Long-chain chloroalkane utilization by a marine protozoan

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A long-chain chloroalkane, 1-chlorooctadecane, was biotransformed by a marine amoeba, *Trichosphaerium* I-7, and utilized by this organism as a carbon source. Haloalkane metabolism was accompanied by marked cell darkening which may represent xenobiotic-induced melanin biosynthesis. Radiolabelled carbon atoms derived from 1-chloro[1-¹⁴C]octadecane were incorporated into cellular proteins and organic intermediates, including fatty acids. Gas chromatography and mass spectrometry analyses of the fatty acid methyl ester derivatives, however, showed that they were not chlorinated. [1-¹⁴C]Octadecanoic acid methyl ester was detected by mass spectrometry, but hexadecanoic acid methyl ester did not contain detectable ¹⁴C. These data suggest that the amoebae convert the chloroalkane to the corresponding fatty acid by oxidative dehalogenation.

Introduction

Halogenated aliphatic compounds, commonly known as haloalkanes, once considered resistant to biodegradation, are now known to be assimilated by several microbial species. Cleavage of the carbon–halogen bond by alkane dehalogenases converts these xenobiotics to normal metabolic intermediates (reviewed by Neilson, 1990). In some cases dehalogenation does not precede utilization and the direct incorporation of long-chain chlorinated alkanes into chlorinated fatty acids has been reported in both bacteria and fungi (Murphy & Perry, 1983, 1984).

Interest in the biodegradation of halogenated compounds has increased with the growing awareness of the dangers posed by the accumulation of these substances in soil and in ground waters. To date, halocarbon-degrading micro-organisms have been isolated primarily from terrestrial and freshwater ecosystems. The marine environment is also known to contain significant amounts of halogenated aliphatic compounds of both anthropogenic and natural origins. One natural source of halogenated organics is marine macroalgae, which synthesize and release substantial quantities of these compounds (Geigert *et al.*, 1984; Gschwend *et al.*, 1985). These natural inputs of halocarbons into the oceans through the millennia may have led to the development of degradation pathways in marine microbes.

We have recently isolated from macroalgal tissues the first known protozoan which ingests both alkanes and haloalkanes (Kaska *et al.*, 1991). This marine amoeba, *Trichosphaerium* I-7, also degrades and utilizes octadecane as a carbon source. In this study we report that *Trichosphaerium* biotransforms the chloroalkane 1-chloro[1-¹⁴C]octadecane, and incorporates the radio-labelled carbon atoms into proteins and organic intermediates, including cellular fatty acids.

Methods

Growth of Trichosphaerium on 1-chlorooctadecane. Descriptions of the organism and culture conditions for maintaining the amoebae on hydrocarbons and halocarbons as carbon sources have been published previously (Polne-Fuller, 1987; Kaska *et al.*, 1991). Briefly, 1-chlorooctadecane (Aldrich) was melted and 200 µl aliquots were spread in sterile plastic Petri dishes (60 mm diam.). After the halocarbon solidified, filter-sterilized sea water and amoebae were added to these dishes or to control dishes containing autoclaved brewer's yeast. 1-Chloro[1-¹⁴C]octadecane (Sigma) [3.1 mCi mmol⁻¹ (115 MBq mmol⁻¹)] was dissolved in ethanol and 50 µl aliquots containing 2.7 × 10⁷ d.p.m. were spread in sterile plastic Petri dishes (30 mm diam.). The ethanol was allowed to evaporate and filter-sterilized sea water and amoebae were added to the dishes. All dishes were sealed with Parafilm to control evaporation and incubated at 15 °C.

Cells grown in the presence of 1-chloro[1-¹⁴C]octadecane were detached by vigorous pipetting and transferred to new Petri dishes. After the cells had attached to the dish, they were washed with sea water and then collected with a rubber scraper. The cell suspension was centrifuged for 5 min at 5600 g in a Costar µ-centrifuge at 4 °C and the cell pellet frozen at –70 °C.

Abbreviation: FAME, fatty acid methyl ester.

