Resonance Raman Studies of Iron-Only Hydrogenases†

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ABSTRACT: The nature of the iron–sulfur clusters in oxidized and reduced forms of Fe-only hydrogenases from *Desulfovibrio vulgaris*, *Thermotoga maritima*, and *Clostridium pasteurianum* has been investigated by resonance Raman spectroscopy. The results indicate the presence of ferredoxin-like $[4Fe–4S]^{2+,+}$ and $[2Fe–2S]^{2+,+}$ clusters in both *T. maritima* hydrogenase and *C. pasteurianum* hydrogenase I, but only $[4Fe–4S]^{2+,+}$ clusters in *D. vulgaris* hydrogenase. This necessitates a reevaluation of the iron–sulfur cluster composition of *C. pasteurianum* hydrogenase I and indicates that the resonance Raman bands in the oxidized hydrogenase that were previously attributed to the hydrogen activating center [Macor, K. A., Czernuszewicz, R. S., Adams, M. W. W., & Spiro, T. G. (1987) *J. Biol. Chem. 262*, 9945-9947] arise from an indigenous $[2Fe–2S]^{2+}$ cluster. No resonance Raman bands that could be uniquely attributed to the oxidized or reduced hydrogen activating center were observed. This suggests that the hydrogen activating center is a novel Fe center that is unrelated to any known type of Fe–S cluster.

Hydrogenases are a class of enzymes that catalyze the oxidation of molecular hydrogen and/or the reduction of protons, coupled to relevant electron-transfer and energy-yielding processes (Adams et al., 1981; Fauque et al., 1988; Adams, 1990). To date, hydrogenases have been purified to homogeneity from about 20 different species, with representatives from most bacterial genera. A comparison of their properties reveals a fairly heterogeneous group of enzymes that differ in molecular composition, specific activity in catalyzing H_2 oxidation and H_2 production, electron carrier specificity, cofactor content, and sensitivity to inactivation by oxygen. However, they have one common feature, all contain one or more iron–sulfur cluster. Based on metal/selenium content, hydrogenases can be classified into three categories. Both NiFe-hydrogenases and NiFeSe-hydrogenases contain a monomeric nickel center (Fauque et al., 1988; Cammack et al., 1988), and the latter have a single selenocysteine that coordinates the nickel (He et al., 1989; Eidsness et al., 1989). Relatively few hydrogenases are known that lack nickel and contain iron as the only metallic element. This subclass, termed Fe-only or Fe-hydrogenases, comprises hydrogenases isolated from strictly anaerobic bacteria. The best characterized examples are from the mesophilic fermentative anaerobes, *Clostridium pasteurianum* (Adams & Mortenson, 1984; Adams et al., 1989; Meyer & Gagnon, 1991) and *Megasphaera elsdenii* (Grande et al., 1983; Filipiak et al., 1989), the mesophilic sulfate-reducer *Desulfovibrio vulgaris* (Fauque et al., 1989; Patil et al., 1988; Huynh et al., 1984; Hagen et al., 1986a,b), and the extremely thermophilic eubacterium *Thermotoga maritima* (Juszczak et al., 1991).

Previous investigations by a variety of biochemical and spectroscopic techniques have established the presence of at least two types of Fe–S centers in all Fe-hydrogenases (Adams, 1990, and references therein; Juszczak et al., 1991). The mesophilic Fe-hydrogenases all contain at least two Fd-like[1] $[4Fe–4S]^{2+,+}$ clusters (F clusters) and a novel H_2-activating cluster (H-cluster) of ill-defined composition that exhibits a characteristic rhombic EPR signal in oxidized samples (g = 2.10, 2.04, 2.00). In contrast, EPR studies of the extremely thermophilic Fe-hydrogenase from *Thermotoga maritima* indicate the presence of two $[2Fe–2S]^{2+,+}$ clusters in addition to approximately two $[4Fe–4S]^{2+,+}$ clusters, and the oxidized enzyme does not appear to exhibit the EPR signal associated with the H-cluster in mesophilic enzymes (Juszczak et al., 1991). Hence, the relationship, if any, between the H_2-activating center in *T. maritima* hydrogenase with those of mesophilic Fe-hydrogenases remains to be established.

Resonance Raman (RR) spectroscopy provides a direct structural probe for biological Fe–S clusters and a method of determining cluster type that is independent of the magnetic ground state properties. High quality RR spectra and assignments of Fe–S stretching modes have been made for simple iron–sulfur proteins containing a single Fe^{3+} center (Yachandra et al., 1983; Czernuszewicz et al., 1986), $[2Fe–2S]^{2+,+}$ clusters (Han et al., 1989a,b; Fu et al., 1992a), $[3Fe–4S]^{+}$ clusters (Johnson et al., 1983), and $[4Fe–4S]^{3+,2+}$ clusters (Czernuszewicz et al., 1987; Moulis et al., 1984, 1988; Backes et al., 1991). However, with the exception of the two Fe-hydrogenases from *C. pasteurianum* (Macor et al., 1987), RR has not been used to assess the type and properties of the multiple Fe–S centers in Fe-hydrogenases. Here we report and compare the low temperature RR spectra of three Fe-hydrogenases: hydrogenase I (bidirectional) from *C. pasteurianum* and the Fe-hydrogenases from *D. vulgaris* and

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[1] Abbreviations: MCD, magnetic circular dichroism; RR, resonance Raman; Fd, ferredoxin; S^b, bridging or inorganic S; S^t, terminal or cysteinyl S.

