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Fungal Ornithine Esterases: Relationship to Iron Transport†

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ABSTRACT: Extracts of *Fusarium roseum* (ATCC 12822) contain an enzyme which hydrolyzes the ornithine ester bonds of fusarinine C, a cyclic trihydroxamic acid produced by this organism. The methyl ester of N^{δ}-dinitrophenyl-L-ornithine is also a substrate for the enzyme, and an assay was devised using this substrate. The enzyme exhibits a sharp maximum of activity at pH 7.5 and is extremely temperature sensitive. It is strongly inhibited by $HgCl_2$ and *p*-chloromercuribenzoate, and it is competitively inhibited by N^{δ}-dinitrophenyl-D-ornithine methyl ester (K_i = 0.3 mM). Methyl esters of glycine, L-alanine, dinitrophenyl-L-alanine, dinitrophenyl-β-alanine, and N^{δ}-dinitrophenyl-N^{α}-acetyl-L-ornithine are not substrates, although N^{ϵ}-dinitrophenyl-L-lysine methyl ester is as effective as the ornithine derivative. Nonspecific lipases do not hydrolyze ornithine esters, nor does trypsin. The three ester bonds of fusarinine C are progressively hydrolyzed by the enzyme to eventually yield the monomer, fusarinine. The ferric chelate of fusarinine C is not hydrolyzed. An enzyme from *Penicillium* sp. was isolated with identical properties toward N^{δ}-dinitrophenyl-L-ornithine methyl ester as substrate. It also hydrolyzes *N,N′,N″*-triacetylfusarinine C, a cyclic trihydroxamate containing N^{α}-acetylornithine ester bonds, which is produced by this organism. This substrate is hydrolyzed to N^{α}-acetylfusarinine. In contrast to the *Fusarium* enzyme, this enzyme is fully active toward the ferric trihydroxamate chelate. However, replacement of iron by aluminum leads to a completely inactive substrate. Production of the enzyme is severely suppressed by iron in the growth medium. It is proposed that these specific ornithylesterases provide a mechanism of cellular iron release by hydrolysis of the ferric ionophores, and that an iron-exchange step occurs prior to, and is a prerequisite for, hydrolysis of the ester bonds.

It is now well established that microbial iron metabolism is dependent upon low-molecular-weight iron chelating agents, called siderochromes (Neilands, 1973; Emery, 1971a). Under conditions of iron-deficient growth, the ligand is excreted into the medium in large quantities where it solubilizes and chelates iron with an affinity constant of approximately 10^{30}. Active transport systems have been described which carry the siderochrome into the cell (Emery, 1971b; Brown and Ratledge, 1975). Iron is required for many cellular processes, and an unresolved problem is the mechanism by which the organism can dissociate the metal from chelates with such high binding constants. It has frequently been suggested that dissociation depends upon reduction of the iron to the ferrous state, for which siderochromes have little affinity (Neilands, 1973; Brown and Ratledge, 1975). On the other hand, evidence has been presented that in *Escherichia coli* iron release from the siderochrome, ferric enterochelin, is dependent upon a cellular esterase (O'Brien et al., 1971). Enterochelin contains three dihydroxybenzoylserine molecules joined in ester linkage. Hydrolysis of the ester bonds yields dihydroxybenzoylserine, a bidentate ligand which is a much poorer chelator of ferric ion than the hexadentate enterochelin.

Fusarium roseum and other fungi produce siderochromes of the trihydroxamic acid type consisting of three molecules of fusarinine, N^{δ}-(*cis*-5-hydroxy-3-methylpent-2-enoyl)-N^{δ}-hydroxy-L-ornithine, joined by head-to-tail ester bonds. The resulting cyclic trimer, in which the three hydroxamate groups form a hexadentate ligand with ferric ion, is called fusarinine C, or fusigen (Diekmann and Zähner, 1967; Sayer and Emery, 1968). Fusarinine C is believed to be a ferric ionophore in *Fusarium roseum,* but investigation of this system has been hampered by the extreme lability of the aminoacyl ester bonds in the fusarinine compounds. We have now found that *Penicillium sp.* produces a stable derivative of fusarinine C, *N,N′,N″*-triacetylfusarinine C (Moore and Emery, 1976). The purpose of this paper is to describe specific esterases of *Fusarium roseum* and *Penicillium* sp. which hydrolyze ester type siderochromes to the monomeric subunits, fusarinine and N^{α}-acetylfusarinine, respectively, and to consider the role of these unique aminoacylesterases in the release of iron from the siderochromes.

