Vol. 142, No. 3, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 13, 1987 Pages 925-931

OCCURENCE, IDENTIFICATION AND POSSIBLE SIGNIFICANCE OF ORNITHINE LIPID IN THIOBACILLUS FERROOXIDANS

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Received November 28, 1986

SUMMARY : An ornithine containing aminolipid has been found in Thiobacillus ferrooxidans grown in ferrous sulfate medium, which was purified and estimated at four main phases of growth. GLC analysis of ornithine lipid has revealed the existence of mainly $C_{18:1}$ and $C_{22:1}$ fatty acids. The infrared spectra showed the existence of both amide and ester linkages in the aminolipid. The major ester linked fatty acid was $C_{22:1}$. The interaction of ornithine lipid with membrane was investigated by delipidation of the membrane particles,which resulted in the perturbation of the activities of the three enzymes of iron oxidation system. The activities could be restored to the lipid depleted particles by preincubation with a dispersion of purified ornithine lipid together with coenzyme Q_8. The kinetic parameters of the enzyme activities were also affected by delipidation which was significantly altered in the reconstituted particles by this lipid, thus indicating a possible role of ornithine lipid in iron oxidation system.

INTRODUCTION: The chemoautotrophic microorganism, Thiobacillus ferrooxidans was found to contain an amino lipid in its polar lipid fraction (1) which is devoid of phosphorus. The existence of this type of lipid has been encountered earlier in T.ferrooxidans grown in sulfur or thiosulfate medium (2-5) and in some other microorganisms(6-10). Ikawa(11) and Wilkinson et al(8) have suggested that the existence of this lipid might have some biochemical significances, which have yet to be determined.

The present communication reports the presence of an aminolipid,identified to be ornithine lipid in T.ferrooxidans,grown in ferrous sulfate medium and seems to be the first report of its kind in iron oxidising thiobacilli.Since this lipid is membrane associated,we assumed that it modulates the surrounding protein of iron oxidation system and hence attempts have been made to explore the possibility of a requirement for this specific lipid class for the enzymes of iron oxidation in vitro by delipidation and subsequent reconstitution of aminolipid in the delipidated

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0006-291X/87 $1.50

membrane.The kinetic parameters of the iron oxidation enzymes before and after delipidation were also investigated in the membrane pellet in presence of ornithine lipid.

MATERIALS & METHODS: Polar lipids of T.ferrooxidans(NCIM-2580,India) grown in modified 9-K media,were extracted and purified as described previously(1).During qualitative analysis on silica gel plate a distinct spot was observed to migrate very close in between phosphatidyl ethanolamine (PE) and phosphatidyl serine (PS) which was separated and purified by two dimensional chromatography, followed by rechromatography in chloroform,methanol,7-N ammonia(65,30,4;v/v) and was confirmed by different chromogenic spray reagents(1). The aminoacid component of it was further confirmed by complete acidic hydrolysis followed by chromatography,using ornithine and other aminoacids as standard.Purified lipid was preserved in chloroform as reported earlier(1).

Preparation of lysoderivatives: Purified aminolipid was converted to its lysocomponent by alkaline methanolysis(8) and after evaporation of the solvent,suspended in chloroform, purified by TLC in chloroform,methanol,acetic acid,water(65,15,10,4;v/v) using nonmethylated lipid as the standard. Both the lysocomponent and released fatty acid of aminolipid were eluted and stored as usual(1).

Delipidation of the membrane particles: Membrane was prepared by sonication of the whole cell(harvested at latelog phase in 0.05M phophate buffer,pH7.0) at 100 watts for 10 minutes(Braunsonic sonicator, Model No.1510,USA),followed by centrifugation at 35Krpm for 60 min (Beckman ultracentrifuge,model L5-50,provided with 50.2 Ti rotor) after removing the cell debris by low speed centrifugation.Membranes were washed and purified in the same buffer by homogenisation and termed as BDLP (pellet before delipidation). The identity of the membrane was ascertained by measurig cytochrome oxidase as the marker enzyme. Delipidation of BDLP and its isolation were done by the method of Lester and Fleisher(12) and Esfahani et al (13),using 90% acetone in water,followed by centrifugation at17Krpm for 15 min. The delipidated particle(DLP) after washing was suspended in the same buffer.