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T. maritima. The results necessitate a reevaluation of the Fe–S cluster composition of *C. pasteurianum* hydrogenase I and add further insight into the properties of the novel H-cluster in mesophilic Fe-hydrogenases.

MATERIALS AND METHODS

Sample Preparation. Hydrogenase I from *C. pasteurianum* W5 (ATCC 6013) and the Fe-hydrogenases from *T. maritima* (DSM 3109) and *D. vulgaris* (Hildenborough, NCIB 8303) were purified to homogeneity, as judged by SDS–polyacrylamide gel electrophoresis, using the published procedures (Adams & Mortenson, 1984; Juszczak et al., 1991; Patil et al., 1988). The H_2-evolution specific activities of the samples used in this work were 1050, 4000, and 60 units/mg for the hydrogenases from *C. pasteurianum*, *D. vulgaris*, and *T. maritima*, respectively. One unit corresponds to 1 μmol of H_2 evolved/min at 30 °C (80 °C in the case of *T. maritima* hydrogenase). Protein concentrations were determined by the Lowry method (Lowry et al., 1951) with bovine serum albumen as the standard, using correction factors deduced from quantitative amino acid analyses (Adams et al., 1989; Patil et al., 1988; Juszczak et al., 1991). The activities are within 20% of the maximal activities previously reported for these enzymes.

Sample handling was carried out inside a Vacuum Atmospheres glove box under argon (<1 ppm O_2) or on gas handling lines under Ar or H_2. The hydrogenases from *C. pasteurianum* and *T. maritima* were purified anaerobically in the presence of 2 mM dithionite. Reduced samples were prepared in H_2-saturated buffer solutions, 100 mM Tris-HCl, pH 7.8, with 20 mM dithionite and were maintained under a H_2 atmosphere at all times. Oxidation was accomplished by adding microliter amounts of a saturated thionine solution to the samples as purified until a weak but stable blue color was observed. *D. vulgaris* hydrogenase was purified aerobically in the oxidized state. Reduced samples were prepared by exchanging the buffer with Ar-saturated 100 mM Tris-HCl buffer, pH 7.8, and incubating under H_2 gas at room temperature for 4 h with constant stirring. Samples were allowed to reoxidized anaerobically by replacing H_2 with Ar gas and incubating for 1 h. More complete reduction was achieved by adding 20 mM dithionite to the H_2-reduced samples. For RR measurements, the samples were concentrated by ultrafiltration to about 100 μL and then further reduced to 20 μL by evaporation under a stream of Ar (oxidized samples) or H_2 (reduced samples). The final protein concentrations were approximately 1 mM for oxidized samples and 2-3 mM for reduced samples. The preparation and properties of the oxidized and reduced samples of *Spinacea oleracea* (spinach) and *C. pasteurianum* [2Fe–2S] Fd that were used for comparative RR studies have been described elsewhere (Fu et al., 1992a).

Spectroscopic Measurements. Raman spectra were recorded using an Instruments SA Ramanor U1000 spectrometer fitted with a cooled RCA 31034 photomultiplier tube with 90° scattering geometry. Spectra were recorded digitally using photon counting electronics interfaced to an IBM PC-XT microcomputer and HP 7470A plotter. Improvements in signal-to-noise were achieved by multiple scanning; no smoothing function has been applied to the data. Band positions were calibrated using the excitation frequency and CCl_4 and are accurate to ±1 cm^{-1}. Lines from a Coherent Innova 100 10-W argon ion laser or Coherent Innova 200-K2 krypton ion laser were used for excitation, and plasma lines were removed using a Pellin Broca Prism premonochromator. Using a custom-designed sample cell (Drozdzewski & Johnson, 1988), samples under Ar or H_2 were placed on the end of a cold finger of an Air Products Displex model CSA-202E closed cycle refrigerator. Scattering was collected from the surface of a frozen 10-μL droplet. This enables the samples to be cooled down to 19 K, which facilitates improved spectral resolution and prevents laser-induced sample degradation.