Materials and Methods

Substrates. Fusarinine C was isolated from cultures of *Fusarium roseum* as previously described (Sayer and Emery, 1968). Fusarinine C labeled with ^{14}C was prepared by growth of the organism for 3 days with 10 μCi of DL-[U-^{14}C]ornithine and 20 μCi of [2-^{14}C]acetic acid added to the medium. *N,N′,N″*-Triacetylfusarinine C was obtained from the culture supernatant of *Penicillium* sp. (Moore and Emery, 1976). The linear trimer of N^{α}-acetylfusarinine was obtained by extraction of the culture supernatant with phenol–chloroform and purification by paper electrophoresis.

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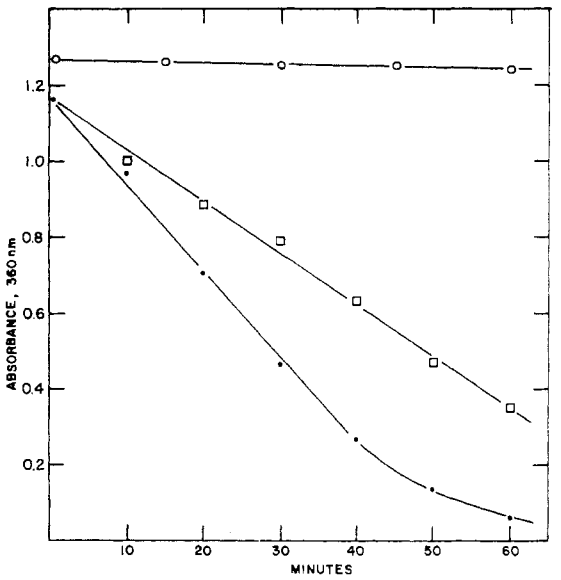

FIGURE 1: Enzymatic hydrolysis of N^{δ}-dinitrophenyl-L-ornithine methyl ester. The assay mixture at a temperature of 20 °C contained 0.1 M potassium phosphate, pH 7.5, and 0.3 mM substrate in a final volume of 6.0 ml. Reaction was initiated by addition of *Fusarium roseum* extract. Aliquots of 1.0 ml were analyzed as described under Methods. (○) No added enzyme; (□) 0.32 mg of protein; (●) 0.64 mg of protein.

Ferric chelates were made by addition of a tenfold excess of ferrous sulfate to an aqueous solution of the compound and aeration for 20 min, followed by phenol–chloroform extraction. The aluminum chelate was prepared in the same way but using a twofold excess of $AlCl_3$ in place of the iron salt and omitting the aeration step. Alternatively, the compound was shaken over a freshly precipitated and washed aqueous suspension of $Al(OH)_3$ for 4 h.

The methyl ester of N^{δ}-dinitrophenyl-L-ornithine was prepared by treatment of the dinitrophenylornithine overnight in 1 N methanolic HCl. Following removal of the solvent by evaporation in vacuo, the ester was extracted into ethyl acetate from a 3% aqueous solution of Na_2CO_3. Esters of the other amino acids or their dinitrophenyl derivatives were prepared similarly.

Hexahydrotriacetylfusarinine C was prepared by hydrogenation of an aqueous solution of *N,N′,N″*-triacetylfusarinine C (ca. 20 mg in 5 ml) over 50 mg of platinum oxide at 40 lb/in.^{2} of pressure. After 45 min the hydrogenation was complete, as evidenced by loss of absorbance at 250 nm. The ferric chelate was prepared as described above.

Preparation of Enzymes. Fusarium roseum (ATCC 12822) and *Penicillium* sp. (Moore and Emery, 1976) were grown in 400 ml of iron-deficient medium as previously described (Emery, 1965). After 3–4 days of growth, the mycelia were collected by filtration and washed twice with 0.05 M phosphate, pH 7.5. The mycelia were resuspended in the same buffer and broken in a Braun MSK homogenizer at 4000 rpm for 90 s using 0.45–0.50 mm glass beads. All subsequent operations were performed at 4 °C. The extract was centrifuged at 41 000*g* for 1 h, and the clear supernatant made to 75% saturation in ammonium sulfate. The precipitated protein was redissolved in the buffer to give a solution containing 10–20 mg/ml of protein. Extracts were stored frozen.

Enzyme Assays. Three methods of enzyme assay were used depending on the substrate.

