# Resonance Raman study of the superoxide reductase from *Archaeoglobus fulgidus*, E12 mutants and a 'natural variant'†

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The resonance Raman (RR) spectra of the oxidized wild-type *Archaeoglobus fuglidus* 1Fe-Superoxide reductase (SOR), E12V and E12Q mutants were studied at different pH conditions upon excitation in resonance with the pH-dependent charge transfer transition to the ferric iron. The wild-type SOR from *Nanoarchaeum equitans* that lacks the highly conserved glutamate residue was investigated as a 'natural variant'. No substantial differences were observed in the RR spectra of the active sites of the *A. fulgidus* proteins. Based on the component analysis in the metal–ligand stretching region the modes involving the Fe–S(Cys) stretching coordinates have been identified. The frequencies of these modes reflect the electronic properties of the Fe–S bond which are related to the catalytic activity of SORs, including reduction of superoxide and product dissociation. Moreover, hydroxide binding to the E12 mutant proteins was demonstrated at high pH. It was further observed that the ferric active site of all three SORs from *A. fulgidus* senses the presence of phosphate, which possibly replaces the hydroxide at high pH.

## Introduction

Superoxide reductases (SORs) constitute a group of mononuclear iron enzymes involved in combating oxidative stress by depleting superoxide levels in strictly anaerobic and microaerophilic prokaryotes.[1–6] Unlike superoxide dismutase which converts $O_2^{\bullet -}$ to $H_2O_2$ and $O_2$, SOR catalyzes the direct reduction of $O_2^{\bullet -}$ to $H_2O_2$ in a one electron–two proton process. The active site of SORs is highly conserved and consists of a pentacoordinated $Fe^{2+}$ center with four equatorial histidines and one axial cysteine in a square pyramidal geometry (center II).[7–9] The free or weakly hydrated sixth axial site of the reduced enzyme is thought to be the binding site for the substrate. In the resting oxidized state of the enzyme this site is occupied by a highly conserved glutamate residue. The 2Fe-SORs contain an additional N-terminal domain which houses a rubredoxin-like center, with $Fe^{3+}$ ligated by four cysteines in a distorted tetrahedral geometry (center I).[10]

The catalytic cycle of SORs has been studied by a variety of stationary and time resolved techniques. The first step is the diffusion-controlled bimolecular reaction of $Fe^{2+}$ with $O_2^{\bullet -}$ to form an $Fe^{3+}$-peroxo intermediate, which is believed to be rapidly protonated. This intermediate decays by a pH-dependent step on a time scale of milliseconds.[2,3,11–14] The decay process, however, varies among SORs from different sources. In *Desulfovibrio vulgaris* (*D. vulgaris*) it appears to lead directly to the glutamate-bound ferric state,[15,16] whereas in *Archaeoglobus fulgidus* (*A. fulgidus*) the formation of a second intermediate is observed, which is assigned to $Fe^{3+}$–OH or $Fe^{3+}$–$OH_2$ species, depending on the pH.[2,17] The decay of this transient species leads to the enzyme resting state, presumably with glutamate as the sixth axial ligand at the active site or, alternatively, a water molecule for those mutants in which glutamate has been replaced. In either case, it is not clear whether formation of the glutamate- or the water/hydroxide-bound ferric species occurs after release of $H_2O_2$ or it is a concerted process in which the new ligand displaces the enzymatic product. However, the transition from the $Fe^{3+}$–OH or $Fe^{3+}$–$OH_2$ species to the glutamate-bound form is observed, within the same time scales, even when a chemical oxidant is used instead of superoxide, suggesting that the release of the product is not concerted with glutamate binding.[2] In all cases studied so far, glutamate binding to the ferric enzyme does not appear to be a crucial step, as mutation of this residue does not affect significantly the kinetics of $O_2^{\bullet -}$ reduction at physiological pH.[2,7,18] The apparent lack of function of this almost strictly conserved residue in the reaction kinetics with superoxide is further supported by the recent characterisation of a fully functional enzyme from *Nanoarchaeum equitans* (*N. equitans*) which naturally lacks the glutamate ligand.[19]

