*THE BIOSYNTHESIS OF FERREDOXIN IN A CELL-FREE SYSTEM**

By Anthony C. Trakatellis and Gerald Schwartz

DEPARTMENT OF BIOCHEMISTRY, MOUNT SINAI SCHOOL OF MEDICINE
OF THE CITY UNIVERSITY OF NEW YORK, AND MEDICAL RESEARCH CENTER
OF BROOKHAVEN NATIONAL LABORATORY

Communicated by Donald D. Van Slyke, April 10, 1969

Abstract.—The investigation reported in this communication was concerned with the biosynthesis of ferredoxin in a cell-free system. A cell-free system, with the ability to incorporate amino acids into peptides and proteins, was developed from *Cl. pasteurianum*, and its requirements were established. After the incorporation of L-alanine-C^{14}, ferredoxin-C^{14} was isolated in crystalline form with the aid of added carrier ferredoxin. Amino acid analyses and absorption spectra strongly indicated that the isolated ferredoxin-C^{14}, crystallized three times, was essentially free from impurities. Furthermore, experiments with Fe^{59} strongly indicated that the iron may combine with the apoprotein after the latter is completely synthesized and released from the polysomes.

Ferredoxin, an iron-containing protein which participates in electron-transfer processes, was isolated originally from *Clostridium pasteurianum*.[1] Subsequently, similar ferredoxins were obtained from other anaerobic bacteria.[2–4]

The *Cl. pasteurianum* ferredoxin has a molecular weight of about 6000, contains 7–8 sulfide groups and 7–8 iron atoms per mole,[5–7] and its complete amino acid sequences have been elucidated.[8]

Materials and Methods.—Cl. pasteurianum cultures: This anaerobic, nitrogen-fixing bacterium was obtained from the American Type Culture Collection, Washington, D. C. Stock cultures of *Cl. pasteurianum* were maintained in tubes of beef liver medium, stored at room temperature, and transferred at 6-month intervals. Seed cultures were grown in potato medium.[9] The medium was prepared by adding a small amount of calcium carbonate to 150 × 18-mm test tubes, a 2-inch deep layer of small pieces of peeled potato, and 15 ml of cold tap water and sterilization at 15 lb steam pressure for 15 min. Immediately prior to use, tubes of potato medium that had been stored at 4°–6° were placed in boiling water for 15 min, cooled rapidly, and inoculated. The cultures were incubated at room temperature in atmospheric air; cultures were transferred to fresh potato medium at 4–5-day intervals. For ribosome preparations, *Cl. pasteurianum* was grown in a synthetic medium similar to that described by Carnahan and Castle.[10] For convenience, and to avoid precipitates formed during heat sterilization, the medium was prepared from the following stock solutions. Solution I (K_2HPO_4, 110 gm; KH_2PO_4, 15 gm; and distilled water to 1 liter). The solution was then autoclaved at 15 lb for 30 min and cooled to room temperature. Solution II ($FeSO_4 \cdot 7H_2O$, 330 mg) was dissolved in 120 ml of distilled water and sterilized by passage through a Millipore filter (25 mm, HA · 45 μ). Solution III (D-biotin 10 mg in 100 ml distilled water) and solution IV (*p*-aminobenzoic acid 10 mg in 100 ml distilled water) were divided into 15-ml aliquots and stored frozen.

The *complete medium* (10 liters) consisted of $MgSO_4 \cdot H_2O$, 1.0 gm; NaCl, 1.0 gm; $Na_2MoO_4 \cdot 2H_2O$, 0.1 gm; $(NH_4)_2SO_4$, 20 gm; distilled water, 9.0 liters; and 1.0 ml of solutions III and IV. It was then autoclaved at 15 lb for 30 min and allowed to cool to room temperature. Then 900 ml of solution I and 70 ml of solution II were added aseptically.

Purified nitrogen was bubbled through the medium for 2 hr prior to inoculation with 100–150 ml of a 24-hr culture of *Cl. pasteurianum* in potato medium. Cultures were

incubated at 34°–36° under N_2 for approximately 18 hr, and the cells were harvested by centrifugation.

Compounds: L-Alanine-C^{14} with a specific activity of 90 mc/mM was obtained from Schwarz BioResearch, Inc. A sterile solution of $Fe^{59}SO_4$, with a specific activity of 10 mc/mg of Fe^{++}, was obtained from Abbott Radio-Pharmaceuticals. Crystalline *Cl. pasteurianum* ferredoxin was purchased from Worthington Company.

