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Resonance Raman studies of the [4Fe-4S] to [2Fe-2S] cluster conversion in the iron protein of nitrogenase

Weiguang Fu, T. Vance Morgan, Leonard E. Mortenson and Michael K. Johnson

Center for Metalloenzyme Studies and School of Chemical Sciences, University of Georgia, Athens, GA 30602, USA

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Resonance Raman spectroscopy has been used to investigate the Fe-S stretching modes of the $[4Fe\text{-}4S]^{2+}$ cluster in the oxidized iron protein of *Clostridium pasteurianum* nitrogenase. The results are consistent with a cubane [4Fe-4S] cluster having effective T_d symmetry with cysteinyl coordination for each iron. In accord with previous optical and EPR studies [(1984) *Biochemistry* 23, 2118–2122], treatment with the iron chelator α,α'-dipyridyl in the presence of MgATP is shown to effect cluster conversion to a $[2Fe\text{-}2S]^{2+}$ cluster. Resonance Raman data also indicate that partial conversion to a $[2Fe\text{-}2S]^{2+}$ cluster is induced by thionine-oxidation in the presence of MgATP in the absence of an iron chelator. This result suggests new explanations for the dramatic change in the CD spectrum that accompanies MgATP-binding to the oxidized Fe protein and the anomalous resonance Raman spectra of thionine-oxidized *Clostridium pasteurianum* bidirectional hydrogenase.

Nitrogenase; Iron protein; Resonance Raman; Iron-sulfur cluster

1. INTRODUCTION

Nitrogenase catalyses the terminal step in biological nitrogen fixation, namely the reduction of dinitrogen to ammonia [1,2]. The enzyme comprises an $\alpha_2\beta_2$ tetramer, known as the MoFe protein, which is the site of substrate reduction, and a γ_2 dimer, known as the iron protein. The iron protein is the site of MgATP binding and hydrolysis and is the obligate electron donor for substrate reduction. The currently held view is that the iron-protein contains a single $[4Fe\text{-}4S]^{2+,+}$ cluster at the interface of the two 30-kDa subunits, with four cysteinyl residues, two from each subunit, coordinating the cluster [3–5]. MgATP binding to the iron-protein induces a conformational change that alters the properties of the [4Fe-4S] center, notably a 100-mV decrease in the midpoint potential [6,7], a change in the anisotropy of the $S = 1/2$ EPR signal of $[4Fe\text{-}4S]^{+}$ cluster [8], and a dramatic change in the CD spectrum of the oxidized protein [9]. Moreover, it greatly enhances the reactivity of the cluster to iron-chelators, and the $[4Fe\text{-}4S]^{2+}$ cluster is degraded via a discrete $[2Fe\text{-}2S]^{2+}$ intermediate by treatment of the oxidized protein with α,α'-dipyridyl in the presence of MgATP [4,10].

Over the past decade, resonance Raman spectroscopy, using excitation into S → Fe charge transfer bands to enhance S-Fe stretching modes, has emerged as an effective probe of cluster type and for monitoring structural perturbations of biological Fe-S clusters [11–16]. However, no resonance Raman studies have been reported for nitrogenase iron-protein. Here we report resonance Raman studies of oxidized iron-protein from *Clostridium pasteurianum* nitrogenase in the presence and absence of MgATP and the iron chelator α,α'-dipyridyl. The results demonstrate the utility of resonance Raman spectroscopy for monitoring the cluster conversions between diamagnetic $[4Fe\text{-}4S]^{2+}$ and $[2Fe\text{-}2S]^{2+}$ centers and provide evidence that this conversion occurs to some extent in the presence of MgATP even in the absence of an iron-chelator. The potential significance of this observation for interpreting spectroscopic studies of nitrogenase iron-protein and other iron-sulfur metalloenzymes is discussed.

