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

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It has become evident within the past 5 years that non-heme iron proteins of the "iron-sulfur" type are ubiquitous among living organisms. We consider the presence in the molecule of "labile sulfur" and iron and the appearance, on reduction, of an EPR signal with an average g value < 2 (principal g value generally 1.94) as the common properties of iron-sulfur proteins. On the other hand, many differences are found in the more detailed properties and function of individual representatives of this class. From Clostridium pasteurianum, for instance, at least two distinct iron-sulfur proteins have been isolated; the protein to which the name ferredoxin was originally given (Mortenson, Valentine, and Carnahan, 1962), and a protein of unknown function (Hardy, Knight, McDonald, and D'Eustachio, 1965), which is not considered to be a ferredoxin. Many proteins of this class have been implicated in photosynthesis or nitrogen fixation. It is therefore of particular interest that in Azotobacter vinelandii, an organism well known for its ability to fix nitrogen, no compound was encountered which

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was considered to be a ferredoxin. Optical absorption spectra published by Bulen and Le Comte (1964) for active azotobacter fractions indicate, however, that such fractions may well include proteins of the iron-sulfur type and an iron-sulfur protein (called here protein I) similar to the above mentioned protein from C. pasteurianum (Hardy, et al., 1965), was in fact previously isolated from A. vinelandii (Shethna, Wilson, Hansen, and Beinert, 1964). We wish to report here the isolation of a second iron-sulfur protein (II) from A. vinelandii, which appears to be more abundant than that found previously (Shethna et al., 1964), and the EPR and optical spectra of which have striking similarities with those of plant type ferredoxins. On the basis of the data to be presented, we come to the conclusion that the question, whether A. vinelandii contains a ferredoxin or not, is not so much a problem of experiment but rather one of nomenclature.

Materials and Methods

In the procedure for the isolation of azotobacter iron-sulfur protein I (Shethna et al., 1966), ammonium sulfate fractionation roughly subdivided the dialyzed butanol extract into fractions containing protein I (0 - 45% saturation), cytochromes (45 - 65%), and flavoprotein (60 - 100%). The bulk of protein II is found associated with the cytochrome fraction and is precipitated between 45 and 60% saturation with ammonium sulfate. In the ensuing DEAE cellulose step the heme proteins are more readily eluted (2.5 mM phosphate pH 7.4) than protein II (5 mM phosphate). The eluate, which in addition to protein II still contains cytochromes, is concentrated by precipitation with ammonium sulfate at 80% saturation, dialyzed, and rechromatographed on Whatman DE-32 cellulose. Details of a procedure for the preparation of proteins I and II of the purity indicated in Table I will be published elsewhere. Each protein migrates as a single band in polyacrylamide gel electrophoresis in the standard system (Davis, 1964), and in the analytical ultracentrifuge.

It is noteworthy that two distinct proteins with the optical and EPR properties described here for proteins I and II were readily obtained from azotobacter

extracts prepared by such treatments as sonic oscillation or application of the French pressure cell, not involving organic solvents.

Results

Analytical data on the azotobacter iron-sulfur proteins and, for comparison, on spinach ferredoxin are found in Table I. As recently reported for protein I (DerVartanian, 1968), and related proteins (Orme-Johnson, Hansen, and Beinert,

Table I: Properties of Azotobacter iron-sulfur proteins I and II and spinach ferredoxin.

Protein	Molecular weight	Iron	Labile sulfur	Absorptivity****
	dalton		g atoms/mole	$M^{-1}cm^{-1} \times 10^{-3}$
Azotobacter I	21,000 ± 1,000*	2.0 ± 0.1	2.1 ± 0.1	15.2 (280); 7.78 (331); 4.73 (419) 4.86 (460); 2.8 (550)
Azotobacter II	24,000 ± 1,000*	2.0 ± 0.1	2.2 ± 0.2	6.83 (280); 8.33 (344); 6.97 (418); 5.30 (460); 2.93 (550)
Spinach*** Ferredoxin	11,650	2	2	10.08 (276); 6.39 (325); 4.84 (420); 4.40 (465)

*Determined by sedimentation-equilibrium, using $\bar{v}$ determined by Mr. Grant Barlow as 0.74 ± 0.01 ml/g; values from DerVartanian, D. V., 1968.

