A RAPID PROCEDURE FOR THE PURIFICATION OF FERREDOXIN FROM CLOSTRIDIA USING POLYETHYLENEIMINE

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Received 8 March 1978

1. Introduction

Different procedures for the purification of ferredoxin from Clostridia have been described [1–6]. They are based on one or several of the following properties of clostridial ferredoxin:

(i) Solubility in acetone–H_2O mixtures up to an acetone concentration of 60% due to the relatively small molecular weight (6000) of ferredoxin.
(ii) Tight binding to DEAE cellulose up to a salt concentration of 0.2 M because ferredoxins are acidic proteins.
(iii) Tight binding to DEAE cellulose in 60% ammonium sulfate due to forces not yet understood.
(iv) Precipitation from ammonium sulfate solutions only at concentrations higher than 70%.

The procedures described to date involve one or several column chromatographic steps which are rather time consuming; at least 2 or more days are required to obtain highly purified ferredoxin with an A_{390}/A_{280} higher than 0.75 [1–6]. One of the major problems experienced is that part of the ferredoxin is denatured during the purification procedure and that dimeric ferredoxin of mol. wt 12 000 can be formed [7]. This is caused by the instability of clostridial ferredoxins in diluted form, especially in the presence of acetone.

In this paper a procedure is described by which highly purified ferredoxin with an A_{390}/A_{280} of 0.76–0.79 can be obtained from Clostridia in very high yields in less than 5 h using only precipitation procedures. The removal of most contaminating proteins via acetone precipitation is followed immediately by precipitation of ferredoxin with polyethyleneimine, thus denaturation or dimerization of the iron–sulfur protein is avoided.

2. Materials and methods

PEI 18 (polyethyleneimine mol. wt 1800) was from Nordmann, Rassmann and Co., Hamburg; polymin-P (polyethyleneimine mol. wt 20 000) water-free, from BASF Ludwigshafen; PEI 600 (polyethyleneimine mol. wt 40 000–60 000) from Dow Chemical Company, Midland, MI. DE 52 (Microgranular, pre-swollen DEAE cellulose) was from Whatman-Biochemicals, Springfield Mill, Maidstone, Kent.

Clostridium pasteurianum was grown on glucose medium [8], *Clostridium kluyveri* on crotonate medium [9], *Clostridium acidi-urici* on urate medium [10] and *Clostridium perfringens* on glutamate medium [11]. After harvesting, the cells were frozen in liquid nitrogen and stored at −70°C.

The molecular weight of ferredoxin was determined by gel chromatography on Sephadex G-50 [7] equilibrated with a 50 mM potassium phosphate buffer, pH 6.8. Non-heme iron and acid-labile sulfur were estimated as in [12] and [13], respectively. The activity of purified ferredoxin was assayed with pyruvate:ferredoxin-oxidoreductase of *Clo. pasteurianum* by following the reduction of ferredoxin at 390 nm in phosphate buffer, pH 7 [14]. The specific activities of ferredoxin given in table 1 were determined as in [4].

3. Results

The procedure for the purification of ferredoxin from 50 g cells (wet wt) of *Clo. pasteurianum*, *Clo. acidi-urici*, *Clo. kluyveri*, or *Clo. perfringens* is below. The procedure can be applied, however, to from 0.5–500 g cells provided it is appropriately scaled down or up. Unless otherwise stated all steps were performed at 0°C under aerobic conditions. The complete data for the purification of *Clo. pasteurianum* ferredoxin are presented in table 1.

3.1. *Step 1: preparation of cell-free extracts*

50 g frozen cells were suspended in 75 ml H_2O containing 50 mg lysozyme and 5 mg deoxyribonuclease. After incubation for 40 min at 37°C the now cell-free lysate was cooled to 0°C and adjusted to pH 6.5 with either 1 M Tris base or 1 M acetic acid. Then H_2O was added until the conductivity of the lysate was equal to that of a 100 mM NaCl solution. The correct adjustment of the pH and of the ionic strength of the lysates proved to be important because they affect the precipitation of ferredoxin with polyethyleneimine in step 3.

3.2. *Step 2: acetone precipitation*

1.2 ml acetone chilled to −20°C was added/g cell-free extract under continuous rapid stirring with a magnetic paddle. The resulting acetone concentration is 55% (v/v). The yellow–brown supernatant containing the ferredoxin had pH 7.2–7.4 and a conductivity equal to that of a 32 mM sodium chloride solution. Acetone concentrations higher and lower than 55% (v/v) have been tried in the procedure. Using acetone conc. 50% the ferredoxin obtained was less pure (A_{380}/A_{280} = 0.6–0.66); conc. 60% gave a reduced yield of ferredoxin.

Ferredoxin is slowly denatured in 55% acetone; therefore it is important to precipitate the ferredoxin from the acetone supernatant immediately after the centrifugation.

