Resonance Raman spectroscopy of *Azotobacter vinelandii* ferredoxin I

Vibrational features of the [3Fe-3S] cluster

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Low temperature resonance Raman spectra have been obtained for *Clostridium pasteurianum* and *Bacillus stearothermophilus* ferredoxins. Several heretofore undetected fundamental bands have been observed and these data have been used to discriminate the vibrational contribution of the [3Fe-3S] cluster to the spectrum of *Azotobacter vinelandii* ferredoxin I. The vibrational features of the [3Fe-3S] core distinguish it from other 3-iron clusters and imply structural differences among this class of iron-sulfur clusters.

Resonance Raman spectroscopy *Low temperature* *Ferredoxin* *Iron–sulfur protein* *[3Fe-3S] cluster* *[4Fe-4S] cluster*

1. INTRODUCTION

Among the 3-iron clusters of iron-sulfur proteins, the one present in *Azotobacter vinelandii* Fd I, which also contains a [4Fe-4S] active center, has been thoroughly characterized by X-ray crystallography [1], EPR and Mössbauer spectroscopy [2], and ^{1}H NMR [3]. Several recent studies have suggested the presence of 3-iron clusters in an increasing number of proteins, as well as the occurrence of [4Fe-4S] ⟷ [3Fe-xS] cluster interconversions [4–10]. On the basis of comparative EPR and Mössbauer studies, it has been proposed that the latter 3-iron clusters are very similar to the [3Fe-3S] cluster of *A. vinelandii* Fd I [2,11]. In contradiction with these conclusions, recent EXAFS investigations of *Desulfovibrio gigas* Fd II [12] and of inactive aconitase [13], as well as iron and inorganic sulfur determinations [4,13], have suggested the existence of a new [3Fe-4S] type of iron-sulfur cluster [4,13].

Significant progress has recently been achieved in RR spectroscopy of iron-sulfur proteins [15], including the identification of [4Fe-4S] and 3-iron clusters in various ferredoxins [9,16]. The latter results emphasize the need of RR data on *A. vinelandii* Fd I in order to gain more information on the structures of 3-iron clusters in frozen aqueous solutions. Here, the vibrational features of the [3Fe-3S] chromophore are determined by comparison of the 457.9 nm excited low temperature RR spectrum of *A. vinelandii* Fd I with those of *C. pasteurianum* and *Bacillus stearothermophilus* Fds, two [4Fe-4S] proteins [17]. The experimental conditions used here ensured higher signal:noise ratios than in [15].

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Abbreviations: Fd, ferredoxin; EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance; RR, resonance Raman; EXAFS, extended X-ray absorption fine structure

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2. MATERIALS AND METHODS

C. pasteurianum W5 (ATCC 6013) ferredoxin [18], *B. stearothermophilus* (ATCC 29609) ferredoxin [19] and ferredoxin I [1] from *A. vinelandii* OP were purified by previously described techniques. Protein purification and handling were invariably carried out with deaerated buffers under argon or nitrogen atmospheres.

For RR measurements, protein solutions in potassium phosphate 0.02 M (pH 7.5) were lyophilized and redissolved in 10–20 μl distilled water. A number of controls, including UV-visible, EPR, and Mössbauer spectroscopy, showed that lyophilization and subsequent dissolution had no deleterious effects on the ferredoxins used in this study. The final concentrations were 20–50 mg protein per ml and 0.2–0.5 M phosphate, 5 μl droplets of sample were rapidly frozen on a microscope cover-slip placed over boiling liquid nitrogen. The samples were immediately introduced into a cryostat flushed with cold helium gas. The temperature of the sample was kept at about 25 K by adjusting the helium flow.

