

Resonance Raman spectroscopic evidence for the presence of 4Fe and 3Fe centers in *Pseudomonas ovalis* ferredoxin and *Mycobacterium smegmatis* ferredoxin

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<i>Raman spectroscopy</i>	<i>Ferredoxin</i>	<i>3Fe center</i>	<i>4Fe center</i>
<i>Pseudomonas ovalis</i>		<i>Mycobacterium smegmatis</i>	

1. INTRODUCTION

Rather recent X-ray crystallographic data have revealed the existence of a novel [3Fe–3S] cluster in *Azotobacter vinelandii* ferredoxin I [1,2]. *Pseudomonas ovalis* ferredoxin and *Mycobacterium smegmatis* ferredoxin have been thought to be analogous ferredoxins to *A. vinelandii* ferredoxin I from the similarity in the primary structures, especially from their cysteine distributions [3–5]. In particular, since *P. ovalis* ferredoxin has the same primary structure from the 2nd to 61st amino acid residues from the N-terminus as that of *A. vinelandii* ferredoxin I, two iron-sulfur centers of these two ferredoxins can be expected to be quite analogous to each other. *M. smegmatis* ferredoxin was characterized to contain two iron-sulfur centers by potentiometric titration and at least one of them was found to be a 3Fe center from EPR spectroscopy [6]. Furthermore, both ferredoxins were identified to have 3Fe and 4Fe centers by proton magnetic resonance spectroscopy [7].

The presence of such 3Fe centers in some iron-sulfur proteins has been verified by Mössbauer spectra [8,9], low temperature magnetic circular dichroism spectra [10], electron paramagnetic spectra [11], and proton magnetic resonance spec-

tra [7,12,13]. Resonance Raman spectra have also been recently applied to infer the kind of iron-sulfur centers of several ferredoxins; *Desulfovibrio gigas* ferredoxin I was shown to contain both 3Fe and 4Fe centers from the resonance Raman spectrum of this ferredoxin [14]. The resonance Raman spectra of the three [2Fe–2S] ferredoxins isolated from spinach, *Phytolacca americana*, and *Halobacterium halobium* had spectral patterns quite similar to each other in the low frequency region [15].

We report here the resonance Raman spectra of two ferredoxins isolated from *P. ovalis* and *M. smegmatis* in both the isolated state and reduced state and show that these two ferredoxins contain both 3Fe and 4Fe centers and that there exist some differences in the fine structures of the iron-sulfur centers in these two ferredoxins.

2. EXPERIMENTAL

Isolation procedures of *P. ovalis* ferredoxin [16] and *M. smegmatis* ferredoxin [6] have been described elsewhere. Protein concentrations were determined by the use of their millimolar extinction coefficients: 26.0 at 406 nm for *M. smegmatis* ferredoxin [6] and 34.0 at 400 nm for *P. ovalis* fer-

redoxin [16]. Absorption spectra were recorded with a Union Giken SM 401 spectrophotometer at ambient temperature.

The Raman spectrometer was equipped with a Narumi model 750z-1200 double monochromator, an NEC GLG3300 Ar⁺ laser, and a cooled HTV (HPK) 649S photomultiplier. Spectra were obtained in a quartz cell using 4579 Å laser excitation. Samples were cooled with cold nitrogen gas during Ar⁺ laser excitation and scattered light at 90° to the incident beam was collected. The spectral slit width was 6 cm⁻¹ and a multiscan averaging technique was employed to improve the signal:noise ratio of the spectrum using a Hitachi Level III microcomputer. Correction of the wave number was made with the plasma line of Ar⁺ laser with reference to the M.I.T. Wavelength Tables [17].

3. RESULTS AND DISCUSSION

Fig.1a shows the resonance Raman spectrum of *P.ovalis* ferredoxin in the isolated state produced by the use of 4579 Å Ar⁺ laser excitation. The spectrum shows 5 clear bands at 260, 332, 345, 365, and 389 cm⁻¹, and a distinct shoulder around 247 cm⁻¹. These band positions and relative intensities are quite similar to those obtained from *D.gigas* ferredoxin I, which have already been proved to contain both 3Fe and 4Fe centers [14]. Upon reduction with excess sodium dithionite, the resonance Raman spectrum of *P.ovalis* ferredoxin changed markedly, as shown in fig.1b, with 5 bands at 242, 332, 346, 362, and 389 cm⁻¹. The authors in [14] showed that the resonance Raman spectrum of the 4Fe center in *Clostridium pasteurianum* ferredoxin changed very little upon reduction, while that of the 3Fe center in *D.gigas* ferredoxin II changed markedly. Based on these observations, the Raman spectra of *P.ovalis* ferredoxin can be explained as follows: the bands at 247 (shoulder) and 332 cm⁻¹ in the isolated state, attributable to the 4Fe center vibration, did not change significantly, while the band at 260 cm⁻¹ disappeared and the bands at 346 and 389 cm⁻¹ decreased markedly in intensity upon reduction. The foregoing results strongly suggest that *P.ovalis* ferredoxin contains both 3Fe and 4Fe centers.

