Biochimica et Biophysica Acta, 657 (1981) 313—320

BBA 69189

PRELIMINARY CHARACTERIZATION OF FERRICHROME SYNTHETASE FROM *ASPERGILLUS QUADRICINCTUS*

WERNER HUMMEL * and HANS DIEKMANN

Lehrgebiet für Mikrobiologie der Universität Hannover, Schneiderberg 50, D-3000 Hannover (F.R.G.)

(Received July 21st, 1980)

Key words: Ferrichrome synthetase; Non-ribosomal peptide synthesis; Iron repression (Aspergillus quadricinctus)

Summary

An enzyme synthesizing the cyclic hexapeptide, ferrichrome, was partially purified from extracts of *Aspergillus quadricinctus* by fractional $(NH_4)_2SO_4$ precipitation and Bio-Gel A 1.5 m filtration. About a 20-fold purification was achieved. The enzyme system incorporated δ-*N*-acetyl-δ-*N*-hydroxyornithine into ferrichrome and catalyzed ATP-PP_i exchange reactions, dependent on the constituent amino acids, glycine and δ-*N*-acetyl-δ-*N*-hydroxyornithine, in the presence of Mg^{2+}. The optimal temperature was 27°C.

K_m values were $3.1 \cdot 10^{-4}$ M for glycine and $5.3 \cdot 10^{-6}$ M for δ-*N*-acetyl-δ-*N*-hydroxyornithine. Both K_m values were significantly lowered in the presence of $1 \cdot 10^{-6}$ M Fe^{3+}. From the inhibition experiments it is concluded that sulfhydryl groups of the enzyme are involved. Both monomers are covalently bound to the enzyme in the course of the reaction. A molecular weight of $1.1 \cdot 10^6$ was determined by gel filtration. As the partially purified protein fraction also catalyzed transacetylation of hydroxyornithine from acetyl CoA, the peptide synthesizing activity may be part of a multienzyme complex.

No ferrichrome synthetase activity can be found when the fungus is grown in the presence of $1 \cdot 10^{-5}$ M Fe^{3+}.

Introduction

Whereas numerous publications have appeared on non-ribosomal peptide synthesis in procaryotes, very little is known about cell-free biosynthesis by a

* Present address: Gesellschaft für Biotechnologische Forschung, Mascheroder Weg 1, D-3300 Braunschweig-Stöckheim, F.R.G.

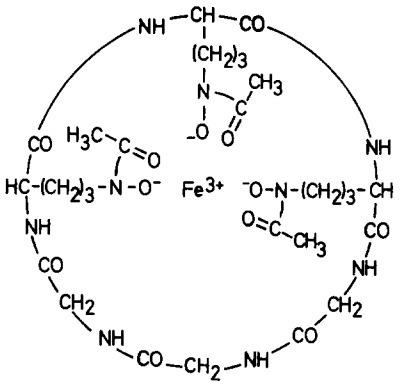

Fig. 1. Chemical structure of ferrichrome [2].

comparable enzyme system in eucaryotes. In a previous paper [1], it was reported that we succeeded in preparing cell-free extracts from *Aspergillus fumigatus* and *Aspergillus quadricinctus* catalyzing the incorporation of L-glycine into ferricrocin and ferrichrome (Fig. 1). To elucidate the mechanism of biosynthesis of cyclic hexapeptides in fungi, we have partially purified and characterized the enzyme from *A. quadricinctus*.

Materials and Methods

Radioactive materials and chemicals. ^{14}C-Labelled amino acids and [^{32}P]PP$_i$ were purchased from New England Nuclear (Dreieichenhain), [^{3}H]acetic anhydride from Amersham Buchler (Braunschweig). All nucleotides and proteins for molecular weight determination were obtained from Boehringer Mannheim, the chemicals for the fermentation media and TLC and electrophoresis from E. Merck (Darmstadt). The sources of other materials are indicated in the text.

Microorganisms and the preparation of cell-free extract. A. quadricinctus Yuill (CBS 135.52) was grown on a glucose-asparagine medium as described in earlier publications [3,4]. Cell-free extracts were prepared as published [1], except that the mycelium was homogenized prior to sonication by a 5 s treatment with an Ultra Turrax (Janke and Kunkel, Staufen).