Resonance Raman spectra were obtained using grazing excitation on optically dense samples [20]. No cell wall was interposed on the path of the excitation beam in the sample area, thus avoiding any troublesome contribution from the Raman scattering of glass in the 300–500 cm^{-1} region. The 457.9 nm emission from an argon laser (Spectra Physics 170.05) was filtered through a 3-prism monochromator (Anaspec) and a narrow-band interference filter. The radiant power reaching the sample was about 1 mW and light scattered was analyzed at about 90° from the illumination beam. The Raman spectrometer (Jobin-Yvon HG 2S-UV) was equipped with a d.c. detection. Typical spectral resolution was 6 cm^{-1} at 300 cm^{-1}. The frequencies were calibrated with the Rayleigh scattering (0 cm^{-1}) and the plasma lines of the Ar laser after removal of the interference filter. Spectra recorded with different samples of a given protein, including some containing ammonium sulfate (2.5 M), the strong 983-cm^{-1} band of which can be used as an internal standard, showed that the frequencies of the RR bands were reproducible within less than 1 cm^{-1}. The signal:noise ratios were improved by summation of 4–12 spectra in a multichannel analyzer (Tracor-Northern 1710). The intensities of the Raman lines were checked after each scan, usually remaining constant over a series of 4–12 scans. Whenever a decrease in intensity was observed, a new scattering site was used. In addition, control UV-visible absorption spectra run on laser-irradiated samples after thawing and diluting gave no indication of active site degradation.

3. RESULTS

The low temperature RR spectrum of *A. vinelandii* Fd I, as isolated in the absence of dithionite (fig.1A), is characterized by two strong bands at 351 cm^{-1} and 397 cm^{-1}, and several weaker bands in the 100–400 cm^{-1} region (table 1). Below 100 cm^{-1} the spectrum is obscured by background scattering, and only some weak and broad features were detected at frequencies higher than 400 cm^{-1} (up to 610 cm^{-1}). A 231-cm^{-1} band

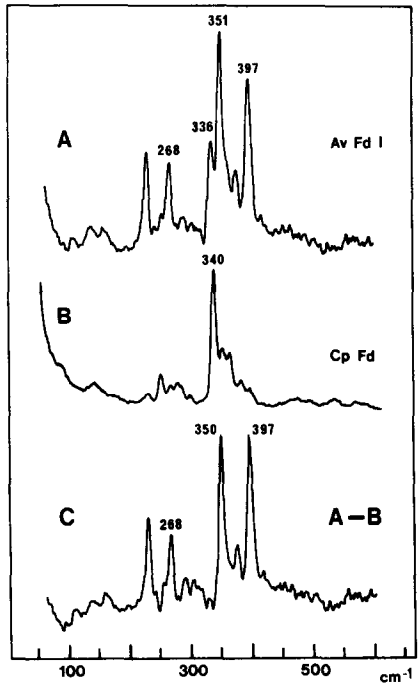

Fig.1. RR spectra of *A. vinelandii* Fd I and of *C. pasteurianum* Fd. Excitation wavelength 457.9 nm; scanning speed 50 cm^{-1}/min; time constant, 1.8 s. Other conditions as in section 2. (A) *A. vinelandii* Fd I at 25 K, 12 scans; (B) *C. pasteurianum* Fd at 30 K, 4 scans; (C) spectrum A minus spectrum B (see text).

Table 1

Resonance Raman frequencies (cm^{-1}) of [4Fe-4S] and [3Fe-xS] clusters in proteins[a]

[4Fe-4S] proteins					[3Fe-xS] proteins		
B st Fd[b] oxidized 22 K	Cp Fd[b] oxidized		Cp Fd[c] oxidized	Assignments[c] (Td)	Av Fd I[b] as isolated 25 K	Dg Fd II[c]	Cp Fd[c] ferricyanide-treated
	30 K	300 K					
	92 vw				112 w		
148 vw,b[d]	145 w				141 w[f]		
					164 w		
226 vwsh					(231 m)[e]		
					244 w		
					256 w[f]		
					268 m	260 m	263 m
					291 m		
					305 vw[f]		
					336 m[f]		
(231 w)[e]	(232 vw)[e]				351 s	345 s	345 s
					363 wsh[f]		
251 w	252 w	250 w	248 m	ν Fe-S* (T_2)	376 m	365 w	365 w
269 w	269 w	270 vw,b			397 s	393 w	393 w
282 w	280 w	282 w	277 w	ν Fe-S* (E)	418 vw		
	286 vwsh						
302 vw	301 vw		298 w				
338 s	340 s	336 s	335 s	ν Fe-S* (A_1)			
354 sh	353 m	348 sh					
360 m	365 m	359 m	357 m	ν Fe-Scys (A_1, T_2)			
		368 wsh					
384 w	384 w	387 vw	395 w	ν Fe-S* (T_2)			
394 wsh	398 w	391 vw					