Fig.1c shows the resonance Raman spectrum of *M.smegmatis* ferredoxin in the isolated state. There are 3 distinct Raman bands at 262, 343, and

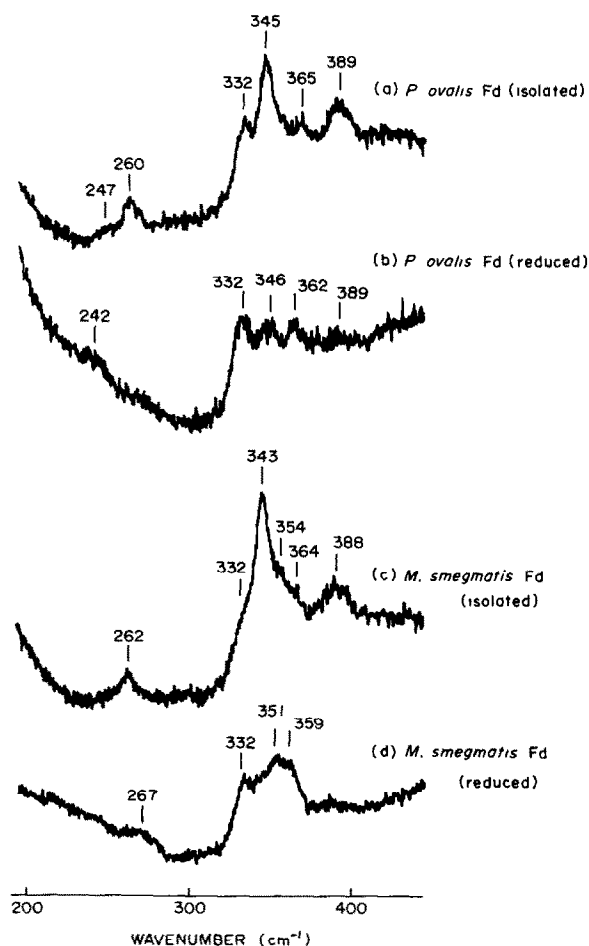


Fig.1. Resonance Raman spectra of *P.ovalis* ferredoxin and *M.smegmatis* ferredoxin. All spectra were recorded using 4579 Å Ar⁺ laser excitation (~40 mW). Spectral band width was 6 cm⁻¹. (a) Isolated state *P.ovalis* ferredoxin; (b) excess dithionite-reduced *P.ovalis* ferredoxin; (c) isolated state *M.smegmatis* ferredoxin; (d) excess dithionite-reduced *M.smegmatis* ferredoxin. The ferredoxin concentrations were 0.66 mM in 0.05 M Tris-HCl (pH 8.0), containing 0.5 M NaCl.

388 cm⁻¹ and very weak shoulders around 332, 354, and 364 cm⁻¹. It follows from the data in [14] that identification of a 4Fe center by Raman spectroscopy can be performed whether or not any intense band around 332 cm⁻¹ is observable. From this fact, it seemed difficult to interpret the Raman spectrum of *M.smegmatis* ferredoxin such that the weak shoulder around 332 cm⁻¹ would signify the presence of 4Fe center. However, the presence of the Raman band around 332 cm⁻¹ became unam-

