Studies of Energy-Linked Reactions

INHIBITION OF OXIDATIVE PHOSPHORYLATION BY DL-8-METHYLDIHYDROLIPOATE

By DAVID E. GRIFFITHS, KELVIN CAIN and ROBERT L. HYAMS

Department of Molecular Sciences, University of Warwick, Coventry CV4 7AL, U.K.

(Received 23 December 1976)

1. DL-8-Methyldihydrolipoate was shown to be a potent inhibitor of mitochondrial oxidative phosphorylation and ATP-driven energy-linked reactions. 2. ADP-stimulated respiration utilizing pyruvate+malate and succinate in both ox heart and rat liver mitochondria is inhibited; oxidative phosphorylation using pyruvate+malate, succinate and ascorbate+NNN'-tetramethyl-*p*-phenylenediamine as substrates is also inhibited; uncoupler-stimulated respiration is unaffected regardless of the substrate used. 3. Mitochondrial oligomycin-sensitive adenosine triphosphatase is inhibited in both the membrane-bound form and the purified detergent-dispersed preparation. 4. ATP-driven transhydrogenase and the ATP-driven energy-linked reduction of NAD⁺ by succinate in ox heart submitochondrial particles are inhibited, whereas the respiratory-chain-driven transhydrogenase is unaffected. 5. DL-8-Methyl-lipoate has no immediate effect on the above reactions, demonstrating the requirement for the reduced form for inhibition. 6. The inhibitory properties of DL-8-methyldihydrolipoate are analogous to those of oligomycin and provide further evidence of a role for lipoic acid in oxidative phosphorylation.

Investigations of the mode of action of dibutylchloromethyltin chloride on mitochondrial oligomycin-sensitive ATPase* in yeast (Cain *et al.*, 1975) and on oxidative phosphorylation and energy-linked reactions in ox heart (K. Cain & D. E. Griffiths, unpublished work), together with the demonstration of the presence of lipoic acid in the inner mitochondrial membrane and ATP synthase preparations, indicate a specific role for dihydrolipoic acid in the terminal reactions of oxidative phosphorylation (Griffiths, 1976*a,b,c*). It is proposed that lipoic acid residues present in the inner mitochondrial membrane serve as a link function between the respiratory chain and the ATP synthase complex and that they play a key role in the ATP-synthesizing and ATP-utilizing reactions catalysed by the ATP synthase complex.

Various analogues of lipoic acid have been synthesized and examined for biological activity in microorganisms (Reed, 1957). One of the most active analogues used was oxidized 8-methyl-lipoate; Stokstad (1954) showed that oxidized 8-methyl-lipoate would inhibit growth in *Streptococcus faecalis* 8043, and moreover the inhibition was competitive with respect to lipoic acid. In addition growth of *Tetrahymena geleii*, which has an absolute requirement for lipoic acid, was competitively inhibited by oxidized 8-methyl-lipoate, and studies with other lipoic acid analogues have been reviewed by Schmidt

et al. (1969). The present paper describes the properties of reduced 8-methyl-lipoate acid as an oligomycin-like inhibitor of oxidative phosphorylation in ox heart and rat liver mitochondria. It is concluded that these results provide further evidence for a role for lipoic acid in oxidative phosphorylation.

Materials and Methods

Materials

The sources of many of the chemicals and reagents used have been described previously (Griffiths & Houghton, 1974). DL-Lipoic acid and DL-dihydrolipoic acid, ADP and hexokinase were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. NAD⁺, NADP⁺ and yeast alcohol dehydrogenase were obtained from Boehringer Corporation (London) Ltd., Ealing, London W.5, U.K. DL-8-Methyl-lipoic acid was a gift from Dr. J. R. Guest, Department of Microbiology, University of Sheffield, and was checked for purity by proton magnetic resonance at 90 MHz. DL-Lipoate and DL-dihydrolipoate were added in a solution of 0.25 M-sucrose/10 mM-Tris/HCl/1 mM-EDTA, pH 8.0. DL-8-Methyl-lipoate was added in the same solution but with the addition of 10% (v/v) ethanol to obtain complete solubility. Reduced DL-8-methyl-lipoate was obtained by adding a solution of DL-8-methyl-lipoate previously titrated with a two-fold excess of dithiothreitol.