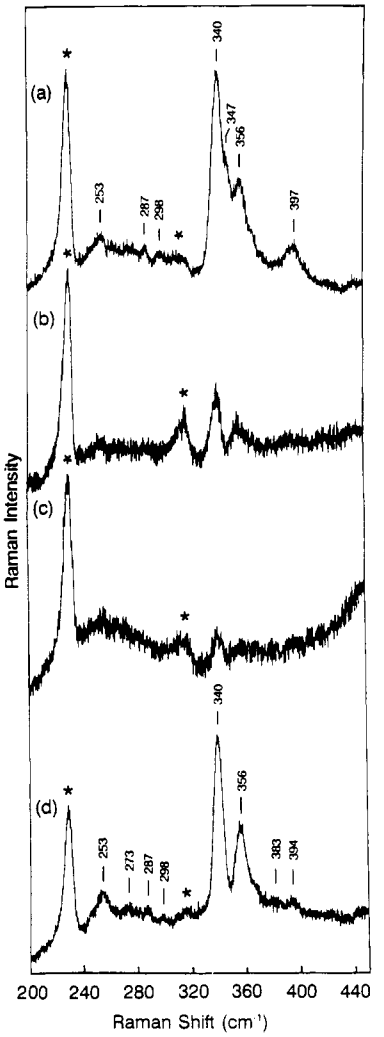

FIGURE 1: Low-temperature RR spectra of *D. vulgaris* hydrogenase (a) as isolated, (b) hydrogen-reduced, (c) hydrogen/dithionite-reduced, and (d) reoxidized under argon. The samples were all ~1 mM in protein and were in 100 mM Tris-HCl buffer, pH 7.8. All the spectra were collected with 457.9-nm laser excitation with a sample temperature of 19 K. The spectrometer was advanced in 0.2 cm^{-1} increments, photon counting for 1 s per point with 6 cm^{-1} resolution. Spectra a, b, c, and d are the sums of 14, 5, 5, and 15 scans, respectively. Bands originating from the frozen buffer solution are indicated by an asterisk.

Parallel EPR studies were carried out on all the samples used for RR studies. X-band EPR spectra were recorded using an IBM/Bruker ER200D spectrometer interfaced to an ESP 1600 data processing system for data storage and manipulation. Low temperatures were obtained with an Oxford Instruments ESR-9 cryostat. Spin quantitations were obtained under nonsaturating conditions by double integration with reference to a 1 mM CuEDTA standard applying the procedures described by Aasa and Vänngård (1975).

RESULTS AND DISCUSSION

The RR spectrum of *D. vulgaris* hydrogenase as isolated under aerobic conditions, using 457.9-nm excitation is shown in Figure 1a. The frequencies and intensity pattern of the

observed Fe–S stretching modes are similar to those of S = 0 $[4Fe–4S]^{2+}$ clusters in bacterial ferredoxins (Czernuszewicz et al., 1987; Moulis et al., 1984, and 1988; Backes et al., 1991). In accord with previous work (Grande et al., 1983; Huynh et al., 1984; Patil et al., 1988), parallel EPR studies on the samples used for RR studies (data not shown) revealed a fast relaxing isotropic resonance at g = 2.02 accounting for 0.2 spins/molecule that has been attributed to a minor contribution of $S = {}^1/_2$ $[3Fe–4S]^+$ clusters. With 457.9-nm excitation, the RR spectra of $[3Fe–4S]^+$ clusters in bacterial ferredoxins are dominated by the totally symmetric $Fe–S^b$ stretching mode of the $Fe_3S^b_4$ core, which occurs at 345–347 cm^{-1} (Johnson et al. 1983). Moreover, the resonance enhancement of Fe–S stretching modes with 457.9-nm excitation is generally significantly larger for $[3Fe–4S]^+$ clusters compared to $[4Fe–4S]^{2+}$ (Johnson et al., 1983). Hence the shoulder at 347 cm^{-1}, which is not observed in the spectra of $[4Fe–4S]^{2+}$ clusters in bacterial ferredoxins, is assigned to the minor component of EPR-detectable $[3Fe–4S]^+$ clusters.

RR spectra of *D. vulgaris* hydrogenase were also obtained for samples reduced under H_2 (Figure 1b), reduced under H_2 followed by anaerobic addition of sodium dithionite (Figure 1c), and for a H_2-reduced sample that was reoxidized by allowing the enzyme to turn over under an argon atmosphere (Figure 1d). H_2 reduction proceeded with progressive loss of the RR spectrum of the $[4Fe–4S]^{2+}$ and $[3Fe–4S]^+$ clusters over a period of several hours. Addition of dithionite caused a further reduction in the intensity of the Fe–S stretching modes and the appearance of the broad bands at about 253 and 450 cm^{-1} associated with excess dithionite in solution. The decrease in Raman intensity was anticipated since previous studies (Spiro et al., 1988; Fu et al., 1992a) have reported weak or negligible resonance enhancement for reduced $[4Fe–4S]^+$ clusters. Reoxidation by allowing the enzyme to turn over under argon atmosphere restores the RR spectrum of the $[4Fe–4S]^{2+}$ clusters but not that of the $[3Fe–4S]^+$ clusters. Parallel EPR studies confirmed the absence of any $[3Fe–4S]^+$ clusters in the anaerobically reoxidized sample. We conclude that either the [3Fe–4S] clusters are not reoxidized under these conditions or they are converted to [4Fe–4S] clusters under reducing conditions. Such cluster conversions are commonly observed for $[3Fe–4S]^+$ clusters that are generated as a result of oxidative degradation of $[4Fe–4S]^{2+}$ clusters during aerobic purification (Beinert & Thomson, 1983).