		10		20		30
<i>A. vinelandii</i>	A F V V T D N	Ⓢ I K Ⓢ K Y T D	Ⓢ V E V	Ⓢ P V D	Ⓢ F Y E G P N F L	
<i>P. ovalis</i>	T F V V T D N	Ⓢ I K Ⓢ K Y T D	Ⓢ V E V	Ⓢ P V D	Ⓢ F Y E G P N F L	
<i>M. smegmatis</i>	T Y V I A E P	Ⓢ V D V K D K A	Ⓢ I E E	Ⓢ P V D	Ⓢ I Y E G A R M L	
<i>T. thermophilus</i>	P H V I	Ⓢ Q P Ⓢ I G V K D Q S	Ⓢ V E V	Ⓢ P V E	Ⓢ I Y D G G D Q F	
		40		50		60
<i>A. vinelandii</i>	V I H P D E	Ⓢ I D Ⓢ A L Ⓢ E P E	Ⓢ P A Q A I F S E D E V P E D M			
<i>P. ovalis</i>	V I H P D E	Ⓢ I D Ⓢ A L Ⓢ E P E	Ⓢ P A Q A I F S E D E V P E D M			
<i>M. smegmatis</i>	Y I H P D E	Ⓢ V D Ⓢ G A Ⓢ E P V	Ⓢ P V E A I Y Y E D D V P D Q W			
<i>T. thermophilus</i>	Y I H P E E	Ⓢ I D Ⓢ G A Ⓢ V P A	Ⓢ P V N A I Y P E E D V P E Q W			

Fig.2. Amino acid sequences of *A.vinelandii* ferredoxin I, *P.ovalis* ferredoxin, *M.smegmatis* ferredoxin, and *T.thermophilus* ferredoxin. Cysteine residues are encircled.

biguous when reduced with excess sodium dithionite, as shown in fig.1d. In a similar manner, as reported in [14], it is possible to interpret the two Raman spectra of *M.smegmatis* ferredoxin as follows: the intensity of the 343 cm^{-1} band, attributable to the 3Fe center vibration, decreased markedly upon reduction, and consequently the shoulder around 332 cm^{-1} became obvious as a peak. Thus, *M.smegmatis* ferredoxin also seems to contain both 3Fe and 4Fe centers.

The weak shoulder around 332 cm^{-1} in the isolated state of *M.smegmatis* ferredoxin, can be interpreted by two possibilities:

- (i) *M.smegmatis* ferredoxin contains many more 3Fe centers than *D.gigas* ferredoxin I, which has been reported to contain 10–40% of 3Fe centers and the remainder 4Fe centers [14].
- (ii) The band intensity at 343 cm^{-1} of *M.smegmatis* ferredoxin is much stronger than that of *D.gigas* ferredoxin I or *P.ovalis* ferredoxin, which fact may be derived from some differences in the fine structure of the Fe-S clusters of the two ferredoxins.

As to this problem, proton magnetic resonance spectra are informative; the 3 ferredoxins isolated from *P.ovalis*, *M.smegmatis*, and *T.thermophilus* showed quite similar spectral intensities [5]. If

P.ovalis ferredoxin contains an equal amount of the 3Fe and 4Fe centers, as would be expected from the similarity of the amino acid sequences as discussed below, it would be reasonable to assume that *M.smegmatis* ferredoxin also contains an equal amount of both Fe-S centers. Thus possibility (ii) seems likely from the results obtained by the proton magnetic resonance spectra. In addition, the amino acid sequences of these ferredoxins may also provide clues to clarifying the two possibilities. These sequences of the 4 homologous ferredoxins are shown in fig.2. The similarity between *P.ovalis* and *A.vinelandii* ferredoxin I is quite obvious: the same sequences are found between the 2nd and the 61st amino acid residues from the N-terminus, in which all the cysteine residues are included. Then, these two ferredoxins should have quite homologous Fe-S clusters. On the other hand, *M.smegmatis* ferredoxin [5], as well as *T.thermophilus* ferredoxin [18], contains a valine residue at the 11th position instead of the cysteine residue as in *P.ovalis* and *A.vinelandii* ferredoxins. This 11th cysteine residue ligates to the 3Fe center in the case of *A.vinelandii* ferredoxin [1]. Absence of the cysteine residue at position 11 may suggest some differences in the structure of the Fe-S clusters between *M.smegmatis* and *P.ovalis* or *A.vinelandii* ferredoxin. This may reflect the difference in intensity of the resonance Raman band at 343 cm^{-1} among these ferredoxins.

In conclusion, the resonance Raman spectra in

the isolated state and the reduced state strongly indicate that *P. ovalis* ferredoxin and *M. smegmatis* ferredoxin contain both 3Fe and 4Fe centers, and furthermore the slightly different resonance Raman spectra may suggest also some differences in the fine structure of the 3Fe center between these two ferredoxins.

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