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CHARACTERIZATION OF THE FERREDOXIN COMPONENT OF THE STEROID 15β-HYDROXYLASE SYSTEM FROM BACILLUS MEGATERIUM.

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The iron-sulfur protein of the 15β-steroid hydroxylase system in Bacillus Megaterium has been purified to apparent homogeneity as judged by SDS-polyacrylamide gel electrophoresis. Its molecular weight, c:a 14000 Dalton, and amino acid composition closely resemble those of the corresponding proteins from bovine adrenal mitochondria (adrenodoxin) and Pseudomonas putida (putidadoxin).

Over the last years several cytochrome P-450 dependent monooxygenase systems have been identified in a multitude of different species, organs and cell organelles (1-22). These systems can be divided into two main groups: those with a two protein system. i.e. a reductase and cytochrome P-450 and those having three proteins, a reductase, a ferredoxin (iron sulfur protein) and cytochrome P-450. Also, it is of interest to note that the three component system has been found in bacteria and mitochondria from several sources (11,15,20,23-25). The two protein system is found in the endoplasmic reticulum in a wide variety of cells. Proteins of the ferredoxin type found in Pseudomonas putida and bovine adrenal mitochondria have been sequenced (26,27). In earlier publications (28-30) we have identified and characterized a three protein system, the 15β-steroid hydroxylase system from Bacillus megaterium. It was shown to contain a ferredoxin reductase and ferredoxin (29) and the terminal component cytochrome P-450_{meg} has been purified to

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homogeneity (30). In this report we describe the purification and partial characterization of the ferredoxin component of this hydroxylase system from B. megaterium.

MATERIAL AND METHODS

Preparation of cellfree extract from B. megaterium.

B. megaterium ATCC 13368 was obtained from American Type Culture Collection (Rochville, Md) and was cultured in a 10 liter Biotec fermentor for 40-45 hours as described earlier (29,30). The harvested cells were frozen and stored at -20°C, thawed and washed before being suspended in 0.1 M potassium phosphate buffer, pH 7.4, to a concentration of 0.4 g of cells per ml. The suspension was sonicated using a MSE 500 W ultrasonic disintegrator at full output for 4 x 20 seconds. The sonicate was then centrifuged at 50000 x g for one hour and the supernatant was collected. DEAE-cellulose, Ultrogel AcA-54, DEAE-Sepharose, Octyl-Sepharose, Sephadex G-25/PD-10 columns and hydroxyapatite were obtained from sources described previously (28-30).

Assay Procedure.

Spectrometric measurements were performed with a Cary model 118 spectrophotometer. Photometric measurements were performed with the Cary 118 instrument or with a Zeiss M4/PM QII photometer.

Protein was measured according to Lowry et al. (31) with bovine serum albumin as standard.

Acid-labile sulfur was determined according to King and Morris (32).

RESULTS AND DISCUSSION

Purification of B. megaterium ferredoxin.

The cellfree extract of B. megaterium (50000 x g supernatant) was subjected to chromatography on a DEAE-cellulose column (4 x 10 cm) equilibrated with 0.1 M potassium phosphate buffer, pH 7.4 (Buffer A). The column was then eluted in a stepwise manner, first with 200 ml of Buffer A, then with 100 ml each of 0.1, 0.2 and 0.4 M KCl in Buffer A. The 0.4 M KCl fraction, which contained all acid-labile sulfur, and which in recombination experiments was found to contain an obligatory function for enzymatic activity, was concentrated to 15 ml using a PM-10 membrane. The concentration protein was then applied to an Ultrogel AcA-54 column (4 x 80 cm) equilibrated with Buffer A