Preparation of δ-N-acetyl-δ-N-hydroxy-L-ornithine. The published method [5] for the preparation of acetylhydroxyornithine by acetylation of hydroxyornithine, that had been obtained by hydrolysis of fusigen, was modified. Hydroxyornithine was chromatographed on Dowex 50W-X8 (Serva Feinbiochemica, Heidelberg) and assayed by iodine oxidation [6]. The yield was 83%.

Samples of the acetylation product were purified by preparative thin layer electrophoresis [7] at pH 5.0, on cellulose (20 V/cm, 35 mA, 30 min). The neutral acetylhydroxyornithine moved 1.2 cm towards the cathode, its location being indicated by spraying with ninhydrin. From each plate (20 × 20 cm) 1 mg acetylhydroxyornithine was recovered.

^{3}H-Labelled acetylhydroxyornithine was prepared by acetylation of 30 mg hydroxyornithine with 10 mCi [2-^{3}H]acetic anhydride (spec. act. 10 mCi/

mmol). The yield was 7.8 mg ^{3}H-labelled acetylhydroxyornithine. The substance was ninhydrin and $FeCl_3$ positive, and did not react with triphenyl tetrazolium chloride or in the iodine oxidation test. The absorption coefficient in the $Fe(ClO_4)_3/HClO_4$-test [8], $\epsilon^{505}_{mM} = 0.94$ was used.

Assay of ferrichrome synthesis. Ferrichrome-synthesizing activity was assayed by measuring the incorporation of radioactivity when one of the two constituent amino acids was labelled. The reaction mixture contained the following in a final volume of 1 ml; 50 μmol Tris-HCl buffer (pH 7.2)/10 μmol $MgCl_2$/12 μmol ATP (neutralized)/2 μmol dithiothreitol (Sigma, St. Louis)/either 1 μCi L-[^{14}C]glycine (0.02 μCi/mmol) plus 0.01 μmol acetylhydroxyornithine or 0.4 μCi [*acetyl*-^{3}H]-acetylhydroxyornithine (10 mCi/mmol) plus 5 μmol glycine and enzyme solution. The mixture was incubated at 22° C for 20 min. The reaction was terminated by adding 20 ml ethanol/2 mg $FeCl_3 \cdot 6\ H_2O$/0.5 mg ferrichrome. Isolation and purification of ferrichrome was performed as described [1]. Radioactivity was measured in a scintillation counter (Isocap 300, Nuclear Chicago, Amsterdam) using Instagel (Packard, Frankfurt) as a solvent and external standardization.

Assay of ATP-[^{32}P]PP_i exchange. Isotopic exchange between pyrophosphate and ATP was measured as described by Calendar and Berg [9] using the modification of Walker et al. [10]. The mixture was incubated at 25°C for 10 min. Radioactive ATP was collected on charcoal filter (Ederol 69/K, Bintzer, Hatzfeld) according to Simlot and Pfänder [11] and counted in Quickzint 501 (Zinsser, Frankfurt).

Results

Preparation of partially purified enzyme. The time course of fermentation is shown in Fig. 2. The mycelium from 20 erlenmeyer flasks (150 ml medium

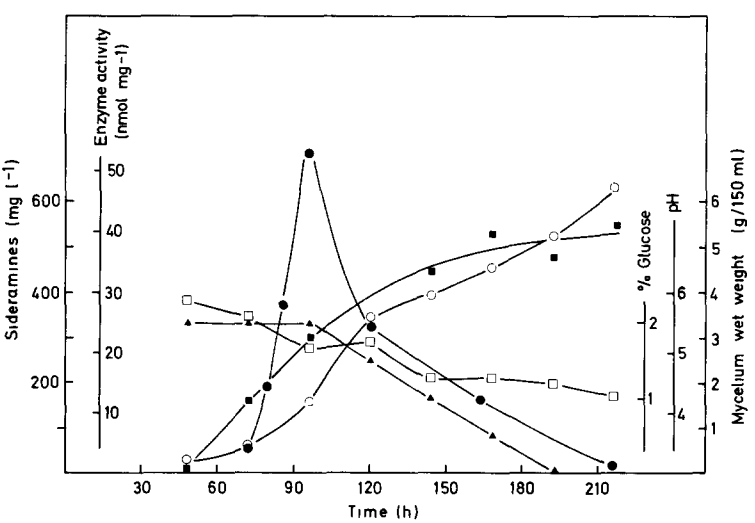

Fig. 2. Time course of fermentation by *Aspergillus quadricinctus*. ■———■, mycelium weight; ●———●, specific enzyme activity; ○———○, total sideramines (mainly ferrichrome); ▲———▲, glucose; □———□, pH value.

