Some properties, including the substrate *in vivo*, of the Δ^9 -desaturase in *Micrococcus cryophilus*

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The Δ^9 -desaturase of the psychrophilic bacterium *Micrococcus cryophilus* is shown to be a membrane-bound enzyme that is probably linked to a cyanide- (and azide-) sensitive respiratory chain with oxygen as the final acceptor. It has a pH optimum of 8.7 and contains an essential thiol group, but has no special ion requirements. The desaturase activity of washed membranes could not be increased by adding supernatant or NADH and NADPH, possibly owing to the endogenous generation of reduced cofactors by the membranes. The substrate for the desaturase is not acyl-CoA and is probably not acyl-acyl-carrier protein. Evidence is presented that the substrate *in vivo* is saturated phospholipid and a scheme for the possible routes of incorporation of exogenous stearic acid into oleoyl-phospholipid is presented.

Desaturase enzymes from a wide range of eukaryotic organisms, including mammals, plants, fish, yeasts, fungi and algae, have been well characterized (for a review, see Fulco, 1974). The Δ^9 -desaturase from rat liver and hen liver have recently been purified to homogeneity (Strittmatter *et al.*, 1974; Prasad & Joshi, 1979, respectively). However, the number of prokaryotic enzymes that have been investigated in detail is much smaller. Those of bacilli have been studied extensively, but in intact bacteria only (Fulco, 1974), and the particulate desaturase systems of *Myobacterium phlei* (Fulco & Bloch, 1964) and *Alcaligenes faecalis* (Kshirsagar & Nair, 1979) have been studied *in vitro*.

The psychrophilic bacterium *Micrococcus cryophilus* has a simple phospholipid fatty acyl composition, consisting of >95% monoenoic fatty acids (palmitoleate and oleate) (Russell, 1971). The double bonds are synthesized aerobically by a Δ^9 -desaturase that is active in cell-free preparations (Russell, 1974, 1978). As part of a study of the adaptation to growth temperature and desaturase activity in *M. cryophilus*, we sought to characterize the enzyme and to relate the findings to membrane fluidity. Therefore, it was of particular interest to determine the cofactor requirement and the nature of the substrate *in vivo*. Evidence is accumulating that

in eukaryotic systems the unsaturated (but not saturated) acyl chains of complex lipids can serve as desaturase substrates (Gurr *et al.*, 1969; Baker & Lynen, 1971; Talamo *et al.*, 1973; Pugh & Kates, 1977, 1978; Stymne & Appelqvist, 1978; Hawke & Stumpf, 1980; Kameyama *et al.*, 1980). In the few bacterial systems that have been investigated the substrate is acyl-CoA (e.g. Fulco & Bloch, 1964; Kshirsagar & Nair, 1979). The present paper describes the characteristics of the Δ^9 -desaturase of *M. cryophilus* and shows that the substrate is not acyl-CoA, but, most likely, phospholipid.

Materials and methods

Radioisotopes and substrates

(1- 14 C)-labelled fatty acids (C_{12} – C_{18} , saturated) and [1- 14 C]stearoyl-CoA were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Spinach acyl-carrier protein and [1- 14 C]stearoyl-acyl-carrier protein were gifts from Dr. J. L. Harwood, Department of Biochemistry, University College, P.O. Box 78, Cardiff, Wales, U.K., and Dr. T. McKeon, Department of Biochemistry, University of California, Davis, CA, U.S.A., respectively.

Maintenance and growth of cultures

M. cryophilus (A.T.C.C. 15174) was cultured at 21°C and maintained at 4°C on a Casamino acids/salts medium (NM medium, pH 7.4) as described previously (Russell, 1974).

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Preparation of lysates and membranes

Bacteria were harvested by centrifugation at 10000 *g* ($r_{av.} = 7.2$ cm) for 15 min. The cell pellets were resuspended in a volume of NM buffer (i.e. NM medium lacking the Casamino acids) corresponding to 10% of the original culture volume. The bacteria were lysed by three passages through a French pressure cell (American Instrument Co. Inc., Silver Springs, MD, U.S.A.) at 4.14×10^4 kPa operating pressure. Cell breakage (>99%) was confirmed by phase-contrast light microscopy.

When they were required, membranes were collected by centrifugation at 105000 *g* ($r_{av.} = 6.3$) for 1 h and resuspended in NM buffer, which was also used for washing membranes.

Lipid extraction and analysis

Lipids were extracted from bacteria, lysates or membranes using the procedure of Garbus *et al.* (1963). In this two-phase system the lower chloroform phase contained phospholipids, fatty acids and wax esters, whereas the upper aqueous methanol phase contained acyl-CoA and acyl-acyl-carrier protein. In some cases acyl-CoA was isolated by t.l.c. of lipid extracts on 0.25 mm-thick plates of silica-gel H (Merck, Darmstadt, Germany) containing 1% potassium oxalate (Holloway & Holloway, 1974), with chloroform/methanol/water (9:7:2, by vol.) as developing solvent.

