Resonance Raman spectroscopic study on the iron–sulfur proteins containing [2Fe–2S] clusters

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Resonance Raman spectra have been obtained for *Spinacea oleracea, Phytolacca americana* and *Halobacterium halobium* ferredoxins. These spectra were very similar to each other in terms of both vibrational frequencies and relative intensities, strongly suggesting that their molecular structures of active centers are very similar. The resonance Raman spectra were, however, fairly different in the frequencies of Fe–S(Cys) stretching modes from the spectrum of beef adrenodoxin previously reported. Probably the nature of Fe–S(Cys)-bonding and/or the manner of cysteine ligation is somewhat different between adrenodoxin and the other [2Fe–2S] ferredoxins.

Resonance Raman spectroscopy *Iron–sulfur protein* *Ferredoxin* *[2Fe–2S] cluster*

1. INTRODUCTION

The [2Fe–2S] iron–sulfur proteins are widely distributed in nature, ranging from non-photosynthetic, aerobic bacteria to higher plants and animals [1]. These proteins are physiologically classified into 3 groups, halobacterial type and chloroplast type of [2Fe–2S] ferredoxins and adrenodoxin. Although the comparative studies of the primary structures of [2Fe–2S] iron–sulfur proteins have been actively performed, the molecular structural similarities and differences of active centers among various [2Fe–2S] iron–sulfur proteins have not been well documented. Only one [2Fe–2S] cluster of *Spirulina platensis* ferredoxin was delineated by X-ray crystallography [2,3]. It is now important to examine whether two ferredoxins consisting of very close amino acid sequences possess very similar [2Fe–2S] clusters and whether two ferredoxins with fairly different amino acid sequences hold somewhat different [2Fe–2S] clusters.

Here, we report the resonance Raman spectra of *Spinacea oleracea* (spinach), *Phytolacca americana*, and *Halobacterium halobium* ferredoxins. These are well known examples of chloroplast type [2Fe–2S] ferredoxin (spinach and *P. americana* ferredoxins) and halobacterial type [2Fe–2S] ferredoxin (*H. halobium* ferredoxin). The amino acid sequences of spinach and *P. americana* ferredoxins are strikingly homologous [4], while the amino acid sequence of *H. halobium* ferredoxin is considerably different from the other two ferredoxins in several regions [5] as shown in fig.1. It will be directly shown by the resonance Raman spectra that all 3 ferredoxins hold very similar [2Fe–2S] clusters. Moreover, the present resonance Raman spectra will be compared to the spectrum of adrenodoxin reported in [6,7] for elucidating the structural differences of [2Fe–2S] clusters.

During the last decade, resonance Raman spectroscopy played an important role in investigating

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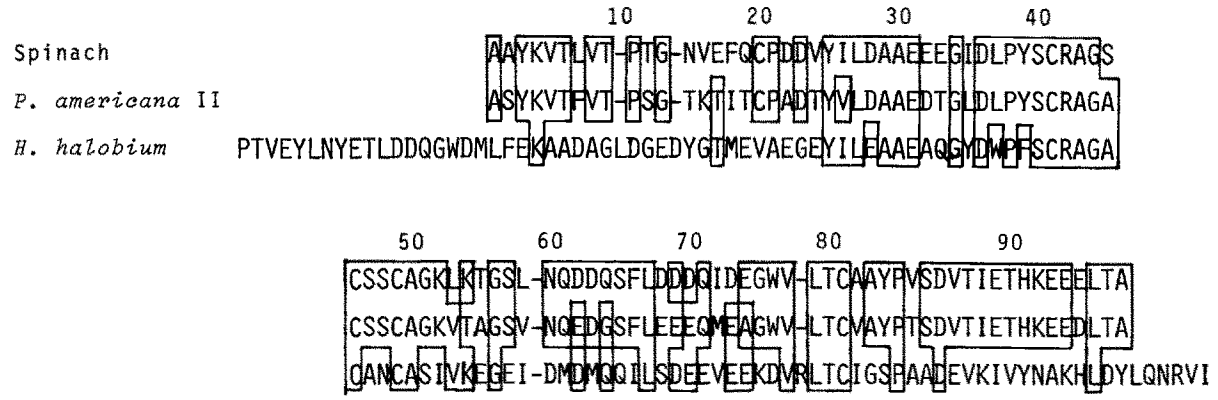

