria were checked each day to establish that they were tightly coupled and all preparations exhibited respiratory control ratios in the range

of 5 to 8 with succinate as substrate and oxygen as the terminal acceptor. Oxygen consumption was monitored in a Yellow Springs Model 53 oxygen analyzer with a water jacketed vessel containing 2.0 ml of solution. Ferricyanide reduction was determined by measuring the change in absorbance at 420 nm in a Gilford Model 2000 spectrophotometer. The basic medium for studies of oxygen consumption and ferricyanide reduction contained 200 mM sucrose, 50 mM KCl, 25 mM tris (pH 7.4), 10 mM potassium phosphate (pH 7.4), 5 mM $MgCl_2$, 2.0 mM ADP, and 10 mM succinate in a total volume of 2.0 ml. When ferricyanide reduction was measured the medium also contained 1.0 mM KCN and 1.0 mM potassium ferricyanide.

Mitochondrial protein concentration was measured by the method of Gornall et. al. (6).

RESULTS AND DISCUSSION

Ferricyanide reduction in the presence of tightly coupled mitochondria and succinate exhibits a rapid initial rate which begins to decrease after 1-3 min. and then establishes a constant inhibited rate (Fig. 1, curve D). If rotenone is included in the medium no inhibition is observed and the rate of ferricyanide reduction with rotenone added is 4-8 times as great as the rate when rotenone is omitted (curve C). In contrast, mitochondrial oxygen consumption with succinate as substrate does not become inhibited and is unaffected by rotenone addition (curve A and curve B).

Since rotenone blocks the oxidation of malate by inhibiting NADH oxidation, it seemed likely that the inhibition of ferricyanide reduction in the absence of rotenone was due to inhibition of succinate dehydrogenase by oxaloacetate. This was confirmed by adding glutamate to the reaction system; no inhibition of ferricyanide reduction was observed when glutamate was present since oxaloacetate was removed by transamination with glutamate (Table 1).

Oxaloacetate inhibition of succinate oxidation in uncoupled mitochondria has been shown to be reversed by ATP addition (2). Reversal of the inhibition in this case is probably due to an increase in the rate of removal of oxaloace-

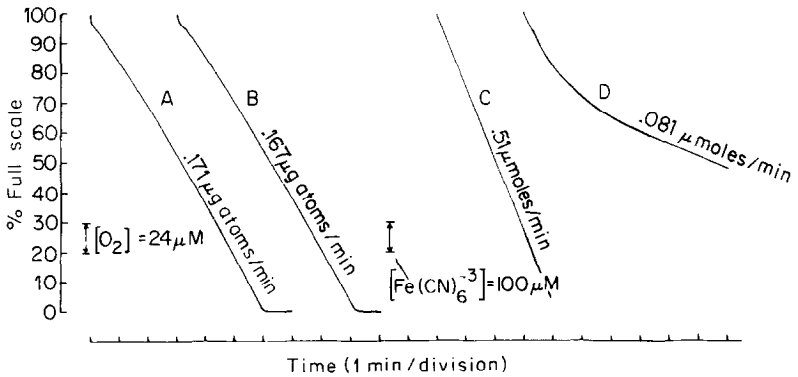

Fig. 1. Oxygen consumption and ferricyanide reduction by tightly coupled liver mitochondria oxidizing succinate in the presence or absence of rotenone. Assay conditions as given in methods. The reactions were started by adding 1.74 mg of mitochondrial protein. Concentration of rotenone was 0.2 μg/ml. Curve A: oxygen consumption with rotenone added: curve B; oxygen consumption, rotenone omitted: curve C; ferricyanide reduction with rotenone added: curve D; ferricyanide reduction, rotenone omitted.

tate by ATP (or GTP) requiring reactions (4). Adding ATP to our inhibited system did not result in a reversal of inhibition (Table 1) but this result would be expected since the mitochondria are tightly coupled and can themselves generate ATP.

