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NON-HEME IRON (IRON–SULFUR) PROTEINS OF *AZOTOBACTER VINELANDII**

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SUMMARY

A non-heme (iron–sulfur) protein has been purified from *Azotobacter vinelandii* Strain OP, which is similar in many properties to the clostridial type ferredoxin. It has 6–7 iron atoms and equivalent labile sulfides in a molecular weight of 13000 ± 1000. Optical and electron spin resonance spectra have been recorded.

INTRODUCTION

In our systematic study of the iron–sulfur proteins of *Azotobacter vinelandii*[1–3] we have now encountered a third major protein of this class. Whereas the two proteins previously described with two iron and two labile sulfur atoms and a molecular weight of 20000–25000 belong to the subgroup of plant type ferredoxins, the protein to be described here has some properties more akin to those of bacterial type ferredoxins. Since *A. vinelandii* is one of the main organisms utilized in work on nitrogen fixation[4–6] and is also under study with respect to its electron transfer system[7] and oxidative phosphorylation[8], we think it may be useful to report our work at this stage, although we have not defined the biological activity of this iron–sulfur protein. This communication describes the purification and some properties of this protein which we call iron–sulfur protein III of *A. vinelandii*.

MATERIALS AND METHODS

Purification. Cells of *A. vinelandii* were cultured in a nitrogen-free medium as described elsewhere[2]. All operations were carried out at 4°. The buffer used throughout was potassium phosphate of pH 7.4. For isolation 1 kg of cell paste was treated with *n*-butanol as described previously[2]. The mixture was centrifuged at 27000 × *g* for 2 h and the aqueous layer was separated (2.2 l).

DEAE-cellulose (Brown Co.) was prepared for use as described[2] and allowed to settle for 2–3 h. The liquid was then decanted and 300 ml of the resulting cellulose suspension were added to the butanol extract. The mixture was stirred for 6–8 h. The cellulose was separated and washed 3 times by centrifugation with 2 l of 0.05 M

* The essential parts of this work were discussed at the 3rd Intern. Conf. on Magnetic Resonance in Biological Systems and Symp. on Non-heme Iron Proteins held at Warrenton, Va., in October, 1968.

buffer. The supernatants were discarded. The cellulose with the adsorbed protein was placed in a 10-cm-wide column and eluted with 0.1 M buffer containing 0.4 M KCl. A dark green band was obtained in approximately 400–600 ml eluate, which was composed mainly of a flavoprotein described earlier[2] and protein III. The eluate was dialyzed with 3 changes against 6 l of water and placed on a DEAE-cellulose (Whatman DE-32) column (1.5 cm × 20 cm) equilibrated against 5 mM buffer. The molarity of the buffer was gradually increased to 0.1 M and then 1 M KCl was added until a KCl concentration of 0.12 M was reached. The green flavoprotein band was eluted slowly. When the column was washed with 50–60 bed volumes of this phosphate–KCl solution, a dark brown band of protein III reached the bottom of the column. The molarity of the KCl was then further increased in steps from 0.12 to 0.3 M and 2-ml fractions were collected. The ratio of absorbance at 280 mμ to that at 400 mμ of the fractions was measured. Fractions with a ratio of 2.3 or lower were pooled and precipitated by adding solid $(NH_4)_2SO_4$ to saturation at 0°. The precipitate was separated by centrifugation in the Spinco centrifuge at 40000 rev./min for 10 min and washed twice with satd. $(NH_4)_2SO_4$. The precipitate was then dissolved in 0.5 M phosphate buffer. The dissolved protein showed a ratio $A_{280\ m\mu}/A_{400\ m\mu}$ of 1.70–1.80. Such preparations were homogeneous in polyacrylamide gel electrophoresis at pH 8.0–9.0 (ref. 9) as shown in Fig. 1. Approx. 10 mg of protein can be obtained from 1 kg of cell paste.

The remaining fractions with ratios of $A_{280\ m\mu}/A_{400\ m\mu}$ not exceeding 6 may be collected from a number of batches and rechromatographed on a Whatman DE-32 column of 1.5 cm width and a length depending on the volume to be treated.

Properties. The properties of protein III, as far as determined, are summarized in Table I.

The light absorption spectra of oxidized and $Na_2S_2O_4$ reduced protein III are shown in Fig. 2 and the EPR spectra in Fig. 3. As isolated, the protein is obtained in a partly reduced state, similar to the high potential iron protein of *Chromatium*[10].

TABLE I

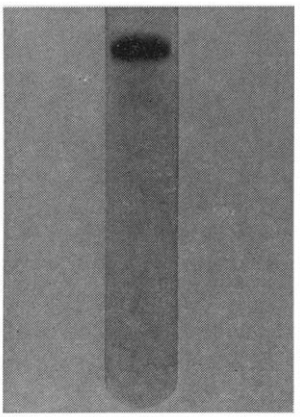
PROPERTIES OF IRON–SULFUR PROTEIN III FROM *A. vinelandii*

Determination of molecular weight and partial specific volume by sedimentation in ^2H_2O and H_2O (ref. 12). Iron determination according to ref. 13. Labile sulfur determination according to ref. 14.

Molecular weight	13000 ± 1000
Partial specific volume	0.684
Iron, atoms/mole of protein	6–7
Labile sulfur, atoms/mole of protein	6–7
Absorptivity at 400 mμ ($M^{-1}\cdot cm^{-1}$)*	
oxidized	4500
reduced	4000
EPR parameters**	
oxidized	$S_m = 2.01$; $g_m = 2.00$; $S_m = 1.975$
reduced	$S_m = 2.03$; $g_m = 2.01$; $S_m = 2.00$

* There is no maximum but a pronounced shoulder. The absorptivity is given per gatom of iron.