TABLE I

PURIFICATION OF FERRICHROME SYNTHETASE

Enzyme activity incorporation test with 12 mM ATP, [*acetyl*-^{3}H]acetylhydroxyornithine as labelled substrate, 21° C.

Fraction	Volume (ml)	Protein (mg/ml)	Enzyme activity		Purification
			total (dpm)	specific (dpm/mg)	
$(NH_4)_2SO_4$ precipitation					
0—40%	3	1.55	1 767 000	380 000	6.6 *
40—65%	3	3.30	316 000	32 000	0.6 *
65—90%	3	3.89	128 000	11 000	0.2 *
Bio-Gel A 1.5 m	1.6	0.22	437 760	1 216 000	3.2 **

* Related to protein in all extract after precipitation with 90% $(NH_4)_2SO_4$.
** Related to 0—40% $(NH_4)_2SO_4$ fraction.

each) was harvested when the concentration of ferrichrome in the medium reached 200 mg/l. After cell disruption by homogenization and sonification the suspension was filtered and centrifuged at 8000 × *g* for 20 min. The supernatant (protein content 1—2 mg/ml) was subjected to fractional $(NH_4)_2SO_2$ precipitation. The fraction precipitated by 40% saturation (Table I) was applied to a Bio-Gel A 1.5 m column (Biorad, München; 100—200 mesh, 2 × 94 cm) and eluted by 0.1 M Tris-HCl buffer, pH 7.2, containing 1 mM dithiothreitol (Fig. 3). The overall purification in the pooled fractions was about 20-fold (Table I).

δ-N-Acetyl-δ-N-hydroxy-L-ornithine as a precursor of ferrichrome biosynthesis. To answer the question whether acetylhydroxyornithine might function as a precursor for ferrichrome synthesis, 1.35 μCi radioactive-labelled acetylhydroxyornithine was added to an incorporation test with a total volume of 5 ml, containing $1 \cdot 10^{-5}$ M glycine and 12.2 mg crude enzyme. After incuba-

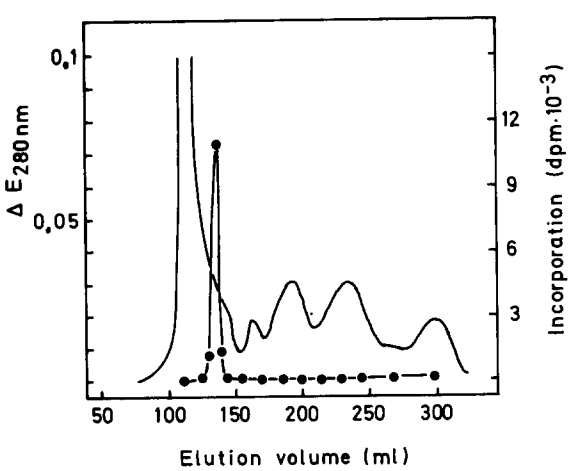

Fig. 3. Filtration of crude ferrichrome synthetase on Bio-Gel A 1.5 m. ●———●, enzyme activity determined in the incorporation test with labelled acetylhydroxyornithine (see text); 12 mM ATP; 21° C.

TABLE II

ACTIVATION OF GLYCINE AND ACETYLHYDROXYORNITHINE BY PURIFIED FERRICHROME SYNTHETASE

AHO, acetylhydroxyornithine.