Correspondence address: M.K. Johnson, Department of Chemistry, University of Georgia, Athens, GA 30602, USA. Fax (1) (404) 542-9454

2. MATERIALS AND METHODS

The iron protein from *C. pasteurianum* nitrogenase was purified under anaerobic conditions in the presence of 1 mM sodium dithionite by a modification of the method of Mortenson [17]. All handling of this enzyme was carried out inside a Vacuum Atmospheres glove box under argon (< 1 ppm O_2). Sodium dithionite was removed from reduced iron protein by anaerobic chromatography using a DEAE-Sepharose column and the resulting samples in 50 mM Tris/HCl buffer, pH 7.7, were concentrated under argon to a final concentration of approximately 2 mM. Oxidation was accomplished by adding μliter aliquots of a saturated thionine solution until a weak but stable blue color was observed. For samples treated with MgATP, a MgATP solution was added to the enzyme to give a final concentration of 50 mM. A freshly prepared MgATP solution containing ATP (Sigma) and $MgCl_2 \cdot 6H_2O$ (Fisher) was used and the pH was adjusted to 7.7 prior to addition. For samples treated with MgATP and α,α'-dipyridyl, oxidized iron protein containing 50 mM MgATP was made 40 mM in α,α'-dipyridyl. The α,α'-dipyridyl solution was prepared in anaerobic Tris/HCl buffer, pH 7.5, immediately prior to use. Excess

reagents were removed by gel filtration after 20 min incubation at room temperature and the protein was reconcentrated to a final concentration of ≈ 2 mM before recording the Raman spectrum.

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. 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 were used for excitation and plasma lines were removed using a Pellin Broca Prism premonochromator. Samples were placed on the end of a cold finger of an Air Products Displex Model CSA-202E closed cycle refrigerator and scattering was collected from the surface of the frozen droplet [18]. This enables the sample to be cooled down to 17 K, which facilitates improved spectral resolution and prevents laser-induced sample degradation.

3. RESULTS AND DISCUSSION

Fig. 1 compares the resonance Raman spectra obtained with 457.9 nm excitation of thionine-oxidized *C. pasteurianum* nitrogenase iron protein in the presence and absence of MgATP and after treatment with MgATP in the presence of the iron chelator α,α'-dipyridyl. The frequencies and relative intensities of the observed Fe-S stretching modes are characteristic of those observed for synthetic and biological $[4Fe\text{-}4S]^{2+}$ clusters [12]. The frequency of the most intense band, which corresponds to the symmetric breathing mode of the Fe_4S_4 cubane, has been found to be a useful indicator of partial non-cysteinyl coordination [16]. This band occurs at 335 cm^{-1} for oxidized iron protein and this frequency is indicative of complete cysteinyl coordination for the cluster.

The resonance Raman spectrum of oxidized iron protein is most similar to that of solution spectra of synthetic cubane clusters such as $[Fe_4S_4(SCH_2Ph)_4]^{2-}$. This synthetic cluster appears to have effective T_d in solution and the Raman spectrum has been assigned by $^{32}S/^{34}S$ isotope shifts and depolarization measurements coupled with normal mode calculations [12]. By analogy, the Raman spectrum for oxidized iron protein can be completely assigned under T_d symmetry, see Table I. A D_{2d} distortion is generally apparent in the Fe_4S_4 cores encountered in bacterial ferredoxins and synthetic analogs in the crystalline state and manifests itself in Raman spectrum by resolvable splittings of degenerate modes [12]. However, it is not possible to rule out a similar distortion for the Fe_4S_4 core in iron protein since the Raman bands are broader despite comparable spectral resolution. This broadening may reflect greater conformational flexibility in the vicinity of the cluster which may itself be a consequence of the cluster bridging between two subunits. In other words, the Raman spectrum may be a composite of numerous slightly different conformational states in the frozen solution.

The presence of MgATP does not affect the frequencies or intensity pattern of the Raman bands associated

