A FERREDOXIN FROM *AGROBACTERIUM TUMEFACIENS*

J. VAN BEEUMEN and J. DE LEY
Laboratorium voor Microbiologie, State University of Gent, Ledeganckstr. 35, 9000 Gent, Belgium

and

D. O. HALL, R. CAMMACK and K. K. RAO
Department of Plant Sciences, University of London, King's College, Half Moon Lane 68, London SE24 9JF, England

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1. Introduction

Ferredoxins are a ubiquitous group of biologically important iron–sulfur-proteins found in all forms of life investigated so far [1,2]. A number of soluble ferredoxins containing 8 Fe + 8 S and 4 Fe + 4 S atoms per molecule have been isolated from various species of bacteria [1–3]. Ferredoxins containing 2 Fe + 2 S atoms per molecule are present in algae and plants, but a few of them have also been isolated from mammalian and bacterial sources [1,2]. This paper reports on the presence, purification and some properties of a 2 Fe + 2 S type ferredoxin from *Agrobacterium tumefaciens*, the microorganism that causes a wellknown plant tumour disease ('Crown-gall') [4].

2. Methods and results

2.1. *Isolation and purification of the ferredoxin*

Agrobacterium tumefaciens, strain II Chrysanthemum (= II Chr) was grown under strong aeration in a 150 litre medium with yeast extract and 1% glucose [5].

The purification of the ferredoxin was carried out starting from 100 g of lyophilized cells. They were suspended in 2 litre of 20 mM Tris–HCl, pH 8.2, and disrupted in an APV Manton-Gaulin press at 600 kg/cm^2. The treatment was stopped when the temperature of the suspension reached 25°C. Cell debris was centrifuged off for 1 h at 15 000 g. A thick suspension of DEAE-cellulose (DE 23, Whatman), precycled with 20 mM Tris–HCl buffer, pH 8.2, containing 0.2 M KCl, was added to the reddish supernatant. After stirring for 10 min, the DE 23 was allowed to settle. The supernatant was decanted and used for the preparation of cytochrome *c*-556 and *c*-552 [6]. The DE 23, containing bound ferredoxin, was poured into a column (2.5 cm × 10 cm) and washed with the above mentioned 0.2 M KCl containing Tris buffer until all the cytochrome material was washed out. The column was then eluted with 0.8 M KCl and the eluate collected in 3 ml fractions. The presence of iron–sulphur proteins was followed by measuring the EPR spectrum of each fraction after reduction with dithionite. Fractions 13–22 which contained the ferredoxin were pooled and subjected to ammonium sulphate fractionation. The 80–100% saturated fraction contained some 3 mg of purified ferredoxin. The salt was removed by ultrafiltration over a UM-2 Diaflo filter (Amicon).

2.2. *EPR spectrum and redox potential*

The EPR spectra were recorded at 77°K on a Varian E4 spectrometer, using a liquid nitrogen insert dewar. The spectrum of the purified ferredoxin in the reduced state is given in fig.1. It shows a signal at $g_{av} = 1.96$, characteristic of an iron–sulphur protein. The axial symmetry of this signal is typical of a 2 Fe + 2 S ferredoxin of the type found in bac-

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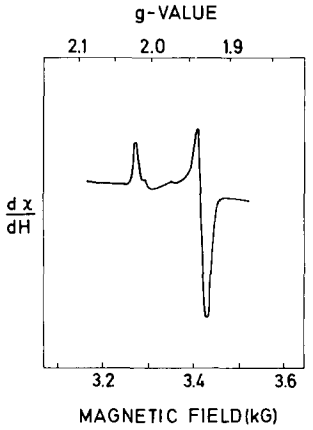

Fig.1. EPR spectrum of reduced two-iron type ferredoxin of *A. tumefaciens*, strain II Chr. The settings were as follows : temperature, 77° K; power, 20 mW; modulation amplitude, 10 gauss.

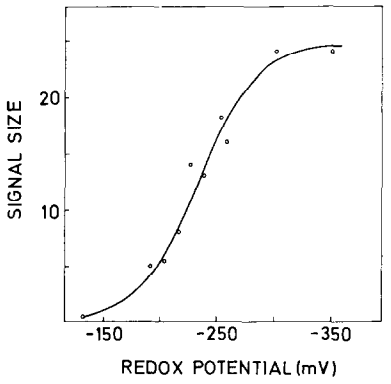

Fig.2. Redox titration curve of *A. tumefaciens* ferredoxin. The EPR signal intensity is shown in arbitrary units. The experiment was carried out at pH 7.4 and 25°C using the mediators mentioned in the text.

teria [1,7] and in mammalian adrenal glands [8].

The redox potential of the ferredoxin was determined by a modification of the redox titration method of Dutton [9]. A 5 ml sample of the ferredoxin in 0.1 M potassium phosphate buffer, pH 7.4, was mixed with the following mediators : 2-hydroxy-1,4-naphthoquinone (E'_0 = −145 mV), anthraquinone-1,5-disulphonate (E'_0 = −170 mV), anthraquinone-2-sulphonate (E'_0 = −225 mV), phenosafranine (E'_0 = −225 mV), safranine T (E'_0 = −289 mV) and benzyl-viologen (E'_0 = −311 mV), all at a final concentration of 0.1 mM. The mixture was maintained at 25°C under purified argon and the potential of the system adjusted with small additions of 0.05 M sodium dithionite or 0.1 M potassium ferricyanide solution. The potential was measured with platinum and calomel electrodes which were calibrated using quinhydrone. After adjustment to a particular redox potential, the mixture was allowed to equilibrate for 2 min. A sample was then withdrawn through a stainless steel transfer tube into an EPR tube flushed with argon, and frozen in liquid nitrogen.

To ensure that the ferredoxin was equilibrated with the mediators, titration curves were determined both by lowering the potential with dithionite and raising it with ferricyanide. Essentially the same result was obtained in each case (fig.2). The shape of the curve indicates that n = 1, as is the case for all 2 Fe ferredoxins for which the number of transferred electrons was measured [1]. The figure shows that the redox potential of *A. tumefaciens* ferredoxin is −223 mV ± 10 mV.

3. Concluding remarks

The ferredoxin from *Agrobacterium* described here adds to the small number of bacterial two-iron ferredoxins known. The lineshape of the EPR signal of the reduced protein is identical to the one measured for the ferredoxins of *Escherichia coli* [10], *Pseudomonas aminovorans* [1], and *Pseudomonas putida* [7]. The latter protein, also called putidaredoxin, catalyses camphor hydroxylation in the same way as the mammalian 2 Fe ferredoxin, adrenodoxin, is involved in the hydroxylation of adrenal steroids [8]. The redox potential of putidaredoxin (−240 mV) and that of adrenodoxin (−270 mV) are very similar to the value for the *Agrobacterium* ferredoxin. This suggests that the latter protein might likewise be involved in hydroxylation reactions although we have no experimental data yet to support this hypothesis.

It may be repeated here that the evolutionary affiliation of the bacterial 2 Fe ferredoxins is very uncertain, mostly due to a lack of amino acid sequence data. Any new discovery of a bacterial two-iron

ferredoxin, whether from an aerobic or an anaerobic organism, may help to throw light on the evolution of these proteins.

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