(*A*) *Assay with N^{δ}-Dinitrophenyl-L-ornithine Methyl Ester.* The incubation mixture contained 0.10 M potassium phosphate, pH 7.5, and 0.3 mM substrate. Enzyme was added to a final volume of 6.0 ml. Temperature was maintained at 20 °C. At zero time, 1.0 ml was removed and immediately extracted with 4.0 ml of ethyl acetate after the addition of 100 mg of Na_2CO_3. The ethyl acetate was clarified by placing it over anhydrous Na_2SO_4 for 20 min, and the absorbance measured at 360 nm. Aliquots were removed at various times and treated similarly. Reaction rates were determined by the difference in absorbance using a millimolar extinction coefficient for N^{δ}-dinitrophenyl-L-ornithine methyl ester of 17.1 (Ramachandran and Sastry, 1962). Controls demonstrated that the ester quantitatively extracted into the organic phase and N^{δ}-dinitrophenyl-L-ornithine, the hydrolysis product, remained in the aqueous phase under these conditions.

(*B*) *Assay with Other Amino Acid Esters.* The incubation conditions were the same as for (A). Aliquots of about 100 μl were streaked on Whatman 3MM paper and subjected to electrophoresis in pyridine–acetate buffer as previously described (Emery, 1965). In the case of dinitrophenyl derivatives, the yellow bands were eluted with methanol and difference in absorbance at 360 nm determined. The underivatized amino acids were detected by spraying with 0.01% ninhydrin, eluting the bands with water, and determining the amino acid concentration by the method of Rosen (1957). The fusarinine compounds (metal free) were detected on the paper by spraying with 1% ferric chloride. The colored bands were eluted with water and assayed by the ferric chloride method (Emery, 1966). This method was also applicable to the aluminum chelate of triacetylfusarinine C.

(*C*) *EDTA Assay of Ferric Chelates.* The incubation mixture contained 0.05 M potassium phosphate, pH 7.5, 0.02 M EDTA,[1] adjusted to pH 7.5 with KOH, and 0.17 mM substrate, e.g., ferric *N,N′,N″*-triacetylfusarinine C, in a final volume of 3.0 ml. Reaction was initiated by addition of enzyme. Substrate was omitted from the reference cuvette. Change in absorbance at 440 nm was followed at 20 °C in a Beckman DU spectrophotometer equipped with a Gilford photomultiplier tube and power supply (Emery, 1976).

Results

Properties of the Fusarium roseum Enzyme. It has always been assumed that the monohydroxamic acids and dihydroxamic acids present in the culture supernatant solutions of *Fusarium roseum* result from spontaneous hydrolysis of the siderochrome, fusarinine C, the ester bonds of which are known to be extremely labile (Sayer and Emery, 1968). However, examination of crude extracts of the organism revealed an enzymatic activity catalyzing hydrolysis of fusarinine C. In order to facilitate enzyme assay, a structural analogue of fusarinine C, N^{δ}-dinitrophenyl-L-ornithine methyl ester, was examined as substrate, and an assay was devised utilizing this substrate (Methods). Figure 1 shows the hydrolysis of this ornithine derivative catalyzed by the crude extract. Utilizing this assay, attempts were made to purify the enzyme. A 75% ammonium sulfate fraction was obtained with a specific activity of 0.1 μmol min^{-1} mg^{-1} of protein, representing a threefold increase in specific activity over the crude extract. Further attempts at purification by chromatographic techniques led to complete loss of activity.

The esterase of *F. roseum* exhibits a temperature sensitivity unusual for an esterase. Preincubation of the enzyme at 40 °C for 20 min is sufficient to cause 85% loss of activity, and even

[1] Abbreviations used are: EDTA, (ethylenedinitrilo)tetraacetic acid; NADH, reduced nicotinamide adenine dinucleotide.

20 °C for 10 min leads to a significant loss of activity. Preincubation at 50 °C for 20 min causes virtually complete loss of activity.

The enzyme shows a sharp pH maximum of activity at pH 7.5. This is in contrast to many other carboxylesterases that show a plateau of maximum activity between pH 7–9 (Stoops, et al., 1969). At pH 6.5 or 9.0, our enzyme is about 50% as active as at pH 7.5. No activity was observed at pH 10.0.

Inhibitors. The following substances produced insignificant (<5%) inhibition at 1 mM concentration: arsenate, cyanide, fluoride, iodoacetamide, and ferrous citrate. At the same concentration, *N*-ethylmaleimide inhibited 26%. The enzyme was very sensitive to mercuric chloride and *p*-chloromercuribenzoate, each of which inhibited more than 90% at 10 μM and 70% at 1 μM. *N*-Bromoacetamide inhibited 98% at 0.1 mM. All inhibitors were tested by preincubation of the enzyme for 10 min prior to addition of substrate. The inhibition by mercuric chloride was not reversed by EDTA.