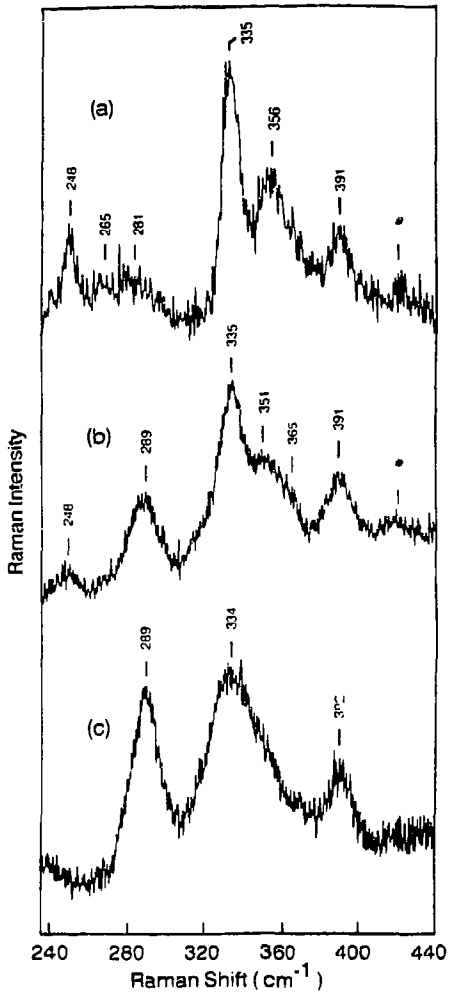

Fig. 1. Low-temperature resonance Raman spectra of *C. pasteurianum* nitrogenase iron protein. The samples were all ≈ 2 mM in protein and were in 50 mM Tris/HCl buffer, pH 7.7. (a) thionine-oxidized iron protein, 9 scans; (b) MgATP-treated, thionine-oxidized iron protein, 4 scans; (c) MgATP- and α,α'-dipyridyl-treated thionine-oxidized iron protein, 20 scans. The spectra were collected with 457.9-nm laser excitation at 17K, photon counting for 1 s at 0.2 cm^{-1} increments using 6 cm^{-1} resolution. The bands marked with # arise from thionine.

Table I

Vibrational frequencies (cm^{-1}) and assignments for oxidized *C. pasteurianum* iron protein and $[Fe_4S_4(SCH_2Ph)_4]^{2-}$ in frozen solutions

T_d Assignment	Iron protein*	$[Fe_4S_4(SCH_2Ph)_4]^{2-}$**
	Mainly terminal ν(Fe-S)	
A_1	391	389
T_2	356	360
	Mainly bridging ν(Fe-S)	
T_2	391	389
A_1	335	336
E	281	273
T_1	265	273
T_2	248	244

*Frozen buffer solution at 17 K.
**Frozen 90/5/5 (vol %) H_2O/dimethylacetamide/Triton X-100 solution at 77 K, Taken from [12].

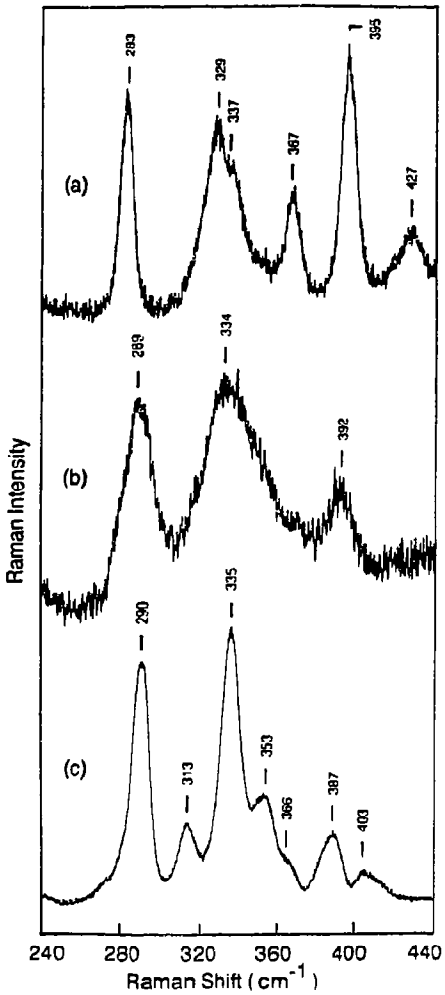

Fig. 2. Low-temperature resonance Raman spectra of (a) oxidized spinach ferredoxin, (b) MgATP- and α,α'-dipyridyl-treated, thionine-oxidized *C. pasteurianum* nitrogenase iron protein, and (c) oxidized *C. pasteurianum* [2Fe-2S] ferredoxin. Samples were all ≈ 2 mM in protein and were in 50 mM Tris/HCl buffer, pH 7.7. All the spectra were obtained using 457.9 nm laser excitation. The data collection parameters and sample temperature are the same as given in Fig. 1. Spectra (a), (b) and (c) are the sums of 5, 20 and 7 scans, respectively.