The assignment of the second intermediate largely relies upon pH-dependent spectral changes. The ferric form of SORs presents an intense $Cys^- \rightarrow Fe^{3+}$ charge transfer (CT) transition at *ca.* 650–670 nm at pH values close to 7. As the pH increases, the CT band shifts to *ca.* 560–590 nm with observed $pK_a$ values of 9–10, depending on the source

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† Dedicated to the memory of Professor Frank Rusnak, Mayo Clinic, Rochester MN, USA.

organism.[2,4,5,8,19,20] Recently, Mathé *et al*. provided compelling resonance Raman (RR) spectroscopic evidence that the 560 nm absorption band corresponds to the formation of high-spin $Fe^{3+}$–OH species at the active site of the SOR from *Desulfoarculus baarsii* (*D. baarsii*).[4] A similar CT band has been transiently observed in pulse radiolysis experiments for the SOR from *A. fulgidus* at physiological pH and was assigned as the second intermediate in the catalytic cycle. On the other hand, while $Fe^{3+}$–OH species have clearly been identified by RR spectroscopy for *D. baarsii* SOR, no RR signal was observed in the $Fe^{3+}$–OH stretching region of the enzyme from *P. furiosus* despite a similar optical transition in alkaline solutions.[4,20] However, binding of hydroxide and glutamate as part of the catalytic pathway, or even for the chemically oxidized enzyme, are not free of uncertainty as other anions present in the medium under *in vivo* or *in vitro* conditions may also bind and compete for $Fe^{3+}$.[2,7] For example, Rodrigues *et al*. reported indirect evidence that phosphate is able to bind to the oxidized mutants lacking the glutamate ligand, but not to the wild type enzyme, since the glutamate presumably inhibits binding of phosphate.[2] Phosphate binding was further supported in that work by the appearance of a new intermediate in the reaction of the wild type SOR with superoxide and the finding that the rate of glutamate binding slows down upon increase of phosphate concentration, which is compatible with competition between the two species.[2] However, it is still not clear whether phosphate binds directly to the ferric site.

In this work we have studied the importance of the highly conserved glutamate residue for the structure of the *A. fulgidus* 1Fe-SOR active site and, therefore, for the catalytic activity of the enzyme. We employed RR spectroscopy in order to characterize the active center of the ferric form of the WT enzyme and the E12V and E12Q mutants that lack the highly conserved glutamate residue. The SOR from *N. equitans*, which also has no glutamate at the same sequence position, was studied as a 'natural variant'.[2,19] The question whether phosphate binds directly to the iron center or in its vicinity is also addressed.

## Experimental procedures

### Expression and purification of proteins

The expression and purification of the recombinant SORs wild type, E12V and E12Q mutant proteins from *A. fulgidus*, and wild type SOR from *N. equitans* were described previously.[2,3,6,19,21] Briefly, the *Escherichia coli* host cells, transformed with respective plasmids, were grown aerobically in supplemented M9 minimal medium.[22] After harvesting, the cells were broken in a mini-cell French press and each SOR was further purified from the soluble cell extract. Protein purity was tested by SDS-PAGE.[23] Fully oxidized proteins, used for subsequent spectroscopic measurements, were obtained by oxidation with potassium hexacholoiridate (Sigma).[2,3,6,19,21]

### Spectroscopy

UV-visible spectra of oxidized (20–30 μM) proteins were recorded on a Shimadzu UV-1603 spectrophotometer at ambient temperature.

Resonance Raman spectra were collected in backscattering geometry using a single stage Raman spectrometer (Jobin Yvon XY, focal length 80 cm) coupled to a confocal microscope (Olympus BX40) and equipped with a back illuminated $N_2$(l)-cooled CCD detector and a 1800-lines $mm^{-1}$ grating. Rayleigh radiation was rejected using holographic super-notch filters (Kaiser). Spectra were typically accumulated for 60 s with laser power at a sample of *ca*. 5–25 mW and spectral increments per data point of *ca*. 0.5 $cm^{-1}$. The 568 and 647 nm lines of a $Kr^+$-laser (Coherent Innova 300) were used for resonance excitation. The choice of excitation line was made on the basis of the maxima of the charge transfer electronic transitions, shown to be dependent on pH and presence/absence of phosphate ions.