Cell extracts were prepared as described by Goodman *et al.* (1990). The frozen pellets were thawed in 5% (w/v) trichloroacetic acid (1 ml total vol.) on ice for 15 min to denature proteinases prior to cell lysis. After centrifugation at 5600 g for 10 min at 4 °C, the supernatant was discarded and the cells extracted with ice-cold acetone (1 ml). The acetone suspension was centrifuged as before and the acetone extract collected. The extraction was repeated and the pooled acetone extracts were analysed by thin-layer chromatography (TLC).

Equal aliquots of the acetone extracts were applied to two silica gel plates. Samples of the original ethanol solution of 1-chloro[1-¹⁴C]octadecane were applied in adjacent lanes on each plate and the plates developed in methylene chloride or methanol. The plates were dried in air and the radioactive spots detected by autoradiography and quantified using a Bioscan imaging scanner (Rock *et al.*, 1988).

The acetone-insoluble precipitates (above) were resuspended in 250 µl of Laemmli sample buffer (Laemmli, 1970). Acid-washed glass beads (250 mg) were added and the mixture was homogenized on a Vortex Genie for 20 min. The liquid was collected, heated to 100 °C for 2 min, and centrifuged (5600 g, 5 min) to remove insoluble debris. SDS-PAGE (10%, w/v, separating gel; 4.5%, w/v, stacking gel) was performed as described by Laemmli (1970). The gel was impregnated with En³Hance (NEN) and dried prior to autoradiography.

Fatty acid analysis. Fatty acid methyl esters (FAMES) were prepared from amoebae grown for 5 d in the presence of 1-chloro[1-¹⁴C]octadecane or for 30 d on unlabelled 1-chlorooctadecane according to the method of Dunlap & Perry (1967). After esterification, the extracts were applied to silica gel plates along with authentic samples of stearic acid (octadecanoic acid) methyl ester (Sigma), and the plates developed in the solvent system light petroleum (b.p. 37.3–55.3 °C)/diethyl ether/acetic acid (90:10:1, by vol.) as described previously (Murphy & Perry, 1983) or in hexane/methylene chloride (6:4, v/v). The compounds were detected with iodine vapour and the radiolabelled FAMES detected by autoradiography or by using a Bioscan imaging scanner. The FAME bands were scraped from the plates and eluted with chloroform.

Gas chromatography/mass spectrometry. Gas chromatography was carried out with a Hewlett-Packard 5790A equipped with a 10 m OV-101 coated fused-silica capillary column of 0.2 mm i.d. Sample sizes of about 1–3 µl of chloroform solutions were injected into a splitless capillary injector at a port temperature of 240 °C. The oven was set at an initial temperature of 50 °C and after 30 s was adjusted manually to 150 °C followed by a 10 °C min⁻¹ temperature ramp to 300 °C. Effluents from the column were analysed by electron ionization using a VG 70-250 HF double-focusing mass spectrometer.

Microspectrophotometry. Living amoebae were analysed with a Nanometrics Nanospec 10S microspectrophotometer adapted to the camera mount of a Leitz Dialux microscope. Visible illumination was provided by a 100 W tungsten-halogen lamp. The cells were scanned over the visible range (450–700 nm) and the transmittance was recorded on a chart recorder.

Results and Discussion

Appearance of Trichosphaerium cultured on 1-chlorooctadecane

The amoebae attached readily to 1-chlorooctadecane and began to ingest the substance within 24 h. In the cultures incubated with small aliquots of 1-chloro[1-¹⁴C]octadecane, the chlorocarbon was no longer visible on the surface of the Petri dishes after 6 d. When exposed to a large excess of unlabelled 1-chlorooctadecane, the cells

Fig. 1. Differential absorbance spectra of a hyaline yeast-grown amoeba vs a dark 1-chlorooctadecane-grown cell (a) or a melanized cell (b).

eroded pits and pockets in the substance. After prolonged culture (>4 weeks) in the presence of 1-chlorooctadecane, the cells gradually accumulated a brown-to-black pigment. This confirmed our previous observation that cells exposed to hydrocarbons or halocarbons show a marked darkening, while yeast-fed cells remain nearly transparent (Kaska *et al.*, 1991).

