Detection of bacterial lipoic acid

A modified gas-chromatographic–mass-spectrometric procedure

Kathleen J. PRATT, Christophe CARLES,* Thomas J. CARNE, Michael J. DANSON†
and Kenneth J. STEVENSON‡

Division of Biochemistry, Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada T2N 1N4

The detection of bacterial lipoic acid by a modified g.c.–m.s. procedure is reported. Cells were hydrolysed in HCl to release protein-bound lipoic acid, which, after extraction into benzene, was reduced with NaBH₄. The dihydrolipoic acid so generated was then isolated by covalent chromatography on dithiol-specific *p*-aminophenylarsenoxide–agarose and, after elution by 2,3-dimercaptopropane-1-sulphonic acid and extraction into benzene, was allowed to O₂-oxidize to the disulphide form. The isolated lipoic acid was allowed to react with diazomethane, and the methyl ester so produced was detected by g.c.–m.s. Analysis of the mass spectrum showed the characteristic molecular ion and seven fragmentation ions, which, along with the identification of those ions retaining the two sulphur atoms, allows the definitive detection of lipoic acid. The methodology has been successfully tested with authentic lipoic acid, the 2-oxoglutarate dehydrogenase multienzyme complex and with whole cells of *Escherichia coli*. In addition, it has been used to search for and identify lipoic acid in the archaebacterium *Halobacterium halobium*. The significance of this discovery and the possible roles of the cofactor in *H. halobium* are discussed.

INTRODUCTION

Lipoic acid is an enzymic cofactor which serves as an acyl carrier in the pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase and branched-chain-2-oxoacid dehydrogenase complexes (Reed, 1974; Perham, 1975; Pettit *et al.*, 1978) and as an aminomethyl carrier in the glycine-cleavage-enzyme system (Kikuchi & Hiraga, 1982). In its role in these multienzyme systems, the lipoic acid becomes fully reduced and is subsequently reoxidized by the enzyme dihydrolipoamide dehydrogenase (EC 1.8.1.4) in an NAD⁺-dependent reaction [reviewed by Williams (1976)].

Dihydrolipoamide dehydrogenase is an integral component of the 2-oxoacid dehydrogenase and glycine-cleavage-enzyme complexes. However, there is accumulating evidence to suggest that the enzyme has a function apart from these multienzyme complexes [reviewed by Danson (1988)]. Thus we have found dihydrolipoamide dehydrogenase in the halophilic and thermoacidophilic archaebacteria (Danson *et al.*, 1984, 1986; Smith *et al.*, 1987) and in the eukaryotic parasite *Trypanosoma brucei* (Danson *et al.*, 1987), all of which lack the 2-oxoacid dehydrogenase complexes and in which the glycine-cleavage system has not been reported. The enzyme appears to be membrane-associated in the thermoacidophiles and in *T. brucei*, and a similar situation has been reported for the eubacterium *Escherichia coli* (Owen *et al.*, 1980).

A necessary step in the elucidation of the function(s) of these membrane-associated dihydrolipoamide dehydrogenases is to determine if their *in vivo* substrate is indeed

lipoic acid. The search for this cofactor in archaebacteria and trypanosomes is thus a priority. In the present paper we report the development of a method, using covalent chromatography, to isolate lipoic acid from cell extracts and subsequent g.c.–m.s. to detect the lipoic acid as its oxidized lipoate methyl ester. The methodology was tested with *E. coli* and with pure proteins, known to possess lipoic acid, and then applied to cells of the archaebacterium *Halobacterium halobium*. Our procedure is compared with the g.c.–m.s. analysis of lipoic acid as methyl 6,8-bis(benzylthio)octanoate (White, 1980).