Fig.1. Amino acid sequences of spinach, *P. americana* and *H. halobium* ferredoxins. Invariant residues are surrounded by lines.

the molecular structures of active centers in various metallo-proteins [8–10]. However, the application to iron–sulfur proteins was rather behind, mainly because of relatively weak resonance enhancement. Recently, high quality resonance Raman spectra of some ferredoxins were presented [11,12]. The authors proposed that resonance Raman spectroscopy has potential in Fe–S cluster identification, even in cases where more than one cluster type is present [11]. The resonance Raman spectra of iron–sulfur proteins provide vibrational spectra of Fe–S clusters in situ without interferences of the Raman bands from apo-proteins. Thus resonance Raman spectroscopy has considerable promise for not only identifying Fe-clusters but also delineating the detailed molecular structure of Fe–S clusters.

2. MATERIALS AND METHODS

The isolation of the ferredoxins from spinach and pokeweed (*P. americana*) was performed by a combination of DEAE-cellulose and Sephadex G-75 column chromatographies essentially as in [13]. The preparation of the ferredoxin from *H. halobium* was based on the same procedures used for plant ferredoxins except for an extraction step. *H. halobium* ferredoxin was extracted from the cells by osmotic shock; the cells were suspended in 10 mM Tris–HCl buffer (pH 7.5) containing 5 mM $MgCl_2$, and stirred after adding DNase until homogenous suspension was obtained. After centrifugation to remove the broken cell debris, crude ferredoxin was obtained from the supernatant by passing through a DEAE-cellulose column. The ferredoxin was eluted with 0.1 M Tris–HCl buffer (pH 7.5) containing 0.7 M NaCl and further purified as above.

The apparatus used to obtain resonance Raman spectra consisted of a Spectra-physics model 164 Ar laser and a JEOL 400D laser Raman spectrophotometer equipped with HTV-photomultiplier tube. A sample was contained in a small cylindrical cell and cooled with cold N_2 gas. The laser light was illuminated from the bottom of the cell and the scattered light was collected at right angles from the incident light. Peak frequencies were calibrated using the spectrum of indene and are believed to be accurate to ± 1 cm^{-1} for well resolved bands. The resonance Raman spectra were repeated at least twice and showed good reproducibility. After the resonance Raman measurement, absorption spectrum and ^{1}H-NMR spectrum were recorded to confirm that the laser light did not decompose or convert an iron–sulfur cluster.

3. RESULTS AND DISCUSSION

The 488-nm excited resonance Raman spectra of the isolated forms of spinach, *P. americana*, and *H. halobium* ferredoxins are shown in fig.2. Although the resonance Raman spectrum of spinach ferredoxin had been already reported [14], we remeasured it for the sake of comparison. The present spectrum of spinach ferredoxin is almost