Omitting ADP or adding oligomycin eliminated oxaloacetate inhibition of ferricyanide reduction and the rate of succinate oxidation was the same whether or not rotenone was added (Table 1). Omitting phosphate also equalized the rate of ferricyanide reduction with and without rotenone but the rate was lower than when ADP was omitted. This lowered rate was probably a result of inhibited uptake of succinate in the absence of phosphate (7). These data led to speculation that inhibition of succinate oxidation by oxaloacetate was somehow energy dependent. However, further experiments demonstrated that omitting ADP or phosphate lowered the oxidation of NADH linked substrates by approximately 50% and this inhibition probably blocks the formation of oxaloacetate (Table 1).

Comparing the rate of ferricyanide reduction (rotenone added) and oxygen consumption (Fig. 1) it was apparent that succinate was oxidized approximately

Table 1. The Effect of Rotenone on Ferricyanide Reduction.

Assay conditions as given in the methods section. Concentrations of other additions were: 10 mM glutamate, 10 mM β-hydroxybutyrate, 0.25 μg/ml oligomycin, 2 mM ATP, and 1.49 mg mitochondrial protein. In the cases where ferricyanide reduction became inhibited (see Fig. 1) the constant inhibited rate was reported.

Substrate	Additions (+) or deletions (-)	Ferricyanide Reduction (μmoles/min/mg protein)	
		Without Rotenone	With Rotenone
Succinate	None	.06	.35
Succinate	+ATP	.09	.35
Succinate	+glutamate	.31	.34
Glutamate	None	.04	---
Succinate	-ADP	.35	.36
Succinate	+Oligomycin	.33	.35
Succinate	-phosphate	.16	.16
β-hydroxybutyrate	None	.08	---
β-hydroxybutyrate	-ADP	.03	---
β-hydroxybutyrate	-phosphate	.04	---

50% faster with ferricyanide than when oxygen was the terminal acceptor. To establish that the increased rate of succinate oxidation was not leading to the oxaloacetate inhibition, succinate oxidation was partially inhibited with malonate and ferricyanide reduction was measured in the presence or absence of rotenone (Fig. 2) Although lowering the rate of succinate oxidation did depress the extent of oxaloacetate inhibition, decreasing ferricyanide reduction (rotenone added) by 50% still resulted in a twofold difference between the rates with and without rotenone. Thus oxaloacetate inhibition of ferricyanide reduction cannot be explained by the difference in rate of succinate oxidation with ferricyanide and oxygen as electron acceptors.

It thus appeared that greater quantities of oxaloacetate were accumulating

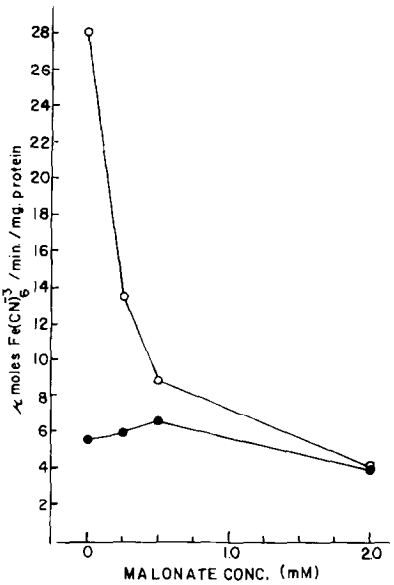

Fig. 2. Effect of malonate on ferricyanide reduction by tightly coupled liver mitochondria oxidizing succinate in the presence or absence of rotenone. Assay conditions as given in methods with malonate added at the indicated concentrations. When ferricyanide reduction became inhibited after 1-3 min. the constant inhibited rate was reported. •, ferricyanide reduction with no rotenone added; o, ferricyanide reduction in the presence of 0.2 μg/ml rotenone.