Some structural analogues of ornithine were examined as potential specific inhibitors. L-Ornithine, D-ornithine, N^{δ}-dinitrophenyl-L-ornithine, and γ-methyl L-glutamate were not significantly inhibitory at 0.3 mM. However, N^{δ}-dinitrophenyl-D-ornithine methyl ester, which is the enantiomer of our synthetic substrate, is an effective competitive inhibitor. Although accurate data are difficult to obtain because of the nature of the assay, a double-reciprocal plot showed a V_{max} of about 0.1 μmol min^{-1} mg^{-1} of protein and a K_m of about 0.1 mM calculated for the substrate. The enantiomer appears to be a competitive inhibitor with a K_i of about 0.3 mM.

Enzymatic Hydrolysis of Fusarinine C. The presence of an ornithine esterase in *F. roseum* suggests that the function of the enzyme in vivo is to hydrolyze fusarinine C, the natural siderochrome which contains ornithine ester bonds. Fusarinine C was, therefore, examined as substrate for the enzyme. Fusarinine C has three positive charges at pH 5.2 (pyridine–acetate electrophoresis buffer) due to the three free α-amino groups of the ornithine residues. Cleavage of each ester bond liberates a negative charge from the newly formed carboxylate group. Thus, the linear trimer (fusarinine B), dimer (fusarinine A), and monomer (fusarine) have two, one, and zero net positive charges, respectively. They are readily separated by paper electrophoresis (Emery, 1965). The enzymatic hydrolysis of [^{14}C]fusarinine C was followed by paper electrophoresis of the reaction mixture and scanning. The results clearly showed that the enzyme hydrolyzes ornithine ester bonds of both fusarinine C and fusarinine B to yield dimer and monomer that predominate at the end of 45 min. Use of larger amounts of enzyme and longer incubation times lead to the monomer, fusarinine, as sole product, showing that the dimer is also hydrolyzed. Controls with no added enzyme, or heated enzyme, showed negligible nonenzymatic hydrolysis under the assay conditions. Hydrolysis could also be clearly demonstrated by spraying the electrophoresis papers with ferric chloride and observing progressive disappearance of the trihydroxamic acids and eventual total hydrolysis to fusarinine.

Substrate Specificity. The possibility that the observed enzymatic activity is due to a nonspecific esterase or lipase was ruled out by examination of the substrate specificity. The methyl esters of the following compounds were examined as substrates for the enzyme: glycine, L-alanine, β-alanine, dinitrophenyl-L-alanine, dinitrophenyl-β-alanine, and N^{δ}-dinitrophenyl-N^{α}-acetyl-L-ornithine. None of these compounds were hydrolyzed to more than 5% of N^{δ}-dinitrophenyl-L-ornithine methyl ester under identical assay conditions. Definitive results could not be obtained with the methyl ester of L-ornithine due to spontaneous lactam formation by this compound. The preference for a free α-amino group in the substrate is indicated by the lack of activity of the N^{δ}-dinitrophenyl-N^{α}-acetyl-L-ornithine ester as well as the observation that *N,N′,N″*-triacetylfusarinine C has only one-third the activity as fusarinine C (Table I). The *sec*-butyl and *tert*-butyl esters of N^{δ}-dinitrophenyl-L-ornithine were also examined and found to have 45 and 23%, respectively, the activity of the methyl ester. N^{ϵ}-Dinitrophenyl-L-lysine methyl ester was as active as the ornithine derivative as substrate for the enzyme.

The enzyme is very different from typical lipases, which usually require neutral, hydrophobic compounds as substrates. Pancreatic lipase and three nonspecific lipases obtained from Dairyland Food Labs, Inc. were found to be completely inactive toward N^{δ}-dinitrophenyl-L-ornithine methyl ester. Trypsin was also inactive. Conversely, the *F. roseum* esterase did not hydrolyze benzoylarginine ethyl ester, a good trypsin substrate.

In view of the fact that enterochelin esterase of *E. coli* has been postulated to function in iron release from the ferric enterochelin chelate (O'Brien et al., 1971), it is most surprising that the *F. roseum* esterase has absolutely no hydrolytic activity toward the ferric chelate of fusarinine C (Table I).

The Esterase of Penicillium sp. While work was in progress on the *F. roseum* esterase, a new siderochrome, *N,N′,N″*-triacetylfusarinine C, was isolated from cultures of *Penicillium* sp. (Moore and Emery, 1976). It was soon found that extracts of this organism possessed ornithine esterase activity and that the properties of the enzyme using N^{δ}-dinitrophenyl-L-ornithine methyl ester as substrate were virtually identical with those described above for the *F. roseum* enzyme. However, the much greater stability of the triacetylated fusarinine C compared to the unacetylated compound facilitated the development of a spectrophotometric assay based on the fact that EDTA can not dissociate ferric ion from trihydroxamic acids, but can effectively compete with monohydroxamic acids and dihydroxamic acids for ferric ion to form the colorless ferric EDTA chelate (Emery, 1976). Thus, decrease of absorbance at 440 nm is a measure of the hydrolysis of ester bonds of the siderochrome.