About 2 μL of 5 mM protein were introduced into a $N_2$(l)-cooled cold finger (Linkam THMS600) mounted on the microscope stage of the Raman spectrometer. All spectra reported here were recorded from frozen protein samples (77 K) in 50 mM buffer (TrisHCl for pH 7, MES for pH 5.5 and glycine for pH 11). Prior to measurements, each protein was oxidized with $K_2IrCl_6$.

After polynomial background subtraction, RR spectra were submitted to band fitting analysis in the region of interest (250–450 $cm^{-1}$). In this analysis a self-made software is used to simulate the experimental spectra as a convolution of the minimum possible number of Lorentzian bands that allows for a good reproduction of the experimental data, accounting for all visible peaks and shoulders, using band positions, widths and intensities as adjustable parameters.[24] The resulting intensities and frequencies of the component Lorentzian bands were used for determination of the intensity weighted frequency $<\nu>_{Fe-S}$ according to eqn (1):[25,26]

$$\langle \nu_{Fe-S} \rangle = \frac{\sum_i I_i \nu_i^2}{\sum_i I_i \nu_i} \qquad (1)$$

## Results and discussion

The absorption spectrum of the fully oxidized wild-type (WT) SOR from *A. fulgidus* at pH 7 is characterized by a strong $Cys^- \rightarrow Fe^{3+}$ CT transition with $\lambda_{max}$ = 666 nm ($\varepsilon$ = 3500 $M^{-1}$ $cm^{-1}$). Upon increasing the pH, the CT band shifts down to 590 nm (Fig. 1) with an apparent $pK_a$ = 9.6.[2] Therefore, RR spectra obtained with CT excitation are expected to be largely dominated by vibrational modes arising from the $Fe^{3+}$–S(Cys) moiety.[5,17,19,20,27] Fig. 2 shows the metal–ligand stretching region of the RR spectra of WT-SOR at pH values of 7 and 11 measured with 647 and 568 nm excitations, respectively. The RR bands in this region originate from modes involving contributions of Fe–S stretching and internal deformations of the cysteine residue. The overall appearance of the spectra is similar to those reported previously for Center II of SORs from different organisms.[5,17,19,20,27] Band assignments adopted here are based on previous works on $^{15}N$ and $^{34}S$ isotopic shifts of globally labelled SORs from *D. vulgaris* and *P. furiosus*.[17,20] In neutral aqueous solution (Fig. 2a) the most prominent band in

 

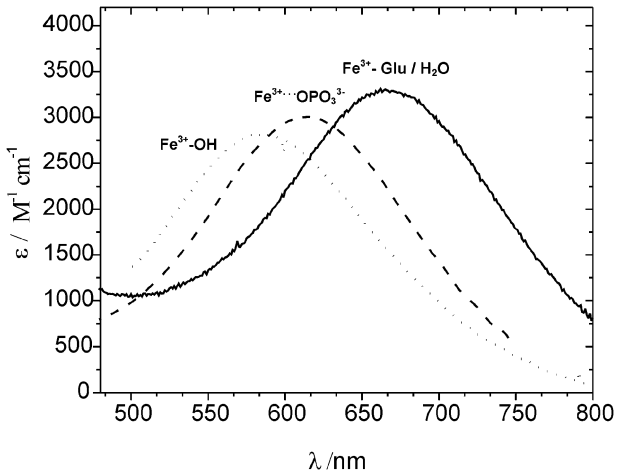


**Fig. 1** pH- and phosphate dependent UV-Vis spectral changes of the ferric *A. fulgidus* SOR: solid line: WT ($Fe^{3+}$–Glu), pH 7 and E12 mutant ($Fe^{3+}$–$H_2O$) proteins at acidic pH; (note that the spectra of E12 mutants have lower extinction coefficient), dashed line: E12 mutants in the presence of phosphate, dotted line: WT and E12 mutant proteins at pH 11.

the low frequency region is found at 298 $cm^{-1}$ and is, therefore, assigned to a mode with the highest contribution of Fe–S stretching. The band at 316 $cm^{-1}$ is assigned to a mode involving a substantial contribution of the $\delta$(S–$C_\beta$–$C_\alpha$) bending in addition to the Fe–S stretching. In contrast to the SORs from *P. furiosus* and *D. vulgaris*,[17,20] a third prominent band in this region is found at 328 $cm^{-1}$ which has a counterpart in the spectra of SORs from *Treponema pallidum* (*T. pallidum*) and *D. baarsii*. Also, this mode is predicted to include the $\delta$(S–$C_\beta$–$C_\alpha$) bending and $\nu$(Fe–S) stretching coordinates.[4,5] Spectral deconvolution reveals the presence of another band at 306 $cm^{-1}$, detected as a shoulder in the experimental spectrum. This band was observed with considerable intensity also in the spectrum of *T. pallidum* SOR and was suggested to be largely of Fe–S stretching character.[5]