Reconstitution of ornithine lipid(OL)to DLP: Purified aminolipid, extracted from the cell was dispersed in 0.025M Citrate-0.05M Phosphate buffer (pH 4.5) by sonication at 4°C, followed by centrifugation at 29K rpm for 1h in SW27 rotor. The clarified dispersion of different amount of this lipid was then reconstituted with DLP by preincubating it with optimum amount of neutral lipid (NL) dispersion(~64.8 µg/ ml final concentration),having UQ_8 (extracted from T.ferrooxidans and purified).The effect of OL on the native membrane was also investigated and compared.

Enzyme studies:Effect of delipidation and reconstitution of lipids in native membrane and DLP were assessed by observing spectrophotometrically the three enzyme activities of iron oxidation system according to the published methods with necessary modifications:

1)Iron oxidase-Determining the decrease in Fe^{2+} ion concentration at 510 nm in Shimadzu-UV-Vis double beam spectrophotometer(14,15);

2)Fe-Cyt C-reductase- Observing the increase in absorbancy at 550 nm for 3 min in Cary model 17D spectrophotometer(14);

3)Cytochrome oxidase-Following spectrophotometrically the decrease in absorbancy at 550 nm for 3 min(Cary model 17D)(14).

The enzyme activities were expressed as µmole or nmole substrate oxidised or reduced h^{-1} or $min^{-1}(mg\ protein)^{-1}$.The extinction coefficient of cytC at 550 nm was assumed as 29.5 mM^{-1} Cm^{-1}.

Analysis:a) IR spectra of aminolipid and its lysocomponents were taken in KBr discs in Perkin-Elmer IR double beam spectrophotometer(Model No.237B);

b) GLC of methyl esters of aminolipid and released fatty acids of methanolysis,prepared according to Mangold et al(16),were fractionated and analysed by the methods as described earlier(1);

c)Lipid phosphorus of aminolipid was estimated according to Ames and Dubin(17);

d) Amino acid part of the lipid was estimated in the acid hydrolysate after removal of HCl by Chinard(18);

e) Presence of glycerol was ascertained by spraying the lipid with alkaline $AgNO_3$ solution after TLC by the method of Trevelyan et al(19).

RESULTS & DISCUSSIONS : Two dimensional chromatography of the polar lipids of T.ferrooxidans yielded a distinct spot lying in between PE and PS,which gave positive responses to I_2 vapour,rhodamine 6G,ninhydrine,hydroxylamine ferric chloride and 2,7-dichlorofluorescine;but remained inert towards p-quinone,molybdenum blue.It didn't respond during phosphate estimation as well as in alkaline AgNO3 solution like ornithylphosphatidyl glcerol of R.capsulate(20).The aminoacid released after acidic hydrolysis showed resemblence with the standard ornithine having same mobility in the chromatogram and similar responses to the aminoacid spray reagents.So,it appeared that the lipid is an aminolipid having ornithine in its structure,devoid of glycerol as well as phosphorus but strongly polar in nature like NPPN lipid of Paracoccus denitrificans(8).

The phospholipid (PL) and OL were isolated and estimated at four main phases of growth; It was observed that PL as well as OL values at midlog phase (Table 1) were very similar to that found by Thiel et al (21) and Wilkinson et al (8).But the most striking feature is that though the relative concentration of OL was more or less same through out the growth like P. denitrificans OL(8) but the ratios of PL to OL in the cell lowered down in late growth phases, suggesting that this aminolipid is quantitatively a major component of polar lipids in T.ferrooxidans.This result was in contrast with Minnikin and Abdolrahimzadeh (22) who reported increase in OL level in aged culture.