The striking difference between the enzyme of *Penicillium sp.* and that of *Fusarium roseum* is that the former enzyme is completely active toward the ferric chelate of triacetylfusarinine C. The hydrolysis of ferric triacetylfusarinine C by a 75% ammonium sulfate fraction of a *Penicillium* sp. extract is shown in Figure 2. The lag phase during the first 10 min is reproducible and is explained by the fact that cleavage of the first ester bond of the substrate yields a linear trihydroxamate that still retains its chelated iron. Subsequent hydrolysis leads to dihydroxamate and monohydroxamate species and concomitant color loss. This is demonstrated by the observation that the lag phase is completely abolished when the ferric chelate of the linear trimer is used as substrate (Figure 2). Starting with either the cyclic or linear ferric trihydroxamate, only the monomer, N^{α}-acetylfusarinine, was present in the incubation mixture after 1 h. Addition of 1 mM NADH had no effect on the hydrolysis rate. The esterase of *F. roseum* is completely inactive toward ferric triacetylfusarinine C.

It is well known that the presence of iron in the growth medium suppresses synthesis of siderochromes (Emery, 1971a). Evidence that the ornithine esterase is physiologically involved in iron transport is the observation that the amount of enzyme extractable from the cells is greatly reduced when the cells are grown in an iron containing medium (Figure 3).

The linear portions of the rate curves are proportional to enzyme concentration, and by use of the EDTA assay with

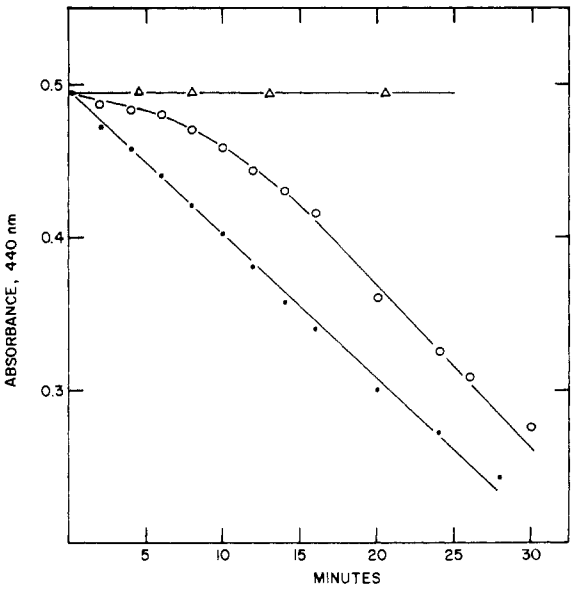

FIGURE 2: Enzymatic hydrolysis of the ferric chelates of acetylfusarinines by the esterase of *Penicillium* sp. The assay mixture contained 0.05 M phosphate (K), pH 7.5, 0.02 M EDTA, and 0.15 mM substrate in a final volume of 3.0 ml. The reaction was initiated by addition of 0.26 mg of a 75% ammonium sulfate fraction of *Penicillium* sp.: (△) no enzyme; (○) *N*, *N'*,*N''*-triacetylfusarinine C (cyclic trimer) as substrate; (●) *N*,*N'*,*N''*-triacetylfusarinine B (linear trimer) as substrate.