3.3. *Step 3: polyethyleneimine precipitation*

Ferredoxin was precipitated from the acetone supernatant by the addition of Polymin P to final conc. 0.5% (v/w). A 10% (w/w) aqueous solution of the polyethyleneimine (adjusted to pH 6.5 with acetic acid) was added with continuous stirring with a magnetic paddle (50 μl 10% Polymin P/g acetone supernatant). As part of the precipitated material always stuch to the flask walls and to the magnetic paddle this step was performed in two 300 ml centrifugation bottles to avoid loss of material on transfer. After centrifugation for 20 min at 15 000 × *g* the light yellow supernatant was quantitatively discarded. If the supernatant A_{390} was below 0.04–0.02 (against H_2O) all the ferredoxin had been precipitated. The brown pellet containing the ferredoxin was extensively

Table 1
Purification of ferredoxin from 50 g wet wt *Clo. pasteurianum* cells

Purification step	Volume (ml)	Protein (mg)	Total units[a]	Units/mg protein
1. Cell-free extract	148	5500	1750	0.31
2. Acetone supernatant	275	59	1190	20.1
3. 60% ammonium sulfate supernatant	10	40	1100	27.5
4. Purified ferredoxin	15	14	1050	75
5. 90% ammonium sulfate precipitated ferredoxin		10	825	82

[a] Ferredoxin was assayed by measuring its stimulation of acetyl phosphate production from pyruvate by a ferredoxin-free extract of *Clo. pasteurianum*. 1 unit is equal to 6 μmol acid hydroxamate formed in the assay/10 min [4]

homogenized with a glass rod under successive addition of small portions of 60% ammonium sulfate (adjusted to pH 6.8 with Tris base). 5–10 ml 60% ammonium sulfate had to be added until an almost clear brown solution was obtained. Any undissolved material was removed by centrifugation at 30 000 × *g* for 10 min.

Instead of Polymin P other polyethyleneimines can also be used to precipitate the ferredoxin from the acetone supernatant. The quantity required being dependent on the type used increased with increasing molecular weight of the polyethyleneimine (PEI 18 < Polymin P < PEI 600) (fig.1). This is only partly because polyethyleneimines with lower molecular weights have relatively more basic groups/mol than those with higher molecular weights (PEI 18: 23; Polymin P: 110; PEI 600: 190). It is important not to add much more polyethyleneimine than necessary to precipitate all of the ferredoxin, or a considerable decrease in yield results.

3.4. *Step 4: adsorption to and desorption from DEAE cellulose*

500–600 mg DE 52 (suspended in 60% ammonium sulfate, pH 6.8) were added to the 60% ammonium sulfate supernatant. After continuous stirring for 45 min all the ferredoxin was adsorbed to the DE 52 which was then sedimented by centrifugation for 10 min at 30 000 × *g*. The supernatant was dark

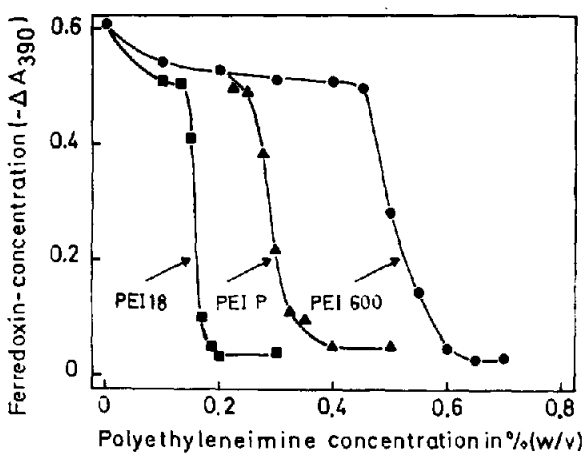

Fig.1. Precipitation of ferredoxin from *Clo. pasteurianum* from the acetone supernatant with polyethyleneimines (■) PEI 18; (▲) Polymin P; (●) PEI 600. For conditions see text.

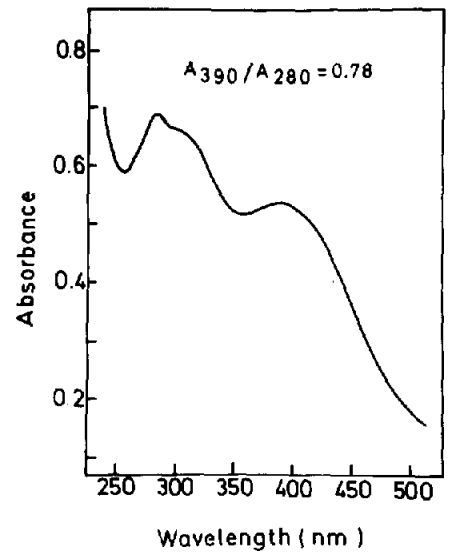

Fig.2. Spectrum of ferridoxin from *Clo. pasteurianum* obtained after step 4 of the purification procedure.

yellow, the DE 52 pellet dark brown. The pellet was washed twice with 35 ml 60% ammonium sulfate to remove contaminating yellow material, then resuspended in 15 ml 10% ammonium sulfate pH 6.8. In 10% ammonium sulfate the ferredoxin dissociates from the DE 52 which could now be separated by centrifugation for 10 min at 30 000 × *g*. The A_{390}/A_{280} of the dark brown supernatant (fig.2) was 0.76–0.79, indicating that the ferredoxin obtained was essentially pure (1).