The IR spectrum of OL(Fig 1) showed a strong absorption band near 1731 cm^{-1},indicating the presence of an ester group.Beside this, two strong peaks at 3300 to 3000 cm^{-1} and 1470 cm^{-1} revealed firmly the presence of amide groups. The weak absorptions at 1680 to 1500 cm^{-1} and the shoulder near 1610 cm^{-1} also indicating the presence of amide groups, though these regions were poorly resolved due to the overlapping of amide, carboxylate and amine salt bands. The strong peak at 3300 to 3000 cm^{-1} indicates the presence of free amino group as NH_3^+. The presence of carboxylated anion was observed by the presence of a shoulder appeared near 1585

Table 1
Effect of culture age on the phospholipid and aminolipid content in the T. ferrooxidans cell

Phases of growth	% of Fe^{2+} consumption*	No. of viable cells ml^{-1}**	% lipid weight as PL	% lipid weight as OL***	Ratio of PL : OL
EL	46.61	$9.0x10^7$	46.36±3.42	12.76±1.19	3.63
ML	68.12	$1.5x10^8$	52.12±7.22	14.38±5.12	3.62
LL	98.72	$5.0x10^9$	23.98±2.12	15.64±2.68	1.57
STN	99.68	$2.7x10^9$	13.28±3.10	12.44±3.26	1.06

*-averages of four independent sets
**-averages of eight plates from four independent sets
***-calculated using a MW of 700 (8)
In case of lipids, results were expressed as Mean ±S.D. of four independent observations

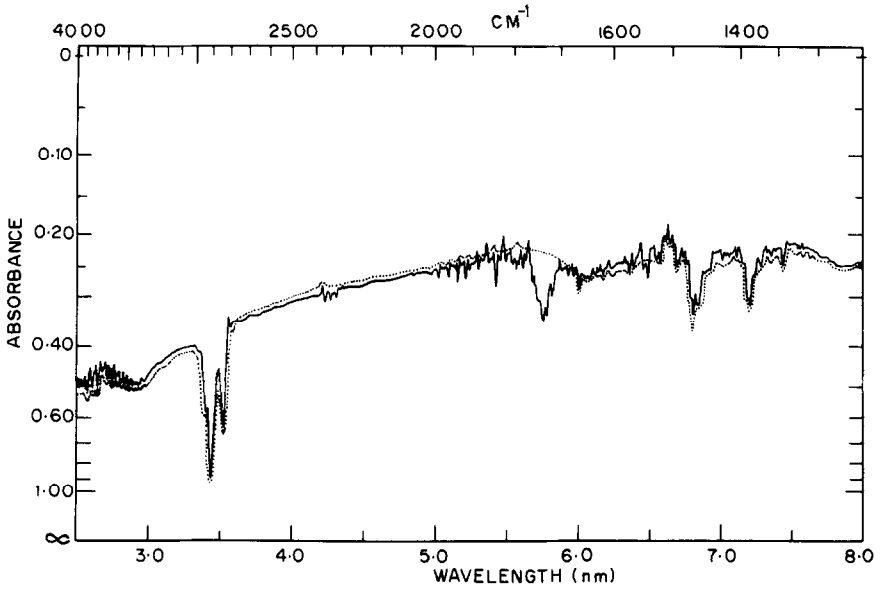

Fig.1. IR spectra of the ornithine containing lipid(___) and its lysoderivative(_ _ _) from T.ferrooxidans cell.