** S_m refers to points of maximal positive or negative slope and g_m to zero slope in the absorption curve[15]. The g values for the oxidized protein measured at 80°K are estimated to lie within ± 0.005 and those of the reduced sample, measured at 13°K, to lie within ± 0.01 of the reported value.



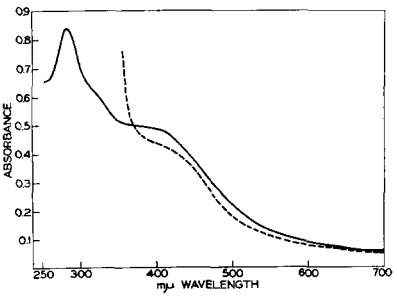


Fig. 1. Polyacrylamide gel electrophoresis pattern of Azotobacter iron–sulfur protein III. The anode was at the bottom.

Fig. 2. Optical absorption spectrum of Azotobacter iron–sulfur protein III. ———, oxidised spectrum on 0.24 mg/ml protein in 25 mM Tris–HCl. (pH 7.4); - - - -, reduced spectrum taken 5 min after addition of excess $Na_2S_2O_4$. Spectra recorded on a Cary 15.

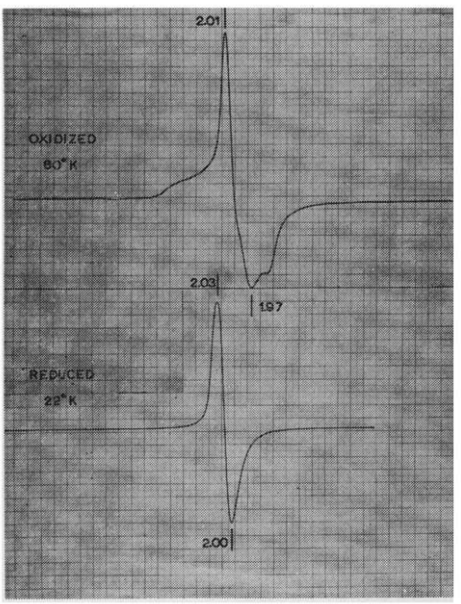


Fig. 3. EPR spectra of Azotobacter iron–sulfur protein III. 5.1 mg of protein (2.15 mM iron) were dissolved per ml of 0.1 M phosphate buffer of pH 7.4. Upper curve, oxidized form: On 0.25 ml of this solution the EPR spectrum was observed repeatedly, while small increments of $K_3Fe(CN)_6$ were added after thawing between observations. When the signal was maximally developed, the spectrum shown was recorded. The microwave power was 18 mW, the modulation amplitude 6 gauss, the scanning rate 200 gauss/min, the time constant 0.5 sec and the temperature 80°K. Lower curve, reduced form: To another 0.25-ml aliquot a small quantity of $Na_2S_2O_4$ was added. The spectrum was recorded at 1 mW of power, 3 gauss modulation amplitude, a scanning rate of 400 gauss/min, time constant of 0.5 sec and a temperature of 22°K.

The optical spectrum of the oxidized form was obtained after passing the protein through a small column of Dowex-1 in Cl^- form, equilibrated against 0.025 M Tris–HCl, pH 7.4, which contained 0.5 M KCl. This column had adsorbed a 20–30-fold molar excess of $K_3Fe(CN)_6$ with respect to the iron content of protein III. On reduction with dithionite there is a slight reduction of absorption at 400 mμ, whereas the EPR spectra show significant differences. Protein III has a high iron content per molecule, as it is typical of the bacterial type ferredoxins. The EPR signals show the strong temperature dependence typical of those of ferredoxins. Whereas the signal of the oxidized form can be seen easily at liquid nitrogen temperature, that of the reduced form is barely detectable above 25°K. We attribute these signals tentatively to iron but the question of valency must be left open. According to double integrations of the EPR spectra, the signals of the oxidized as well as the reduced forms could account for 6–7 % of the iron found in the protein by chemical analysis. Although the unpaired electrons accounted for in EPR signals of ferredoxins are in all known instances less than the number of iron atoms present, the significance of the fractional recoveries observed with protein III is not clear. It is also not clear whether the iron atoms which are responsible for the signal of the oxidized form are the same as those showing the signal of the reduced form.

Protein III does not appear to be a part of the nitrogenase fractions I or II as named by BULEN AND LECOMTE[4]. This interpretation was based on the experience gained during isolation of these fractions from *A. vinelandii* by the method of HWANG[11]. Fractions I and II were removed by centrifugation of heat and streptomycin sulfate treated crude extract at 40000 rev./min (Spinco) for 1 h. The supernatant so obtained was dialyzed against water without anaerobic precautions. A heavy precipitate was formed of the remaining denatured nitrogenase which was removed by centrifugation at 27000 $\times g$ for 15 min, and from this supernatant, protein III could be isolated on a Whatman DE-32 column as described earlier.

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After submission of this manuscript a report appeared by YOCH *et al.*[16] describing a protein from *A. vinelandii*, which these authors choose to call ferredoxin and which, according to the reported properties, is very similar if not identical to protein III described here. There is a discrepancy in molecular weight, which YOCH *et al.*[16] tentatively report as 20000. HALL AND EVANS[17], on the basis of our report at the Symposium on Non-heme Iron Proteins at Warrenton, Va., in October 1968, list some properties of protein III in their review article, but erroneously refer to ref. 3 of the present paper, which deals only with proteins I and II.

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