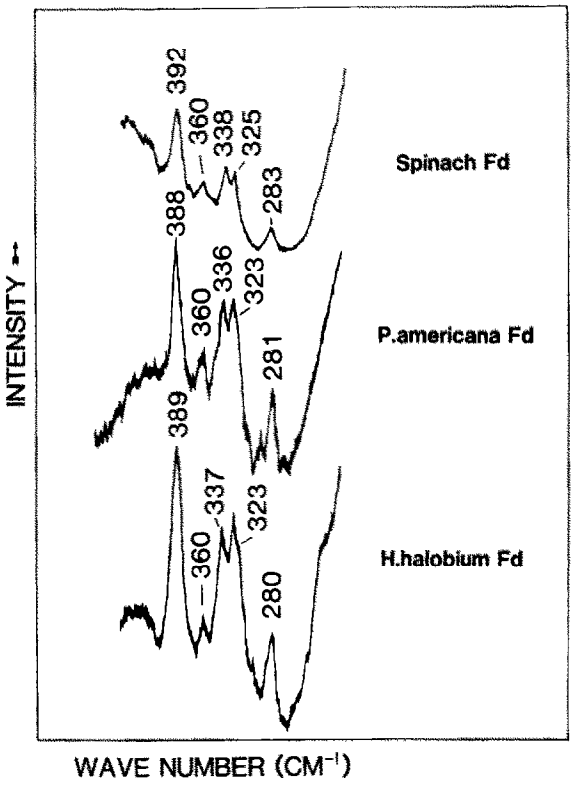

Fig.2. The resonance Raman spectra of the isolated forms of spinach, *P. americana*, and *H. halobium* ferredoxins. Experimental conditions: excitation wavelength = 488.0 nm; laser power = 120 mW; spectral slit width = 6 cm^{-1} for *P. americana* and *H. halobium* ferredoxins, and 8 cm^{-1} for spinach ferredoxin; time constant = 3.2 s.

identical with that previously reported. However, the spectral resolution is slightly better for the present spectrum; the band near 330 cm^{-1} was observed as a broad band in the previously reported spectrum, while the same band could be detected as a doublet peak in the present spectrum as shown in fig.1.

The resonance Raman spectra of [2Fe–2S] ferredoxins from spinach, *P. americana*, and *H. halobium* are very similar in terms of both band frequencies and relative intensities to each other. All the resonance Raman spectra exhibit the prominent bands at 281, 324, 337, and 390 cm^{-1} and very weak feature at 360 cm^{-1}. The resonance Raman spectra were also measured with 514.5 nm excitation. The relative intensity of the band at 390 cm^{-1} decreased with 514.5 nm excitation.

Table 1 summarizes the vibrational frequencies of the resonance Raman spectra of iron–sulfur proteins containing [2Fe–2S] clusters. The overall spectral properties of the reported resonance Raman spectra of iron–sulfur proteins containing [2Fe–2S] clusters are fairly close to each other although there are some appreciable differences in band frequencies and relative intensities between the resonance Raman spectrum of adrenodoxin and those of the other [2Fe–2S] ferredoxins. The resonance Raman spectra of [2Fe–2S] iron–sulfur proteins can be characterized by the marked bands near 285, 335, and 390 cm^{-1}. By replacing the inorganic sulfurs with seleniums, the authors in [6] were able to assign the band at 347 cm^{-1} to a Fe–S(Cys) stretching mode and the bands at 292 and 393 cm^{-1} to Fe–S(labile) stretching modes. These spectra of [2Fe–2S] iron–sulfur proteins are clearly distinct from the spectra of iron–sulfur proteins containing [4Fe–4S] or [3Fe–3S] clusters [11,12]. The most prominent difference between the resonance Raman spectra of iron–sulfur proteins containing [2Fe–2S] clusters and those containing the other clusters is that the resonance Raman spectra of [2Fe–2S] iron–sulfur proteins show a medium band near 285 cm^{-1} while no band is observed in the region of 275–300 cm^{-1} for [3Fe–3S] or [4Fe–4S] iron–sulfur proteins. The second difference is the relative intensity of the resonance Raman band near 390 cm^{-1}. In the 488-nm excited resonance Raman spectra of [2Fe–2S] iron–sulfur proteins the relative intensity of the band at 390 cm^{-1} is fairly strong. In contrast, the intensity of the corresponding band near 390 cm^{-1} is weak in the 488-nm excited resonance Raman spectra of [4Fe–4S] or [3Fe–3S] iron–sulfur proteins. Thus it has been pointed out [11] that resonance Raman spectroscopy possesses considerable potential in Fe–S cluster identification. However, only two [2Fe–2S] iron–sulfur proteins, adrenodoxin and spinach ferredoxin, have been examined by resonance Raman spectroscopy so far. These results, including the data of bacterial type of [2Fe–2S] iron–sulfur protein, provide further support to their indication.