In accord with previous studies (Hagen et al., 1986a), the reoxidized sample exhibited the characteristic rhombic EPR signal of the oxidized H-cluster, g = 2.11, 2.05, 2.00. Despite the observation that this resonance accounted for 0.3 spins/molecule in the samples used for Raman spectroscopy, the RR spectra of the reoxidized *D. vulgaris* hydrogenase obtained with excitation wavelengths between 406.7 and 514.5 nm can be rationalized *exclusively* in terms of conventional, Fd-like $[4Fe–4S]^{2+}$ clusters. This is illustrated in Table 1, where the bands are assigned to Fe–S stretching modes by analogy with oxidized *C. pasteurianum* Fd (Czernuszewicz et al., 1987). The totally symmetric $\nu(A_1)$ $Fe–S^b$ stretching frequency of the $Fe_4S^b_4$ core, 340 cm^{-1}, is just above the range previously established for cubane $[4Fe–4S]^{2+}$ clusters with complete cysteinyl coordination, 333–339 cm^{-1} (Conover et al., 1990). However, complete cysteinyl coordination for two Fd-like $[4Fe–4S]^{2+,+}$ clusters in all Fe-hydrogenases is suggested by the presence of eight conserved cysteines that are arranged with the same spacings as in 8Fe Fds (Voordouw & Brenner, 1985; Stokkermans et al., 1989; Voordouw et al., 1989; Meyer & Gagnon, 1991).

Table I: Fe–S Stretching Frequencies (cm^{-1}) and Vibrational Assignments for the $[4Fe–4S]^{2+}$ Clusters in Oxidized *C. pasteurianum* Ferredoxin and *D. vulgaris* Hydrogenase

mode $D_{2d}(T_d)$	*C. pasteurianum* Fd[a]	*D. vulgaris* H_2ase
	Mainly Terminal ν(Fe–S)	
A_1	395 (3.9)	394
$E(T_2)$	363 (2.0)	356
$B_2(T_2)$	351 (0.7)	
	Mainly Bridging ν(Fe–S)	
$E(T_2)$	395 (3.9)	394
$B_2(T_2)$	380 (5.6)	383
A_1	338 (7.0)	340
$A_1(E)$	298 (4.9)	298
$B_1(E)$	276 (4.5)	287
$E(T_1)$	276 (4.5)	273
$A_2(T_1)$	266 (4.0)	273
$B_2(T_2)$	251 (6.2)	253
$E(T_2)$		

[a] Vibrational assignments with ^{34}S-bridging isotope shifts in parentheses are taken from Czernuszewicz et al. (1987) with the minor changes suggested by Fu et al. (1992b).

The RR spectra of thionine-oxidized *C. pasteurianum* hydrogenase I, *T. maritima* hydrogenase, and reoxidized *D. vulgaris* hydrogenase with excitation at 457.9 nm are compared in Figure 2. The RR spectra of the well-characterized oxidized $[2Fe–2S]^{2+}$ clusters in *S. oleracea* (spinach) Fd (Figure 2a) and *C. pasteurianum* [2Fe–2S] Fd (Figure 2d) recorded under analogous conditions are also shown to facilitate comparison. Parallel EPR studies (data not shown) showed that the oxidized *C. pasteurianum* hydrogenase I sample exhibited the rhombic EPR signals associated with the oxidized H-cluster, g = 2.10, 2.04, and 2.00, that accounted for 0.7 spins/molecule. In contrast, the oxidized *T. maritima* hydrogenase sample exhibited only an intense isotropic signal centered around g = 2.00 that accounted for 0.3 spin/molecule. It is unlikely to be a radical species since it is fast relaxing and only observable below 20 K. Moreover, parallel MCD studies give no indication of the presence of $S = {}^1/_2$ $[3Fe–4S]^+$ centers in the thionine-oxidized samples of this hydrogenase.[2] Hence the origin of this paramagnetic species is uncertain at present.

The RR spectra of *C. pasteurianum* hydrogenase I (Figure 2b) and *T. maritima* hydrogenase (Figure 2c) are readily analyzed as superimpositions of contributions from Fd-like $[4Fe–4S]^{2+}$ clusters and $[2Fe–2S]^{2+}$ clusters, respectively. The 250-cm^{-1} bands in both hydrogenases are characteristic of asymmetric $\nu(T_2)$ $Fe–S^b$ stretching modes of the $Fe_4S_4^b$ core of synthetic and biological $[4Fe–4S]^{2+}$ clusters. However, the spectra of both hydrogenases are clearly dominated by bands arising from $[2Fe–2S]^{2+}$ clusters. The two intense bands at 289 and 394 cm^{-1} in *C. pasteurianum* hydrogenase I are coincident with the two strongest bands in the spectrum of *S. oleracea* Fd. These two bands have been assigned to $B_{3u}{}^t$ and $A_g{}^b$ Fe–S stretching modes under idealized D_{2h} symmetry for the $Fe_2S_2{}^bS_4{}^t$ cluster (Han et al., 1989a,b; Fu et al., 1992a). Strong bands at these particular frequencies do not appear in the RR spectra of $[4Fe–4S]^{2+}$ clusters or of any other known Fe–S cluster type. Therefore, in accord with previous RR studies of *C. pasteurianum* hydrogenase I (Macor et al., 1987), the spectra demonstrate the presence of $[2Fe–2S]^{2+}$ clusters in the oxidized enzyme. There is also a close correspondence in the frequencies and relative intensities of the Raman bands

[2] R. C. Conover, A. Juszczak, G. Smith, M. W. W. Adams, and M. K. Johnson, unpublished observations.