Phospholipids were separated from neutral lipids by t.l.c. of the chloroform phase on 0.25 mm-thick plates of silica-gel H (Merck) with petroleum ether/diethyl ether/acetic acid (85:15:1, by vol.) as the developing solvent. This procedure was essential when measuring desaturation using [$1-^{14}\text{C}$]stearate as the substrate to remove unincorporated fatty acid from phospholipid; this step also separated fatty acids from the major neutral lipid, namely wax ester. Fatty acid methyl esters were prepared from phospholipids by transmethylation on silica gel using 2M-sodium methoxide or 2.5% H_2SO_4 in dry redistilled methanol (Christie, 1973). The constituent fatty acids of acyl-CoA or acyl-acyl-carrier protein were isolated by saponification with 2.8 ml of 10% KOH in methanol per 1.2 ml of aqueous methanol phase. After heating at 80°C for 1 h, 2.8 ml of 3.6M- H_2SO_4 was added and the fatty acids extracted with three portions of light petroleum (b.p. 30–40°C). Fatty acid methyl esters were prepared by methylating with 2.5% H_2SO_4 in dry redistilled methanol. If the fatty acid methyl esters were to be analysed by argentation/t.l.c., 2 mg of stearate and 4 mg of oleate were added before (trans)-methylation.

Desaturase assay

Incubations usually consisted of 1 ml of an aqueous suspension of bacteria, lysate or mem-

branes (0.4–1.0 mg of protein), together with an appropriate amount of substrate and other additions as indicated below. Reactions were performed in 15 mm $\times$ 125 mm test tubes, which were shaken vigorously in a water bath at 21°C to ensure adequate aeration. When required, anaerobic conditions were obtained by bubbling N_2 gas through the suspension. Reactions were terminated by the addition of 3.75 ml of chloroform/methanol (2:1, v/v) and the lipid extracted and washed as described above.

Desaturation was measured by argentation/t.l.c. analysis on 0.25 mm-thick plates of silica gel HF₂₅₄₋₃₆₀ type 60 (Merck) containing 10% (w/w) AgNO_3 and with light petroleum/diethyl ether (23:4, v/v) as developing solvent. Plates were prepared and activated by heating at 110°C for 45 min on the day of use. Fatty acid methyl esters were located using a u.v. lamp and the silica gel scraped directly into scintillation vials. PCS scintillation cocktail (10 ml; Amersham-Searle, Arlington Heights, IL, U.S.A.) was added and the radioactivity was measured by using an Intertechnique PG 4000 liquid-scintillation counter with external-standards-ratio facility for quench correction. Samples were counted within 12 h of adding scintillant, to avoid colour quenching by Ag^+ . Desaturation was expressed either as percentage desaturation or as the amount of unsaturated fatty acid synthesized (pmol/mg of protein per min) calculated by using the specific radioactivity of the isotopic precursor.

In some cases fatty acid methyl esters were analysed by radio-g.l.c. analysis on columns of 15% (w/w) ethylene glycol succinate silicone (EGSS-X; Supelco, Philadelphia, PA, U.S.A.) as detailed previously (Sandercock & Russell, 1980).

Preparation of saturated ($1-^{14}\text{C}$)-fatty acyl-labelled phospholipid

Initially, saturated phospholipids were prepared by extracting phospholipids from bacteria that had been incubated under aerobic conditions with [$1-^{14}\text{C}$]stearate followed by chemical reduction of the fatty acyl chains using H_2/PtO_2 (Kates, 1972). This method, of course, produced phospholipids in which all the acyl chains, whether radioactive or not, were saturated.

In later experiments an alternative approach was adopted to produce phospholipids containing essentially non-radioactive unsaturated acyl chains with a proportion of radioactive saturated acyl chains. This was achieved by bubbling N_2 gas through 50 ml of a late-exponential-phase culture of *M. cryophilus* for 1 h, after which time 9.25 kBq of [$1-^{14}\text{C}$]stearate was added and the culture incubated for a further 30 min under anaerobiosis. In these conditions the radioactive stearate is incorporated into phospholipid acyl chains, but remains as stearate. The lipids were

extracted and the phospholipid isolated by preparative t.l.c. as described above, except that 0.5 mm-thick plates were used. When necessary the phospholipid was separated into phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol (cardiolipin) by t.l.c. on 0.5 mm-thick plates of silica-gel H developed in chloroform/methanol/acetic acid/water (170:30:20:7, by vol.). Phospholipids were eluted from the silica gel by extensive washing with chloroform/methanol/diethyl ether (1:1:1, by vol.), and stored at -20°C until required.

Estimation of $^{32}\text{P}/^{14}\text{C}$ ratio of phospholipids

Before carrying out double-isotope incorporation experiments, the kinetics of phospholipid labelling with $[^{32}\text{P}]\text{P}_i$ were established. The incorporation of $[^{32}\text{P}]\text{P}_i$ reached a plateau after approx. 4 h, when it was assumed that a steady state had been reached. This time period then was used in double labelling experiments and the amount of ^{32}P used was adjusted so as to give approximately equivalent numbers of ^{32}P and ^{14}C counts in the extracted phospholipids, in order to minimize the radioactivity counting errors due to overlap of the isotopic energy spectra. The amount of overlap of ^{32}P counts in the ^{14}C channel was approx. 25%; any increase due to quenching was assessed by adding known amounts of ^{32}P to samples and recounting. The recorded counts were adjusted for quenching by using the external-standards ratio method, and for decay of the ^{32}P by counting daily a fixed amount of the isotope in a separate vial.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin dissolved in 0.1 M-Tris/HCl buffer (the same concentration of Tris/HCl as present in NM buffer) as standard, because Tris/HCl alone gives a positive reaction in the assay.