[a] Experimental conditions as in section 2 and in fig.1,2

[b] Here

[c] From [15]. Samples cooled with cold N_2 gas

[d] Relative intensities from spectra excited at 457.9 nm. s, strong; m, medium; w, weak; b, broad; v, very; sh, shoulder. Unless otherwise specified in the text, the relative positioning, in this table, of frequencies of different clusters should not be taken as inferring any correlation between their vibrational origins

[e] Raman band of ice

[f] Frequencies ascribed to the [4Fe-4S] cluster. Other modes belong to the [3Fe-3S] center

arises at least in part from Raman scattering of ice. Since *A. vinelandii* Fd I as isolated in the absence of dithionite contains both a $[4Fe\text{-}4S]^{2+}$ and a $[3Fe\text{-}3S]^{3+}$ cluster [1,2], the contribution of the former cluster in the RR spectrum must be identified in order to elucidate the vibrational features of the latter. We therefore recorded a low temperature RR spectrum of *C. pasteurianum* Fd, a thoroughly characterized 2[4Fe-4S] protein [17].

Oxidized *C. pasteurianum* Fd at 30 K yields a 457.9 nm excited RR spectrum (fig.2A) dominated by a strong 340-cm^{-1} band, corresponding to the 335-cm^{-1} band previously observed at a higher temperature [9,15,16]. Similar frequency shifts, which are probably due in part to temperature effects, were also observed for other modes of *C. pasteurianum* Fd (table 1). Indeed, at 300 K and under identical instrumental conditions, the major band of *C. pasteurianum* Fd was recorded at 336 cm^{-1} (fig.2B, table 1). It is worth noticing that *C. pasteurianum* Fd exhibit 10 RR bands in the 200–400 cm^{-1} region (table 1), 4 of which were previously undetected [9,15]. Temperature effects cannot be invoked as the same number of bands is observed, though with a poorer signal:noise ratio, at room temperature (fig.2B, table 1). The additional RR bands are neither due to a slight inequivalence of the two [4Fe-4S] clusters present in *C. pasteurianum* Fd since *B. stearothermophilus* Fd, which contains a single [4Fe-4S] cluster [19], shows a RR spectrum as complex as that of *C. pasteurianum* Fd in the same conditions (fig.2C, table 1). Hence, the $[4Fe\text{-}4S]^{2+}$ ferredoxin center displays no less than 10 Raman-active modes in the 200–400 cm^{-1} region, which indicates that the symmetry point group of this type of cluster must

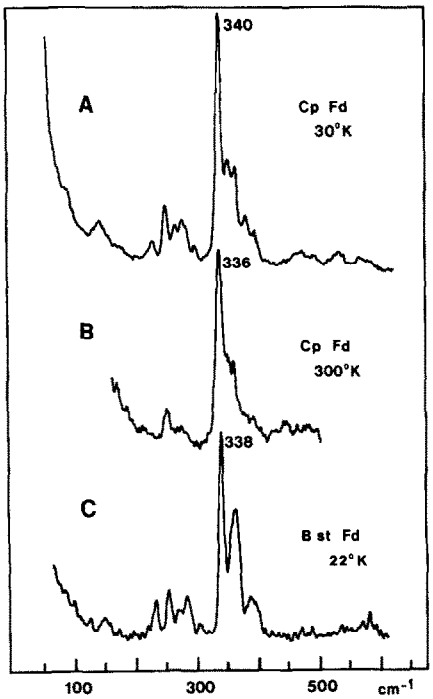

Fig.2. RR spectra of: (A) *C. pasteurianum* Fd at 30 K, 4 scans; (B) *C. pasteurianum* Fd at 300 K, 12 scans; (C) *B. stearothermophilus* Fd at 22 K, 13 scans. Same conditions as in fig.1.

be lower than T_d (6 Raman-active stretching modes) [9,15] and might be closer to D_{2d} (11 Raman-active stretching modes) irrespective of the temperature and the state of the solution, fluid or frozen.