EXPERIMENTAL

Materials

Reacti-Vials were obtained from Pierce Chemical Co., Rockford, IL, U.S.A. All chemicals were of the highest purity available. 2,3-Dimercaptopropane-1-sulphonic acid, 2,3-dimercaptopropanol and α -D-lipoic acid (DL-6,8-thioctic acid) were from Sigma Chemical Co., St. Louis, MO, U.S.A. Diazald (*N*-methyl-*N*-nitroso-*p*-toluenesulphonoamide) was from Aldrich Chemical Co., Milwaukee, WI, U.S.A. *Escherichia coli* K12, mid- to late-exponential phase, was purchased from Grain Processing Co., Muscatine, IA, U.S.A. *Halobacterium halobium* (C.C.M. 2090) was kindly provided by Dr. W. D. Grant (Department of Microbiology, University of Leicester, Leicester, U.K.). The organism was grown aerobically in liquid shake culture in the medium described by Payne *et al.* (1960).

Abbreviation used: Nbs₂, 5,5'-dithiobis-(2-nitrobenzoic acid).

* Current address: Laboratoire de Biochimie et Technologie Laitières, CNRS, 78350-Jouy-en-Josas, France.

† Current address: Department of Biochemistry, University of Bath, Claverton Down, Bath BA2 7AY, U.K.

‡ To whom all correspondence and reprint requests should be addressed.

Preparation of 2-oxoglutarate dehydrogenase multienzyme complex from *E. coli*

2-Oxoglutarate dehydrogenase multienzyme complex was isolated from *E. coli* K12 by the method of Reed & Mukherjee (1969).

Synthesis of *p*-aminophenylarsenoxide-agarose

p-Aminophenylarsenoxide-agarose was prepared by allowing a 2-fold excess of *p*-aminophenyldichloroarsine to react with CNBr-activated Sepharose 4B in 0.1 M-NaHCO₃, pH 9.0, according to the procedure of Stevenson & Landman (1971). Coupling was approx. 10 µmol of arsenical/g of wet resin. The Sepharose 4B was activated by the procedure of Wilchek *et al.* (1984). *p*-Aminophenyldichloroarsine was synthesized as described by Stevenson *et al.* (1978). Under the coupling conditions the dichloroarsine (R-AsCl₂) was transformed to the arsenoxide (R-AsO).

Preparation of diazomethane

Diethyl ether solutions of diazomethane (ethanol-free) were prepared from Diazald as outlined by Vogel (1967) and were stored at -20 °C (for a maximum of 2 weeks).

Quantification of reduced lipoic acid using Nbs₂

Aliquots containing reduced lipoic acid were added to 950 µl of 50 mM-potassium phosphate buffer, pH 8.0. Nbs₂ (50 µl of a 10 mM-solution) was introduced and the production of thionitrobenzoate was monitored at 412 nm. In this case, 1 mol of Nbs₂ reacts with 1 mol of dihydrolipoic acid to yield 2 mol of thionitrobenzoate and 1 mol of oxidized lipoic acid.

Covalent chromatography of lipoic acid

Covalent chromatography of *p*-aminophenylarsenoxide-agarose was used to isolate lipoic acid from cells before g.c.-m.s. On account of the sensitivity of the g.c.-m.s. system and the disruptive effects of organic polymers, special attention was given to all apparatus used in these procedures. Glassware was acid-washed (H₂SO₄/HNO₃ = 3:1, v/v) and rinsed with deionized water, and plastic apparatus (beakers, pipette tips, rotary-evaporator connecting tubes) was avoided, as was the use of vacuum grease on stop-cocks of separatory funnels.

Before elution of lipoic acid from *p*-aminophenylarsenoxide-agarose, all solutions were extensively purged with N₂ to maintain the lipoic acid in the reduced state.

(a) Procedure using authentic lipoic acid. Commercial DL-(oxidized) lipoic acid (4.07 mg, 20 µmol) was suspended in 1.0 ml of 6 M-HCl and hydrolysed in a sealed evacuated Pyrex tube for 11 h at 100 °C. The sample was evaporated to dryness on a rotary evaporator (water pump) at 50 °C over 3 h, suspended in 1 ml of 0.25 M-NaHCO₃ and transferred to a 10 ml distillation flask. Reduction was achieved by adding NaBH₄ (12 mg) while purging with N₂ for 30 min. Benzene (2 ml) was added, and the aqueous layer was adjusted to pH 1 with 5 M-HCl. The entire suspension was transferred to a 15 ml separation funnel and, after vigorous agitation, the benzene layer, containing the reduced lipoic acid,

was removed. The aqueous layer was extracted twice more with benzene. After purging with N₂, the combined extracts were evaporated to dryness by rotary evaporation.