The colour and granular appearance of the pigment which accumulated in the amoebae are reminiscent of melanin. Melanins are widely distributed in plants, animals and protists such as fungi, including yeasts (Butler *et al.*, 1989; Roy *et al.*, 1989; Gadd *et al.*, 1990). Melanization in fungi can be induced and/or accelerated by the presence of xenobiotics such as organotin compounds (Newby & Gadd, 1988), and recently a role for fungal melanin in the biosorption of tributyltin chloride was suggested (Gadd *et al.*, 1990). The protective role of melanin as an antioxidant biopolymer against peroxidation of polyunsaturated fatty acids in the vertebrate liver has also been reported (Scalia *et al.*, 1990). The use of melanins as scavengers of activated oxygen species by micro-organisms, such as *Trichosphaerium*, has not yet been investigated.

In order to determine whether the dark pigment in *Trichosphaerium* showed properties characteristic of melanins, the dark chlorocarbon-grown cells and hyaline yeast-grown cells were analysed by microspectrophotometry in the visible range (450–700 nm). A differential absorption spectrum revealed a single broad peak with a maximum at 575–600 nm (Fig. 1). The height of the peak varied with the thickness of the cells analysed. This profile is similar to the spectrum previously reported for a suspension of yeast melanin (Butler *et al.*, 1989).

Fig. 2. Autoradiogram of thin-layer chromatography plates. 1-Chloro-[1- 14 C]octadecane dissolved in ethanol was applied to lanes 1 and 3, acetone-soluble extracts of amoebae cultured for 6 d in the presence of 1-chloro[1- 14 C]octadecane were applied to lanes 2 and 4. Lanes 1 and 2 were developed in methylene chloride, lanes 3 and 4 were developed in methanol.

Trichosphaerium cells containing authentic melanin were prepared by the addition of melanin powder (Sigma) to a culture of yeast-fed cells. The amoebae engulfed the particles and within 24 h had acquired a brown-to-black colouration similar to that in chlorocarbon-grown cells. Differential absorbance spectra of melanized vs hyaline amoebae were highly similar to the corresponding spectra of dark chlorocarbon-grown cells (Fig. 1). These data suggest that *Trichosphaerium* may synthesize melanin in response to prolonged utilization of halo-carbons as a carbon source. Additional studies will be required, however, to further characterize this pigment deposition.

Biotransformation of 1-chlorooctadecane

Trichosphaerium cultured for 6 d in the presence of 1-chloro[1- 14 C]octadecane incorporated 14 C into cellular proteins. SDS-PAGE of acetone-insoluble macromolecules followed by autoradiography showed the presence of numerous radiolabelled protein bands. Utilization of the terminal carbon atom, which was chlorinated in the original alkane, in protein biosynthesis, provides indirect evidence that the carbon-chloride bond was severed. Analysis of the acetone-soluble cellular components by TLC showed the presence of several radioactive substances with higher polarity than the original chloroalkane (Fig. 2). When the plate was developed in methylene chloride, radiolabelled substances (not identified) remained at the origin and components with R_F values of 0.61 and 0.86 were

detected. Duplicate plates developed in methanol contained a labelled substance at $R_F = 0.44$ and a streak between $R_F = 0.61$ and 0.94, but little or no labelled substances remained at the origin. These data confirm that 1-chloro[1- 14 C]octadecane is biotransformed by the amoebae.