**As for *, but $\bar{v}$ assumed as 0.74 ml/g.

***Taken from Tagawa, K., and Arnon, D. I., 1968 .

****Per g atom of iron, corresponding wavelength in mμ in parenthesis.

1968), protein II takes up one reducing equivalent per mole, i.e., per two g atoms of iron, and the electron added is accounted for ($\pm$ 10%) in the EPR signal.

The isolation of protein I was originally monitored by EPR spectroscopy. We realized that the EPR signal of the final product was not identical in detail with that of the starting material. This could be interpreted as being due to selection of one species out of a larger number (≥ 2) of similar proteins or to modification of the metal complex exhibiting the signal. That the first alternative is correct can be seen from a comparison of the EPR spectra of the products with that of the original butanol extract, which is the source material for the isolation of both iron-sulfur proteins. These spectra are shown in Fig.

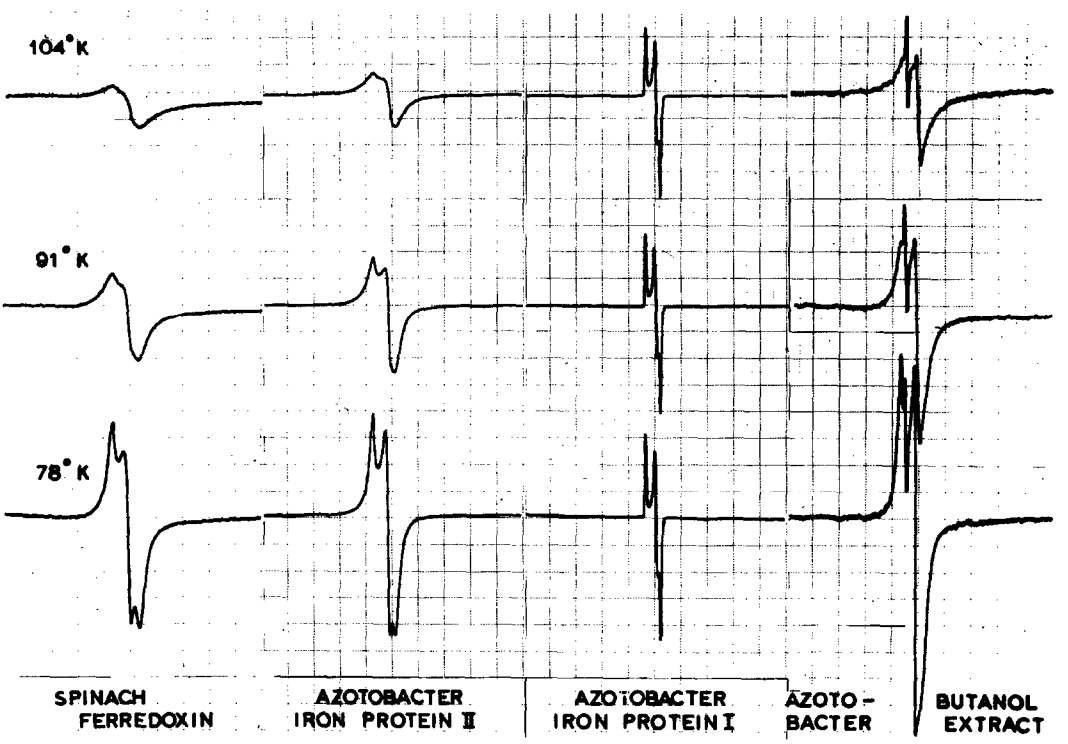

Figure 1: Temperature dependence of EPR signals of iron-sulfur proteins. EPR spectra (first derivative of absorption vs. magnetic field on the abscissa) are shown at 3 different temperatures as indicated. The conditions of EPR spectroscopy were: Microwave power, 27 mwatt; modulation amplitude, 6 gauss; scanning rate, 500 gauss/min.; time constant, 0.5 sec. The total scan represents approximately 2500 gauss, with the field increasing from left to right. The prominent peaks in the spectra are located at these field values at 9,255.3 MHz: spinach ferredoxin: 3227, 3341, 3404, and 3485 gauss; azotobacter iron-sulfur protein II: 3243, 3375, 3420, and 3470 gauss; and azotobacter iron-sulfur protein I: 3274, 3376, 3402, and 3431 gauss.