cm^{-1} and a band near 1400 cm^{-1} and the result is consistent with previous reports (3,4,21). GLC of fatty acids of OL revealed the presence of mainly $C_{22:1}$ and $C_{18:1}$ fatty acids, the existence of former was uncommon in its kind (3,8,21). $C_{18:3}$ was also present in an appreciable amount besides the other minor fatty acids, like $C_{20:1}$, $C_{20:2}$, $C_{16:3}$, $C_{16:1}$, $C_{16:0}$ and $C_{20:0}$. GLC spectrum after alkaline methanolysis revealed the presence of $C_{18:1}$ fatty acid in the lyso ornithine while $C_{22:1}$ was present in the released fatty acid. The methanolysis also split the ester linkages as revealed by the weak absorption band at 1731 cm^{-1} in IR spectrum of lysocomponent with concomitant release of mainly $C_{22:1}$ fatty acid. All these observations help us to elucidate the structure of OL, where probably the α-amino group of ornithine was attached by an amide linkage to a $C_{18:1}$ fatty acid residue,unlike $C_{16:1}$ fatty acid of T.thiooxidans (3) or, $C_{20:1}$ fatty acid of P.denitrificans OL (8,21) and this $C_{18:1}$ fatty acid in turn was esterified next with a $C_{22:1}$ fatty acid moiety.

The existence of OL might have some taxonomical implications, since it was found in wide varieties of microorganisms and hence there were several speculations about its role (2-5,8,11,21). Preliminary experiments indicated that 90% aquous acetone removed 40-70% polar lipids from the native membrane,resulting in a change of the activities of iron oxidation enzymes,mostly vast perturbation of cytochrome oxidase activities (Madhumita Das Gupta, Ph.D. Thesis,1985, Calcutta University). Furthermore,it was also observed that preincubation of the lipid depleted membrane with a mixture of phospholipid and neutral lipid,

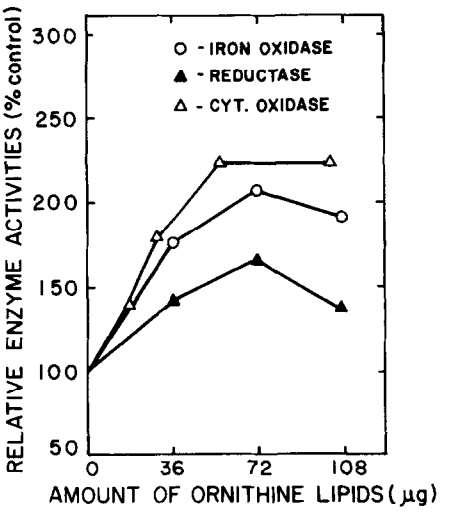

Fig.2. Effect of ornithine lipid concentration in restoration of enzyme activities in delipidated membrane.

having coenzyme Q_8 isolated from I.ferrooxidans cells could restore the lost enzyme activities in DLP relative to that observed in native membrane (Madhumita Das Gupta,Ph.D.Thesis,1985,Calcutta University).

Fig.2 revealed that the iron oxidation system in DLP was increased with the increase in the amount of OL reconstitution together with optimum NL upto a certain level;72.14 μg OL was the optimum amount to enhance 2.0 and 1.7 folds increase in iron oxidase and reductase, while 60.1 μg OL was the optimum to enhance 2.2 fold increase in cytochrome oxidase activities- thus suggesting that the particular amount of OL is deemed ncessary for catalytic activities of these enzymes. OL without NL had no effect on enzyme activiites when measured in DLP.

The kinetic parameters of three enzymes altered differently due to delipidation (Fig 3). K_m and V_{max} values of iron oxidase remained more or less unaltered (BDLP-1.34×10^{-5}M, 3.174×10^{-3}moles h^{-1} mg $protein^{-1}$; DLP-0.93×10^{-5}M,3.38×10^{-3} moles h^{-1}mg $protein^{-1}$); reductase showed higher values for both parameters (BDLP-0.72×10^{-6}M,10.75×10^{-9} moles min^{-1} mg $protein^{-1}$;DLP-4.4×10^{-6}M,20×10^{-9} moles min^{-1} mg $protein^{-1}$); while in cytochrome oxidase,the K_m values increased but the V_{max} remained unaltered (BDLP-2.1×10^{-6}M, 0.9×10^{-9} moles min^{-1} mg $protein^{-1}$,DLP-6.25×10^{-6}M,0.83×10^{-9}moles min^{-1} mg $protein^{-1}$).