p-Aminophenylarsenoxide-agarose (2 ml) was dispersed into 50 ml of 50 mM-sodium phosphate buffer, pH 7.5, and purged with N₂. The matrix was recovered by centrifugation and placed in a reaction vessel equipped with a stirring bar and an inlet for N₂. The reduced lipoic acid was dissolved in 10 ml of 50 mM-sodium phosphate, pH 7.5, and the pH was adjusted to 9 with NaOH. A portion was removed for quantification of thiol with Nbs₂ and the remainder was added to the agarose gel and mixed overnight by gentle purging with nitrogen. The gel was washed extensively on a sintered-glass filter with 50 mM-sodium phosphate buffer, pH 7.5, resuspended in buffer and 0.5 g of 2,3-dimercaptopropane-1-sulphonic acid was added to cleave the cyclic dithioarsenite bond between the reduced lipoic acid and the immobilized arsenical. After 3 h of purging with N₂ the eluant was collected by filtration and the matrix was further washed with phosphate buffer. The pH of the combined filtrates was adjusted to pH 3 to protonate the lipoic acid. Extraction of the filtrate three times with benzene removed the lipoic acid from the aqueous solution. Any 2,3-dimercaptopropane-1-sulphonic acid extracted into benzene was removed by washing the benzene once with 0.1 M-HCl. Reduced lipoic acid present in the benzene layer was allowed to oxidize overnight (in a fume hood) by purging with oxygen, a procedure which resulted in a dried extract.

(b) Isolation of lipoic acid from whole cells. The general procedure outlined above was to isolate lipoic acid from whole cells of *E. coli* and *H. halobium*. Cells (100–200 g) were hydrolysed by refluxing in 1–2 litres of 6 M-HCl accompanied by constant purging with N₂ for 2 h. The hydrolysate was extracted three times with an equal volume of benzene. Separation of the aqueous and non-aqueous layers on occasion required brief centrifugation at 4000 g in 150 ml-capacity Correx centrifuge bottles. The combined benzene layers were dried by rotary evaporation and the extract was transferred to a 50 ml pear-bottom flask. The residue was resuspended in 20 ml of 50 mM-NaHCO₃ and the pH was adjusted to pH 9 with NaOH. Lipoic acid present was reduced by adding 1.7 g of NaBH₄ in small amounts over a period of 3 h to minimize frothing. The pH was subsequently lowered to pH 3 with HCl to decompose excess borohydride. The solution was quickly made 30% (v/v) with respect to ethanol, 3 ml of 0.5 M-Na₂HPO₄ was added and the pH was re-adjusted to pH 9 with NaOH. This solution was immediately added to 6 g of *p*-aminophenylarsenoxide-agarose and was gently agitated overnight on a wrist-action shaker.

The matrix was then washed with 100 ml of 50 mM-sodium phosphate, pH 7.5, in 30% (v/v) ethanol, resuspended in 15 ml of the same buffer containing 0.5 g of 2,3-dimercaptopropane-1-sulphonic acid and gently shaken for 3 h. The eluant was collected by filtration, and the matrix was further washed with 20 ml of 50 mM-sodium phosphate buffer, pH 7.5, containing 30% (v/v) ethanol. The pH of the combined filtrates was adjusted to pH 3 with HCl and the solution was extracted three times with 50 ml of benzene to remove the lipoic acid. The combined benzene extracts were further

washed with 50 ml of 0.1 M-HCl to remove any extracted 2,3-dimercaptopropane-1-sulphonic acid.

The liponic acid in the benzene extract was allowed to oxidize by purging with oxygen overnight, a procedure resulting in a dried extract.

Isolation of liponic acid from 2-oxoglutarate multienzyme complex

Purified 2-oxoglutarate dehydrogenase multienzyme complex (40 mg) was hydrolysed with 2 ml of 6 M-HCl for 11 h at 110 °C to release protein-bound liponic acid. After being evaporated to dryness, the sample was methylated directly with diazomethane as outlined below and subjected to g.c.-m.s.