Results and discussion

Desaturase activity was investigated initially by determining the fate of saturated fatty acids (C_{12} – C_{18}) added to growing cultures of *Micrococcus cryophilus*. Exogenous radioactive saturated fatty acids were taken up by bacteria extremely rapidly, and within a few seconds radioactivity was present in phospholipid acyl chains. The incorporation reached a plateau after 5 min when 2.3% of the exogenous fatty acid was present as phospholipid acyl chains. Moreover, these acyl chains were largely unsaturated and the appearance of unsaturated fatty acids in various cell lipids is shown in Figs. 1(a) and 1(b), expressed in terms of the percentage desaturation (Fig. 1a) or the amount of unsaturated fatty acid synthesized (Fig. 1b). Clearly, the major proportion of unsaturated fatty acids was found in the phospholipid, which became progressively more desaturated in a linear fashion for approx. 10 min. After 2 h the percentage desaturation of phospholipid radioactivity was 90–95% (not shown), thus approaching the proportion of unsaturated acyl chains normally present in the bacterial phospholipids (Russell, 1971). There was, in addition to desaturation, elongation of C_{12} – C_{16} fatty acids and

Fig. 1. The desaturation of lipid fractions in *M. cryophilus*

Cultures were incubated with $[1-^{14}\text{C}]\text{stearate}$ (1.85 kBq/ml of culture) and at intervals samples were extracted by using the Garbus procedure (Garbus *et al.*, 1963). Phospholipids and fatty acids were isolated from the lower chloroform phase by t.l.c. and the phospholipid acyl chains and fatty acids were converted into fatty acid methyl esters by (trans)methylation. The aqueous methanol upper phase, containing the bulk of the acyl-CoA and acyl-acyl-carrier protein was saponified, and the fatty acids were extracted and methylated as detailed in the Materials and methods section. Unsaturated fatty acids were separated by argentation/t.l.c. and desaturation of each lipid expressed as the percentage desaturation (a) or amount of unsaturated fatty acid (UFA) synthesized (b). ■, Phospholipid; ▲, aqueous methanol phase; ●, unesterified fatty acid.

Table 1. *The effect of cell breakage and fractionation on the desaturation of stearate by M. cryophilus*

Bacterial culture (50ml), or the cell fraction derived from 50ml of culture, was incubated with 18.5kBq of [1-¹⁴C]stearate at 21°C for 2h and the desaturation of phospholipid acyl chains measured by argentation/t.l.c. as detailed in the Materials and methods section. Results are expressed as means $\pm$ s.d. (*n* = number of independent experiments or subcellular fractionations normalized to 1 ml of original culture). Abbreviations: UFA, unsaturated fatty acid; n.d., not determined.

Experimental system	Relative incorporation (%)	Desaturation (%)	UFA (pmol/ml of original culture)
Intact bacteria	100	90.7 $\pm$ 5.0 (12)	2456.0 $\pm$ 804.0 (6)
Lysate	11.4 $\pm$ 7.8 (10)	41.3 $\pm$ 10.0 (10)	188.0 $\pm$ 169.0 (6)
Membranes	8.0 $\pm$ 4.6 (9)	36.0 $\pm$ 18.1 (9)	119.0 $\pm$ 105.0 (5)
Supernatant	3.1 $\pm$ 1.4 (5)	1.2 $\pm$ 1.8 (7)	n.d.
Membranes + supernatant	4.8 $\pm$ 2.1 (5)	37.6 $\pm$ 18.8 (4)	59.0 $\pm$ 43.0 (4)
Washed membranes	2.2 $\pm$ 0.4 (3)	27.0 $\pm$ 4.2 (3)	18.0 $\pm$ 2.8 (3)
Washed membranes + supernatant	3.2 $\pm$ 0.6 (3)	15.0 $\pm$ 8.5 (3)	16.0 $\pm$ 7.1 (3)

retroconversion of stearate (Sandercock & Russell, 1980).

The appearance of unsaturated fatty acids in the aqueous methanol phase of the two-phase extraction procedure (see the Materials and methods section), which contains the acyl-CoA and acyl-acyl-carrier protein, was much slower and even after 30 min was 10-fold less. This point is discussed below when considering phospholipid as the substrate for desaturation.

The amount of unsaturated fatty acids synthesized was directly proportional to the amount of protein in the concentration range 0–1 mg/ml. Above 1 mg/ml this direct proportionality did not hold, and above 10 mg/ml, enzyme activity decreased markedly. This was presumably due to a limitation of available cofactors and dissolved O₂. In contrast, desaturase activity expressed as percentage desaturation (rather than as the amount of unsaturated fatty acid synthesized) was independent of protein concentration except at extremely low concentrations. At such low concentrations the amount of exogenous radioactive stearate would be significantly large compared with the endogenous pool, which, therefore, would be diluted. In view of these data, assays (1 ml) contained 0.4–1.0 mg of protein.