when ferricyanide was the electron acceptor than when oxygen served as the terminal acceptor. This could occur if the rate of synthesis of oxaloacetate were accelerated or if the rate of removal were decreased when ferricyanide was added. To differentiate between these two alternatives, mitochondria were preincubated with malate for 2 min. to generate intramitochondrial oxaloacetate. Then succinate was added and the degree of inhibition of ferricyanide reduction or oxygen consumption was taken as a relative measure of the oxaloacetate level (Fig. 3). When rotenone was added along with succinate after the 2 min. malate preincubation, further oxaloacetate formation was stopped and the lowering in the degree of inhibition demonstrated the rate of removal of oxaloacetate. In reactions where no rotenone was added, the degree of inhibition gave a measure of the oxaloacetate level which was a result of the rate of formation and the rate of removal.

With further malate oxidation blocked, oxaloacetate was removed from both

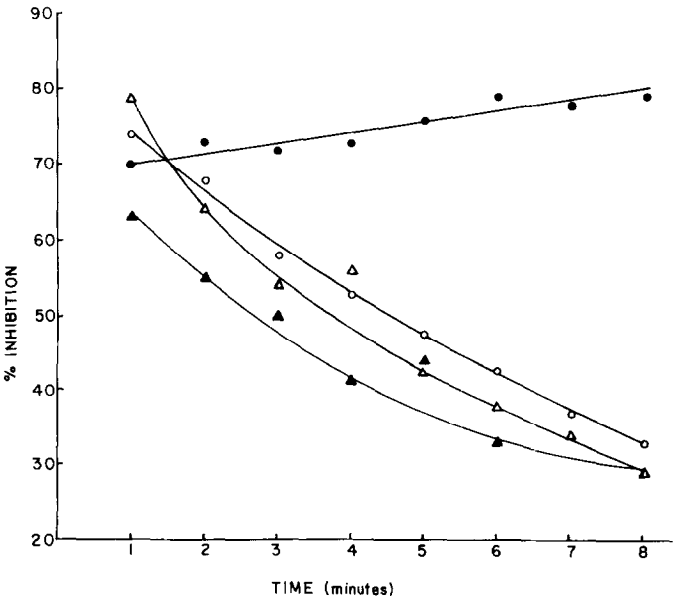

Fig. 3. Oxaloacetate inhibition of oxygen consumption and ferricyanide reduction by tightly coupled liver mitochondria oxidizing succinate in the presence or absence of rotenone. Inhibited systems were preincubated for exactly 2.0 min. in a media consisting of 200 mM sucrose, 50 mM KCl, 25 mM tris (pH 7.4), 10 mM potassium phosphate (pH 7.4), 5 mM $MgCl_2$, 2 mM ADP, and 1 mM malate so that oxaloacetate would be generated. After the 2.0 min. preincubation, 10 mM succinate or 10 mM succinate plus 0.2 µg/ml rotenone were added and the rates of oxygen consumption or ferricyanide reduction were determined. For ferricyanide reduction reactions 1 mM KCN and 1 mM ferricyanide were added concurrent with succinate or succinate plus rotenone. Control reactions were treated in the same manner as oxaloacetate inhibited reactions except that rotenone was added before the preincubation period so that no oxaloacetate was generated. Control reactions were used as a basis for calculating percent inhibition. ●, ferricyanide reduction, no rotenone added; o, ferricyanide reduction, rotenone added to block further formation of oxaloacetate; ▲, oxygen consumption, no rotenone added; △, oxygen consumption, rotenone added to block the further formation of oxaloacetate.

the oxygen and ferricyanide systems as evidenced by the decline in the degree of inhibition of succinate oxidation. In addition, the rate of decline in oxaloacetate level in the oxygen system was nearly the same whether or not malate oxidation was blocked. However, no reversal of inhibition of ferricyanide reduction was observed when oxaloacetate formation was not blocked, indicating that the rate of formation of oxaloacetate was at least as fast as the rate of removal.

These data indicate that when succinate is the substrate and oxygen the terminal acceptor, malate oxidation must be inhibited because adding rotenone did

not accelerate the rate of removal of oxaloacetate. However, when ferricyanide is the electron acceptor this control over malate oxidation is lost and oxaloacetate continues to be synthesized and inhibits succinate oxidation.