Reconstitution of OL dispersions to DLP resulted in dramatic improvement of maximum velocity with concomitant increase in $K_m$values in both iron oxidase and reductase (Iron oxidase -1.92×10^{-5}M $FeSO_4$,8×10^{-3} moles h^{-1} mg $protein^{-1}$;Reductase-18.18×10^{-6}M Cyt C, 133.35×10^{-9} moles min^{-1} mg $protein^{-1}$),indicating high turnover of both enzymes with a lower affinity for substrate.Contrast to these, OL reconstitution showed stringent effect on

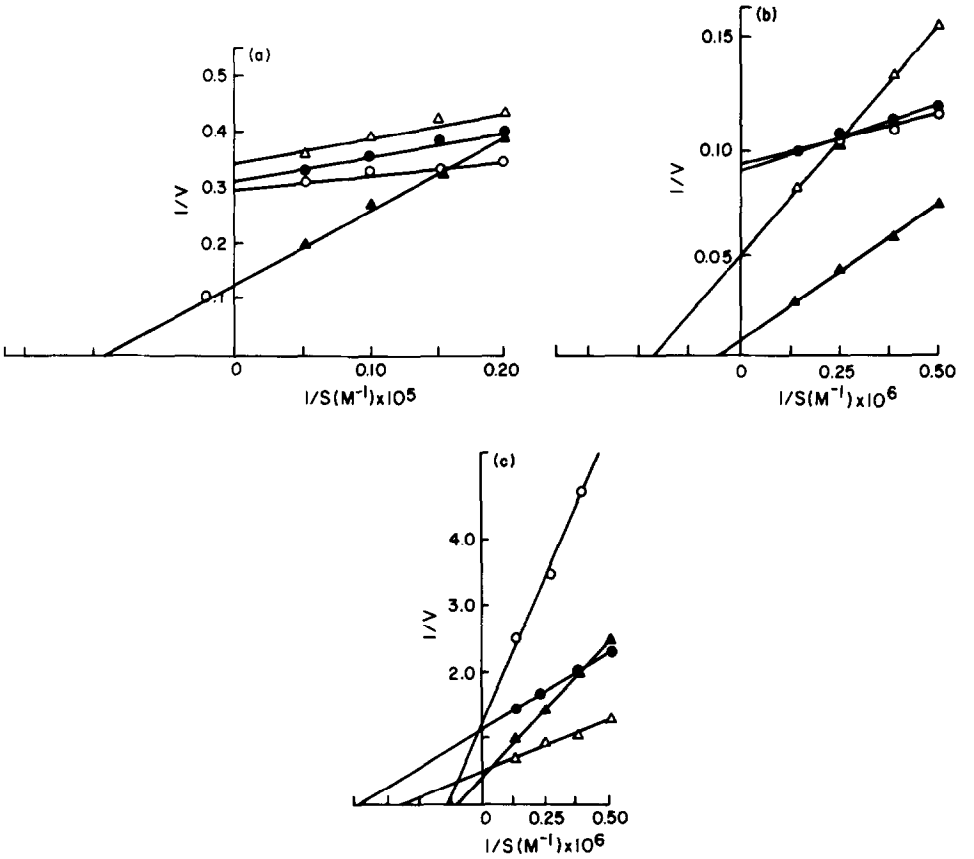

Fig.3. Kinetic parameters of a)Iron Oxidase, b)Cytochrome-C-Reductase, and c)Cytochrome Oxidase in BDLP and DLP in presence and absence of OL.
For Iron Oxidase and Cytochrome Oxidase— ●-BDLP, O-DLP, △-BDLP+OL, ▲-DLP+OL;
For Reductase—●-BDLP, △- DLP, O-BDLP+OL, ▲-DLP+OL.

cytochrome oxidase; it could not improve the K_m (10×10^{-6}M), but increased the V_{max} values (2.5×10^{-9} moles min^{-1} mg $protein^{-1}$) of cytochrome oxidase, indicating enhanced enzyme activities. Substitution of OL to native membrane slightly lowered both K_m and V_{max} values in iron oxidase, same K_m and V_{max} in reductase, and didn't at all affect the K_m in cytochrome oxidase, but doubles the V_{max} values.