The incorporation of [1-¹⁴C]stearate into phospholipids and the formation of oleoyl acyl chains by various cell fractions is summarized in Table 1. The level of incorporation of exogenous fatty acid into phospholipid of growing bacteria varied considerably from one experiment to another; the percentage of desaturation varied much less. However, a consistent pattern was observed when intact bacteria were compared with broken cells and cell fractions in that both incorporation of fatty acid phospholipid and desaturation declined with the amount of experimental manipulation, for example, the number of passages through the French pressure

cell during cell breakage or the amount of ultracentrifugation (Table 1). A similar sensitivity to experimental manipulation has been observed with other systems (Gurr *et al.*, 1969). The decrease in desaturase activity was most marked when expressed in terms of the absolute amount of unsaturated fatty acid synthesized, because this value reflected the large drop in incorporation as well as the fall in percentage desaturation.

The supernatant contained little desaturase activity, but unlike several other desaturase systems (e.g., see Kshirsagar & Nair, 1979), the activity of membranes was not increased by the re-addition of supernatant, whether or not the membranes had been washed (Table 1). Similarly, the desaturase activity of lysate and membranes were comparable. Thus, in *M. cryophilus* the supernatant does not contain cofactors essential for desaturation. Consistent with this observation was the fact that the desaturase activity of membranes could not be increased by the addition of the cofactors ATP, CoA, NADH or NADPH (Table 2). The incorporation of stearate into phospholipid was stimulated almost 3-fold, however, so that the percentage desaturation of the phospholipid acyl chains was decreased because there was no change in the amount of unsaturated fatty acid produced. Both ATP and CoA were required to obtain this decrease, but reduced nicotinamide nucleotides seemed less important (Table 2). It is presumed that the desaturase of *M. cryophilus* requires some form of reduced cofactor: if it does not, then it is radically different from those desaturases characterized to date (Gurr & James, 1980). It may be that the cofactor required is something other than a reduced nicotinamide nucleotide. The concentrations of cofactors used (see Table 2) were comparable with those known to be effective in other systems (e.g., see Safford *et al.*, 1975), and were adequate to effect the incorporation of fatty acid into phospholipid.

Table 2. *The effect of cofactors on the desaturation of stearate by membranes of M. cryophilus*

Incubations consisted of 0.37–0.75 mg of membranes and 7.4 kBq of [^{14}C]stearate in 1 ml of NM buffer containing cofactors as indicated at the following concentrations: ATP, 1 mM; NADH, 0.5 mM; NADPH, 0.36 mM; CoA, 0.5 mM. Reactions were terminated after 10 min by the addition of 3.75 ml of chloroform/methanol (2:1, v/v), and the phospholipids isolated and desaturation measured by argentation/t.l.c. as described in the Materials and methods section. Relative incorporation was estimated from the total radioactivity in saturated and unsaturated fatty acid methyl esters. The results are expressed as means $\pm$ S.D. of six independent experiments. Abbreviation: UFA, unsaturated fatty acid.

Additions	Relative incorporation (%)	Desaturation (%)	UFA (pmol/min per mg of protein)
None	100	19.4 $\pm$ 8.2	3.4 $\pm$ 2.3
ATP + CoA + NADH + NADPH	355.1	4.3 $\pm$ 1.5	3.6 $\pm$ 1.9
ATP + CoA + NADH	300.0	6.9 $\pm$ 2.2	3.7 $\pm$ 1.9
ATP + CoA + NADPH	424.6	4.1 $\pm$ 1.5	3.9 $\pm$ 1.2
ATP + CoA	279.7	9.8 $\pm$ 3.9	4.0 $\pm$ 2.6
ATP + NADH + NADPH	88.4	16.2 $\pm$ 5.6	2.6 $\pm$ 2.6
NADH + NADPH	129.0	17.6 $\pm$ 8.1	4.5 $\pm$ 2.9
CoA + NADH + NADPH	127.5	12.8 $\pm$ 6.1	2.6 $\pm$ 1.2

Raising the cofactor concentrations by 5-fold had no further effect on desaturation. The fact that this effect on incorporation was observed with membranes implied that not only were the fatty acid-activating enzymes membrane-bound, but that the membranes were able to generate reduced cofactors. During cell breakage in the French pressure cell membrane vesicles are formed (Lancaster & Hinkle, 1977); thus cofactors and substrates could have been trapped within them.

The effect of various inhibitors on the formation of oleoyl-phospholipid is summarized in Table 3. In common with some other bacterial desaturases (e.g., see Kshirsagar & Nair, 1979) cyanide inhibited, but high concentrations were required; some bacterial desaturases are resistant to cyanide (e.g., see Fulco & Bloch, 1964). As expected, desaturation required O_2 . Thiol-specific reagents inhibited desaturation, L-cysteine more so than reduced glutathione, but the inhibition was not marked. The metal ion chelator, EDTA, had no effect even at high concentrations, but the more specific ferrous ion chelator, *o*-phenanthroline, was partially inhibitory. The desaturases of *Mycobacterium phlei* and *Alcaligenes faecalis* require ferrous ions and in both cases are inhibited by *o*-phenanthroline (Fulco & Bloch, 1964;

Table 3. *Inhibitors of the desaturation of stearate by M. cryophilus*

Reactions, consisting of 1 ml of a bacterial lysate (0.5–1.3 mg of protein) containing 7.4 kBq of [^{14}C]stearate and one of the inhibitors at the concentrations indicated, were carried out at 21°C for 10 min. Desaturation and the amount of UFA synthesized was measured by argentation/t.l.c. as described in the Materials and methods section, and the results are expressed as the mean percentage inhibitions ($\pm$ S.D.) relative to a control lysate lacking inhibitor of three to five independent experiments. Abbreviation: UFA, unsaturated fatty acid.