1. The signals in the second column are those of azotobacter iron-sulfur protein II, in the third, those of protein I, and in the fourth, those of a butanol extract from azotobacter. The spectra are shown, from top to bottom, as recorded at 104, 91 and 78°K. The temperature sensitivity of the EPR signal of protein II is striking and almost equals that of the spinach ferredoxin signal (first column) whereas it is clearly different from that of protein I. Line width and separation in the spectrum of protein II are also more similar to those in the spinach ferredoxin signal than to those of protein I, which again resembles in this respect the protein from C. pasteurianum. The signals obtained from the butanol extract (last column) clearly show that both the temperature sensitive signal of protein II as well as the - even at higher temperature - sharp spikes of protein I are represented. In the crude extract a small radical signal at g = 2 is superimposed on the signals of the iron proteins. Since EPR spectroscopy, as applied in the original purification procedure, was routinely carried out at 98°K, it is obvious from Fig. 1 that, on the basis of this criterion, protein II could easily be missed or discounted as a minor contaminant.

The optical spectra of the oxidized forms of spinach ferredoxin and the two azotobacter proteins are compared in Fig. 2. While proteins I and II have a maximum or a shoulder at 550 mμ which is absent in the spinach ferredoxin spectrum, the spinach ferredoxin and protein II spectra are more similar in the 400 - 500 mμ region.

No function for either azotobacter protein is known as yet. Protein II neither substitutes for spinach ferredoxin in the TPN photoreduction by chloroplasts nor clostridial ferredoxin in nitrogen fixation by C. pasteurianum. It is, however, possible that the isolation procedures, though yielding apparently intact and homogeneous iron-sulfur proteins, may not be suitable for preservation of a specific activity. Characterization of both proteins is being continued.

These findings as well as related work in this area, pursued in several laboratories, clearly call for a clarification and definition of nomenclature.

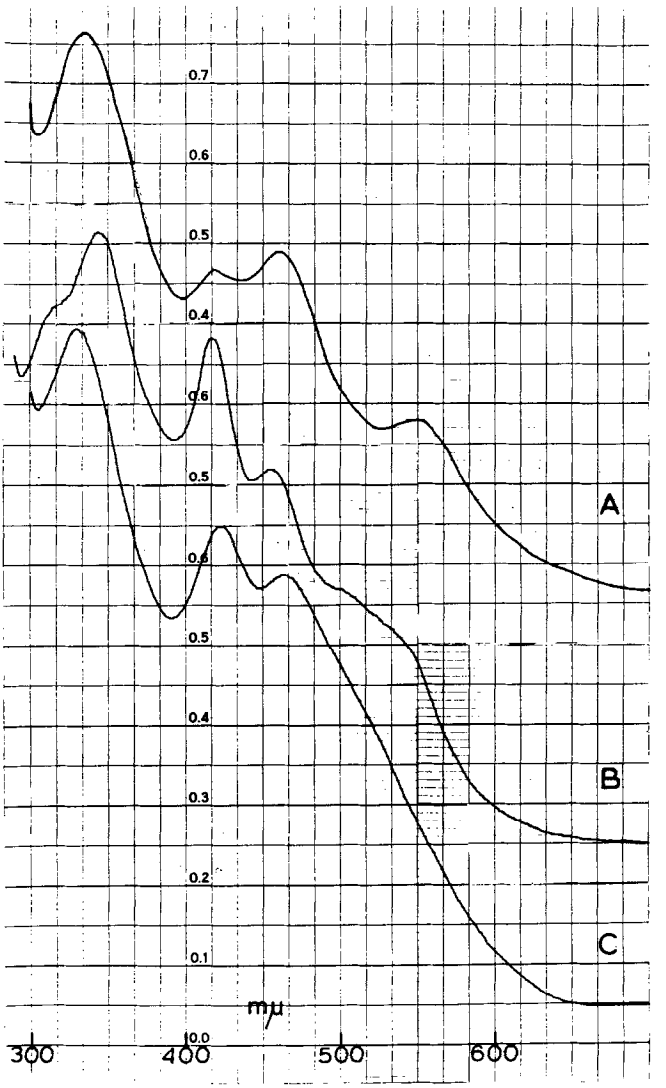

Figure 2: Optical absorption spectra of iron sulfur proteins from 300 to 700 mμ: A, azotobacter iron-sulfur protein I; B, azotobacter iron-sulfur protein II; and C, spinach ferredoxin. The solutions of the azotobacter proteins in phosphate buffer contained 100 ± 5 mμ g atoms of bound iron per ml, that of spinach ferredoxin 1.35 times this amount. The light path was 1.0 cm. For better comparison, the spectra are staggered. The absorbance values printed on the recorder paper immediately below the peak at ~420 mμ of each curve refer to the absorbance values applying to this curve.

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