BBA 45579

ON THE NATURE OF ENDOGENOUS SUBSTRATE IN RAT-LIVER MITOCHONDRIA

JADWIGA BRYŁA, ZBIGNIEW KANIUGA AND BARBARA FRACKOWIAK
Department of Biochemistry, Warsaw University, Warszawa 22 (Poland)
(Received January 30th, 1967)

SUMMARY

1. Oxidation and phosphorylation coupled with endogenous substrate oxidation was studied in comparison with fatty-acid oxidation. Similar sensitivities to arsenite and malonate were observed for the oxidation of both endogenous substrate and fatty acid. The P:O ratio for the oxidation of endogenous substrate and fatty acids was found to be approx. 2.5. Endogenous substrate oxidation as well as fatty-acid oxidation was stimulated by NAD^+.

2. The endogenous substrate was activated by both systems described by VAN DEN BERGH for the activation of fatty acids. The carnitine-stimulated oxidation of endogenous substrate was observed only in the dinitrophenol-sensitive system.

3. The results presented indicate that fatty acids constitute the endogenous substrate in rat-liver mitochondria.

INTRODUCTION

Endogenous substrates of isolated mitochondria are now regarded to be an important factor affecting experimental results. They can influence: (a) the rate of exogenous substrate oxidation[1–3], (b) the yield of oxidative phosphorylation coupled with the exogenous substrate oxidation[4–12], (c) the energy-dependent NAD^+ reduction[13–18], and (d) respiratory control[19,20].

There are many reports on the composition and function of various components of the mitochondrial fraction. However, little is known about the nature and function of the mitochondrial endogenous substrate. The nature of this substrate was first investigated by SCHNEIDER, STRIEBICH AND HAGEBOOM[21], who observed that citrate accumulated in liver mitochondria isolated from fluoroacetate-treated rats. BELLAMY[1], using mitochondria from various organs, found that endogenous respiration includes oxidation through the tricarboxylic acid cycle. Various authors have suggested that fatty acids are endogenous substrates[2,12,17,18,22,23]. However no satisfactory proof is available in support of this suggestion.

This paper presents some observations on oxidation and phosphorylation coupled with the endogenous substrate oxidation in comparison with the fatty-acid oxidation by rat-liver mitochondria.

MATERIAL AND METHODS

Rat-liver mitochondria were prepared in 0.25 M sucrose as described by MYERS AND SLATER[24].

The standard reaction medium contained 15 mM KCl, 2 mM EDTA, 50 mM Tris–HCl buffer, 5 mM $MgCl_2$, 30 mM phosphate, 0.1 mM ADP, 60 mM glucose, 100–150 Cori units of hexokinase and 6–10 mg of protein in a final vol. of 1 ml. Other additions were: 0.1 mM octanoate, 0.1 mM palmitate, 1–10 mM arsenite, 1–10 mM malonate, 1 mM succinate, 2 mM NAD^+. The final pH was 7.4, and the reaction time was 16 min at 25°. Fatty-acid oxidation was studied in the presence of 1 mM carnitine *plus* 1 % (w/v) bovine serum albumin. Fatty acids, rotenone and oligomycin were added as ethanolic solutions. The final ethanol concentration did not exceed 2 %.

Protein was determined by the biuret method as described by CLELAND AND SLATER[25] using egg albumin as a standard.

P:O ratios were determined by the method of SLATER AND HOLTON[26] in which O_2 uptake was measured manometrically, and phosphorylation estimated by allowing the ATP formed to be used in the synthesis of hexose monophosphate. Phosphorylation was measured enzymatically by Method II of SLATER[27].

Polarographic determination of the rates of O_2 consumption was done with the Clark electrode by the procedure of CHAPPELL[28]. The reaction mixture (1.5 ml) was the same as that described for manometric experiments except that it did not contain ADP, glucose or hexokinase. Additions to this medium as indicated in the figure were made in small volumes (10–50 μl).

The reagents used were commercial preparations: ADP and ATP, Boehringer; NAD^+, Reanal; rotenone, Penick Co.; oligomycin, Sigma; carnitine, Light.

Yeast hexokinase was prepared according to DARROW AND COLOWICK[29] without the final crystallization step.