Inhibitor	Concentration (M)	Inhibition (%) of phospholipid desaturation	Inhibition (%) of UFA biosynthesis (pmol/min per mg of protein)
KCN	10^{-4}	3.6 $\pm$ 1.0	51.1 $\pm$ 3.6
	10^{-3}	16.0 $\pm$ 0.5	74.8 $\pm$ 1.1
Sodium azide	5×10^{-4}	2.4 $\pm$ 0.6	9.0 $\pm$ 1.2
	5×10^{-3}	16.1 $\pm$ 8.6	59.0 $\pm$ 2.0
Glutathione (reduced)	5×10^{-3}	15.2 $\pm$ 4.6	28.0 $\pm$ 6.4
L-Cysteine	5×10^{-3}	35.4 $\pm$ 18.2	49.0 $\pm$ 7.8
EDTA	10^{-3}	0.3 $\pm$ 0.2	4.0 $\pm$ 1.2
<i>o</i> -Phenanthroline	5×10^{-3}	32.1 $\pm$ 4.1	43.0 $\pm$ 2.0
Anaerobiosis*	—	88.0 $\pm$ 4.8	88.4 $\pm$ 7.0

* Anaerobic experiments were performed with intact bacteria as described in the Materials and methods section; the inhibition in this case is relative to control bacteria under aerobic conditions.

Kshirsagar & Nair, 1979). However, in *M. cryophilus* ferrous, cupric, zinc and magnesium ions had no significant effect on stearate desaturation, although Zn^{2+} and Fe^{2+} did stimulate (20–25%) fatty acid incorporation into phospholipid.

It is concluded from these experiments that the Δ^9 -desaturase of *M. cryophilus* is probably linked to a cyanide- (and azide-) sensitive respiratory chain with O_2 as the final acceptor; it may contain an essential thiol group, but has no special ion or cofactor requirements.

Effect of pH

The apparent pH optimum of the desaturase was approx. 8.7, whether activity was expressed as percentage desaturation or as the amount of unsaturated fatty acid. This value is similar to that of the stearyl-CoA desaturase in *Fusarium oxysporum*, which has a pH optimum of 8.2–8.3 (Wilson & Miller, 1978). The optima of these two enzymes are in marked contrast with those of most

bacterial (and mammalian) desaturases, which have optima close to neutrality (e.g. Oshino *et al.*, 1966; McDonald & Kinsella, 1973; Kshirsagar & Nair, 1979). To standardize the experiments *in vitro* with those carried out with intact bacteria (that are grown at pH 7.4), a pH value of 7.4 was adopted for all experiments, especially as a high level of desaturase activity (approx. 65% of maximum) was obtained at this pH.

Acyl chain length specificity

The effect of substrate chain length on desaturation depended on the way in which it was assessed. When expressed as the percentage desaturation of phospholipid acyl chains incorporated from exogenous fatty acid, it was observed consistently that there was a minimum at C_{16} . This minimum reflected a true difference in percentage desaturation, because, within any one experiment, there was no significant difference between the incorporation of fatty acids of different chain lengths into phospholipid. *M. cryophilus* elongates C_{14} and C_{16} fatty acids (Sandercock & Russell, 1980), which complicates interpretation of the data. Thus the experiments were repeated in the presence of 10 mM-NaAsO₂, which inhibits elongation (Sandercock & Russell, 1980), but the same result was obtained. This may reflect the fact that arsenite does not completely inhibit elongation. In any case the explanation of this result is unclear.

An alternative method of assessing chain-length specificity was to determine the amount of unsaturated product having the same chain length as the radioactive precursor by using radio-g.l.c. analysis, and to ignore the desaturation of any elongation products. When assessed in this manner the activity of the desaturase in *M. cryophilus* increased with acyl chain length up to C_{18} . Similar results have been obtained by others for the desaturases of goat mammary gland, hen liver, yeast and *Chlorella* (Brett *et al.*, 1971). However, this method of assessment is valid only if reactions removing the precursor are the same for all fatty acid substrates. It is known that the preferred products in *M. cryophilus* are $C_{16:1}$ and $C_{18:1}$ fatty acids, and, therefore, elongation of C_{12} and C_{14} fatty acids must effectively compete with desaturation, thus contributing to the apparent increased activity of the desaturase with increasing chain length of the substrate. Some of the problems of determining chain-length specificity using substrates that may be metabolized by several routes have been discussed previously (Jeffcoat *et al.*, 1977). Strictly speaking, chain-length specificity studies should not be undertaken until a purified enzyme system is available, when complicating side-reactions such as elongation can be eliminated.