As the $[4Fe\text{-}4S]^{2+}$ cluster of *A. vinelandii* Fd I has been shown by X-ray crystallography to assume essentially the same structure as the $[4Fe\text{-}4S]^{2+}$ clusters of other proteins [1], its vibrational properties must be very similar to those outlined above for other [4Fe-4S] clusters of ferredoxins [9,15,16, here]. On this basis, a simple visual comparison of the RR spectra of *C. pasteurianum* Fd (fig.1B,2A) and of *A. vinelandii* Fd I (fig.1A) allows us to attribute the 336-cm^{-1} band to the [4Fe-4S] cluster and the 268-, 351- and 397-cm^{-1} bands to the [3Fe-3S] cluster in the latter spectrum. Fig.1C, which shows the contribution of the [3Fe-3S] cluster, was obtained by subtracting the spectrum of *C. pasteurianum* Fd (fig.1B), first downshifted by 4 cm^{-1}, from that of *A. vinelandii* Fd I shown in fig.1A. The intensities of the 336-cm^{-1} band of the latter and of the 340-cm^{-1} band of the former spectrum were equalized.

4. DISCUSSION

The RR spectrum of the [3Fe-3S] cluster of *A. vinelandii* Fd I (fig.1C) is clearly different from those of *D. gigas* Fd II and of ferricyanide-treated *C. pasteurianum* Fd in [9,16]. Though both types of spectra display a strong band at about 350 cm^{-1}, an obvious discrepancy concerns the activity of the approximate 395 cm^{-1} mode, which gives rise to *A. vinelandii* Fd I to a 397-cm^{-1} band 8–10-times stronger than the 393-cm^{-1} bands of the two other proteins [9,16], taking the approximate 350-cm^{-1} bands as internal standards (fig.1). Also, the 268-cm^{-1} band of *A. vinelandii* Fd I (fig.1C) is relatively stronger than the approximate 260-cm^{-1} bands of *D. gigas* Fd II and of ferricyanide-treated *C. pasteurianum* Fd [9,16]. These spectral differences can be ascribed neither to different resonance conditions, the excitation wavelength (457.9 nm) being the same in all experiments, nor to distinct temperature conditions inasmuch as no such effects were observed for [4Fe-4S] (table 1), [2Fe-2S] and [1Fe] centers (unpublished), nor to any degradation of our samples, as lyophilization and subsequent dissolution had no deleterious effects on the ferredoxins used here. They most probably reflect structural differences which cannot be explicited in any detail as the vibrational modes of the 3-iron clusters are not sufficiently characterized at this time. It is noteworthy however, that the RR spectrum of the [3Fe-3S] cluster of *A. vinelandii* Fd I (fig.1C) displays more numerous medium and strong bands than did the spectra of other 3-iron centers [9,15,16] and this general enhancement can possibly be related to a lower symmetry of the [3Fe-3S] structure compared to other 3-iron clusters. In this respect, it should be mentioned that X-ray structural investigations of *A. vinelandii* Fd I have shown that the flattened twist boat conformation of the [3Fe-3S] core has only pseudo 2-fold and pseudo-mirror symmetry elements [1]. On the other hand, higher local symmetries are expected for the Fe_3S_4 models proposed for the 3-iron clusters of inactive aconitase and of *D. gigas* Fd II [13], and are displayed by synthetic compounds [21–23] which may be structurally relevant

to some of the 3-iron centers found in proteins. Thus our RR data support the existence, in frozen aqueous solution, of at least two types of 3-iron centers in iron-sulfur proteins, as previously inferred [4,13].