RESULTS

P:O ratio

P:O ratios for the endogenous substrate oxidation were similar to the P:O ratios (corrected and uncorrected) for the fatty-acid oxidation (Table I).

Effects of arsenite and malonate

Arsenite and malonate are inhibitors commonly used for the elimination of endogenous substrate oxidation[7,13,17]. Figs. 1 and 2 illustrate the effects of various concentrations of arsenite and malonate on the oxidation of the endogenous substrate and fatty acids. The oxidation of the endogenous substrate and palmitic or octanoic acids is inhibited by these compounds to similar extents.

Effect of NAD^+

WEINBACH[6] found that the addition of NAD^+ or $NADP^+$ to NAD^+-depleted mitochondria restores their endogenous activity. Other authors[13,14,17] found that endogenous substrate oxidation occurs in the presence of NAD^+. The results presented in Table II indicate that NAD^+ stimulates the oxidation of both endogenous substrate and palmitic acid.

TABLE I

OXIDATIVE PHOSPHORYLATION COUPLED WITH THE OXIDATION OF ENDOGENOUS SUBSTRATE AND PALMITIC AND OCTANOIC ACIDS

O_2 uptake was measured manometrically. Conditions as described in MATERIAL AND METHODS. 7.4 and 7.6 mg of protein were used in Expts. 1 and 2, respectively.

Expt. No.	*Substrate*	*—ΔO (μatoms)*	*Δ esterified P (μmoles)*	*P:O ratio*	*P:O ratio corrected**
1	Endogenous	1.94	4.94	2.55	
	Octanoic acid	3.85	9.43	2.44	2.35
2	Endogenous	1.52	3.70	2.40	
	Palmitic acid	7.50	17.24	2.30	2.26

* P:O ratio calculated after subtracting ΔO and Δ esterified P for endogenous substrate.

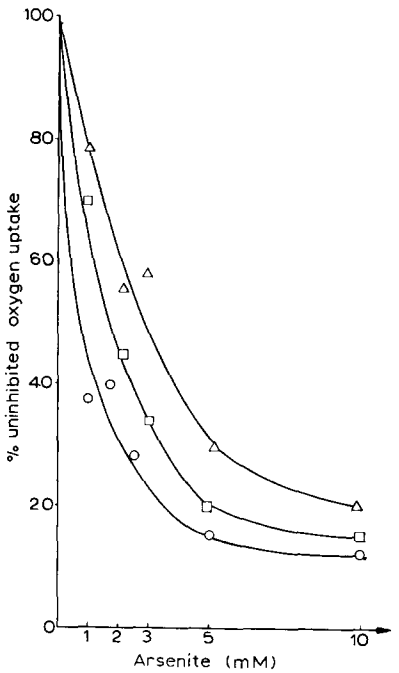

Fig. 1. Inhibition of the oxidation of endogenous substrate and octanoic and palmitic acids by arsenite. O—O, endogenous substrate; □—□, octanoic acid; △—△, palmitic acid. Respiration was measured manometrically.

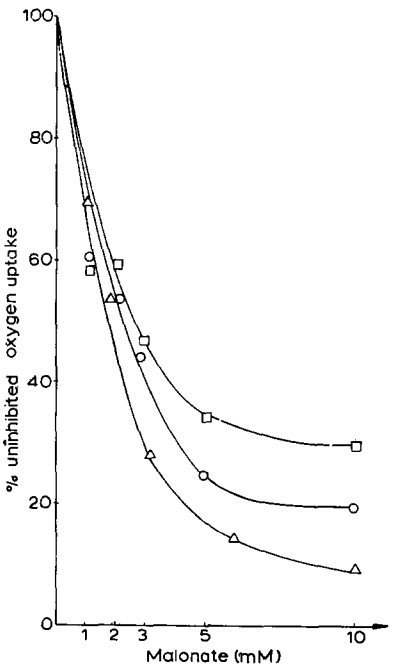

Fig. 2. Inhibition of the oxidation of endogenous substrate and octanoic and palmitic acids by malonate. O—O, endogenous substrate; □—□, octanoic acid; △—△, palmitic acid. Respiration was measured manometrically.

Activation of endogenous substrate

In 1965 VAN DEN BERGH[30] described dinitrophenol-sensitive (in the presence of phosphate) and dinitrophenol-insensitive (in the absence of phosphate but in the presence of dinitrophenol) systems for fatty-acid activation.