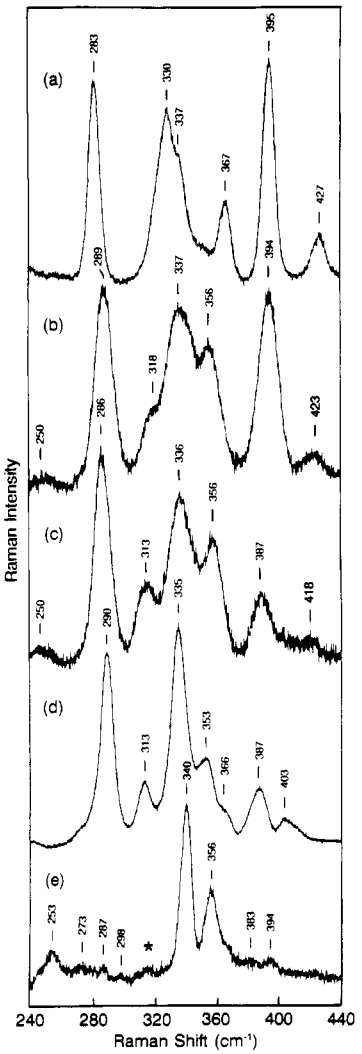

FIGURE 2: Low-temperature RR spectra of (a) oxidized spinach Fd, (b) thionine-oxidized *C. pasteurianum* hydrogenase I, (c) thionine-oxidized *T. maritima* hydrogenase, (d) oxidized *C. pasteurianum* [2Fe–2S] Fd, and (e) *D. vulgaris* hydrogenase reoxidized under argon. The sample concentrations, buffer solution, sample temperature, excitation wavelength, data handling and collection parameters are the same as given in the legend to Figure 1. Spectra a, b, c, d, and e are the sums of 6, 30, 30, 6, and 12 scans, respectively. Bands originating from the frozen buffer solution have been subtracted after normalizing to the ice-band at 230 cm^{-1}.

of oxidized *T. maritima* hydrogenase with those of *C. pasteurianum* [2Fe–2S] Fd. Hence, in accord with EPR studies of the reduced hydrogenase (Juszczak et al., 1991), RR provides unambiguous evidence for one or more $[2Fe–2S]^{2+}$ clusters in this oxidized hydrogenase.

The RR spectrum of reoxidized *D. vulgaris* hydrogenase (Figure 2e) is very similar to that reported for *C. pasteurianum* hydrogenase II (Macor et al., 1987), which also contains two $[4Fe–4S]^{2+,+}$ clusters in addition to the H-cluster (Rusnak et al., 1987; Zambrano et al., 1989), but quite distinct from those of the other two Fe-hydrogenases investigated in this work. The intense contributions from $[2Fe–2S]^{2+}$ centers that dominate the RR spectra for *C. pasteurianum* hydrogenase I and *T. maritima* hydrogenase are not seen in *D. vulgaris* hydrogenase or *C. pasteurianum* hydrogenase II. Previous workers had assigned the [2Fe–2S]-type RR bands in *C. pasteurianum* hydrogenase I to the oxidized H-cluster (Macor et al., 1987). The failure to observe similar RR bands in oxidized *D. vulgaris* hydrogenase (this work) or *C. pasteurianum* hydrogenase II (Macor et al., 1987), both of

Table II: Fe–S Stretching Frequencies (cm^{-1}) and Vibrational Assignments for the $[2Fe–2S]^{+}$ Clusters in Reduced *C. pasteurianum* [2Fe–2S] Fd, *S. oleracea* Fd, *T. maritima* Hydrogenase, and *C. pasteurianum* Hydrogenase I

mode[a]		*C. pasteurianum* 2Fe Fd[b]	*S. oleracea* Fd[b]	*C. pasteurianum* H_2ase I	*T. maritima* H_2ase
D_{2h}	C_{2v}				
$B_{2u}{}^{b}$	B_1	390	404	390	401
$A_g{}^{b}$	A_1	370	385	374	375
$B_{3u}{}^{b}$	A_1	310	313	316 or 304	308 or 300
$B_{1u}{}^{t}$	B_2	328	325	no[c]	no[c]
$B_{2g}{}^{t}$	B_2	no[c]	no[c]	no[c]	no[c]
$A_g{}^{t}$	A_1	310	313	304 or 316	300 or 308
$B_{1g}{}^{b}$	B_1	280	278	260	265
$B_{3u}{}^{t}$	A_1	267	263		