So, the present studies indicated that OL is deemed necessary for the cytochrome oxidase activities mainly in T.ferrooxidans, suggesting that probably this aminolipid have some influences on the respiratory enzymes of iron oxidation system. This signified that probably in situ OL regulates the activities of these enzymes. In bacteria, this type of requirement of specific lipids for diglyceride kinase(23), crystalline pyruvate oxidase(24) and the respiratory enzymes of E.coli (24) have been reported. Fleischer et al (25) have also established the requirement of PL for the optimal activity of mitochondrial enzymes.

Thus it can be concluded that OL partly controls the iron oxidation system by showing major effects on cytochrome oxidase, which is the unique characteristic of the bacterium T.ferrooxidans and as such seems to be novel.

REFERENCES

1. Ghosh, M. and Mishra, A. K. (1985) Arch. Microbiol. 143, 297-301.
2. Shively, J. M. and Knoche, H.W. (1969) J. Bacteriol. 98, 829-830.
3. Knoche, H. W. and Shively, J. M. (1972) J. Biol. Chem. 247, 170-178.
4. Knoche, H. W. and Shively, J. M. (1969) J. Biol. Chem. 244, 4773-47778.
5. Dees, C. and Shively, J. M. (1982) J. Bacteriol. 149, 798-799.
6. Gorchein, A. (1964) Biochim. Biophys. Acta. 84, 356-358.
7. Kawai, Y., Moribayashi, A. and Yano, I. (1982) J. Bacteriol. 152, 907-910.
8. Wilkinson, B. J., Sment, K. A. and Mayberry, W. R. (1982) Arch. Microbiol. 131, 338-343.
9. Wilkinson, S. G. (1972) Biochim. Biophys. Acta. 270, 1-17.
10. Houtsmuller, U. M. T. and Van Deenan, L. L. M. (1964) Biochim. Biophys. Acta. 84, 96-98.
11. Ikawa, M. (1967) Bacteriol. Rev. 31, 54-64.
12. Lester, R. L. and Fleischer, S. (1961) Biochim. Biophys. Acta. 47, 358-377.
13. Esfahani, M., Rudkin, B. B., Cutler, C. J. and Waldron, P. E. (1977) J. Biol. Chem. 252, 3194-3198.
14. Blaylock, B. A. and Nason, A. (1963) J. Biol. Chem. 238, 3453-3462.
15. Sugio, T., Tano, T. and Imai, K. (1981) Agric. Biol. Chem. 45, 1791-1799; 405-412.
16. Mangold, H. K. (1969) Thin Layer Chromatography, Stahl E. (ed). A laboratory handbook, 2nd ed. pp. 363-421, Allen and Unwin, London.
17. Ames, B.N. and Dubin, T. (1960) J. Biol. Chem. 235, 769-775.
18. Chinard, F. P.(1952) J. Biol. Chem. 199, 91-95.
19. Trevelyan, W. E., Procter, D. P. and Harrison, J. S. (1950) Nature, 166, 444-445.
20. Wood, B. J. B., Nichols, B. W. and James, A. T. (1965) Biochim. Biophys. Acta. 106, 261-273.
21. Thiele, O.W., Biswas, C. J. and Hunneman, M. (1980) Eur. J. Biochem. 105, 267-274.
22. Minnikin, D. E. and Abdolrahimzadeh, H. (1974) FEBS Lett. 43, 257-260.
23. Schneider, E. G. and Kennedy, E. P. (1973) J. Biol.Chem. 248, 3739-3741.
24. Cunnigham, C. C. and Hager, L. P. (1971) J. Biol. Chem. 246, 1575-1582.
25. Fleischer, S., Brierly, G., Klouwen, H. and Slautterback, D. B. (1962) J. Biol. Chem. 237, 3264-3272.