3.4.1. Yields

Based on a molar absorption coefficient at 390 nm of 30 000 $cm^{-1}M^{-1}$ [6,7] and mol. wt 6000, 12–15 mg ferredoxin were obtained after step 4 from 50 g *Clo. pasteurianum* (wet wt), 25 mg ferredoxin from 50 g *Clo. acidi-urici*, 3 mg ferredoxin from 50 g *Clo. kluyveri* and 2.5 mg from 50 g *Clo. perfringens*. The 50 g wet cells were equivalent to approx. 10 g dried cells and contain 5 g protein.

3.4.2. Molecular properties

Ferredoxin from *Clo. pasteurianum* was analyzed in detail in order to ascertain that it did not contain the dimeric form [7]. The ferredoxin was found to have mol. wt 6000 and to contain 8 mol non-heme

iron and an equal amount of acid-labile sulfur/mol. It had the same specific activity in the pyruvate : ferredoxin oxidoreductase assay as that isolated as in [1]. The ferredoxin contained trace amounts of rubredoxin (< 1%) which could, however, be removed via precipitation of the ferredoxin in 90% ammonium sulfate (adjusted to pH 6.8 with Tris base) [15].

3.5. *Step 5: ammonium sulfate precipitation*

To precipitate the ferredoxin the dark brown supernatant was dialyzed overnight against 100 vol. 90% ammonium sulfate, pH 6.8 (75% for ferredoxin from *Clo. acidi-urici*). The precipitated ferredoxin was washed twice with 1–2 ml 90% ammonium sulfate, pH 6.8, in order to remove remaining traces of rubredoxin and then resuspended in 0.5 ml 90% ammonium sulfate, pH 6.8. It is important to dialyze the ferredoxin against 90% ammonium sulfate rather than to increase the concentration by the addition of solid ammonium sulfate. When solid ammonium sulfate was added a considerable part of the ferredoxin was always denatured.

3.5.1. Storage

Ferredoxin from *Clo. pasteurianum* was stable after precipitation when stored as a concentrated suspension (30 mg/ml) in 90% ammonium sulfate, pH 6.8, at either −15°C or 4°C. After 2 years storage at −15°C it had the same specific activity in the pyruvate : ferredoxin oxidoreductase assay and the A_{390}/A_{280} was 0.76–0.78. Anaerobic conditions were not required.

4. Discussion

With the procedure described highly purified ferredoxin with a A_{390}/A_{280} between 0.76 and 0.79 was isolated from Clostridia in less than 5 h. The ferredoxin can be precipitated by dialysis against 90% ammonium sulfate in another 4–5 h and then stored at 4°C or −16°C. Published procedures require at least 2, and some 3–4 days [1–6] for obtaining ferredoxin with A_{380}/A_{280} 0.75.

25–30 mg ferredoxin were obtained/100 g *Clo. pasteurianum* cells (wet wt). This is almost twice the yield obtained from the same species with one of the other procedures (12–16 mg/100 g wet cells). The yield obtained for *Clo. acidi-urici* is equal to that in [6]. The costs of the new method are low. Polyethyleneimines are inexpensive and only small amounts of DEAE cellulose are required (less than 1/100 of the amount required in previously described procedures).

Acknowledgement

This work was supported by a grant from the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

References

[1] Mortenson, L. E. (1964) Biochim. Biophys. Acta 81, 71–77.
[2] Rabinowitz, J. C. (1972) Methods Enzymol. 24, 431–446.
[3] Mayhew, S. G. (1971) Anal. Biochem. 42, 191–194.
[4] Lovenberg, W., Buchanan, B. B. and Rabinowitz, J. C. (1963) J. Biol. Chem. 238, 3899–3913.
[5] Tagawa, K. and Arnon, D. I. (1962) Nature 195, 537–543.
[6] Hong, J. S. and Rabinowitz, J. C. (1970) J. Biol. Chem. 245, 4982–4987.
[7] Gersonde, K., Trittelvitz, E., Schlaak, H. E. and Stabel, H. H. (1971) Eur. J. Biochem. 22, 57–65.
[8] Scherer, P. A. and Thauer, R. K. (1978) Eur. J. Biochem. in press.
[9] Bergmeyer, H. U., Holz, G., Klotzsch, H. and Lang, G. (1963) Biochem. Z. 338, 114–121.
[10] Rabinowitz, J. C. (1963) Methods Enzymol. 6, 703–713.
[11] Nees, S., Veh, R. W., Schauer, R. and Ehrlich, K. (1975) Hoppe Seyler's Z. Physiol. Chem. 356, 1027–1042.
[12] Doeg, K. A. and Ziegler, D. M. (1962) Arch. Biochem. Biophys. 97, 37–40.
[13] Siegel, L. M. (1965) Anal. Biochem. 11, 126–132.
[14] Uyeda, K. and Rabinowitz, J. C. (1970) J. Biol. Chem. 246, 3111–3119.
[15] Lovenberg, W. and Sobel, B. E. (1965) Proc. Natl. Acad. Sci. USA 54, 193–199.