[a] Symmetry labels under idealized C_{2v} symmetry for localized-valence $Fe_2S_2{}^{b}S_4{}^{t}$ core and the corresponding mode under idealized D_{2h} symmetry for the oxidized $Fe_2S_2{}^{b}S_4{}^{t}$ core. [b] Assignments taken from Fu et al. (1992a). [c] Not observed. The $B_{1u}{}^{t}$ mode predominantly involves Fe(III)–S^t stretching and does not undergo significant enhancement with the 530.9-nm excitation (Fu et al., 1992a). The $B_{2g}{}^{t}$ mode predominantly involves Fe(II)–S^t stretching and is not enhanced with visible excitation (Fu et al., 1992a).

which exhibit the characteristic rhombic EPR spectrum associated with the oxidized H-cluster, argues against such an assignment. This leaves two possibilities. Either the $[2Fe–2S]^{2+}$ center(s) in *C. pasteurianum* hydrogenase I are products of oxidative degradation of [4Fe–4S] clusters, as is the case for nitrogenase Fe-protein (Anderson & Howard, 1984; Fu et al., 1991), or the accepted cluster composition based on EPR (Adams, 1990), MCD (Zambrano et al., 1989), and Mössbauer (Wang et al., 1984) studies is incorrect, and the F centers are a mixture of $[4Fe–4S]^{2+,+}$ and $[2Fe–2S]^{2+,+}$ clusters.

To address this question, the RR spectra of the reduced hydrogenases were investigated. The results discussed above for hydrogen/dithionite-reduced *D. vulgaris* hydrogenase (Figure 1) show that little or no resonance enhancement is expected from the reduced H-cluster or the reduced $[4Fe–4S]^{+}$ clusters. In contrast, our recent RR studies of [2Fe–2S]-containing Fds have shown that high quality RR spectra of reduced $[2Fe–2S]^{+}$ centers can be obtained with visible excitation. Moreover, the relative intensity and frequencies of the RR spectra of $[2Fe–2S]^{+}$ clusters are highly conserved in the different classes of [2Fe–2S]-proteins [including Rieske-type proteins which have histidyl as opposed to cysteinyl coordination of the localized valence Fe(II) site (Kuila et al., 1992)], indicating that RR provides an effective method for identifying this type of cluster in reduced multicluster metalloenzymes (Fu et al., 1992a). Consequently, if reduced $[2Fe–2S]^{+}$ clusters can be observed in reduced *C. pasteurianum* hydrogenase I that has been isolated anaerobically in the presence of dithionite, without ever being exposed to oxygen or thionine, then [2Fe–2S] clusters must be indigenous redox centers rather than oxidative degradation products. Figure 3 compares RR spectra obtained with 530.9-nm excitation for hydrogen/dithionite-reduced *T. maritima* hydrogenase and *C. pasteurianum* hydrogenase I with those of $[2Fe–2S]^{+}$ clusters in reduced *S. oleracea* Fd and reduced *C. pasteurianum* [2Fe–2S] Fd. With the exception of the bands at 336 and 419 cm^{-1} in *C. pasteurianum* hydrogenase I, there is a one-to-one correspondence in the RR features of these four proteins. The spectra of the two hydrogenases can be assigned by direct analogy to those of the two Fds (Fu et al., 1992a), (see Table II). In reduced [2Fe–2S] Fds, the Fe(III)–S^t B_{1u} mode, which occurs around 325 cm^{-1}, is not clearly observed with 530-nm excitation but becomes progressively more enhanced as the excitation wavelength decreases (Fu et al.,