* Abbreviation: ATPase, adenosine triphosphatase.

Methods

Ox heart mitochondria were prepared essentially as described by Löw & Vallin (1963), except that the treatment with N_2 was omitted; the ox heart mitochondria were stored at -20°C in 0.25 M-sucrose/10 mM-Tris/HCl/1 mM-EDTA, pH 7.5, at a protein concentration of 40 mg/ml. Submitochondrial particles were always freshly prepared from frozen mitochondria as described by Beyer (1967); the sedimented submitochondrial particles were resuspended at a protein concentration of 40 mg/ml in 0.25 M-sucrose/10 mM-Tris/HCl/1 mM-EDTA, pH 7.5. Rat liver mitochondria were prepared as described by Chappell & Hansford (1969) and suspended in 0.25 M-sucrose. The complex-V preparation was prepared as described by Hatefi *et al.* (1974), and the proton-translocating ATPase preparation as described by Serrano *et al.* (1976); the 38–45 p fraction described by the latter authors was used in all experiments.

Oxygen-uptake experiments were carried out at 30°C in a Clark-type oxygen electrode in a reaction medium containing 0.25 M-sucrose, 10 mM-Tris/HCl, 5 mM- KH_2PO_4 , 10 mM-KCl, 5 mM- MgCl_2 , pH 7.5, and mitochondrial protein (2–3 mg for ox heart mitochondria and 6–7 mg for rat liver mitochondria). Other additions to the reaction medium are described in Fig. 1 legend. Respiration experiments were always carried out with freshly prepared mitochondria.

Oxidative phosphorylation was measured by using a glucose/hexokinase trap system as described previously (Griffiths, 1976c). Phosphorylation was determined by disappearance of P_i . The reaction media contained, in a final volume of 1.1 ml, 0.25 M-sucrose, 22 mM-glucose, 10 mM-Tris/HCl, 5 mM- KH_2PO_4 , 2 mM- MgCl_2 , 0.5 mM-EDTA, 20 units of hexokinase (EC units; $1 \mu\text{mol}/\text{min}$) 2 mM-ADP, 2 mg of mitochondrial protein, pH 7.5. Substrate concentrations and other details are described in the legends to Tables 1 and 2.

ATP-driven transhydrogenase was measured essentially as described by Griffiths & Robertson (1966b) in 3 ml of reaction medium containing 0.25 M-sucrose, 50 mM-Tris/HCl, 5 mM- MgCl_2 , $0.66 \mu\text{M}$ -rotenone, 100 mM-ethanol, $66 \mu\text{M}$ - NAD^+ , 300 μg of yeast alcohol dehydrogenase, 1 mM-KCN, 0.5–1 mg of submitochondrial particles, $330 \mu\text{M}$ - NADP^+ , 1.33 mM-ATP, pH 8.0. NADP^+ reduction was followed at 340 nm in a Unicam SP.1800 spectrophotometer at 30°C . The submitochondrial particles were preincubated with and without inhibitors for 3 min before adding the NADP^+ ; 3 min later the reaction was initiated with ATP. Succinate-driven transhydrogenase was measured in exactly the same manner, except that KCN was omitted and succinate (1.33 mM) replaced the ATP.

The ATP-driven reduction of NAD^+ by succinate

was assayed by the method of Griffiths & Robertson (1966a) in 3 ml of medium containing 0.25 M-sucrose, 6 mM- MgCl_2 , 50 mM-Tris/HCl, 1 mM-KCN, 1 mM- NAD^+ , 5 mM-succinate, pH 8.0 at 30°C . The submitochondrial particles (0.5–1 mg) were preincubated with and without inhibitors for 3 min before initiation of the reaction with 2 mM-ATP; NAD^+ reduction was measured as described for the transhydrogenase reactions.

Mitochondrial ATPase activity was measured in 50 mM-Tris/HCl/2 mM- MgCl_2 /5 mM-ATP, pH 8.5 (final vol. 1 ml) at 30°C . The assays were performed as described by Griffiths & Houghton (1974). Protein and P_i were determined as described by Griffiths (1976c). Specific preincubation procedures are detailed in the relevant Tables.