Raising the pH to a value of 11 produces a significant redistribution of intensities in the RR spectrum of WT SOR, while band positions display shifts between 1 and 4 $cm^{-1}$ (Fig. 2 and Table 1). The fact that the strongest bands are found at 315 and 332 $cm^{-1}$ at pH 11 but at 298 $cm^{-1}$ at pH 7 suggests a subtle strengthening of the Fe–S(Cys) bond in the alkaline form. However, the Fe–S(Cys) stretching coordinate is distributed over several modes due to strong kinematic coupling with the internal deformations of the cysteine residue, impairing a straightforward comparison.

A more reliable parameter for comparing the strength of the Fe–S(Cys) bond is the so-called intensity weighted frequency, $<\nu>_{Fe-S}$ (Eqn (1)), which represents an average frequency of all modes with appreciable Fe–S stretching contribution, weighted by their respective intensites.[26] The values of $<\nu>_{Fe-S}$ determined for SORs under different conditions are summarized in Table 1. For the WT *A. fulgidus* protein we obtain 312 and 320 $cm^{-1}$ at pH 7 and 11, respectively, confirming a strengthening of the Fe–S bond under alkaline conditions. According to Badger's law, the intensity weighted frequency is inversely proportional to the metal–S bond

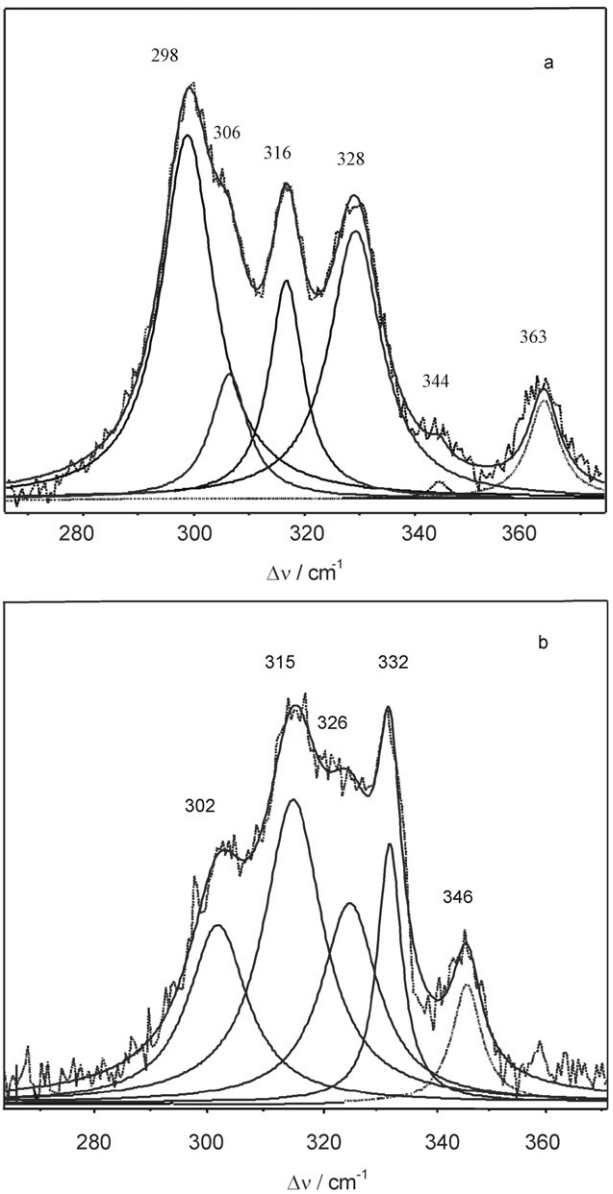


**Fig. 2** Experimental and component resonance Raman spectra of the WT SOR from *A. fulgidus*, (a) at pH 7, measured with 647 nm and (b) at pH 11, measured with 568 nm excitation, at 77 K.