Substrate	Protein (mg)	Exchange	
		(dpm)	(nmol)
—	0.103	448	10.9
Glycine $1 \cdot 10^{-3}$ M	0.103	2046	49.9
AHO $1 \cdot 10^{-5}$ M	0.103	2128	51.9
Glycine $1 \cdot 10^{-3}$ M + AHO $1 \cdot 10^{-5}$ M	0.103	559	13.6

tion at 21°C for 15 min and careful purification of ferrichrome by TLC on silica gel, firstly with the solvent system chloroform/methanol/water (65 : 25 : 4), secondly *n*-propanol/acetic acid/water (4 : 1 : 1) (for experimental details see Ref. 1), about 1% of the radioactivity was found in ferrichrome. With higher concentrations of glycine ($5 \cdot 10^{-3}$ M) up to 15% incorporation was achieved.

Amino acids dependent ATP-PP_i exchanges. When aminoacyl adenylates are formed in the first step of the reaction sequence, exchange of radioactivity from [^{32}P]PP_i to ATP may be observed. Results of exchange experiments are shown in Table II. It is remarkable that exchange is almost negligible when both amino acids are added together. This may indicate that during ferrichrome biosynthesis the concentrations of aminoacyl adenylates are extremely low.

Variations of the ferrichrome synthetase test. The test shown in Table III indicated that all components of the test mixture, except dithiothreitol which had an protecting effect, were absolutely necessary for ferrichrome synthesis. Mg^{2+} could be replaced by the same concentration of Mn^{2+}, but not by Ca^{2+}, Zn^{2+}, Co^{2+} or Fe^{2+}. ATP cannot be replaced by GTP, CTP or UTP. When L-alanine was added instead of glycine or L-serine was added together with glycine, no synthesis of a hexapeptide containing L-Ala or L-Ser was observed. Further-

TABLE III

OMISSION TEST FOR FERRICHROME SYNTHETASE

AHO, acetylhydroxyornithine.

Test	Incorporation *		Activity (%)
	(dpm)	(nmol)	
Complete	650	59.0	100
Dithiothreitol	260	23.6	40.1
Mg^{2+}	22	2.0	3.3
ATP	9	0.8	1.4
AHO	16	1.5	2.4
Protein	12	1.1	1.8

* Test contains in 1 ml: 1.02 mg raw extract protein/0.1 μCi [^{14}C]glycine (20 μmol)/0.1 M Tris-HCl, pH 7.2.

TABLE IV

K_m VALUES AND MAXIMAL VELOCITIES FOR GLYCINE AND ACETYLHYDROXYORNITHINE IN THE PRESENCE OF $1 \cdot 10^{-6}$ M Fe^{3+} (0.188 MG PROTEIN/TEST)

AHO, acetylhydroxyornithine.

Amino acid	Fe^{3+}	K_m (M)	V (nmol · min^{-1})
Glycine	—	$3.08 \cdot 10^{-4}$	0.80
Glycine	$1 \cdot 10^{-6}$ M	$2.53 \cdot 10^{-4}$	0.80
AHO	—	$5.34 \cdot 10^{-6}$	1.05
AHO	$1 \cdot 10^{-6}$ M	$3.72 \cdot 10^{-6}$	1.05

more, no ferrichrome synthesis took place when the linear di- or tri-peptides of glycine (Fluka, Neu-Ulm) replaced glycine.

In amino acid activation reactions it is quite usual to observe a sharp peak of activity dependent on ATP concentration. We found a concentration of 11 mM ATP optimal in crude extracts, but 2 mM optimal after purification by gel filtration. In both cases, higher concentrations of ATP are strongly inhibitory.

While testing the temperature dependence of the enzyme we were surprised to find that at 22°C (optimal) the activity is a 1000-fold higher in crude extracts than at 37° C. Optimal temperature for the partially purified enzyme was 27°C (more than a 3-fold increase in activity compared to that at 37°C).

K_m values and activation by Fe^{3+}. K_m and V values for glycine and acetylhydroxyornithine were determined by using the ferrichrome synthetase test and varying the concentrations of the amino acids. Data were plotted according to Lineweaver and Burk [12]. Results are given in Table IV.

While studying the effect of Fe^{3+} on ferrichrome synthetase (enzyme) synthesis, we had to check the possible influence of Fe^{3+} on the ferrichrome synthetase reaction. In crude extracts we observed a 50% activation of ferrichrome synthesis at $1 \cdot 10^{-6}$ M Fe^{3+}. Addition of Fe^{3+} to purified protein in experiments for K_m and V determinations revealed a significant lowering of K_m values for both amino acids, leaving the V values unchanged (Table IV).