Fig. 1. Inhibition of ADP-stimulated respiration in ox heart and rat liver mitochondria by DL-8-methyldihydrolipoate

Mitochondrial respiration was recorded as described in the Materials and Methods section, with the following additions: (a) 2 mg of ox heart mitochondria; A, $10 \mu\text{mol}$ of pyruvate + $1 \mu\text{mol}$ of malate; B, 300 nmol of ADP; C, 16 nmol of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; D, 40 nmol of DL-8-methyldihydrolipoate; E, 80 nmol of dithiothreitol; F, 40 nmol of DL-8-methyl-lipoate; (b) 7.2 mg of rat liver mitochondria; A, $10 \mu\text{mol}$ of pyruvate + $1 \mu\text{mol}$ of malate; B, 750 nmol of ADP; C, 0.3 nmol of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; D, 40 nmol of DL-8-methyldihydrolipoate; E, 80 nmol of dithiothreitol; F, 40 nmol of DL-8-methyl-lipoate.

Results and Discussion

ADP-stimulated respiration with pyruvate+malate as substrate is inhibited by DL-8-methyldihydrolipoate in both ox heart and rat liver mitochondria. Figs. 1(a) and 1(b) show that DL-8-methyldihydrolipoate totally inhibits ADP-stimulated respiration in ox heart and rat liver mitochondria at concentrations of 20 nmol and 5.5 nmol/mg of protein respectively. These concentrations are approx. 2-fold in

excess of the amounts required to give complete inhibition. In addition the 8-methyl-lipoate sample used is a mixture of two diastereoisomers (Bullock *et al.*, 1956); consequently the concentrations giving inhibition can be regarded as overestimations of the true inhibitory value for 8-methyldihydrolipoate. The oxidized derivative has no effect on the respiration stimulated by ADP, and control experiments with dithiothreitol, lipoic acid, lipoic acid reduced with

Table 1. *Inhibition of oxidative phosphorylation by DL-8-methyldihydrolipoate*

Phosphorylation was measured as described in the Materials and Methods section. Ox heart mitochondria (20 mg in 1.0 ml of 0.25 M-sucrose/10 mM-Tris/HCl/1 mM-EDTA, pH 7.5) were preincubated with and without the indicated additions for 30 min, at 4°C; 0.1 ml of this reaction medium (2 mg of protein) was then added to the phosphorylation medium, immediately followed by the indicated substrates. The reaction was terminated after 20 min with 1 ml of 10% (w/v) trichloroacetic acid.

Substrate	Additions (nmol/mg of protein)	P _i uptake (μmol/20 min per mg)	Inhibition (%)
15 mM-Pyruvate + 1 mM-malate	None	2.4	0
	DL-8-Methyl-lipoate (50)	2.13	12
	DL-8-Methyldihydrolipoate (25)	0.47	71
	DL-8-Methyldihydrolipoate (50)	0.27	79
	Dithiothreitol (60)	2.4	0
	Dithiothreitol (120)	2.35	2
20 mM-Succinate	None	2.4	0
	DL-8-Methyl-lipoate (25)	2.1	12
	DL-8-Methyldihydrolipoate (25)	0.49	80
	DL-8-Methyldihydrolipoate (50)	0.49	80
	Dithiothreitol (60)	2.4	0
	Dithiothreitol (120)	2.4	0
20 mM-Ascorbate + 2 mM-NNN'- tetramethyl- <i>p</i> -phenylenediamine + 2.5 μg of antimycin A/mg of protein	None	2.16	0
	DL-8-Methyl-lipoate (25)	1.75	20
	DL-8-Methyldihydrolipoate (25)	0.47	78
	DL-8-Methyldihydrolipoate (50)	0.43	81
	Dithiothreitol (60)	2.16	0
	Dithiothreitol (120)	2.15	0

Table 2. *Effect of preincubation on the inhibition of oxidative phosphorylation by DL-8-methyldihydrolipoate*

Details for this experiment were as described in Table 1, except that the mitochondria were further preincubated for 10 min at 30°C in the phosphorylation medium before initiating the reaction with the substrates.