Nature of the desaturase substrate

The possible substrates for desaturases include acyl-CoA, acyl-acyl-carrier protein and the acyl chains of phospholipids. In preliminary experiments it was established that exogenous [$1-^{14}C$]stearate was incorporated into all of these potential substrate pools; moreover each contained a proportion of unsaturated acyl chains. Thus a direct approach to the identity of the desaturase substrate in *M. cryophilus* was to test membrane fractions with each potential substrate.

Acyl-CoA

When [$1-^{14}C$]stearoyl-CoA was incubated with a lysate or membranes there was little or no desaturation of the substrate and a large proportion (up to 66%) was hydrolysed to unesterified fatty acid. Up to 10% of the acyl-CoA was incorporated into phospholipid and remained as saturated fatty acyl chains. This lack of desaturation of acyl-CoA was consistent with the observation (cf. Table 2) that ATP and CoA had no effect on the amount of unsaturated phospholipid synthesized from [$1-^{14}C$]stearate by membranes or a lysate.

Acyl-acyl-carrier protein

Very similar results were obtained by using acyl-acyl-carrier protein, in that desaturation of acyl-acyl-carrier protein was not observed and the incorporation into phospholipid was in the form of saturated acyl chains. In contrast, when a semi-pure preparation of spinach acyl-carrier protein was added to membranes incubated with [$1-^{14}C$]stearate, desaturation was stimulated to a small extent (3–18%), whether or not NAD(P)H and ATP were included. An objection to these experiments is that both the acyl-carrier protein and acyl-acyl-carrier protein were from plant sources and it has been shown that the products of *Escherichia coli* fatty acid synthetase differ when the native acyl-carrier protein is substituted with plant acyl-carrier protein (Simoni *et al.*, 1967).

Phospholipid

When intact bacteria were incubated with [$1-^{14}C$]stearate the fatty acid substrate was incorporated initially into saturated phospholipid; this was followed by the appearance of radioactivity in unsaturated phospholipid, whereas the radioactivity in saturated phospholipid declined proportionately (Fig. 2). These kinetics suggested that saturated phospholipid might be the substrate for desaturation and are reminiscent of the precursor-product relationships observed with oleate incorporation in several plant systems (e.g., see Stymne & Appelqvist, 1978; Hawke & Stumpf, 1980). One difference in *M. cryophilus*, however, was the lack of a lag period in the appearance of unsaturated phospholipid as observed in *Chlorella vulgaris* (Gurr *et al.*, 1969).

Fig. 2. The incorporation and desaturation of exogenous stearate acid by *M. cryophilus*

[1-¹⁴C]Stearate (92.5 kBq) was added to an exponentially growing culture of *M. cryophilus* at 21°C. At 15 s intervals 1 ml portions were mixed with 3.75 ml of chloroform/methanol (2:1, v/v) to stop the reaction and isolate the lipids. Phospholipids were isolated by t.l.c. and fatty acid methyl esters prepared by transmethylation. Saturated and unsaturated fatty acid (FA) methyl esters were isolated by argentation/t.l.c., and their radioactivities were estimated by liquid-scintillation spectrometry.

The most direct approach to the question of whether phospholipid served as substrate was to add saturated phospholipid to membrane preparations. Two preparations of saturated phospholipid were used, one fully reduced chemically and the other containing essentially non-radioactive unsaturated acyl chains and radioactive saturated acyl chains (for details see the Materials and methods section). In a few experiments there was up to 30% desaturation of the substrate, but in most cases there was none, whether the exogenous phospholipid was added as liposomes, as a suspension in ethanol/water (1:1, v/v) or dissolved in chloroform/methanol (1:1, v/v). Attempts to improve access of the phospholipid to the membrane-bound desaturase by sonication or by the addition of deoxycholate (0.1% final concentration) produced the same result. Essentially the same results were obtained with the two types of saturated phospholipid substrate. At the normal assay temperature of 21°C the fully saturated phospholipids would have been below their gel-to-liquid-crystalline phase-transition temperature (T_m). It is known that the physical structure of phospholipid substrates is important for desaturase activity in other systems (e.g., see Talamo *et al.*, 1973). However, raising the temperature to 45°C (> T_m for dipalmitoyl-phospholipid) or 60°C (> T_m for distearoyl-phospholipid) had no effect on desaturation after allowing for the inhibitory effect

of the elevated temperature (measured by stearate desaturation).

It was of interest to test the desaturase of *M. cryophilus* with isolated phosphatidylethanolamine, phosphatidylglycerol and cardiolipin, since it was known that the (dioleoyl)phosphatidylcholine of *C. vulgaris* was active only in mixed-lipid dispersions (Gurr *et al.*, 1969), whereas, conversely, the phospholipid desaturase of *Candida lipolytica* utilizes only individual phospholipids (Pugh & Kates, 1973). However, the results with individual phospholipids of *M. cryophilus* were the same as those obtained by using a total phospholipid extract.

An important difference between the putative phospholipid desaturase of *M. cryophilus* and those of other micro-organisms or plants is that in *M. cryophilus* the substrate must of necessity be a saturated (rather than an unsaturated) phospholipid, since polyunsaturated fatty acids are not present in the native phospholipids.