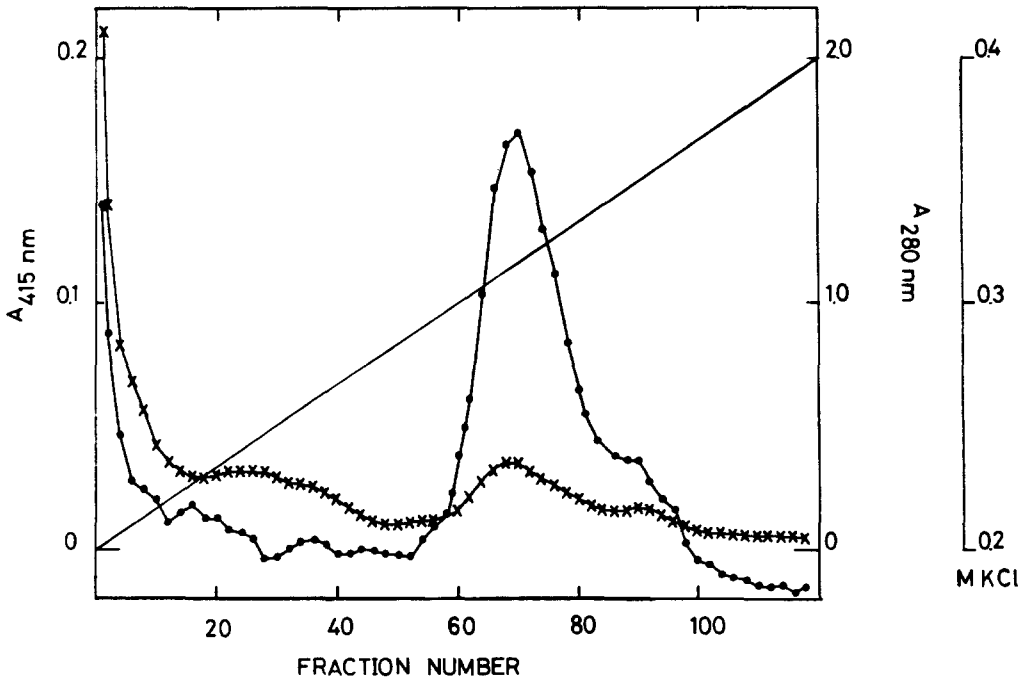


Fig. 1. DEAE-Sepharose chromatography of B. megaterium ferredoxin. •—• absorbance at 415 nanometers. X—X absorbance at 280 nanometers. For details, see text.

and eluted with the same buffer in about 5 ml fractions. Acid-labile sulfur protein was eluted as a single peak and the corresponding fractions were pooled, diluted twice with

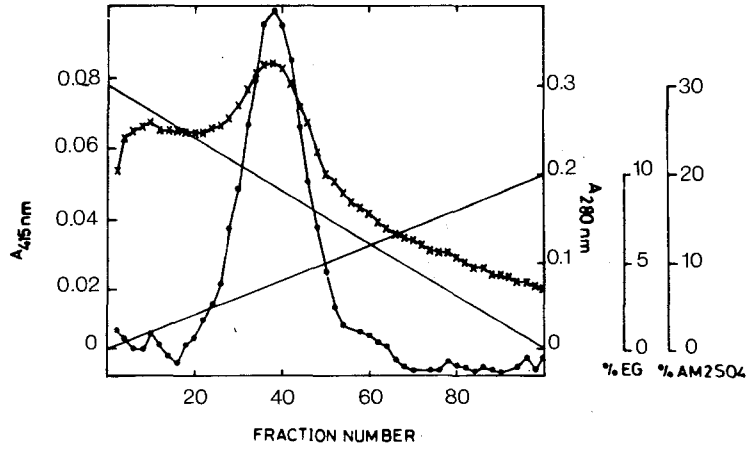
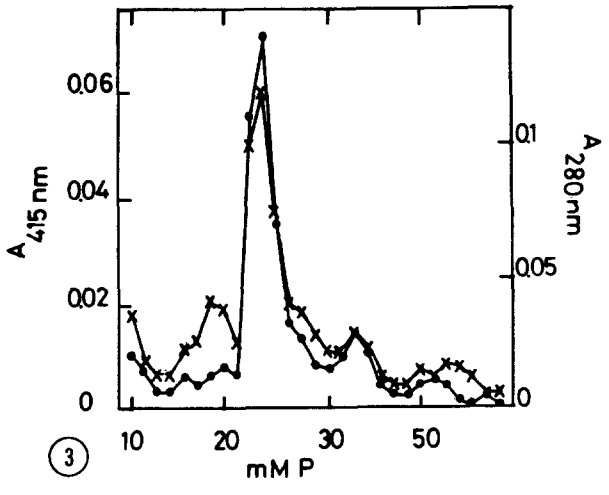


Fig. 2. Octyl-Sepharose chromatography of B. megaterium ferredoxin. •—• absorbance at 415 nanometers. X—X absorbance at 280 nanometers. For details, see text.