Preparation of ribosomes: The harvested bacterial cells were ground in a mortar with alumina. Hoagland medium A (0.005 *M* Tris, 0.025 *M* KCl, 0.005 *M* $MgCl_2$, 0.25 *M* sucrose, pH 7.2) was then added to the bacterial paste. The suspension was centrifuged at 2° in a refrigerated centrifuge at 2400 × *g* for 10 min to remove alumina, cell debris, and unbroken cells. The supernatant fluid was then centrifuged for 20 min at 25,000 rpm in a Spinco ultracentrifuge with a no. 40 rotor. The clear supernatant fraction was then subjected to a final centrifugation at 40,000 rpm (rotor no. 40, Spinco ultracentrifuge) for 2 hr to yield a ribosome pellet and the S100 supernatant fraction. All these procedures were conducted at 0°–2°.

Fractionation of ribosomal preparations into polysomes of varying aggregate size: The sedimentation patterns of ribosomal preparations were obtained as has been described in previous publications.[11, 12]

Cell-free amino acid incorporating system: This system has been described in detail in previous publications.[13] Briefly, ribosomes were suspended in an ice-cold buffer solution (0.045 *M* Tris, 0.0075 *M* $MgCl_2$, 0.075 *M* NaCl, and 0.08 *M* NH_4Cl, pH 7.2) containing 0.8 μmole of β-mercaptoethanol, 15 μmoles of phosphoenolpyruvic acid (tri-sodium salt), 50 μg pyruvate kinase, 1.0 μmole ATP, 0.5 μmole GTP, 5 μmoles of the labeled C^{14} amino acid, and a mixture of 19 amino acids, exclusive of the amino acid-C^{14} used in the assay. This mixture supplied 5 μmoles of each amino acid per assay tube. The ribosome suspension was incubated in the presence of G25-S100 fraction obtained by gel filtration of the S100 fraction through a Sephadex G-25 1.2 × 50-cm column, as has been described earlier.[13] The gel filtration separates the amino acids and nucleotides from the proteins and tRNA's of the S100 fraction. The protein-tRNA fraction (the first peak appearing upon chromatography) constitutes the G25-S100 fraction.

Measurement of radioactivity: The preparation of samples for measurement of radioactivity by a Packard Tri-carb liquid scintillation spectrometer has been described in detail in previous publications.[13] Samples of crystalline ferredoxin were counted by dissolving the ferredoxin in water and by using Bray's scintillation liquid. Finally, radioactivity from Fe^{59} was measured with an auto-gamma Packard spectrometer system.

Isolation of ferredoxin: Ferredoxin was purified essentially by the method of Lovenberg and Sobel.[14] Amino acid analyses of acid hydrolysates were performed in a Beckman-Spinco amino acid analyzer equipped with a digital readout system (Model CRS-10AB, Infotronics Corp., Houston) according to the method of Spackman *et al.*[15] The absorption spectrum of oxidized ferredoxin was measured in a Cary recording spectrophotometer (Model 14).

Results and Discussion.—Cell-free amino acid incorporating system from Cl. pasteurianum: Ribosomes (Fig. 1) from cells harvested at the middle of the logarithmic phase were prepared as described in *Materials and Methods* and used in the cell-free system. The requirements of the cell-free amino acid incorporating system are summarized in Table 1. The incorporating system is dependent on ATP, GTP, and amino acids. This was expected because the soluble fraction (S100) was chromatographed through a G-25 Sephadex column prior to its use in the incorporation assay. This procedure presumably separates the small-molecular-weight amino acids and nucleotides from the proteins and tRNA's of the soluble fraction. Ribosomes and the factors of the soluble fraction (enzymes, tRNA's) were also necessary in the incorporating system for obvious reasons.

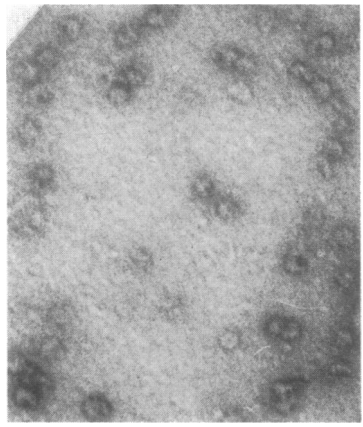

FIG. 1.—Electron microscopy picture of *Cl. pasteurianum* ribosomes, negatively stained with uranyl zinc acetate. The presence of polysomes is also evident. Magnification × 126,000.