Fatty acid analysis

Long-chain chloroalkanes are known in bacterial and fungal systems to be terminally oxidized to chlorinated fatty acids and directly incorporated into cellular lipids (Murphy & Perry, 1983, 1984). The cellular fatty acids of *Trichosphaerium* after growth on yeast, 1-chlorooctadecane or 1-chloro[1- 14 C]octadecane were esterified and analysed by TLC. Radioactively labelled substances which co-migrated with authentic samples of octadecanoic acid methyl ester were found in extracts of cells grown on the labelled chloroalkane. The FAMES were then analysed by gas chromatography (GC) and mass spectrometry. FAMES were recognized by the presence of the characteristic fragment ion peaks at m/z 74 and m/z 87 (McLafferty, 1973; Pavia *et al.*, 1979). The GC selected ion profiles of m/z 74 and m/z 87 were superimposable. The FAMES in *Trichosphaerium* after growth on 1-chlorooctadecane showed four peaks, identified as the methyl esters of hexadecanoic acid (65%), octadecanoic acid (18%), hexadecenoic acid (10%) and tetradecanoic acid (7%). The mass spectra closely matched the standard spectra in the EPA/NIH Mass Spectral Data Base (Heller & Milne, 1978). This fatty acid composition was identical to that of cells grown on yeast. No peaks with retention times characteristic of chlorinated FAME (Murphy & Perry, 1983) were present in the gas chromatograph from chlorocarbon-grown cells.

These results indicate that oxidation of 1-chloro-[1- 14 C]octadecane to the fatty acid occurs at the C-1 in *Trichosphaerium*, and that the reactions include a dehalogenation of the alkane. If this is indeed the case, then the expected product would be [1- 14 C]octadecanoic acid. Mass spectral analysis of the octadecanoic acid methyl ester prepared from cells grown on 1-chloro-[1- 14 C]octadecane should then have fragment ion peaks two atomic mass units higher than those of the 12 C species. Both the fragments detected in the prominent peaks at m/z 74 and m/z 87 contain the C-1, and in the mass spectrum of octadecanoic acid methyl ester from cells grown on 1-chloro[1- 14 C]octadecane, these fragment peaks are accompanied by a small isotope peak at m/z 76 (3.5% corrected abundance) and at m/z 89 (3% corrected abundance) (Fig. 3a). These data are consistent with the hypothesis that approximately 3% of the octadecanoic acid methyl esters in this preparation

Fig. 3. (a) Partial mass spectrum of octadecanoic acid methyl ester prepared from amoebae grown for 6 d on 1-chloro[1- ^{14}C]octadecane. The fragment ion peak masses are indicated for the peaks. Arrows indicate the fragment ion peaks two atomic mass units larger than the m/z 74 and m/z 87 peaks which result from decomposition of [1- ^{14}C]octadecanoic acid methyl ester. (b) Partial mass spectrum of octadecanoic acid methyl ester prepared from amoebae grown on 1-chlorooctadecane.

contained ^{14}C at the C-1 position. The isotope peaks at m/z 76 and m/z 89 were not detectable in mass spectra of the octadecanoic acid methyl ester from cells grown on unlabelled 1-chlorooctadecane (Fig. 3b). The mass spectra of hexadecanoic acid methyl ester from amoebae grown on ^{14}C -labelled and on unlabelled 1-chlorooctadecane were identical, and no peaks at m/z 76 or m/z 89 were present above background (data not shown). This indicates that the hexadecanoic acid methyl ester did not contain detectable ^{14}C at the C-1 position. Hexadecanoic acid formed from octadecanoic [1- ^{14}C] acid by β -oxidation would not be expected to be radiolabelled because the ^{14}C would be released as part of an acetate unit.

Conclusions

The marine amoeba *Trichosphaerium* ingests and biodegrades the haloalkane 1-chlorooctadecane and utilizes it as a carbon source. The results indicate that the 1-chlorooctadecane undergoes oxidative dehalogenation to form octadecanoic acid which is incorporated into cellular lipids or degraded by β -oxidation. Eventually, numerous cellular components contain carbon atoms originally derived from the chlorocarbon.

The intertidal regions which these protozoa inhabit in nature experience halocarbon input from macroalgal decay and from anthropogenic sources such as halogenated solvents and pesticides in stream runoff. As important indigenous members of marine ecosystems, protozoa may play a critical, but underestimated role in

the steady-state biotransformation of these halocarbons. Further studies are required to determine the repertoire of compounds which marine amoebae can metabolize.

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