Davis et. al.(8) found that NADH and succinate compete for the electron transport chain in submitochondrial particles and oxidation of succinate inhibited the rate of NADH oxidation. Greengard et. al. (9) also reported a control over malate oxidation during the oxidation of succinate. They found that the higher the succinate oxidation rate the less malate was oxidized to oxaloacetate.

These results coupled with the present data demonstrate that succinate oxidation exerts a control over malate oxidation when oxygen is the terminal acceptor Why then is this control of malate oxidation lost when ferricyanide is added as an artificial electron acceptor? We have considered two possibilities: (1) the level of oxidized NAD is lower in the ferricyanide reaction thus accelerating the oxidation of malate, and (2) the resistance to electron flow from malate to ferricyanide is less than that to oxygen. The first alternative was tested by measuring the change in fluorescence after a transition from a steady state level of NAD during succinate oxidation to fully reduced NADH when rotenone and glutamate were added. The fluorescence change was the same when either oxygen or ferricyanide were the terminal acceptor (unpublished observation) which indicates that the level of NAD was the same in both systems.

The idea that resistance to electron flow might be less from malate to ferricyanide than from malate to oxygen arose from the fact that succinate was oxidized at a more rapid rate in the presence of ferricyanide than when oxygen served as the electron acceptor. This possibility was tested by partially inhibiting electron transport with antimycin to increase the resistance to electron flow. Inhibiting ferricyanide reduction by as much as 50% did not relieve the oxaloacetate inhibition of succinate oxidation (unpublished observation) and a twofold difference in ferricyanide reduction was observed between the rate in the presence or absence of rotenone.

Although we do not presently have data to explain why control of malate

oxidation is lost when ferricyanide is used as an electron acceptor there are several levels at which control could be exerted. Estabrook (10) and Sottocassa and Sandri (11) have shown that ferricyanide accepts electrons from cytochrome c in intact mitochondria and cytochrome c is more oxidized when ferricyanide is added than when oxygen is the terminal acceptor. This raises the possibility that one of the electron carriers between NADH and cytochrome c may control malate oxidation when it is relatively more reduced. Another possibility is that some component of the electron chain between cytochrome c and oxygen or the third site of oxidative phosphorylation may exert control over malate oxidation since these components would not be active in the presence of KCN and ferricyanide.

REFERENCES

1. Zeylemaker, W. P., Klaasse, A.D. M., and Slater, E. C., Biochim. Biophys. Acta 191, 229 (1969).
2. Oestreicher, A. B., Van Den Berg, S. G., and Slater, E. C., Biochim. Biophys. Acta 180, 45 (1969).
3. Wojczak, A. B., Biochim, Biophys. Acta 172, 52 (1969).
4. Papa, S., Lofrumento, N. E., Paradies, G., and Quagliariello, E., Biochim. Biophys. Acta 180, 35 (1969).
5. Johnson, D. and Lardy, H., In Estabrook, R. W., and Pullman, M. E. (eds), "Methods in Enzymology" vol. 10, Academic Press, New York, 1967. P 95.
6. Gornall, A. G., Bardawill, C. P. and David, M. M., J. Biol. Chem. 177, 751 (1949).
7. Chappell, J. B., and Haarhoff, K. N., In Slater, E. C., Kaniuga, Z., and Wojczak, L. (eds), "Biochemistry of Mitochondria", Academic Press, New York, 1967. P 75.
8. Davis, E. J., Blair, P. V., Mahoney, A. J., Biochim. Biophys. Acta 172, 574 (1969).
9. Greengard, P., Minnaert, K., Slater, E. C., and Betel, I., Biochem. J. 73, 637 (1959).
10. Estabrook, R. W., J. Biol. Chem. 236, 3051 (1961).
11. Sottocasa, G. L., and Sandri, G., Biochim. Biophys, Acta 223, 36 (1970).