The addition of $FeSO_4$, up to 1 μg per assay tube, did not affect (stimulate or inhibit) the incorporation of L-alanine-C^{14}. The time, course of L-alanine-C^{14} incorporation is depicted in Figure 2. The rate of L-alanine-C^{14} incorporation decreases progressively with incubation time, and the profile of the curve describing the course of incorporation is similar to those obtained with cell-free systems from bacterial or mammalian cells.

Biosynthesis of ferredoxin in the cell-free system: Ribosomes from *Cl. pasteurianum* were incubated in the cell-free system, in the presence of L-alanine-C^{14} and 0.2 μg of $FeSO_4$ per assay tube, at 37° for 45 min. At the end of the incubation time, all assay tubes were pooled and stored in a freezer. Ribosomes from approximately 750 gm of cells, harvested from 10 cultures (10 liters each), were pooled together and used in the incorporation assays. After the frozen material was thawed, 20 mg of crystalline ferredoxin were added to serve as a carrier. This was followed by extraction with one volume of acetone, and the ferredoxin was isolated in crystalline form from the solution by the method of Lovenberg and Sobel.[14] The complete experiment is described schematically in Figure 3. After centrifugation of the acetone extract, the supernatant fluid was applied to a 0.9 × 28-cm DEAE-cellulose column (DEAE, Mannex standard capacity 0.9 ± 0.1 meq/gm). The column was then washed with 5 vol of water and eluted with 0.15 *M* Tris-chloride buffer. During this elution step, the

TABLE 1. *Requirements of cell-free amino acid incorporating system.*

Description of the system	L-Alanine-C^{14} incorporated per 0.2 mg RNA (%)*
Complete system†	100
Complete system plus $FeSO_4$‡	98
Complete system minus ribosomes	0
Complete system minus S100-G25 fraction	24
Complete system minus amino acid mixture	28
Complete system minus ATP generating system	13
Complete system minus GTP	27

* The cpm of L-alanine-C^{14} incorporated per 0.2 mg of RNA of the complete system taken as 100% incorporation.

† Complete system as has been described in *Materials and Methods.*

‡ 1 μg of $FeSO_4$ per assay tube.

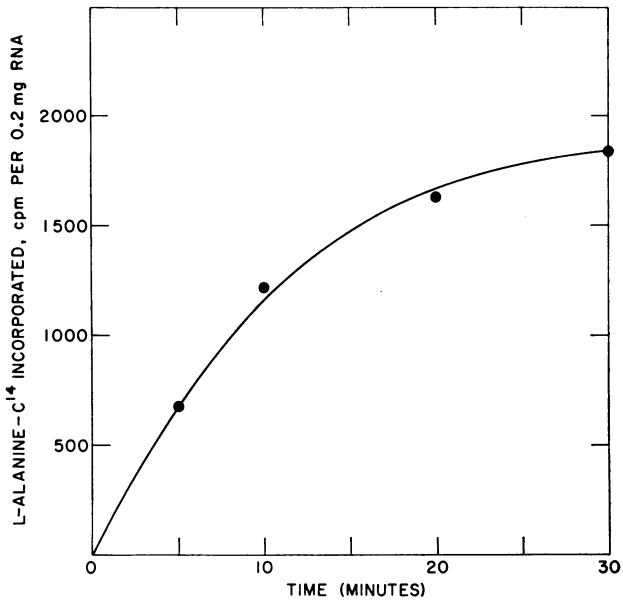

FIG. 2.—Time course of L-alanine-C^{14} incorporation in a cell-free system with ribosomes and soluble factors isolated from *Cl. pasteurianum*.

absorbancy and radioactivity of the effluent were followed. When the absorbancy of the effluent dropped from 1.0 OD (1856 cpm/ml) to 0.04 OD (10 cpm/ml), the ferredoxin was eluted with 0.15 *M* tris-chloride, pH 7.3 containing 0.65 *M* NaCl. The eluate (total volume 25 ml. total cpm = 21,500) was then de-