Inhibition by SH-group blockers and covalent binding of amino acids. To investigate inhibitory effects of sulfhydryl blockers, dithiothreitol was omitted from the ferrichrome synthetase test mixture. Both reagents tested, *p*-chloromercuribenzoate and *N*-ethylmaleinimide were strongly inhibitory, leading to 50% reduction of enzymatic activity at $1.1 \cdot 10^{-6}$ M and $1.0 \cdot 10^{-5}$ M, respectively.

In the previous publication it was shown that addition of ribonuclease to the test system had no effect on ferrichrome synthesis [1]. In order to demonstrate covalent binding of glycine and acetylhydroxyornithine, as was postulated in analogy to the reaction mechanism in gramicidin S [13], thyrocidine and bacitracin biosynthesis, labelled precursors were added to the reaction for ferrichrome synthesis singly or together. The mixture was incubated at 22°C for 30 min and the reaction stopped by abrupt cooling at 0°C. The solution was then applied to a Bio-Gel P60 column (1 × 30 cm) and eluted with 0.1 M Tris-HCl, pH 7.2, 1 mM dithiothreitol buffer. The exclusion volume was 6 ml;

monomeric amino acids were eluted at 17 ml. Protein eluted around the exclusion volume was collected and the radioactivity in this pool counted. 10 μCi [^{14}C]glycine alone yielded 9760 dpm in the protein peak; 5 μCi [^{3}H]acetylhydroxyornithine yielded 24 780 dpm; the same amounts of both components added together yielded only 32 dpm. The label was retained when the protein was precipitated by trichloracetic acid. This fact mainly seems to prove covalent binding of both amino acids. With both amino acids present, the very low label might be explained by an instant release of ferrichrome after completion.

Molecular weight of ferrichrome synthetase and test for transacetylase activity. The same column that had been used for preparative isolation of ferrichrome synthetase (Fig. 3) was calibrated with bovine serum albumin, aldolase, catalase and ferritin. The peak of ferrichrome synthetase has K_{av} value of 0.12 which corresponds to a molecular weight of about $1.1 \cdot 10^6$.

The rather high molecular weight gave rise to the speculation whether the protein possibly catalyses other reactions besides peptide bond synthesis. Earlier work in Emery's [14] and our laboratory [5] had shown that in the fungi tested, ornithine is hydroxylated to hydroxyornithine first and then actylated to the hydroxamate. Therefore, we have tested the partially purified protein for transacetylase activity according to the method of Ong and Emery [14].

After incubation at 27°C for 20 min formation of 1.1 nmol hydroxamate was determined. The same amount of protein formed in a parallel synthetase test under the same conditions 2.2 nmol ferrichrome.

Repression of ferrichrome synthetase biosynthesis by Fe^{3+}. *A. quadricinctus* was grown in glucose-asparagine medium containing increasing amounts of Fe^{3+} ($1 \cdot 10^{-8}$ up to $1 \cdot 10^{-4}$ M). The mycelia were collected by centrifugation after 3 days and disrupted by ultrasonic treatment. Protein was precipitated by $(NH_4)_2SO_4$ (0.90 s). The supernatant from the fermentation broth was assayed for ferrichrome content.

The activity of ferrichrome synthetase was repressed to 50% (compared with medium without addition of Fe^{3+}) at $2.0 \cdot 10^{-7}$ M Fe^{3+}. At this iron concentration the extracellular ferrichrome content is lowered by 15% only. At $1 \cdot 10^{-5}$ M Fe^{3+} in the medium, no ferrichrome synthetase could be detected, the residual ferrichrome content was 15%.

Discussion

The partial purification (removing endogeneous substrates) of ferrichrome synthetase allowed a preliminary characterization of the enzyme from *A. quadricinctus*. It activates and binds glycine and δ-*N*-acetyl-δ-*N*-hydroxyornithine prior to the formation of the hexapeptide ring. The introductory reaction is the reversible formation of aminoacyl-AMP from amino acid and ATP. The overall reaction may be formulated as

$$3\ \text{Gly} + 3\ \text{acetylhydroxyornithine} \xrightarrow{\text{ATP,Mg}^{2+}} \text{ferrichrome} + 6\ H_2O$$

Because of the low purity of the enzyme we did not persue the activation by Fe^{3+} at the moment. Likewise, K_m values and the optimal concentration of ATP must be rechecked after complete purification.