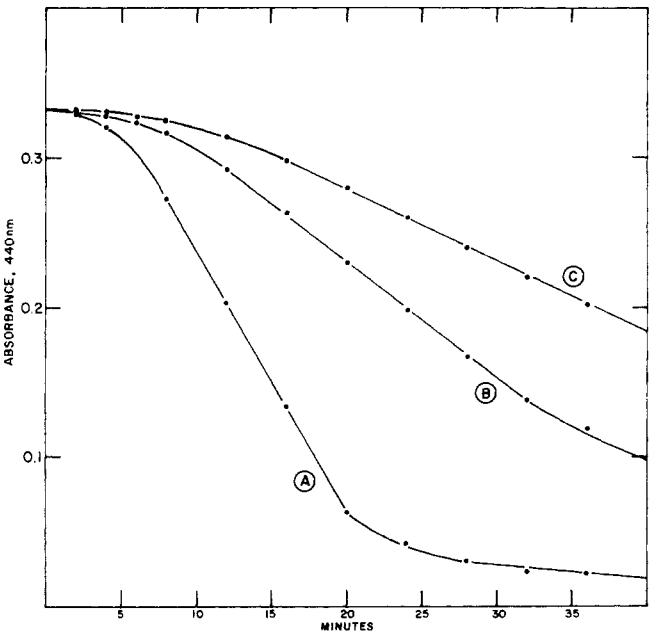

FIGURE 3: Effect of iron on the production of the ornithine esterase of *Penicillium* sp. Cells were grown for 3 days under identical conditions except for the addition of ferric citrate to the culture medium. Equal volumes (ca. 15 ml) of packed cells were broken and a 75% ammonium sulfate fraction prepared. Assay conditions were the same as for Figure 2 except that 0.52 mg of protein was used and 0.10 mM *N*,*N'*,*N''*-triacetylfusarinine C was substrate: (A) no added iron; (B) 5.0 μM iron; (C) 10.0 μM iron.

ferric triacetylfusarinine C as substrate a V_{max} of 0.031 μmol min^{-1} mg^{-1} of protein and a K_m of 0.3 mM were calculated. These values may suggest that the analogue, N^{δ}-dinitrophenyl-L-ornithine methyl ester, is a somewhat better substrate for the enzyme of *F. roseum* (see above).

The activities of the esterases of *F. roseum* and *Penicillium* sp. toward various substrates were compared, and the results

TABLE I: Relative Activities of Different Substrates with the Ornithine Esterases of *Fusarium roseum* and *Penicillium* sp.[a]

Substrate	*Fusarium roseum* Enzyme	*Penicillium* sp. Enzyme
N^{δ}-Dinitrophenyl-L-ornithine methyl ester	100	90
Fusarinine C	18	190
Ferric fusarinine C	0	1
N,*N'*,*N''*-Triacetylfusarinine C	5	6
Ferric *N*,*N'*,*N''*-triacetylfusarinine C	0	31
Aluminum *N*,*N'*,*N''*-triacetylfusarinine C	—	0
Ferric hexahydro-*N*,*N'*,*N''*-triacetylfusarinine C	—	18

[a] All activities are expressed in nmol min^{-1} mg^{-1} of protein. The 75% ammonium sulfate fraction of each enzyme was used. Refer to Methods for assay procedures: Method A for assay with N^{δ}-dinitrophenyl-L-ornithine methyl ester; method B for metal-free ligands; method C for ferric chelates.

are shown in Table I. The most significant conclusions are: (1) The enzymes are about equally active toward the synthetic substrate, N^{δ}-dinitrophenyl-L-ornithine methyl ester. (2) The enzyme of *Penicillium* is considerably more active toward the ferric chelate of its physiological substrate, *N*,*N'*,*N''*-triacetylfusarinine C, than toward the ligand itself. This is in sharp contrast to the enzyme of *Fusarium*, which is completely inactive toward the ferric chelate of its trihydroxamate, fusarinine C. (3) The *Penicillium* enzyme is about ten times more active toward fusarinine C than is the *Fusarium* enzyme. (4) Hydrogenation of the three double bonds of triacetylfusarinine C leads to a small, but significant, decrease in the rate of hydrolysis of the ferric chelate by the *Penicillium* enzyme. (5) Replacement of iron by aluminum completely abolishes the substrate activity of the *Penicillium* siderochrome.

The lack of activity of the aluminum chelate is very surprising (see Discussion). This is not due to inhibition of the enzyme, because the enzyme remains active toward the iron chelate in the presence of an equimolar concentration of the aluminum chelate, nor does addition of 0.1 mM $AlCl_3$ affect the activity of the extract. Aluminum appears to form a normal chelate with the three hydroxamate groups of the siderochrome, as evidenced by the release of three protons upon addition of aluminum chloride to a solution of the metal free ligand. The aluminum chelate is extractable into organic solvents, and the metal resists hydrolysis at pH 10.

Discussion

The properties of the esterases described in this paper support the conclusion that the physiological function of the enzymes is to specifically hydrolyze esters of ornithine or derivatives of ornithine. Particularly noteworthy is the substrate specificity. Esters of short chain amino acids or their DNP derivatives are not hydrolyzed. Furthermore, the ornithine moiety must be of the levorotatory configuration, N^{δ}-dinitrophenyl-D-ornithine methyl ester being an effective competitive inhibitor of the enzyme. This is in contrast to pig liver carboxylesterase, which hydrolyzes L- and D-tyrosine ethyl esters equally rapidly (Stoops et al., 1969). It can not be fortuitous that such enzymes are found in organisms that synthesize iron chelating agents containing ornithine esters.