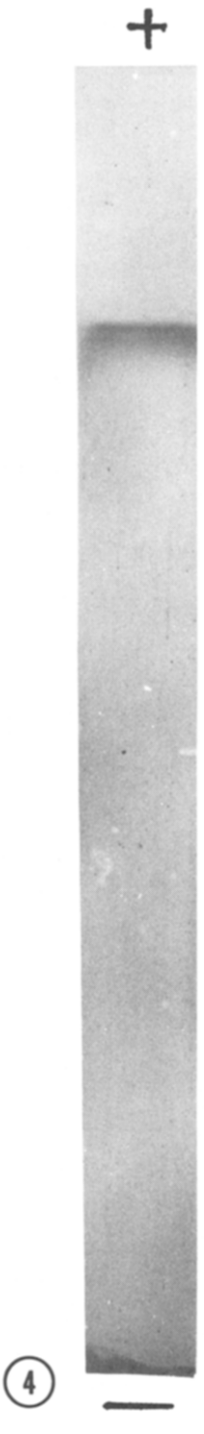


Fig. 3. Hydroxy-apatite chromatography of B. megaterium ferredoxin. •——• absorbance at 415 nanometers. X——X absorbance at 280 nanometers. For details, see text.

Fig. 4. A sample from the 20mM hydroxyapatite eluate subjected to sodiumdodecylsulfonate polyacrylamide gel electrophoresis. The amount of protein added was 2 ugrams.

redistilled water and applied to a DEAE-Sepharose column (1 x 4 cm) equilibrated with 50 mM potassium phosphate buffer, pH 7.4 (Buffer B). The column was first washed with 1 volume of Buffer B and then with 2-3 volumes of 0.2 M KCl in Buffer B. The sample was then eluted using a linear gradient of 0.2-0.4 M KCl in Buffer B. A total elution volume of 200 ml was collected in 2 ml fractions. As shown in Fig. 1, the ferredoxin (measured at 415 nm) is eluted as a single peak at around 0.3 M KCl. No other acid-labile sulfur protein containing fractions were found. The acid-labile sulfur containing fractions were pooled, made 20% (w/v) with regard to glycerol and 30% (w/v) with regard to ammonium sulfate. The solution was then applied to an Octyl Sepharose column (1 x 4 cm) equilibrated with 30% ammonium sulfate and 20% glycerol in Buffer A. The column was then el,uted with a linear gradient of decreasing ionic strength (30% to 0% ammonium sulfate) and of increasing concentration of ethylene glycol (0 to 10% w/w) in Buffer A. Total elution volume was 200 ml and fraction volume about 2 ml. As shown in Fig. 2, the acid-labile sulfur containing fraction is eluted as a single peak, and no other acid labile sulfur containing fractions were found. The proper fractions from the Octyl Sepharose column were then desalted on Sephadex G-25/PPM-10 columns, equilibrated with 10 mM potassium phosphate buffer, pH 7.4, and applied to a hydroxyapatite column equilibrated with 5 mM potassium phosphate buffer, pH 7.4. The column was eluted in a stepwise manner using different concentrations of potassium phosphate buffers, pH 7.4. The elution was carried out using 20 ml of each ionic strength and fraction volumes were 2-3 ml.

As shown in Fig. 3 the ferredoxin was eluted as a single peak with 20 mM phosphate buffer. No other ISP:s have been found in the eluate. The purified proteins from this peak move as a single

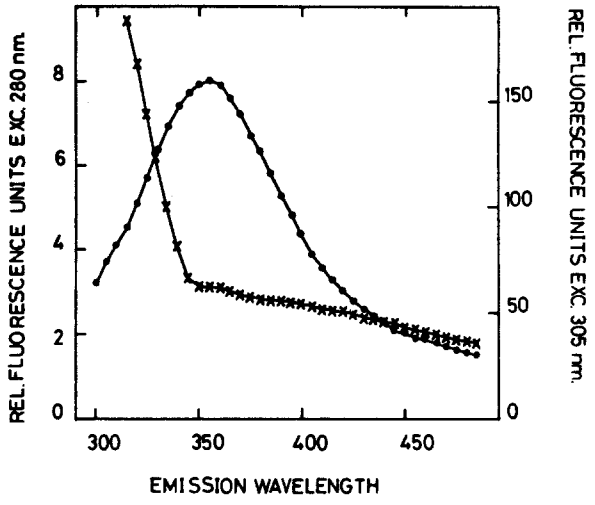


Fig. 5. Fluorescence spectra of pure B. megaterium ferredoxin. Fluoprescence spectra were recorded at 20°C using a JASCO FP-550 spectrofluorometer. Protein concentration was 0.1 mg of protein per ml in 20 mM potassium phosphate buffer, pH 7.4. ●——● exication at 280 nanometers. X——X excitation at 305 nanometers.

band on sodiumdodecyl polyacrylamide gel electrophoresis, with an apparent molecular weight of around 14000 as shown in Fig. 4.