We have no interpretation for the unusual temperature optimum at 27°C. No

indications for a similar behaviour of enzymes catalyzing the synthesis of gramicidin S, tryrocidine and bacitracin could be found in the literature. On the other hand, Ong and Emery [14] mentioned the increased inactivation of δ-*N*-hydroxyornithine: acetyl CoA-δ-*N*-transacetylase from *Ustilago sphaerogena* at temperatures above 20°C.

The molecular weight of ferrichrome synthetase ($1.1 \cdot 10^6$) is even higher than that which was found before [15] for fusigen synthetase from *Fusarium cubense* ($8 \cdot 10^5$). From studies of the biosynthesis of gramicidin S and valinomycin it was concluded that each protein chain that activates a single amino acid has a molecular weight of about $7 \cdot 10^4$ or $8 \cdot 10^4$, respectively [16,17]. According to this consideration the molecular weight of ferrichrome synthetase is much higher than necessary for the activation of 3 glycine and 3 acetylhydroxyornithine molecules and the cyclization steps. One might speculate that ferrichrome synthetase is a multienzyme complex carrying other catalytic properties. Indeed, first tests for transacetylase activity were positive. We are now looking particularly for an ornithine hydroxylating enzyme.

The repression of enzyme biosynthesis by Fe^{3+} is still another example for the well known regulation of enzymes that participate in the synthesis of siderophores (iron-complexing metabolites) [18—23].

Acknowledgement

This work was supported by a grant from the Deutsche Forschungsgemeinschaft.

References

1 Müller, H.-G. and Diekmann, H. (1977) Arch. Microbiol. 113, 243—246
2 Rogers, S.J., Warren, R.A.J. and Neilands, J.B. (1963) Nature (London) 200, 167
3 Diekmann, H. (1967) Arch. Microbiol. 58, 1—5
4 Diekmann, H. and Krezdorn, E. (1975) Arch. Microbiol. 106, 191—194
5 Anke, T. and Diekmann, H. (1974) Arch. Microbiol. 95, 227—236
6 Tomlinson, G., Cruickshank, W.H. and Viswanatha, T. (1971) Anal. Biochem. 44, 670—679
7 Wollenweber, P. (1967) in Dünnschicht-Chromatographie (Stahl, E., ed.), pp. 33—42, Springer, Berlin
8 Atkin, C.L. and Neilands, J.B. (1968) Biochemistry 7, 3734—3739
9 Calendar, R. and Berg, P. (1966) Biochemistry 5, 1681—1690
10 Walker, J.E., Otani, S. and Perlman, D. (1972) FEBS Lett. 20, 162—166
11 Simlot, M.M. and Pfänder, P. (1973) FEBS Lett. 35, 201—203
12 Lineweaver, H. and Burk, D. (1934) J. Am. Chem. Soc. 56, 658—666
13 Bauer, K., Roskoski, R., Jr., Kleinkauf, H. and Lipmann, F. (1972) Biochemistry 11, 3266—3271
14 Ong, D.E. and Emery, T.F. (1972) Arch. Biochem. Biophys. 148, 77—83
15 Müller, H.-G. (1976) Dissertation Universität Tübingen
16 Akers, H.A., Lee, S.G. and Lipmann, F. (1977) Biochemistry 16, 5722—5729
17 Anke, T. and Lipmann, F. (1977) FEBS Lett. 82, 337—340
18 Brot, N. and Goodwin, J. (1968) J. Biol. Chem. 243, 510—513
19 Young, I.G. and Gibson, F. (1968) Biochim. Biophys. Acta 177, 401—411
20 Anke, T. and Diekmann, H. (1972) FEBS Lett. 27, 259—262
21 Anke, H., Anke, T. and Diekmann, H. (1973) FEBS Lett. 36, 323—325
22 Anke, H. and Diekmann, H. (1974) Arch. Microbiol. 95, 213—225
23 Schafft, M. and Diekmann, H. (1978) Arch. Microbiol. 117, 202—207