Enterochelin (enterobactin) is a siderochrome of the phenolic type produced by *E. coli*. Enterochelin is composed of 3 mol of dihydroxybenzoylserine in head-to-tail ester linkage to give a cyclic triester. An esterase is present in *E. coli* capable of hydrolyzing the ester bonds (O'Brien et al., 1971). These workers suggest that the physiological function of enterochelin esterase is to hydrolyze the ferric chelate of enterochelin in order to facilitate release of the metal. Subsequent to hydrolysis of the chelate, the metal can be reduced to the ferrous state for incorporation into protoporphyrin by ferrochelatase (Porra et al., 1972). Reduction of the metal can only be accomplished when it is chelated to the bidentate hydrolysis products, but not when the metal is complexed to the hexadentate enterochelin. It is virtually impossible to maintain iron in the ferrous state in the presence of oxygen and siderochrome ligands. Thus, hydrolysis of the chelate provides the thermodynamic drive to allow metal reduction and transfer.

In direct conflict with the results of O'Brien et al. is a report that the identical enzyme has *no* activity toward ferric enterochelin, but only hydrolyzes the metal-free substrate (Bryce and Brot, 1972). To date, this discrepancy has not been resolved.

A similar paradox arises in our own work. Although we believe that the esterases described in this paper are for the physiological purpose of iron release, in agreement with O'Brien et al., we find that the enzyme of *Fusarium roseum* is completely inactive toward the ferric chelate, in direct contrast to the enzyme of *Penicillium* sp. We believe that these conflicting results can be explained if one postulates that removal of the metal is a prerequisite for ester bond hydrolysis. Because of the sensitivity of our enzymes to mercury, we favor a reductive mechanism. In our assay there are no reducing equivalents present, and NADH does not increase the rate of hydrolysis, so the iron must begin and end in the ferric state. According to this mechanism, there is a transient reduction of the iron by a sulfhydryl group on the protein. Ester bond hydrolysis is concerted with the dissociation of the ferrous ion from the ligand. The enzyme then becomes reduced again by concomitant reoxidation of the metal. The metal is trapped as the ferric chelate of EDTA in our assay. Under physiological conditions, however, we assume that the metal is incorporated into protoporphyrin (Porra et al., 1972). Because of the crude nature of our preparation, it is not possible to say whether catalysis of metal exchange resides in the esterase itself or in a different protein. It is interesting that similar exchange mechanisms have been invoked to explain iron mobilization in higher organisms (Harrison et al., 1974).

The mechanism postulated above, that is, release of the metal prior to ester bond hydrolysis, explains why some enzyme preparations are active with the metal chelate and others are not. The former, such as the enzyme from *Penicillium* sp., are capable of the reductive removal of the metal. The metal exchange activity is more labile in the case of the *Fusarium roseum* enzyme and is lost during isolation; hence, this enzyme cannot hydrolyze the ferric chelate. If this explanation is correct, it suggests that the exchange activity of enterochelin esterase may also be lost during preparation of the enzyme (cf. Bryce and Brot, 1972). It may be significant in this regard that O'Brien et al. (1971) report that enterochelin esterase can be separated into two inactive components during column chromatography of the enzyme. Full activity toward the ferric enterochelin was restored upon mixing the two fractions. They did not investigate the possibility that one of the components retains esterase activity toward the metal free enterochelin.

Support for the above hypothesis comes from the observation that the esterase of *Penicillium* sp. is completely inactive toward the aluminum chelate of *N,N',N''*-triacetylfusarinine C, in contrast to the ferric chelate. It is unlikely that replacement of Fe^{3+} by Al^{3+} alters the chemical properties of the ester bonds, which are not involved in the chelation. Detailed proton magnetic resonance studies by Neilands and his collaborators have shown that replacement of iron by aluminum in the trihydroxamate, ferrichrome, only slightly alters the conformation of the compound (Llinás et al., 1972). Ferrioxamine E is structurally very similar to the fusarinines, with amide bonds instead of ester bonds, and the crystal structure of this compound shows that the amide bonds are remote from the chelation center (van der Helm and Poling, 1976). Examination of a molecular model of triacetylfusarinine C also suggests that the ester bonds are not close to the metal. Furthermore, hydrogenation of the three double bonds of triacetylfusarinine C would be expected to alter the conformation of the molecule in the vicinity of the ester bonds to a much greater extent than substitution of iron by aluminum, yet hydrogenation has a much smaller effect on reactivity with the enzyme than does substitution of iron by aluminum (Table I). We propose that the presence of a metal in the vicinity of the esteratic site of the enzyme is inhibitory, and reductive removal of the metal is a prerequisite for hydrolysis. Since the aluminum cannot be reduced, ester bond hydrolysis cannot occur. It is interesting in this regard that the specific ferrichrome uptake system of *Ustilago sphaerogena* is completely active with alumichrome, but the cells are then unable to dissociate the aluminum from the chelate (Emery, 1971b). Efforts are underway to demonstrate that the siderochrome esterase activity of *Penicillium* sp. is dependent upon a metal exchange reaction.