Methylation of oxidized liponic acid in preparation for g.c.-m.s.

Oxidized liponic acid (both untreated standard and samples after covalent chromatography on *p*-amino-phenylarsenoxide-agarose) was methylated by the following procedure. Samples were dissolved in 2 ml of diethyl ether, and saturating amounts of cold diazomethane in diethyl ether were added (the yellow colour persisted after the methylation). The solution was kept at 4 °C for 10 min and was then evaporated to dryness. The residue was dissolved in dichloromethane, transferred to a Reacti-Vial, dried, and stored at -70 °C. Aliquots (1 µl of 5 µl) in dichloromethane were subjected to g.c.-m.s.

G.c.-m.s. of oxidized DL-lipoic acid methyl ester

Oxidized DL-lipoic acid methyl ester (20 nmol in 1 µl of dichloromethane) was applied via on-column injection to a Kratos-MS 80 g.c.-m.s. equipped with a 30 m long × 0.322 mm diameter glass capillary DB1 column with a 0.25 µm coating. The helium flow rate was 1.2 ml/min. The initial and final temperatures were 60 and 250 °C respectively, and the rate of variation of temperature was 10 °C/min. The mass spectrometer operated in the electron-impact mode with an ionizing voltage of 70 eV and an ion-source temperature of 250 °C. The mass-spectra resolution was 1000.

RESULTS

G.c.-m.s. of oxidized liponic acid methyl ester

Initially, pure oxidized liponic acid was methylated with diazomethane and subjected to g.c.-m.s. analysis to provide a standard with which to compare later samples. The gas chromatogram (Fig. 1a) consists of a single peak with an elution time of 18.3 min. The mass spectrum of this peak is shown in Fig. 1(b). The assignment of the various peaks of the spectrum is given in Table 1, which shows that, in addition to the molecular ion at $m/z = 220$, there are characteristic fragmentation ions at m/z values of 189, 161, 155, 123 and 105. Moreover, the natural abundance of sulphur-34 is 4.22% (Dyke *et al.*, 1971); therefore, ions possessing two sulphur atoms ($m/z = 220$, 189, 161 and 105) have usually large (≥ 0.1) $(m+2)/m$ peak-intensity ratios, compared with ratios of ≤ 0.04 for peaks of $m/z = 155$ and 123, which are devoid of this element.

Authentic liponic acid taken through the procedure involving the arsenical-agarose yielded a gas chromatogram with three peaks. The material eluting at

Fig. 1. G.c.-m.s. of oxidized liponic acid methyl ester prepared from commercial liponic acid

Oxidized liponic acid was methylated with diazomethane and subjected to analysis as described in the Experimental section. (a) Gas chromatogram monitored by mass spectroscopy. The trace represents the total ion chromatogram (TIC). The profiles obtained by plotting the mass ions of $m/z = 220$ and 123 as a function of time are indicated by 220 and 123 respectively. Oxidized liponic acid methyl ester was eluted at 18.3 min. (b) Mass spectrum of the oxidized liponic acid methyl ester showing the molecular ion at $m/z = 220$ and fragmentation ions at m/z 189, 161, 155, 123, 105, 95 and 81. The assignment of these ions is given in Table 1.

18.3 min possessed a mass spectrum which was unequivocally identified as methylated liponic acid.

Detection of liponic acid in purified protein and *E. coli* cells

The isolation of liponic acid and subsequent detection by g.c.-m.s. were tested with samples known to contain liponic acid, namely the purified 2-oxoglutarate dehydrogenase multienzyme complex and whole *E. coli* cells. In the case of the pure protein the sample was methylated after acid hydrolysis, whereas with the whole cells the liponic acid was isolated by covalent chromatography before methylation. The gas chromatograms of these two samples are shown in Figs. 2(a) and 3(a) respectively. The peak of oxidized liponic acid methyl ester was identified by monitoring the presence of the fragmentation ion ($m/z = 123$) and the molecular ion

Table 1. Expected mass-spectral fragmentation pattern of oxidized lipoic acid methyl ester