A major problem in these experiments was the extensive deacylation (up to 67%) of the phospholipid substrate. Such phospholipase activity was stimulated by sonication or by 0.1% deoxycholate and could be reduced by approx. 50% using 1 mM-EDTA. It was concluded that in view of this phospholipase activity and the problem of access of the exogenous substrate to the membrane-bound desaturase an alternative approach should be used.

The method adopted was to measure the desaturation of saturated phospholipid formed endogenously in bacteria incubated with [1-¹⁴C]stearate under anaerobic conditions. Upon transfer of cultures to aerobic conditions phospholipid desaturation proceeded immediately and was unaffected by the presence of a large excess (up to 200-fold) of exogenous [U-¹²C]stearate (Fig. 3). Since it was known that exogenous stearate was incorporated rapidly into phospholipid (cf. Fig. 1), it was assumed that such an excess of stearate would not only dilute the unincorporated exogenous [1-¹⁴C]stearate, but also equilibrate rapidly with endogenous pools of acyl-CoA and/or acyl-acyl-carrier protein and thus lower their specific radioactivity. Therefore, if phospholipid deacylation and entry of acyl chains into one or other of these pools was involved in the desaturation of phospholipid, the excess of exogenous [U-¹²C]stearate should lower the desaturation rate. The fact that it had no effect is regarded as evidence that intact phospholipid acyl chains are desaturated.

To attempt to verify this point the desaturation of phospholipids 'doubly labelled' with [1-¹⁴C]stearate and [³²P]P_i was followed after a change from anaerobic to aerobic conditions (Fig. 4). During the 30 min after the switch to aerobiosis the ¹⁴C/³²P ratio fell by 9.0% and 15.7% in the presence of respectively 40 µg and 400 µg of [U-¹²C]stearate/ml

Fig. 3. The effect of exogenous stearate on the desaturation of endogenous saturated phospholipid in *M. cryophilus*

An exponentially growing culture of *M. cryophilus* was made anaerobic by bubbling N_2 through the medium for 60 min, and then incubated with 11.1 kBq of $[1-^{14}C]$ stearate per ml of culture for 1 h with anaerobic conditions maintained. The culture was divided into three portions and $[U-^{12}C]$ stearate was added to two of them to give final concentrations of 40 μg and 400 μg of stearate per ml of culture respectively. All three cultures were transferred immediately to aerobic conditions and 1 ml samples removed at intervals for the estimation of the desaturation of phospholipid acyl chains measured by argentation/t.l.c. ■, Control; ●, 40 μg of stearate per ml; ▲, 400 μg of stearate per ml.

Fig. 4. Effect of exogenous $[U-^{12}C]$ stearate on the $^{14}C/^{32}P$ ratio of endogenous phospholipids containing saturated acyl chains upon transfer from anaerobic to aerobic conditions

Two exponentially growing cultures of *M. cryophilus* were made anaerobic by bubbling N_2 through the medium for 60 min. Each culture was incubated with 11.1 kBq of $[1-^{14}C]$ stearate and 74.8 kBq of $[^{32}P]P_i$ per ml of medium for 4.5 h with anaerobic conditions maintained. $[U-^{12}C]$ stearate was added to the cultures to give a final concentration of 40 μg (●) or 400 μg (▲) per ml of medium respectively; the cultures were transferred immediately to aerobic conditions and the $^{14}C/^{32}P$ ratio of the phospholipids in 1 ml samples measured as described in the Materials and methods section. The results are means $\pm$ S.D. of four independent experiments, after normalization of the $t = 0$ mean values. The line of best fit was determined by regression analysis.

of culture (Fig. 4). In the same period of time at least 60% of the radioactive acyl chains became desaturated, which would have decreased the $^{14}C/^{32}P$ ratio by considerably more than 9.0% or 15.7% if the acyl chains had entered a pool that equilibrated with the large excess of non-radioactive stearate added. The turnover of lipid phosphorus during this time would have been $<2\%$ (N. J. Russell, unpublished work) and so it is assumed that the decrease in $^{14}C/^{32}P$ ratio was due to acyl chain turnover independently of desaturation that occurred on the phospholipid. A control experiment in which $[1-^{14}C]$ stearate was added together with $[^{32}P]P_i$ at the start of the experimental protocol (see the legend of Fig. 4) showed that the change from aerobiosis to anaerobiosis and back to aerobiosis (plus an excess

of exogenous stearate) had no effect in itself on the $^{14}C/^{32}P$ ratio.

It is not known why endogenous saturated phospholipid was desaturated rapidly in all experiments in contrast with exogenous saturated phospholipid, which generally was not desaturated. The more efficient desaturation of endogenous phospholipid has been observed also with *C. vulgaris* (Gurr *et al.*, 1969) and safflower seeds (Slack *et al.*, 1979). It is puzzling why, in *M. cryophilus*, the endogenous saturated phospholipid formed from stearyl-CoA or stearyl-acyl-carrier protein was not subsequently desaturated. The explanation for this discrepancy may be that exogenous substrates are incorporated into a saturated phospholipid pool that forms micelles that are unable to make favourable contact with the desaturase. It should also be noted that of necessity the experiments in which the desaturation of endogenous phospholipid was followed were carried

Fig. 5. Possible routes in incorporation of stearate into oleoyl-phospholipid in *M. cryophilus*. Abbreviation: ACP, acyl-carrier protein.