Substrate	Additions (nmol/mg of protein)	P _i uptake (μmol/20 min per mg)	Inhibition (%)
15 mM-Pyruvate + 1 mM-malate	None	2.35	0
	DL-8-Methyl-lipoate (25)	0.55	77
	DL-8-Methyldihydrolipoate (25)	0.29	88
	Dithiothreitol (60)	2.35	0
20 mM-succinate	None	2.35	0
	DL-8-Methyl-lipoate (25)	0.4	83
	DL-8-Methyldihydrolipoate (25)	0.32	86
	Dithiothreitol (60)	2.35	0
20 mM-Ascorbate + 2 mM-NNN'- tetramethyl- <i>p</i> -phenylenediamine + 2.5 μg of antimycin A/mg of protein	None	2.15	0
	DL-8-Methyl-lipoate (25)	0.42	81
	DL-8-Methyldihydrolipoate (25)	0.41	81
	Dithiothreitol (60)	2.15	0

Fig. 2. Inhibition of ATP-driven reactions by DL-8-methyl-dihydrolipoate

(a) ATP- and succinate (SUCC)-driven reduction of NADP⁺ were assayed as described in the Materials and Methods section with 0.75 mg of ox heart submitochondrial particles. The following additions were carried out: A, control, no additions; B, 40 nmol of DL-8-methyl-lipoate; C, 20 nmol of DL-8-methyl-dihydrolipoate; D, 40 nmol of DL-8-methyl-dihydrolipoate; E, succinate control, no additions; F, 40 nmol of DL-8-methyl-dihydrolipoate. (b) ATP-driven reduction of NAD⁺ by succinate was carried out as described in the Materials and Methods section with 0.75 mg of ox heart submitochondrial particles. The following additions were made: A, control, no additions; B, 20 nmol of DL-8-methyl-lipoate; C, 0.8 nmol of DL-8-methyl-dihydrolipoate; D, as C but with a 6 min preincubation with the inhibitor instead of the usual 3 min; E, 1.0 nmol of DL-8-methyl-dihydrolipoate; F, 2.0 nmol of DL-8-methyl-dihydrolipoate.

borohydride and lipoic acid reduced with dithiothreitol show that they are all non-inhibitory.

Uncoupled respiration is unaffected by DL-8-methyl-dihydrolipoate, demonstrating that pyruvate oxidation and the respiratory chain are unaffected by the compound. Similar studies with succinate as the substrate show that only the ADP-stimulated respiration is inhibited by DL-8-methyl-dihydrolipoate. Therefore DL-8-methyl-dihydrolipoate inhibits oxygen uptake in coupled mitochondria in a manner analogous to the classical energy-transfer inhibitor oligomycin, and the inhibition is also reversed by the action of uncoupling agents.

Respiration studies with ox heart submitochondrial particles have shown that respiration with NADH, succinate and ascorbate + *NNN'*-tetramethyl-*p*-phenylenediamine as substrates is unaffected by DL-8-methyl-dihydrolipoate. However, the phosphorylation experiments shown in Tables 1 and 2 clearly demonstrate that phosphorylation at all three sites of ATP synthesis is inhibited by DL-8-methyl-dihydrolipoate. Table 1 shows that a concentration of 25 nmol of the reduced derivative/mg of protein produces 70–80% inhibition of phosphorylation; again dithiothreitol has no effect. However, DL-8-methyl-lipoate gives 10–20% inhibition. A possible explanation for this result is that the 8-methyl-lipoate is being slowly reduced (or reductively acylated) under the conditions of the experiment (30 min preincubation at 4°C) and that it is the product which is inhibitory. This hypothesis was examined by giving a further 10 min preincubation with the inhibitor at 30°C before adding the substrate. Table 2 shows that this procedure increases the degree of inhibition to values approaching those given by the reduced derivative. DL-8-Methyl-lipoate did not produce any inhibition in the ADP-stimulated-respiration experiments, presumably because it was only preincubated for 2 min before adding the ADP. In contrast, in the phosphorylation experiments the mitochondria were preincubated with the DL-8-methyl-lipoate for a maximum period of 40 min. DL-8-Methyl-dihydrolipoate therefore appears to act on the ATP synthase complex. This hypothesis is confirmed by the following experiments on energy-linked ATP-driven reductions.