length.[25,26] This concept has been extensively applied to type 1 copper proteins probed by excitation in resonance with the (Cys)S(p$\pi$)-Cu(d$\pi$) CT band. For changes of $<\nu>_{Cu-S}$ which are similar in magnitude to the $<\nu>_{Fe-S}$ changes reported here, Cu–S(Cys) bond lengths vary $<0.05$ Å.[28] Although small, these changes can be correlated with redox potentials in blue copper proteins.[25] Interestingly, the $<\nu>_{Metal-S}$ value for type 1 Cu is typically *ca.* 400 $cm^{-1}$ while for SORs-Fe it is *ca.* 320 $cm^{-1}$. This suggests longer metal–S(Cys) bonds in SORs compared to blue copper centers, as also confirmed by EXAFS data.[20] One should note that the strength of the metal–S(Cys) bond is modulated by the protein environment through mechanical constrains and through the local electrostatics. Moreover, perturbations at distant sites of the protein can propagate through the structure and influence the properties of the metal site.

**Table 1** Parameters of the component spectra for the *A. f.* WT and E12 mutants, and *N. e.* WT SOR, at different pH: band position $\nu/cm^{-1}$, width $\Delta\nu/cm^{-1}$, and normalized intensity, *I*, together with intensity weighted frequency $<\nu>_{Fe-S}/cm^{-1}$. The spectra were recorded either with 568 nm or 647 nm (asterisk) excitation

| Protein | pH | $\nu/cm^{-1}$ | $\Delta\nu/cm^{-1}$ | *I* | $<\nu>_{Fe-S}/cm^{-1}$ |
|---|---|---|---|---|---|
| WT *A.f.* | *7 | 298 | 11 | 1 | 312 |
| | | 306 | 8 | 0.3 | |
| | | 316 | 8 | 0.6 | |
| | | 328 | 11 | 0.73 | |
| | 11 | 302 | 11 | 0.63 | 320 |
| | | 315 | 12 | 1 | |
| | | 326 | 12 | 0.65 | |
| | | 332 | 6 | 0.87 | |
| | 11 + $PO_4^{3-}$ | 316 | 8 | 0.7 | 325 |
| | | 324 | 6 | 0.4 | |
| | | 332 | 7 | 1 | |
| E12V *A.f.* | 7 | 286 | 13 | 0.27 | 310 |
| | | 297 | 12 | 0.94 | |
| | | 304 | 12 | 0.69 | |
| | | 315 | 9 | 0.87 | |
| | | 327 | 10 | 1 | |
| | 11 | 284 | 10 | 0.61 | 307 |
| | | 294 | 13 | 0.85 | |
| | | 294 | 14 | 0.97 | |
| | | 303 | 10 | 0.9 | |
| | | 314 | 10 | 1 | |
| | | 328 | | | |
| | *11 + $PO_4^{3-}$ | 300 | 10 | 0.99 | 313 |
| | | 305 | 11 | 0.77 | |
| | | 318 | 7 | 0.82 | |
| | | 329 | 10 | 1 | |
| | *4.3 | 298 | 14 | 1 | 311 |
| | | 306 | 12 | 0.43 | |
| | | 316 | 6 | 0.52 | |
| | | 328 | 11 | 0.71 | |
| E12Q *A.f.* | 7 | 286 | 5 | 0.3 | 311 |
| | | 298 | 11 | 0.45 | |
| | | 306 | 12 | 0.56 | |
| | | 314 | 6 | 1 | |
| | | 329 | 8 | 0.24 | |
| | 11 | 283 | 10 | 1 | 303 |
| | | 295 | 11 | 0.75 | |
| | | 304 | 13 | 0.79 | |
| | | 314 | 8 | 0.87 | |
| | | 328 | 7 | 0.56 | |
| | *11 + $PO_4^{3-}$ | 299 | 7 | 0.85 | 313 |
| | | 305 | 8 | 0.86 | |
| | | 317 | 7 | 1 | |
| | | 330 | 11 | 0.89 | |
| | *5.5 | 300 | 10 | 1 | 313 |
| | | 306 | 10 | 0.59 | |
| | | 317 | 8 | 0.6 | |
| | | 328 | 10 | 0.95 | |
| WT *N.e.* | 7 | 286 | 14 | 0.36 | 308 |
| | | 292 | 8 | 0.29 | |
| | | 302 | 11 | 0.41 | |
| | | 310 | 10 | 0.56 | |
| | | 317 | 8 | 1 | |