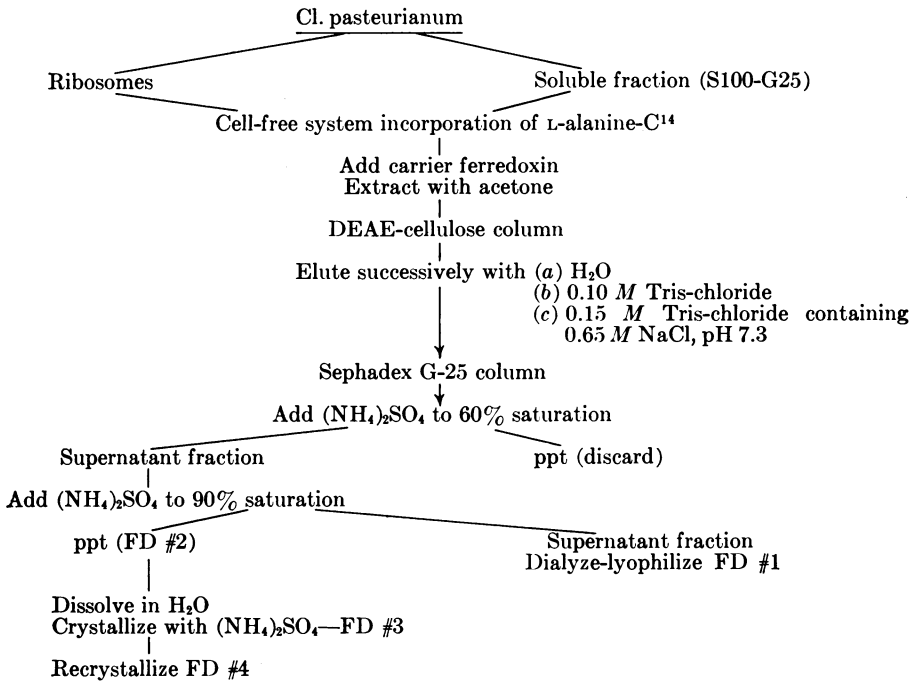

FIG. 3.

salted on a 2 × 37-cm G-25 Sephadex column equilibrated with distilled water. The desalted solution was brought to 60 per cent saturation with ammonium sulfate, and any precipitate was removed by centrifugation. The clear supernatant fluid was then brought to 90 per cent saturation with $(NH_4)_2SO_4$, and the precipitated ferredoxin was collected after centrifugation. The supernatant fluid was dialyzed in Visking 30/32 dialysis tubing in the cold against 8 liters of water (four 2-liter portions) and then lyophilized. The material obtained was marked ferredoxin (Fd –1). The precipitated ferredoxin (Fd #2) was dissolved in a small volume of 0.05 *M* Tris-chloride pH 7.3 and crystallized by the slow addition of saturated $(NH_4)_2SO_4$ (Fd #3). Finally, the crystalline Fd #3 was recrystallized once more to yield Fd #4. The specific activities of Fd #1, 2, 3, and 4 in cpm/mg were 2736, 2840, 2920, and 2936, respectively. The specific activities of preparations 3 and 4 were very close, whereas a small increase in activity was observed from preparation 1 to 2 to 3, 4. Amino acid analyses of samples 2, 3, and 4 are depicted in Table 2. As one would expect, the analysis of Fd #2, 3, and 4 gives the correct amino acid composition for *Cl. pasteurianum* ferredoxin. More important, however, was the complete absence of even traces of the amino acids histidine, arginine, leucine, and methionine that are not found in ferredoxin. Finally, the isolated crystalline ferredoxin gave the same characteristic absorption spectrum with the ferredoxin used as carrier. The data of specific radioactivity, amino acid analysis, and absorption spectra strongly indicated that the isolated ferredoxin, crystallized three times, was essentially free from impurities.

Biosynthesis of Fe^{59}-ferredoxin: For this experiment, *Cl. pasteurianum* was grown in the presence of $Fe^{59}SO_4$ (10 μc/10 liters of culture medium). The cells were harvested by centrifugation, and about 15 gm of cells were used for obtaining polysome profiles (Fig. 4). The rest of the cells (approximately 60 gm) were used to prepare the Fe^{59}-ferredoxin. Again, as in the previous experiment,

TABLE 2. *Amino acid composition* of carrier and isolated ferredoxins.*

Amino acid	Theory	Carrier ferredoxin found	Fd #2	Fd #3	Fd #4
Lysine	1	1.4	1.3	1.1	1.1
Histidine	—	—	—	—	—
Arginine	—	—	—	—	—
Aspartic	8	8.4	8.2	8.3	8.1
Threonine	1	1.0	1.0	1.1	1.1
Serine	5	3.3†	3.5†	3.9†	4.1†
Glutamic acid	4	4.1	4.2	4.0	4.0
Proline	3	3.0	3.0	3.0	3.0
Glycine	4	4.1	4.1	4.0	4.0
Alanine	8	6.8	6.9	7.6	7.7
Valine	6	5.4	5.3	5.8	5.8
Isoleucine	5	4.1	4.1	4.4	4.5
Leucine	—	—	—	—	—
Tyrosine	1	0.5	0.6	0.7	0.7
Phenylalanine	1	1.0	1.0	1.0	1.0
Cysteine	8	‡	‡	‡	‡

* Number of amino acid residues per molecule.
† Not corrected for hydrolytic destruction.
‡ Not determined.