When subjected to fluorescence spectrometry, the purified protein gives indication of a tryptophan residue in a rather hydrophilic environment as shown in Fig. 5.

The absolute spectra of the oxidized and reduced purified forms of the ferredoxin shown in Fig. 6 indicative of a bacterial type ferredoxin.

Table 1 shows the amino acid composition of B. megaterium ferredoxin, as compared to the corresponding proteins from Pseudomonas putida and the steroid 11β-hydroxylase system from bovine adrenal mitochondria.

Fig. 4 shows the hydroxyapatite ferredoxin containing fraction on SDS-polyacrylamide gel electrophoresis giving a molecular weight of 14000. This fits well with the value for molecular weight computed from the amino acid composition (Table 1). This gives a sulfur to protein molar ratio of 1.8 and, as described in

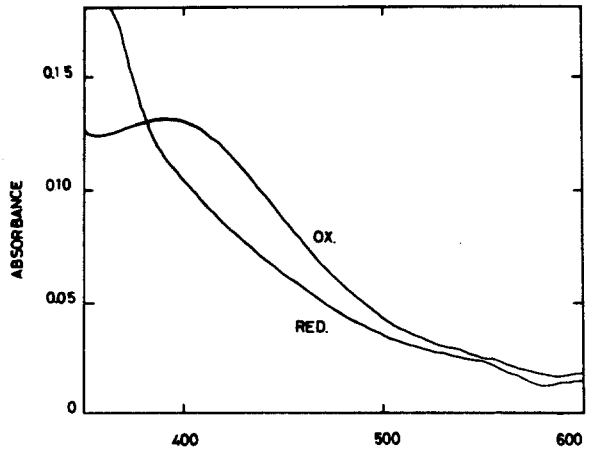


Fig. 6. Absolute oxidized and reduced spectra of B. megaterium ferredoxin. The sample contained 25 nanomoles of acid-labile sulfur/ml.

an earlier publication, the ferredoxin has a sulfur to protein molar ratio of 0.9 (29). This indicates that the B. megaterium ferredoxin is a 2 sulfur-2 iron protein.

The EPR-spectrum of partially purified B. megaterium ferredoxin has been given in an earlier publication (29). It had g-values at 1.90, 1.93 and 2.06. This procedure gives a purification factor of 2000 and a total field of 5%.

TABLE I
THE AMINO ACID COMPOSITION OF B. MEGATERIUM FERREDOXIN AS COMPARED TO PSEUDOMONAS PUTIDA AND BOVINE ADRENAL MITOCHONDRIA FERREDOXINS

Amino acid	B. meg.	P. putida	Adrenal mitoch.
asp	15	15	20
thr	6	5	10
ser	6	7	7
glu	11	12	12
pro	6	4	1
gly	10	9	8
ala	11	10	7
cys	5	6	5
val	7	15	7
iso	7	6	8
leu	7	7	13
phe	4	2	4
his	2	2	3
lys	5	3	5
arg	4	5	4
tyr	3	3	1
met	3	3	3
trp	1	1	0
MW	13,500	12,500	13,100

Values for P. putida and adrenal mitochondria from ref. 33.

The results presented in this paper show a strong resemblance between the ferredoxin from B. megaterium and the corresponding proteins from P. putida and bovine adrenal mitochondria. In an earlier paper we reported the interchangeability between the B. megaterium ferredoxin and adrenal mitochondrial ferredoxin in the electrion transport systems of the B. megaterium steroid 15β-hydroxylase system and the mitochondrial steroid 11β-hydroxylase system, respectively (30).

It is hoped that further work will help to elucidate the reason for the similarities between the B. megaterium and bovine adrenal mitochondrial systems.