with the $[4Fe\text{-}4S]^{2+}$ cluster, suggesting that little, if any, structural perturbation of the cluster accompanies MgATP binding. However, two additional bands at 289 and 391 cm^{-1} are observed. (The latter is superimposed on one of the bands observed for $[4Fe\text{-}4S]^{2+}$ cluster and is apparent as a change in the relative intensity of this feature.) The origin of these additional bands became apparent from the effect of α,α'-dipyridyl on the MgATP-bound iron protein. The resulting spectrum consisted of broad bands centered at 289, 334, and 392 cm^{-1} and the frequencies and intensity pattern are characteristic of those observed for oxidized $[2Fe\text{-}2S]^{2+}$ clusters. This is illustrated in Fig. 2 which compares the resonance Raman spectra of α,α'-dipyridyl-treated, MgATP-bound iron protein with those of $[2Fe\text{-}2S]^{2+}$ clusters in spinach Fd and *C. pasteurianum* [2Fe-2S]Fd obtained with the same excitation wavelength. Rational assignments based normal mode calculations that reproduce $^{32}S/^{34}S$ and $^{54}Fe/^{56}Fe$ isotope shifts have been made for $[2Fe\text{-}2S]^{2+}$ clusters in proteins and in analog complexes [14,15], and the characteristic feature that clearly distinguishes this type of cluster from $[3Fe\text{-}4S]^{+}$ and $[4Fe\text{-}4S]^{2+}$ centers is the appearance of an intense band between 280 and 291 cm^{-1}. Once again, the broadness of the Raman bands for the [2Fe-2S] cluster in iron-protein compared to those in ferredoxins suggests much greater conformational flexibility in the vicinity of the cluster.

The oxidized $[4Fe\text{-}4S]^{2+}$ cluster in the Fe protein, therefore, appears to be unique among biological [4Fe-4S] clusters in its ability to undergo degradation to a [2Fe-2S] center. This type of cluster conversion is completely different from the now well-established $[4Fe\text{-}4S]^{2+}$ to $[3Fe\text{-}4S]^{+}$ conversion that is induced in many proteins by exposure to O_2 or ferricyanide [16,19] and may well be a consequence of a $[4Fe\text{-}4S]^{2+}$ cluster that bridges between two subunits. These results confirm the optical and EPR studies of Anderson and Howard [4] which indicated that a [2Fe-2S] cluster is formed during the degradation of the [4Fe-4S] cluster in iron protein by exposure to an iron chelating agent in the presence of MgATP. They also add one important piece of information, namely that partial $[4Fe\text{-}4S]^{2+}$ to $[2Fe\text{-}2S]^{2+}$ conversion can be induced on binding of MgATP to the oxidized protein in the *absence* of α,α'-dipyridyl. We estimate that the extent of conversion is between 10% and 30%. Since the resonant enhancements of Fe-S stretching modes associated with $[2Fe\text{-}2S]^{2+}$ clusters are several times greater than that observed for $[4Fe\text{-}4S]^{2+}$ clusters with 457.9 nm excitation, it is possible to detect conversion of only small fraction of the clusters using this technique.

This result has important implications for the interpretation of previous spectroscopic studies of MgATP binding to nitrogenase iron protein and resonance Raman studies of anaerobically oxidized [4Fe-4S]-containing metalloenzymes. In relation to the first point, it offers an alternative interpretation for the dramatic change in the CD spectrum that is observed on MgATP binding to oxidized nitrogenase Fe-proteins [9]. Proteins containing oxidized $[2Fe\text{-}2S]^{2+}$ clusters exhibit much more intense CD spectra than those containing $[4Fe\text{-}4S]^{2+}$ clusters [20]. Consequently the observed changes in the CD spectrum on binding of MgATP may not result from a conformational change in the vicinity of the Fe-S cluster, but rather from partial [4Fe-4S] to [2Fe-2S] cluster conversion. The importance of this observation lies in the fact that CD data is the only direct evidence for MgATP interaction with the oxidized protein. Further CD studies are planned to test this hypothesis. With respect to the second point, we have noted that the Raman spectrum obtained for thionine-oxidized iron-protein in the presence of

MgATP closely resembles that reported for thionine-oxidized *C. pasteurianum* bidirectional hydrogenase [21]. In this case the features characteristic of the [2Fe-2S] cluster were tentatively attributed to a component of the novel hydrogen activating cluster. In light of the results presented herein, a plausible alternative explanation is that these features arise from partial degradation of one of the multiple [4Fe-4S] clusters on thionine-oxidation. Detailed Raman studies of a range of Fe-only hydrogenases suggest that this is indeed the case and these new results will be reported elsewhere.

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