TABLE II

EFFECT OF NAD^+ ON THE OXIDATION OF ENDOGENOUS SUBSTRATE AND PALMITIC ACID

O_2 uptake was measured manometrically. Conditions as described in MATERIAL AND METHODS. 7.6 mg of protein was used.

Substrate	*ΔO (μatoms)*	
	Without NAD^+	*With NAD^+*
Endogenous	1.65	2.90
Palmitic acid	6.05	7.18

In Table III these two systems are compared for endogenous substrate oxidation. As shown in the second line, oligomycin inhibits the endogenous substrate oxidation in the presence of phosphate. On the other hand, oxidation of the endogenous substrate in the absence of phosphate (*plus* DNP) is insensitive to oligomycin. The dinitrophenol-sensitive oxidation is only 50 % inhibited by 1 mM arsenite and not completely by 5 mM arsenite. On the contrary the dinitrophenol-insensitive process is completely dependent on the operation of the Krebs cycle (Lines 3 and 4). The addition of dinitrophenol (last line) to a system containing phosphate causes complete inhibition of the endogenous substrate oxidation as the addition of oligomycin *plus* arsenite does (Lines 5 and 6).

TABLE III

COMPARISON OF THE DINITROPHENOL-SENSITIVE AND DINITROPHENOL-INSENSITIVE SYSTEMS FOR ENDOGENOUS SUBSTRATE OXIDATION

O_2 uptake was measured manometrically. Reaction medium for the dinitrophenol-sensitive system (Column A) was the same as described under MATERIAL AND METHODS; and that for the dinitrophenol-insensitive system (Column B) did not contain phosphate, ADP, glucose or hexokinase, but contained 0.05 mM dinitrophenol. 6.5 mg of protein was used. Other additions were: 0.50 μg of oligomycin and arsenite as indicated.

Additions	*O_2(μl)*	
	A	*B*
None	15.5	7.3
Oligomycin	5.8	8.1
Arsenite (1 mM)	7.4	2.8
Arsenite (5 mM)	2.1	0.0
Oligomycin + arsenite (1 mM)	3.6	
Oligomycin + arsenite (5 mM)	0.6	
Dinitrophenol	0.3	

Stimulation by carnitine

Carnitine increases the rates of long-chain fatty acid oxidation by mitochondria prepared from several tissues[31,32] and stimulates the oxidation of endogenous substrate in rat heart and locust flight-muscle mitochondria[33].

Fig. 3 shows that carnitine stimulates the endogenous substrate oxidation by rat-liver mitochondria in the dinitrophenol-sensitive system (Fig. 3A) but does not stimulate the endogenous substrate oxidation in the dinitrophenol-insensitive system (Fig. 3B).

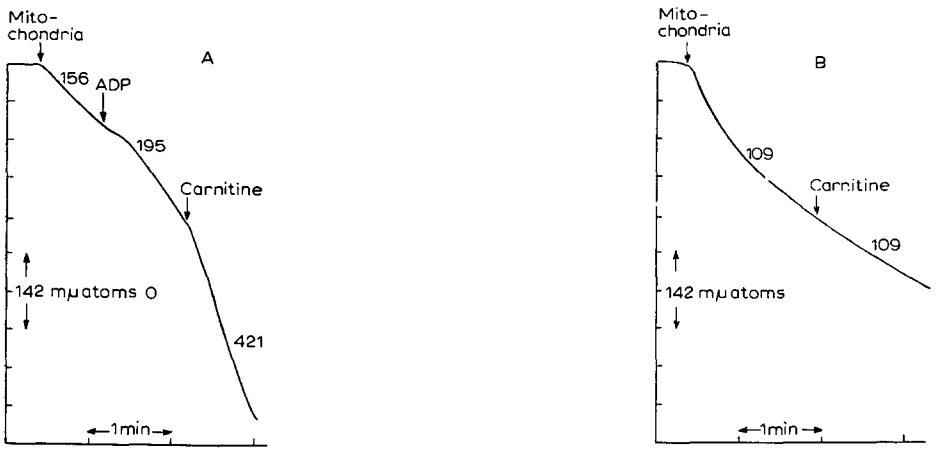

Fig. 3. Effect of carnitine on the endogenous substrate oxidation in dinitrophenol-sensitive (A) and dinitrophenol-insensitive (B) systems. O_2 uptake was measured with the Clark 'oxygen electrode'. The reaction medium for the dinitrophenol-sensitive system was as described in MATERIAL AND METHODS, and that for the dinitrophenol-insensitive system contained 0.05 mM dinitrophenol instead of phosphate. 3 μmoles of ADP and 1.5 μmoles of carnitine were added as indicated. 9.5 and 7.7 mg of mitochondrial protein were used in Expts. A and B, respectively.The numbers on the tracing express the O_2 consumption as mμatoms/min.