Fragmentation ion	<i>m/z</i> value
Molecular ion	220
189
161
155
123
105
95
81

(*m/z* 220). In both samples their presence coincides at an elution time of 18.3 min, and the full mass spectra (Figs. 2*b* and 3*b*) confirm the presence of oxidized lipoic acid methyl ester. Taking the g.c.-m.s. analysis of hydrolysed *E. coli* cells as an example, the mass spectrum (Fig. 3*b*) shows clearly defined peaks at *m/z* = 220, 189, 161, 155 and 123, as well as peaks at *m/z* 105 and 95. In addition, the relative intensities are very similar to those observed with pure lipoic acid (Fig. 1*b*). Moreover, the high (*m*+2)/*m* ratio of the molecular ion (*m/z* = 220) is consistent with this species possessing two sulphur atoms. The definitive identification of lipoic acid in these samples thus validates the methodology as a means of detecting this cofactor in proteins and whole-cell hydrolysates.

Identification of lipoic acid in *Halobacterium halobium*

The procedure for the isolation and identification of lipoic acid from whole cells was applied to *H. halobium*. The presence of lipoic acid in *H. halobium* was unequivocally demonstrated, as shown by the gas chromatogram and mass spectra in Figs. 4(*a*) and 4(*b*). The *m/z* 123 and 220 ions coexisted in the mass spectrum of the g.c. peak eluted at 18.3 min. All mass-ion peaks found in the standard were present in similar ratios in the *H. halobium* sample. Moreover, from the (*m*+2)/*m* peak-intensity ratios, the presence of two sulphur atoms is clearly seen in the molecular-ion peak (*m/z* 220) although the background prevents similar analysis of the fragmentation ions.

Fig. 2. G.c.-m.s. of oxidized lipoic acid methyl ester from purified 2-oxoglutarate dehydrogenase multienzyme complex

(*a*) Gas chromatogram monitored by mass spectroscopy of acid-hydrolysed 2-oxoglutarate dehydrogenase multienzyme complex. TIC represents the total ion chromatogram. The profiles obtained by plotting the mass ions of *m/z* = 220 and 123 as a function of time are indicated by 220 and 123 respectively. (*b*) Mass spectrum of the peak eluted at 18.3 min containing oxidized lipoic acid methyl ester. The assignment of molecular and fragmentation ions is given in Table 1.

DISCUSSION

The use of g.c.-m.s. for the identification of lipoic acid in tissue was first developed by White and his colleagues (White, 1980; White *et al.*, 1980). The method consisted of acid hydrolysis of the tissue to release protein-bound lipoic acid, extraction of lipoic acid into dichloromethane and subsequent derivatization with benzyl bromide to yield methyl 6,8-bis(benzylthio)octanoate before g.c.-m.s. analysis. The important features of the mass spectrum of this derivative are the strong mass ion at *m/z* 311 and a weak fragmentation ion at *m/z* 137. However, few other fragmentation ions exist, thereby limiting unequivocal identification of the derivatized lipoic acid.

The method of White (1980) was used to study the biosynthesis of lipoic acid in *E. coli*, an organism known to produce the cofactor for a number of multienzyme systems. However, one of our major objectives was to

Fig. 3. G.c.-m.s. of oxidized liponic acid methyl ester enriched from *E. coli*

(a) Gas chromatogram monitored by mass spectroscopy of fraction enriched by covalent chromatography on *p*-aminophenylarsenoxide-agarose. TIC represents the total ion chromatogram. The profiles obtained by plotting the mass ions of $m/z = 220$ and 123 as a function of time are indicated by 220 and 123 respectively. (b) Mass spectrum of the peak eluted at 18.3 min (indicated by arrow) containing oxidized liponic acid methyl ester. The assignment of molecular fragmentation ions is given in Table 1.