In Fig. 2(a), the effect of DL-8-methyl-dihydrolipoate on the ATP-driven transhydrogenase reaction is shown; 26.5 nmol of the inhibitor/mg of protein produces 75% inhibition of the transhydrogenase reaction, and 100% inhibition is produced by 53 nmol/mg of protein. DL-8-Methyl-lipoate is non-inhibitory, and control experiments have shown no inhibition with dithiothreitol. The succinate-driven reaction is not inhibited by DL-8-methyl-dihydrolipoate. DL-8-Methyl-dihydrolipoate is therefore a good inhibitor of the ATP-driven transhydrogenase, but the concentrations required to inhibit the transhydrogenase reaction are higher than those required to inhibit the ADP-stimulated respiration; a possible explanation for this is discussed below.

The ATP-driven reduction of NAD⁺ by succinate is highly sensitive to DL-8-methyl-dihydrolipoate (Fig. 2b); a concentration of 2.7 nmol/mg of protein is sufficient to inhibit the reaction completely. Lower concentrations produce a biphasic inhibition; the degree of inhibition increases progressively as the reaction proceeds after addition of ATP. For example, a concentration of 1.0 nmol/mg of protein (curve C in Fig. 2b) produces approx. 60% inhibition; however, after 3 min the inhibition is virtually 100%.

Table 3. *Inhibition of mitochondrial ATPase by DL-8-methyldihydrolipoate*

ATPase was measured as described in the Materials and Methods section; the reaction was started by the addition of 100 μ g of protein. Additions of inhibitors were made to 1 ml of 0.25 M-sucrose/10 mM-Tris/HCl/1 mM-EDTA, pH 7.5, containing 1 mg of protein. The reaction mixture was then incubated at 4°C for 30 min before removal of a 100 μ l portion (100 μ g of protein) for the ATPase assay.

Preparation	Additions (nmol/mg)	ATPase activity (μ mol of P_i /min per mg)	Inhibition (%)
Ox heart submitochondrial particles	None	2.3	0
	DL-8-Methyl-lipoate (100)	2.3	0
	DL-8-Methyldihydrolipoate (40)	1.23	46
	DL-8-Methyldihydrolipoate (100)	0.91	61
	Oligomycin (15)	0	100
Complex V	None	6.4	0
	DL-8-Methyl-lipoate (100)	5.7	11
	DL-8-Methyldihydrolipoate (20)	3.8	41
	DL-8-Methyldihydrolipoate (50)	2.3	64
	Oligomycin (15)	0	100
Proton-translocating ATPase	None	5.7	0
	DL-8-Methyl-lipoate (100)	5.47	4
	DL-8-Methyldihydrolipoate (20)	3.76	34
	DL-8-Methyldihydrolipoate (50)	2.5	56
	Oligomycin (15)	0	0

Table 4. *Effect of further preincubation on ATPase inhibition by DL-8-methyldihydrolipoate*

The details for this experiment were as described for Table 3, except that the submitochondrial particles were further preincubated at 30°C for 10 min with and without 5 mM-succinate before assaying for ATPase activity.

Preparation	Additions (nmol/mg)	ATPase activity (μ mol of P_i /min per mg)	Inhibition (%)
Ox heart submitochondrial particles Without succinate	None	2.3	0
	DL-8-Methyl-lipoate (100)	2.1	9
	DL-8-Methyldihydrolipoate (40)	1.2	49
	DL-8-Methyldihydrolipoate (100)	0.83	63
With 5 mM-succinate	None	2.3	0
	DL-8-Methyl-lipoate (100)	1.7	26
	DL-8-Methyldihydrolipoate (40)	0.83	64
	DL-8-Methyldihydrolipoate (100)	0.48	79

Further, increasing the preincubation time (curve D in Fig. 2b) with the inhibitor does not alter the characteristics of the inhibition. DL-8-Methyl-lipoate is non-inhibitory, and control experiments showed that dithiothreitol had no effect.

The mitochondrial oligomycin-sensitive ATPase complex (ATP synthase) is inhibited by DL-8-methyldihydrolipoate in both the membrane-bound form and the purified detergent-dispersed preparations (Table 3). The maximum inhibition obtainable in all three preparations appears to be about 60%. In all three cases preincubation with DL-8-methyldihydrolipoate is necessary to produce inhibition; preincubation with an oxidizable substrate increases the amount of inhibition (Table 4).