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REFERENCES

[1] Ghosh, D., O'Donnell, S., Furey, W. jr, Robbins, A.H. and Stout, C.D. (1982) J. Mol. Biol. 158, 73–109.
[2] Emptage, M.H., Kent, T.A., Huynh, B.H., Rawlings, J., Orme-Johnson, W.H. and Münck, E. (1980) J. Biol. Chem. 255, 1793–1796.
[3] Sweeney, W.V. (1981) J. Biol. Chem. 256, 12222–12227.
[4] Beinert, H. and Thomson, A.J. (1983) Arch. Biochem. Biophys. 222, 333–361.
[5] Münck, E. (1982) in: Iron-Sulfur Proteins (Spiro, T.G. ed) Metal Ions in Biology, vol.4, pp.147–175, Wiley, New York.
[6] Le Gall, J., Moura, J.J.G., Peck, H.D. jr and Xavier, A.V. (1982) in: Iron-Sulfur Proteins (Spiro, T.G. ed) Metal Ions in Biology, vol.4, pp.177–248, Wiley, New York.
[7] Thomson, A.J., Robinson, A.E., Johnson, M.K., Cammack, R., Rao, K.K. and Hall, D.O. (1981) Biochim. Biophys. Acta 637, 423–432.
[8] Kent, T.A., Dreyer, J.-L., Kennedy, M.C., Huynh, B.H., Emptage, M.H., Beinert, H. and Münck, E. (1982) Proc. Natl. Acad. Sci. USA 79, 1096–1100.
[9] Johnson, M.K., Spiro, T.G. and Mortenson, L.E. (1982) J. Biol. Chem. 257, 2447–2452.
[10] Moura, J.J.G., Moura, I., Kent, T.A., Lipscomb, J.D., Huynh, B.H., Le Gall, J., Xavier, A.V. and Münck, E. (1982) J. Biol. Chem. 257, 6259–6267.
[11] Huynh, B.H., Moura, J.J.G., Moura, I., Kent, T.A., Le Gall, J., Xavier, A.V. and Münck, E. (1980) J. Biol. Chem. 255, 3242–3244.
[12] Antonio, M.R., Averill, B.A., Moura, I., Moura, J.J.G., Orme-Johnson, W.H., Teo, B.-K. and Xavier, A.V. (1982) J. Biol. Chem. 257, 6646–6649.
[13] Beinert, H., Emptage, M.K., Dreyer, J.-L., Scott, R.A., Hahn, J.E., Hodgson, K.O. and Thomson, A.J. (1983) Proc. Natl. Acad. Sci. USA 80, 393–396.
[14] Beinert, H. (1983) Anal. Biochem. 131, 373–378.
[15] Spiro, T.G., Hare, J., Yachandra, V., Gewirth, A., Johnson, M.K. and Remsen, E. (1982) in: Iron-Sulfur Proteins (Spiro, T.G. ed) Metal Ions in Biology, vol.4, pp.407–423, Wiley, New York.
[16] Johnson, M.K., Hare, J.W., Spiro, T.G., Moura, J.J.G., Xavier, A.V. and Le Gall, J. (1981) J. Biol. Chem. 256, 9806–9808.
[17] Sweeney, W.V. and Rabinowitz, J.C. (1980) Annu. Rev. Biochem. 49, 139–161.
[18] Moulis, J.-M. and Meyer, J. (1982) Biochemistry 21, 4762–4771.
[19] Mullinger, R.N., Cammack, R., Rao, K.K., Hall, D.O., Dickson, D.P.E., Johnson, C.E., Rush, J.D. and Simopoulos, A. (1975) Biochem. J. 151, 75–83.
[20] Lutz, M. (1977) Biochim. Biophys. Acta 460, 408–430.
[21] Henkel, G., Tremel, W. and Krebs, B. (1981) Angew. Chem. Int. Ed. Engl. 20, 1033–1034.
[22] Hagen, K.S. and Holm, R.H. (1982) J. Am. Chem. Soc. 104, 5496–5497.
[23] Hagen, K.S., Christou, G. and Holm, R.H. (1983) Inorg. Chem. 22, 309–314.