DISCUSSION

In the present paper, the identification of fatty acids as the endogenous substrate is based on such experimental results as: similar P:O ratios, similar sensitivity to arsenite and malonate, stimulation by NAD^+ and carnitine (in the presence of phosphate), and the existence of two systems for its activation, known to operate with fatty acids.

Equal P:O ratios (close to 2.5) for oxidation of the endogenous substrate and palmitic acid are similar to those expected for the oxidation of $FADH_2$ *plus* NADH. They are in agreement with the data of WEINBACH[6] for the endogenous substrate oxidation, with the results of HIRD AND WEIDEMANN[34] for the oxidation of low concentrations of butyrate, hexanoate and octanoate, and with those of STEVENSON[35] for palmitate oxidation by moth flight-muscle mitochondria.

The NAD^+-stimulated endogenous substrate oxidation (Table II) indicates participation of NAD^+ in the endogenous substrate oxidation postulated by WEINBACH[6].

Two systems described by VAN DEN BERGH[30] for activation of fatty acids have been successfully applied for the endogenous substrate activation. Complete inhibition of the endogenous substrate oxidation by dinitrophenol or arsenite *plus* oligomycin in a system containing phosphate, and inhibition of the endogenous substrate oxidation by arsenite in a system free of phosphate, suggest that high-energy intermediates of oxidative phosphorylation cannot activate endogenous fatty acids in rat-liver mitochondria without intervention of nucleotide triphosphate. These observations confirm the results of VAN DEN BERGH[36].

Since the endogenous substrate oxidation is not exhausted even after 45 min incubation in the presence of ADP and phosphate, it can be assumed, in agreement

with MINNAERT's suggestion[22], that the endogenous fatty acids are formed in mitochondria continuously, *e.g.* by hydrolysis of lipids.

The carnitine-stimulated endogenous substrate oxidation by rat-liver mitochondria in the dinitrophenol-sensitive system (Fig. 3A) is in agreement with the observation of BODE AND KLINGENBERG[33] with rat-heart and locust flight-muscle mitochondria. However, it is contrary to the lack of stimulation by carnitine of endogenous substrate oxidation in the kidney mitochondria[37]. The carnitine-stimulated endogenous substrate oxidation that we have observed seems to be a result of the activated endogenous fatty-acid oxidation products transferred as acylcarnitines through the carnitine barrier to the site of β-oxidation. This suggestion is in accord with the current view[38–41] that the fatty acid activation by the ATP-dependent enzyme occurs, at least in part, outside the carnitine barrier of the mitochondria, and that the β-oxidation of fatty-acids takes place inside this barrier. The lack of stimulation of the endogenous substrate oxidation by carnitine in the dinitrophenol-insensitive system (Fig. 3B) seems to indicate that activation of this substrate, dependent on α-oxoglutarate-linked substrate level phosphorylation, occurs inside the carnitine barrier of the mitochondria.

Fatty acids are different from other mitochondrial respiratory substrates in two respects. Firstly, they require activation before oxidation[30,39,42], secondly, they are powerful uncoupling agents[43–47]. Both these properties allow us to consider endogenous fatty acids as control points in possible regulatory mechanism as suggested by VAN DEN BERGH[39]. Moreover, endogenous substrate may be present as a pool of substrate reserve available to maintain a necessary minimal oxidative source of energy required for maintenance of structural integrity[2] and mitochondrial stability[6].

ACKNOWLEDGEMENTS

The authors are indebted to Professor IRENA CHMIELEWSKA for encouragement and discussion, to Professor E. C. SLATER for the gift of oligomycin, to Dr. J. L. HOWLAND for the Clark electrode and to Dr. L. WOJTCZAK for carnitine and fatty acids.

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