The results of these experiments establish that DL-8-methyldihydrolipoate is an energy-transfer inhibitor which has the same general mode of action as oligomycin and related energy-transfer inhibitors. The compound is only active in the reduced form, and the oxidized compound is only active after preincubation, which presumably leads to reduction or a reductive transformation. The effectiveness of DL-8-methyldihydrolipoate is determined not only by its oxidation state but also by the oxidoreduction and energy-conservation capability of the enzyme preparation used. The incubation conditions and preincubation procedures that facilitate energy-conservation reactions also enhance the effectiveness of DL-8-methyldihydrolipoate as an inhibitor. For example, in

studies of ADP-stimulated respiration in mitochondria, inhibition is observed in 30s. Similarly, aerobic preincubation in the presence of substrate enhances the ATPase-inhibitory effect of DL-8-methyldihydrolipoate and converts DL-8-methyl-lipoate into an inhibitory form. The inhibition of NAD⁺ reduction by succinate shows that the degree of inhibition increases as the reaction progresses, presumably owing to an enzymic transformation.

The possibility must be considered that a metabolic product of DL-8-methyldihydrolipoate is formed which is a more effective inhibitor than the parent compound. The data in the present paper, coupled with the known growth-inhibition properties of DL-8-methyl-lipoate (Stokstad, 1954), and its capacity to compete with lipoic acid in lipoic acid-requiring reactions (Reed, 1957; Schmidt *et al.*, 1969) all point to a key role for lipoic acid in oxidative phosphorylation. 8-Methyl-lipoate is thus a powerful probe for the analysis of the intermediary reactions in oxidative phosphorylation catalysed by the ATP synthase complex and for the evaluation of the role of lipoic acid in energy-linked reactions.

References

- Beyer, R. E. (1967) *Methods Enzymol.* **10**, 191
- Bullock, M. W., Hand, J. J. & Stokstad, E. L. R. (1956) *J. Am. Chem. Soc.* **79**, 1975–1978
- Cain, K., Partis, M. D. & Griffiths, D. E. (1975) *International Symposium on Electron-Transfer Chains and Oxidative Phosphorylation*, p. 47, University of Bari, Bari
- Chappell, J. B. & Hansford, R. B. (1969) in *Subcellular Components Preparation and Fractionation* (Birnie, G. D. & Fox, S. M., eds.), p. 43, Butterworths, London
- Griffiths, D. E. (1976a) *Post-Congress Bioenergetic Workshop on Electron Transport, Oxidative Phosphorylation and Photophosphorylation, Hamburg*, p. 10
- Griffiths, D. E. (1976b) in *Genetics and Biogenesis of Chloroplasts and Mitochondria* (Bucher, Th., Neupert, W., Sebald, W. & Werner, S., eds.), pp. 175–185, North-Holland, Amsterdam
- Griffiths, D. E. (1976c) *Biochem. J.* **160**, 809–812
- Griffiths, D. E. & Houghton, R. L. (1974) *Eur. J. Biochem.* **46**, 157–167
- Griffiths, D. E. & Roberton, A. M. (1966a) *Biochim. Biophys. Acta* **113**, 13–26
- Griffiths, D. E. & Roberton, A. M. (1966b) *Biochim. Biophys. Acta* **118**, 453–464
- Hatefi, Y., Stiggall, D. L., Galante, Y. & Hanstein, W. G. (1974) *Biochem. Biophys. Res. Commun.* **61**, 313–321
- Löw, H. & Vallin, I. (1963) *Biochim. Biophys. Acta* **69**, 361–374
- Reed, L. J. (1957) *Adv. Enzymol. Relat. Subj. Biochem.* **18**, 319–347
- Schmidt, U., Grafen, P., Altland, K. & Goedde, H. W. (1969) *Adv. Enzymol. Relat. Areas Mol. Biol.* **32**, 423–469
- Serrano, E., Kanner, B. I. & Racker, E. (1976) *J. Biol. Chem.* **251**, 2453–2461
- Stokstad, E. L. R. (1954) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **13**, 712–714