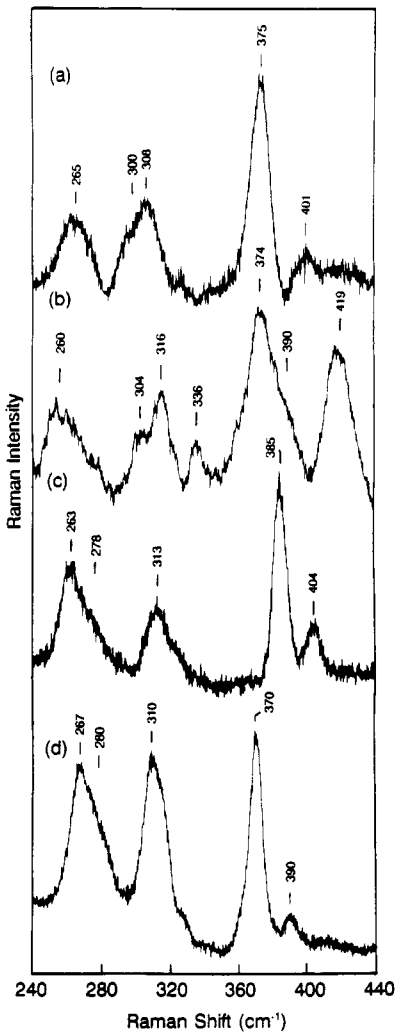

FIGURE 3: Low-temperature RR spectra of (a) hydrogen/dithionite-reduced *T. maritima* hydrogenase, (b) hydrogen/dithionite-reduced *C. pasteurianum* hydrogenase I, (c) dithionite-reduced spinach Fd, and (d) dithionite-reduced *C. pasteurianum* [2Fe–2S] Fd. The samples were 2–3 mM in protein and were in 100 mM Tris-HCl buffer, pH 7.8, with a 20-fold excess sodium dithionite. All other experimental conditions are the same as given in the legend to Figure 1 except for using 530.9-nm laser excitation. Spectra a, b, c, and d are the sums of 45, 80, 10, and 15 scans, respectively. Bands originating from the frozen buffer solution have been subtracted after normalizing to the ice-band at 230 cm^{-1}.

1992a). This mode could not be clearly identified in the hydrogenases since higher energy exciting lines resulted in increased fluorescence backgrounds and greater enhancement of residual amounts of unreduced $[4Fe–4S]^{2+}$ and $[2Fe–2S]^{2+}$ clusters. The weak band at 336 cm^{-1} in reduced *C. pasteurianum* hydrogenase I is assigned to the $\nu(A_1)$ Fe–S^b mode of a trace of oxidized $[4Fe–4S]^{2+}$ clusters. The previous RR studies on less reduced samples (Macor et al., 1987) suggest that at least one of the $[4Fe–4S]^{2+,+}$ clusters has a lower potential than the $[2Fe–2S]^{2+,+}$ center. Recent studies have revealed that the 419-cm^{-1} band that appears with variable intensity in the spectra of reduced *C. pasteurianum* hydrogenase I samples originates from glycerol. Glycerol was present in the buffers used during purification and was not always completely removed by the dialysis procedure used in preparing Raman samples. Hence the additional bands in the spectrum of reduced *C. pasteurianum* hydrogenase I are readily accounted for and it is clear that one or more $[2Fe–2S]^{2+,+}$ clusters are present as intrinsic components of both *T. maritima* hydrogenase and *C. pasteurianum* hydrogenase I.

In the case of *T. maritima* hydrogenase, the available EPR data indicate the presence of two spectroscopically distinct $[2Fe–2S]^{2+,+}$ clusters (Juszczak et al., 1991). The number of $[2Fe–2S]^{2+,+}$ clusters in *C. pasteurianum* hydrogenase I remains to be determined. On the basis of sequence homology between *D. vulgaris* hydrogenase and *C. pasteurianum* hydrogenase I and the observation of eight conserved cysteines in a classical 8Fe Fd arrangement, it is likely that both contain two $[4Fe–4S]^{2+,+}$ clusters (Voordouw & Brenner, 1985; Stokkermans et al., 1989; Voordouw et al., 1989; Meyer & Gagnon, 1991). The additional Fe–S clusters in *C. pasteurianum* hydrogenase I are most likely contained in the additional N-terminal domain of approximately 110 amino acids which are not present in the large subunit of *D. vulgaris* hydrogenase (Meyer & Gagnon, 1991). This region contains eight cysteines that are not arranged in a recognizable Fd-type motif but are in principle capable of coordinating two [2Fe–2S] clusters or one [4Fe–4S] and one [2Fe–2S] cluster.

The question then arises as to why the $[2Fe–2S]^{+}$ center(s) in reduced *C. pasteurianum* hydrogenase I have apparently escaped detection up to now by EPR (Adams, 1990), Mössbauer (Wang et al., 1984), and MCD (Zambrano et al., 1989) spectroscopies. The EPR spectrum of hydrogen/dithionite-reduced *C. pasteurianum* hydrogenase I in the $S = {}^1/_2$ region consists of a broad featureless resonance that has been attributed to spin–spin interaction between multiple $[4Fe–4S]^{+}$ clusters (Adams, 1990). Temperature dependence studies indicate that the resonance persists albeit with broadening up to about 60 K.[3] EPR signals from $[2Fe–2S]^{+}$ clusters can usually be observed without significant broadening at 70 K. However, their relaxation properties will be enhanced by spin–spin interaction with other nearby paramagnetic Fe–S clusters. Hence intercluster spin–spin interaction is most likely responsible for the anomalous relaxation characteristics of the $S = {}^1/_2$ $[2Fe–2S]^{+}$ cluster(s) in reduced *C. pasteurianum* hydrogenase I. Low temperature MCD spectroscopy can provide a method of distinguishing paramagnetic $[2Fe–2S]^{+}$ and $[4Fe–4S]^{+}$ clusters, since each known type of cluster exhibits a characteristic pattern of bands (Zambrano et al., 1989; Conover et al., 1990; Fu et al., 1992a; Onate et al., 1993). On the basis of this premise, the low temperature MCD spectrum of the reduced hydrogenase I in *C. pasteurianum* is certainly dominated by the contributions from $S = {}^3/_2$ and $S = {}^1/_2$ $[4Fe–4S]^{+}$ clusters (Zambrano et al., 1989). However, the similarity in the low temperature MCD intensities of $[4Fe–4S]^{+}$ and $[2Fe–2S]^{+}$ clusters make it difficult to detect one $[2Fe–2S]^{+}$ cluster in the presence of three $[4Fe–4S]^{+}$ clusters. Reexamination of the reported MCD spectra (Zambrano et al., 1989), in light of the recent MCD studies of [2Fe–2S] proteins (Fu et al., 1992a), leads to the conclusion that one $[2Fe–2S]^{+}$ cluster can be accommodated by the available MCD data. The presence of $S = {}^1/_2$ $[2Fe–2S]^{+}$ clusters is much more apparent in the low temperature MCD spectra of reduced *T. maritima* hydrogenase, and the spectra are consistent with the EPR results (Juszczak et al., 1991) which were interpreted in terms of two $[2Fe–2S]^{+}$ and two $[4Fe–4S]^{+}$ clusters.[2] Therefore, on the basis of low temperature MCD studies, we conclude that *C. pasteurianum* hydrogenase I contains one $[2Fe–2S]^{2+,+}$ and