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REFERENCES

1. Madyasthu, K.M., Meehan, T.D., and Coscia, C.J. (1976) Biochemistry, 15, 1097-1102.
2. Murphy, P.J., and West, C.A. (1969) Arch. Biochem. Biophys., 133, 395-407.
3. Powell, J., Potts, M., Weklych, R., and Conn, E.E. (1974) J. Biol. Chem., 249, 5019-5026.
4. Yoshida, Y., Aozoma, Y., Kumavha, H., and Kubutu, S. (1977) Biochem. Biophys. Res. Commun. 78, 1005-1010.
5. Lebault, J.M., Lode, E.T., and Coon, M.J. (1971) Biochem. Biophys. Res. Commun., 42, 413-419.
6. Cerniglia, C.E., and Gibson, D.T. (1978) Arch. Biochem. Biophys., 186, 121-127.
7. Ferris, J.P., MacDonald, L.M., Patric, M.A., and Martin, M.A. (1976) Arch. Biochem. Biophys. 175, 443-452.
8. Appleby, C.A. (1967) Biochim. Biophys. Acta, 147, 399-402.
9. Cardini, G., and Jurtshuk, P. (1968) J. Biol. Chem., 243, 6070-6072.
10. Matson, R.S., Hare, R.S., and Fulco, A.J. (1977) Biochim. Biophys. Acta, 487-494.
11. Katagiri, M., Ganguli, B.N., and Gunsalus, I.C. (1968) J. Biol. Chem. 243, 3543-3546.
12. Kulkarni, A.P., Smith, A., and Hodgson, E. (1976) Comp. Biochem. Physiol., 54B, 509-513.
13. Agosin, M. (1971) Mol. Cell. Biochem. 12, 33-44.
14. Ghazarian, J.G., Jefcoate, C.R., Knutson, J.C., Orm-Johnson, W.H., and DeLuca, H.F. (1974) J. Biol. Chem., 249, 3026-3033.
15. Ghazarian, J.G., Hsu, P.Y., and Peterson, B.L. (1977) Arch. Biochem. Biophys., 184, 596-604.

16. Wainic, (1978) Pharmac. Ther., A 2, 359-372.
17. Lu, A.Y.H., and West, S.B. (1978) Pharm. Ther., A 2, 337-358.
18. Bryson, M., and Sweet, M.L. (1968) J. Biol. Chem., 243, 2799-2804.
19. Takemori, S., Sato, H., Gomi, T., Sukara, K., and Katigiri, M. (1975) Biochem. Biophys. Res. Commun. 67, 1151-1157.
20. Suzuki, K., and Kimura, T. (1965) Biochem. Biophys. Res. Commun., 19, 340-345.
21. Sato, R., Atsuta, Y., Iamai, Y., Tanigrichi, S., and Okuda, K. (1977) Proc. Natl. Acad. Sci., USA, 74, 5477-5481.
22. Taniguchi, S. Hoshita, N., and Okuda, K. (1973) Eur. J. Biochem., 40, 607-617.
23. Pedersen, J.I., Oftebro, H., and Vänngård, T. (1977) Biochem. Biophys. Res. Commun., 76, 666-673.
24. Pedersen, J.I., Ghazarian, J.G., Orme-Johnson, N.R., and DeLuca, H. (1976) J. Biol. Chem. 254, 3933-3941.
25. Gunsalus, I.C., Pedersen, T.C., and Sligar, S.G.(1975)Am. Rev. Biochem., 44, 377-407.
26. Tanaka, M., Haniu, M., Yasonubu, K.T., Dus, K., and Gunsalus, I.C. (1974) J. Biol. Chem., 249, 3684-3701.
27. Tanaka, M., Haniu, M., and Yasonubu, K.T. (1970) Biochem. Biophys. Res. Commun., 39, 1182-1188.
28. Berg, A., Carlström, K., Gustafsson, J.-Å., and Ingelman-Sundberg, M. (1975) Biochem. Biophys. Res. Commun., 66, 1414-1423.
29. Berg, A., Gustafsson, J.-Å., Ingelman-Sundberg, M., and Carlström, K. (1976) J. Biol. Chem., 251, 2831-2838.
30. Berg, A., Ingelman-Sundberg, M., and Gustafsson, J.-Å. (1978) J. Biol. Chem., 254, 5264-5271.
31. Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem., 250, 3567-3570.
32. King, T.E., and Morris, R.O. (1967) Methods Enzymol., 10, 634-639.
33. Tsai, R.L., Gunsalus, I.C., and Dus, K. (1971) Biochem. Biophys. Res. Commun., 45, 1300-1306.