Fig. 4. G.c.-m.s. of oxidized liponic acid methyl ester enriched from *Halobacterium halobium*

(a) Gas chromatogram monitored by mass spectroscopy of fraction enriched by covalent chromatography on *p*-aminophenylarsenoxide-agarose. TIC represents the total ion chromatogram. The profiles obtained by plotting the mass ions of $m/z = 220$ and 123 as a function of time are indicated by 220 and 123 respectively. (b) Mass spectrum of the peak eluted at 18.3 min (indicated by arrow) containing oxidized liponic acid methyl ester. The assignment of molecular and fragmentation ions is given in Table 1.

search for liponic acid in species where 2-oxoacid dehydrogenase multienzyme complexes are not found and in which the cofactor may occur only in small amounts or even be absent. For this purpose, we endeavoured to develop a selective isolation procedure for liponic acid followed by g.c.-m.s. analysis where the mass spectrum possessed a larger number of characteristic features, thereby ensuring a definitive identification. The mass spectrum of oxidized liponic acid methyl ester provides seven characteristic fragmentation ions in addition to the molecular ion. Moreover, three fragmentation ions ($m/z = 189$, 161 and 105) and the molecular ion (m/z 220) possess ($m + 2$) peaks characteristic of the two sulphur atoms and the natural abundance of sulphur-34.

Although g.c.-m.s. provides a combination of features permitting the definitive identification of liponic acid, the success of the procedure is dependent on prior enrichment of the cofactor, particularly when cell extracts are being

investigated. We have introduced covalent chromatography with an immobilized trivalent organoarsenical selectively to isolate molecules, including liponic acid, possessing *vicinal* thiols. Immobilization is through cyclic dithioarsenite formation with the reduced *vic*-thiols. These complexes are, in turn, effectively decomposed by 2,3-dimercaptopropane-1-sulphonic acid, which releases reduced liponic acid and forms a more stable complex with the trivalent arsenical. The negatively charged sulphonated derivative was employed to enhance the polar character of the more common reagent 2,3-dimercaptopropanol, thereby restricting its extraction into benzene. Elution with 2,3-dimercaptopropane-1-sulphonic acid and subsequent extraction into benzene then yield a suitably 'clean' sample for g.c.-m.s.

We have successfully tested the procedures with authentic liponic acid, the 2-oxoglutarate dehydrogenase multienzyme complex and with whole *E. coli* cells. We have used this methodology to make the first definitive

identification of lipoic acid in an archaebacterium, namely the extreme halophile *H. halobium*. There has been a previous suggestion that the purple membrane of *H. halobium* may contain lipoic acid (Griffiths *et al.*, 1977). Lipoate content was assayed by titration with dibutylchloro[³H]methyltin chloride and subsequent identification by t.l.c. analysis and by a bioassay. However, neither method is absolutely specific for lipoic acid, and the possibility of the detection of a structurally related dithiol species could not be ruled out. Moreover, intensive structural and mechanistic analysis of purple membrane has not confirmed the presence of lipoic acid, and current theories of energy transduction by this system take no account of such a cofactor [Engelhard *et al.* (1985) and references cited therein].

The significance of our finding lipoic acid in *H. halobium* rests on our previous discovery that this archaebacterium possess the enzyme dihydrolipoamide dehydrogenase, which catalyses the oxidation-reduction of this dithiol-disulphide substrate (Danson *et al.*, 1984, 1986). The presence of this enzyme had been unexpected, in that the multienzyme complexes of which it is normally an integral component are absent from the archaebacteria (Aitken & Brown, 1969; Kerscher & Oesterhelt, 1982). Thus we have identified the free dihydrolipoamide dehydrogenase and its substrate, lipoamide (as lipoic acid), in *H. halobium*. In the thermoacidophilic archaebacterium *Thermoplasma acidophilum* (Smith *et al.*, 1987) and in *Trypanosoma brucei* (Danson *et al.*, 1987), the non-complexed dihydrolipoamide dehydrogenase is plasma-membrane-bound, and it will be necessary to establish whether or not any lipoic acid occupies the same cellular location. Finally, we have indicated (Danson, 1988) the accumulating evidence that dithiol-disulphide interchanges may play a general role in membrane-related processes such as solute transport, energy transduction and cell-surface-receptor activation. Whether or not such processes are mediated through a cofactor such as lipoic acid and the enzyme dihydrolipoamide dehydrogenase is unknown; however, the methodology reported here may be a starting point for investigating such possibilities.

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