Resonance Raman spectroscopy has also the potential of probing molecular structural differences among [2Fe–2S] clusters. The molecular evolution of chloroplast and bacterial type ferredoxins have been extensively studied [1]. Spinach

Table 1
The vibrational frequencies of the resonance Raman bands observed in the resonance Raman spectra of the isolated forms of various iron–sulfur proteins containing [2Fe–2S] clusters

Sample	Frequencies (cm^{-1})			Ref.
Adrenodoxin	297(m)	350(s)	397(m)	[6]
	288(m)	345(s)	390(m)	[7]
Se-adrenodoxin	263(m)	350(s;doublet)		[6]
Spinach Fd	284(m)	330(broad)	365(vw)395(m)	[14]
	283(m)	325(s)338(s)	360(vw)392(s)	
P. americana Fd	281(m)	323(s)336(s)	360(vw)388(s)	
H. halobium Fd	280(m)	323(s)337(s)	360(vw)389(s)	

s, strong; m, medium; vw, very weak

and *P. americana* ferredoxins are located very closely in the phylogenetic tree of ferredoxins. The amino acid sequences of these two ferredoxins are strikingly homologous (fig.1). About 70% of amino acid residues are invariant between the two ferredoxins. The close similarity of the resonance Raman spectra strongly suggests that not only the primary structures, but also the molecular structures of [2Fe–2S] clusters are very close to each other between spinach and *P. americana* ferredoxins. *H. halobium* ferredoxin is situated far from spinach and *P. americana* ferredoxins in the phylogenetic tree of ferredoxins. Halobacteria do not live by photoreduction system of NADP [15]. The amino acid sequence of *H. halobium* ferredoxin is quite different from that of spinach and *P. Americana* ferredoxins in the various regions and it has extra residues; 22 residues at the amino N-terminal and 5 at the carboxyl C-terminal region, respectively ([5]; fig.1). However, the amino acid sequences of the regions near the 4 cysteines (Cys-41, Cys-46, Cys-49, and Cys-81) chelating two iron atoms are relatively similar between *H. halobium* and chloroplast-type ferredoxins. Thus the question remains whether *H. halobium* ferredoxin possesses the [2Fe–2S] clusters similar to those of chloroplast type ferredoxins such as spinach ferredoxin. The present result of the resonance Raman spectra clearly reveals that the molecular structure of the [2Fe–2S] clusters of *H. halobium* ferredoxin are very close to those of spinach and *P. americana* ferredoxins. Probably the structure of [2Fe–2S] clusters is conserved among chloroplast type and halobacterial type ferredoxins.

Although the resonance Raman spectra of spinach, *P. americana* and *H. halobium* ferredoxins are very close to each other, the resonance Raman spectral differences between these ferredoxins and adrenodoxin are significant. The resonance Raman spectrum of adrenodoxin showed the Fe–S(Cys) stretching mode to be 347 cm^{-1} as described above. On the other hand, the corresponding resonance Raman bands for the other 3 [2Fe–2S] iron–sulfur proteins are seen to be about 330 cm^{-1} as a doublet peak. Probably the Fe–S(Cys) stretching mode shifted and split into two bands. These shifts and the splitting of the Fe–S(Cys) stretching mode suggest that the nature of Fe–S(Cys) bonding and/or the mode of cysteine ligation, are slightly different from each other between adrenodoxin and the ferredoxins. In contrast to the Fe–S(Cys) stretching mode, the Fe–S(labile) stretching modes did not show a large shift, indicating that the inner structures of [2Fe–2S] clusters are close to each other.

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