In order to avoid a futile cycle, the esterase activity of these organisms must be separated from the biosynthetic enzymes. O'Brien et al. (1971) concluded that compartmentalization of the esterase and biosynthetic enzymes occurs in *E. coli*. Since the function of the siderochromes is to transport extracellular iron into the cell, it is logical to assume that synthesis occurs in the outer membrane and the ligand is secreted directly from the cell. Chelation of iron allows re-entry into the cytoplasm where the esterase can catalyze release of the metal.

Acknowledgments

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Sulfatides of *Mycobacterium tuberculosis:* The Structure of the Principal Sulfatide (SL-I)†

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ABSTRACT: The gross structural features of five families of multiacylated trehalose 2-sulfates elaborated by *Mycobacterium tuberculosis* strain H37Rv are described. The principal sulfatide SL-I is a 2,3,6,6′-tetraacyl-α,α'-D-trehalose 2′-sulfate, whose component carboxylate substituents (and homology) were previously established. In the present study the specific locations of the acyl substituents were assigned. The desulfated glycolipid (SL-I-CF) was methanolyzed on a column of diethylaminoethylcellulose (free base form), affording tri-, di-, and monoacylated trehalose mixtures. The most abundant diacyltrehalose generated was identified as 6,6′-bis(2,4,6,8,10,12,14,16-octamethyl-17-hydroxydotriacontanoyl)trehalose (6,6′-bis(C_{40}-hydroxyphthioceranoyl)trehalose), along with lower and higher homologues. A small amount (about 15%) of the unhydroxylated analogue (phthioceranate) was also recognized. From the monoacylated carbohydrate mixture (chiefly 6-(C_{40}-hydroxyphthioceranoyl)trehalose) surviving trehalose monopalmitate(s) were isolated by preparative gas chromatography of the trimethylsilylated products. Trehalose 2-palmitate was identified as the principal component. Small amounts of the 3 isomer may also be present, but no 6-palmitate was detectable. Gentle acidic solvolysis, which minimizes the possibility of acyl migrations, afforded a different diacyltrehalose, identified by mass spectrometry of the permethylated derivative as principally 2-palmitoyl(stearoyl)-3-phthioceranoyltrehalose. A variant in which hydroxyphthioceranate substitutes at the 3 position was also recognized. The results indicate that the biological acylation processes at the trehalose core are not entirely specific, but instead yield an SL-I family, for the chief member of which a logical structural expression is deduced.

The sulfatides (sulfolipids (Middlebrook et al., 1959)) of *Mycobacterium tuberculosis* comprise some five or more families[1] of multiacylated trehalose sulfates. These contain, in addition to palmitic–stearic acids, several unique multimethyl-branched substituents: phthioceranic and hydroxyphthioceranic acids (see below). In our earlier studies, individual glycolipid sulfates were separated and identified as multiesters of trehalose 2-sulfate. The gross distribution and proportions of the various acyl substituents within the individual sulfatides were determined and reported in part; and the chemical structures of the phthioceranic and hydroxyphthioceranic families of homologues were elucidated (Goren, 1970b; Goren et al., 1971); see Table I.

In the interim, we have learned that the sulfatides may play an important role in the pathogenesis of tuberculosis, probably promoting intracellular survival of the tubercle bacillus by antagonizing phagosomal–lysosomal fusion within phagocytic cells (Goren et al., 1974; Goren, D'Arcy Hart, Young, and Armstrong, in preparation). With these and other biological activities demonstrated (Kato and Goren, 1974), studies to delineate complete structural expressions for the more abundant sulfatides were more vigorously pursued. In the present study we summarize the gross structures of five mycobacterial sulfatides so far characterized and report the structure(s) of the principal sulfatide family (SL-I[2]) of the strain H37Rv.

Materials and Methods

Surface culturing of *M. tuberculosis,* strain H37Rv, lipid extractions, and column chromatographic separation of the various sulfatide families on diethylaminoethylcellulose

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[1] Designated "families" because of the extensive homology characterizing the acyl substituents and also because of a degree of randomness in the specific location of individual acyl functions, as documented in the present study.

[2] Abbreviations used are: SL-I to -III, the intact sulfated glycolipids, SL-CF for the desulfated analogues; DEAE-cellulose, diethylaminoethylcellulose; Me_3Si, trimethylsilyl; GLC, gas–liquid chromatography; TLC, thin-layer chromatography; mu, mass units; ir, infrared; NMR, nuclear magnetic resonance; E–M, ether–methanol; MS, mass spectroscopy.