[3] A. T. Kowal, M. W. W. Adams, and M. K. Johnson, unpublished results

[4] W. Fu, L. C. Brinkmann, L. E. Mortenson, T. V. Morgan, and M. K. Johnson, unpublished observations.

three $[4Fe–4S]^{2+,+}$ clusters, in addition to the H-cluster. Mössbauer spectra have been only been reported for oxidized *C. pasteurianum* hydrogenase I (Wang et al., 1984), and it is clearly not possible to exclude contributions from diamagnetic $[2Fe–2S]^{2+}$ clusters to the broad and unresolved quadrupole doublets of the multiple diamagnetic $[4Fe–4S]^{2+}$ clusters. Even if Mössbauer spectra were available for the reduced enzyme, it would probably be difficult to detect a single $S = {}^1/_2$ $[2Fe–2S]^+$ cluster in the presence of three paramagnetic ($S = {}^1/_2$ and $S = {}^3/_2$) $[4Fe–4S]^+$ clusters using this technique. The above discussion serves to demonstrate that the presence of a single $[2Fe–2S]^{2+,+}$ cluster in *C. pasteurianum* hydrogenase I, which is required for interpretation of the RR data presented herein, is not inconsistent with the existing spectroscopic data from other techniques.

Finally we turn our attention to the implications of RR results for the nature of the novel H-cluster. The absence of [2Fe–2S] clusters in *D. vulgaris* hydrogenase and *C. pasteurianum* hydrogenase II makes these enzymes the best candidates to assess the RR characteristics of the H-cluster. We conclude that the reduced H-cluster has negligible resonance enhancement. The oxidized H-cluster either exhibits negligible resonance enhancement or is indistinguishable from a conventional $S = 0$ $[4Fe–4S]^{2+}$ cluster in terms of RR characteristics. The latter seems unlikely in view of the paramagnetic $S = {}^1/_2$ ground state for the oxidized H-cluster. Hence we favor the former explanation and conclude that the oxidized H-cluster most likely exhibits weak or negligible RR spectrum as a result of substantial non-sulfur Fe coordination and/or a cluster nuclearity greater than four Fe atoms. In the latter case, it is worth noting that the oxidized [8Fe–8S] and [Mo7Fe–8S] clusters in nitrogenase MoFe-proteins (Kim & Rees, 1992) exhibit only very weak resonance enhancement of Fe–S stretching modes with visible excitation lines.[4]

CONCLUSIONS

RR studies of oxidized and reduced forms of the Fe-hydrogenase from *T. maritima* and hydrogenase I from *C. pasteurianum* indicate the presence of Fd-like $[4Fe–4S]^{2+,+}$ and $[2Fe–2S]^{2+,+}$ clusters as intrinsic redox components of the anaerobically isolated enzymes. Although RR alone cannot assess the stoichiometry of these clusters, these results combined with the available analytical, EPR, and MCD data (Adams, 1990; Zambrano et al., 1989; Juszczak et al., 1991) indicate two $[4Fe–4S]^{2+,+}$ and two $[2Fe–2S]^{2+,+}$ clusters in *T. maritima* hydrogenase and three $[4Fe–4S]^{2+,+}$ and one $[2Fe–2S]^{2+,+}$ cluster in *C. pasteurianum* hydrogenase I. In contrast, after anaerobic redox cycling, only $[4Fe–4S]^{2+}$ clusters are observable by RR in *D. vulgaris* Fe-hydrogenase, and the available EPR and Mössbauer results indicate that two $[4Fe–4S]^{2+,+}$ clusters mediate electron transport in this enzyme (Grande et al., 1983; Huynh et al., 1984; Hagen et al., 1986a). The absence of any RR bands that can be uniquely attributed to either the reduced or oxidized H-cluster indicates negligible resonance enhancement with visible excitation, as a result of substantial non-sulfur Fe coordination and/or a cluster with Fe nuclearity in excess of four. This suggests that the H-cluster is a novel Fe center that is unrelated to any known type of Fe–S center.

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