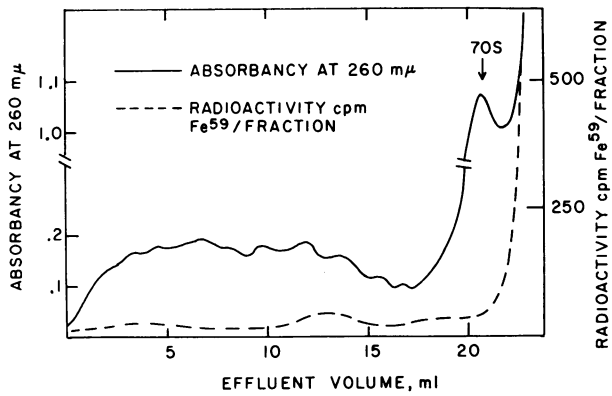

FIG. 4.—Sedimentation pattern of ribosomes isolated from 15 gm of *Cl. pasteurianum* cells grown in the presence of $Fe^{59}SO_4$. The ribosomes were suspended in Hoagland's medium A and 2 ml of the suspension was layered over 27 ml of linear sucrose gradient (0.3 M − 1.0 M) and centrifuged at 25,000 rpm in a SW25 rotor for 3 hr and 30 min. Following centrifugation, fractions were collected through the bottom of the tube with the aid of the Beckman fractionating system. Absorbancy at 260 mμ and radioactivity of fractions were determined.

20 mg of crystalline ferredoxin were added as carrier, and the ferredoxin was isolated in the crystalline form (recrystallized three times) and counted with an auto-gamma Packard spectrometer system. The total cpm of the ferredoxin recovered was 210,000 and the specific radioactivity 28,000 cpm/mg. Despite this high activity, the polysome profiles depicted in Figure 4 do not show any appreciable radioactivity. This strongly indicates that the growing apoferredoxin chains on the polysomes do not contain any iron. This suggests that the combination of the iron-apoprotein takes place after the latter is completely synthesized and released from the polysomes.

The authors wish to express their appreciation to Drs. M. Montjar, R. Drew, and H. Johnson for their help and also wish to acknowledge the excellent technical assistance of Miss Roberta Klimaski.

* This work was supported by the U.S. Atomic Energy Commission.

[1] Mortenson, L. E., R. C. Valentine, and J. E. Carnahan, *Biochem. Biophys. Res. Commun.*, **7**, 448 (1962).

[2] Valentine, R. C., R. L. Jackson, and R. S. Wolfe, *Biochem. Biophys. Res. Commun.*, **7**, 453 (1962).

[3] Valentine, R. C., W. J. Brill, and R. D. Sayers, *Biochem. Biophys. Res. Commun.*, **12**, 315 (1963).

[4] Buchanan, B. B., W. Lovenberg, and J. C. Rabinowitz, these PROCEEDINGS, **49**, 345 (1963).

[5] Lovenberg, W., B. B. Buchanan, and J. C. Rabinowitz, *J. Biol. Chem.*, **238**, 3899 (1963).

[6] Mortenson, L. E., *Biochim. Biophys. Acta*, **81**, 71 (1964).

[7] Malkin, R., and J. C. Rabinowitz, *Ann. Rev. Biochem.*, **36**, 113 (1967).

[8] Tomaka, M., T. Nakasima, A. Benson, H. F. Mower, and H. T. Yasunobu, *Biochem. Biophys. Res. Commun.*, **16**, 422 (1964).

[9] Jensen, H. L., and D. Spencer, *Proc. Linnean Soc. N.S. Wales*, **72**, 73 (1947).

[10] Carnahan, J. E., and J. E. Castle, *J. Bacteriol.*, **75**, 1214 (1958).

[11] Trakatellis, A. C., A. E. Axelrod, and M. Montjar, *Nature*, **203**, 1134 (1964).

[12] Trakatellis, A. C., A. E. Axelrod, and M. Montjar, *J. Biol. Chem.*, **239**, 4237 (1964).

[13] Trakatellis, A. C., these PROCEEDINGS, **59**, 854 (1968).

[14] Lovenberg, W., and B. F. Sobel, these PROCEEDINGS, **54**, 193 (1965).

[15] Spackman, D. H., W. H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).