out with intact bacteria, whereas acyl-CoA and acyl-acyl-carrier protein were added to cell-free extracts to facilitate their access to cellular enzymes. The suggested possible routes of unsaturated phospholipid biosynthesis in *M. cryophilus* are summarized in Fig. 5. Exogenous lipids and endogenous acyl-CoA enter a saturated phospholipid pool ('pool 1') that is unavailable for desaturation, whereas endogenously generated acyl-ACP enters a pool ('pool 2') that is capable of being desaturated. The present experiments do not rule out acyl-acyl-carrier protein as the substrate for desaturation, and it is possible that endogenous saturated phospholipid synthesized under anaerobic conditions by route B is converted into unsaturated phospholipid via acyl-acyl-carrier protein (by reversal of route B) on oxygenation, instead of the more direct route A involving a phospholipid substrate (Fig. 5). The rapid incorporation of exogenous stearate into oleoyl-phospholipid is consistent with a biosynthetic route via acyl-acyl-carrier protein rather than acyl-CoA. The amount of radioactivity incorporated into acyl-CoA and acyl-acyl-carrier protein pools from exogenous [$1\text{-}^{14}\text{C}$]stearate is small, so it must be assumed that the flux through acyl-acyl-carrier protein would be much faster if this interpretation of the data is correct.

References

- Baker, N. & Lynen, F. (1971). *Eur. J. Biochem.* **19**, 200–210
- Brett, D., Howling, D., Morris, L. J. & James, A. T. (1971) *Arch. Biochem. Biophys.* **143**, 535–547
- Christie, W. W. (1973) *Lipid Analysis*, 1st. edn., pp. 87–90, Pergamon Press, Oxford
- Fulco, A. J. (1974) *Annu. Rev. Biochem.* **43**, 215–241
- Fulco, A. J. & Bloch, K. (1964) *J. Biol. Chem.* **239**, 993–997
- Garbus, J., De Luca, H. F., Loomans, M. E. & Strong, F. M. (1963) *J. Biol. Chem.* **238**, 59–63
- Gurr, M. I. & James, A. T. (1980) *Lipid Biochemistry: An Introduction*, 3rd edn., pp. 42–53, Chapman and Hall, London
- Gurr, M. I., Robinson, M. P. & James, A. T. (1969) *Eur. J. Biochem.* **9**, 70–78
- Hawke, J. C. & Stumpf, P. K. (1980) *Arch. Biochem. Biophys.* **203**, 296–306
- Holloway, C. T. & Holloway, P. W. (1974) *Lipids* **9**, 196–200
- Jeffcoat, R., Brawn, P. R., Safford, R. & James, A. T. (1977) *Biochem. J.* **161**, 431–437
- Kameyama, Y., Yoshioka, S. & Nozawa, Y. (1980) *Biochim. Biophys. Acta* **618**, 214–222
- Kates, M. (1972) *Techniques of Lipidology. Isolation, Analysis and Identification of Lipids*, 1st edn., pp. 451–452, North-Holland Publishing Co., Amsterdam
- Kshirsagar, S. S. & Nair, P. M. (1979) *Biochim. Biophys. Acta* **618**, 214–222
- Lancaster, J. R. Jr. & Hinkle, P. C. (1977) *J. Biol. Chem.* **252**, 7657–7661
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- McDonald, T. M. & Kinsella, J. E. (1973) *Arch. Biochem. Biophys.* **156**, 223–231
- Oshino, N., Imai, Y. & Sato, R. (1966) *Biochim. Biophys. Acta* **128**, 13–28
- Prasad, M. R. & Joshi, V. C. (1979) *J. Biol. Chem.* **254**, 6362–6369
- Pugh, E. L. & Kates, M. (1973) *Biochim. Biophys. Acta* **316**, 305–316
- Pugh, E. L. & Kates, M. (1977) *J. Biol. Chem.* **252**, 68–73
- Pugh, E. L. & Kates, M. (1978) *Lipids* **14**, 159–165
- Russell, N. J. (1971) *Biochim. Biophys. Acta* **231**, 254–256
- Russell, N. J. (1974) *J. Gen. Microbiol.* **80**, 217–225
- Russell, N. J. (1978) *Biochim. Biophys. Acta* **531**, 179–186
- Safford, R., Jeffcoat, R. & James, A. T. (1975) *Biochim. Biophys. Acta* **409**, 86–96
- Sandercok, S. P. & Russell, N. J. (1980) *Biochem. J.* **188**, 585–592
- Simoni, R. D., Criddle, R. S. & Stumpf, P. K. (1967) *J. Biol. Chem.* **242**, 573–581
- Slack, C. R., Gratton, P. R. & Browse, J. (1979) *Biochem. J.* **179**, 649–656
- Strittmatter, P., Spatz, L., Corcoran, D., Rogers, M. J., Setlow, B. & Redline, R. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 4565–4569
- Stymne, S. & Appelqvist, L. (1978) *Eur. J. Biochem.* **90**, 223–229
- Talamo, B., Chang, N. & Bloch, K. (1973) *J. Biol. Chem.* **248**, 2738–2742
- Wilson, A. C. & Miller, R. W. (1978) *Can. J. Biochem.* **56**, 1109–1114