RR spectra and, specifically, the $<\nu>_{metal-S}$ values do not provide sufficient criteria for discriminating the origin of the changes of the bond strength. However, relative changes in the $<\nu>_{Me-S(Cys)}$ values for comparable species do provide strong evidence for the weakening/strengthening of the Me–S(Cys) bond, which is normally associated with differences in bond lengths, as documented for a number of Fe–S and Cu–S sites.[18,25,28–30]

Replacement of the conserved glutamate residue of SOR by valine or glutamine (E12V and E12Q mutants, respectively) does not produce significant alterations of the RR spectra in terms of band positions, band widths and relative intensities (Fig. 3). Note that the RR spectra of E12V and E12Q shown in Fig. 3 were recorded at pH values of 4.3 and 5.5, respectively, because the $pK_a$ values for the "alkaline" transitions of the mutants are lowered to *ca.* 6.5 and are, therefore, to be compared with the neutral form of the WT protein (Fig. 2a). Only very minor differences in terms of relative intensities and band positions were detected through component analysis of the RR spectra of the mutants in comparison to the WT, as summarized in Table 1. The intensity weighted frequencies also show negligible differences, within 1 $cm^{-1}$. This finding is consistent with previously reported EPR spectra of the E12 mutants and the WT, that show the same ferric high spin resonances with axial distortion in all three proteins.[2] Thus, it can be safely concluded that, similarly to other SORs,[20] the Fe–S bond length is relatively insensitive to the presence of the conserved glutamate in the active site of *A. fulgidus* proteins (Table 1), at least for the acidic form. Notably, the alkaline form of the E12V mutant displays a RR spectrum which differs only slightly in terms of relative intensities with respect to the corresponding acidic form (Fig. 4a and Table 1). This contrasts with the more drastic effect of the pH on the RR spectrum of the WT protein. In agreement with this observation the $<\nu>_{Fe-S}$ values change 8 $cm^{-1}$ upon alkalinization of the WT but only 4 $cm^{-1}$ for E12V. The E12Q mutant represents the most sensitive pH-induced intensity changes (Table 1).

In addition to the bands with Fe–S(Cys) stretching character centred at *ca.* 320 $cm^{-1}$, RR spectra of the studied SORs from *A. fulgidus* reveal pH-insensitive, weak bands at 379, 400, 620, 644, 660 $cm^{-1}$ (data not shown). These bands were assigned to cysteine deformations, overtones and combination modes in *P. furiosus* SOR, based on $^{34}S$, $^{15}N$ and $^{54}Fe$ isotopic shifts.[20] A more intense band that appears at 752 $cm^{-1}$ in the spectra of *A. fulgidus* SORs, corresponds to the mode with a predominantly cysteinyl C–S stretching character, as observed in the spectra of *P. furiosus* protein.[4,20]

SOR coded in the genome of the hyperthermophilic archaeon *N. equitans* does not have the conserved glutamate residue as the 6th coordinating ligand of the ferric active site. All other conserved iron ligand residues are present, indicating an active site which is essentially equivalent to that of the E12 mutants, as shown by the three-dimensional model based on the 3D structure of *P. furiosus* SOR and the electronic spectra.[19] As for the E12 mutants from *A. fulgidus*, *N. equitans* SOR presents an upshifted "alkaline" transition with a $pK_a$ value of *ca.* 6.5.[19] Thus, this enzyme can be regarded as a 'natural variant'. However, the RR spectrum of *N. equitans* SOR measured at pH 7, *i.e.* in the "alkaline" form, displays only a low level of similarity with the spectra of *A. fulgidus* and other 1Fe- and 2Fe- SORs.[4,5,17,20] The presence of several poorly resolved bands of comparable intensities (Fig. 4b, Table 1) can be partially attributed to heterogeneity of the sample.[17] Band fitting analysis indicates a number of vibrational modes centered at 300 $cm^{-1}$ (Fig. 4b), thus down-shifted with respect to the other SORs reported here and previously studied. The most intense mode is at 317 $cm^{-1}$, similar to the

 

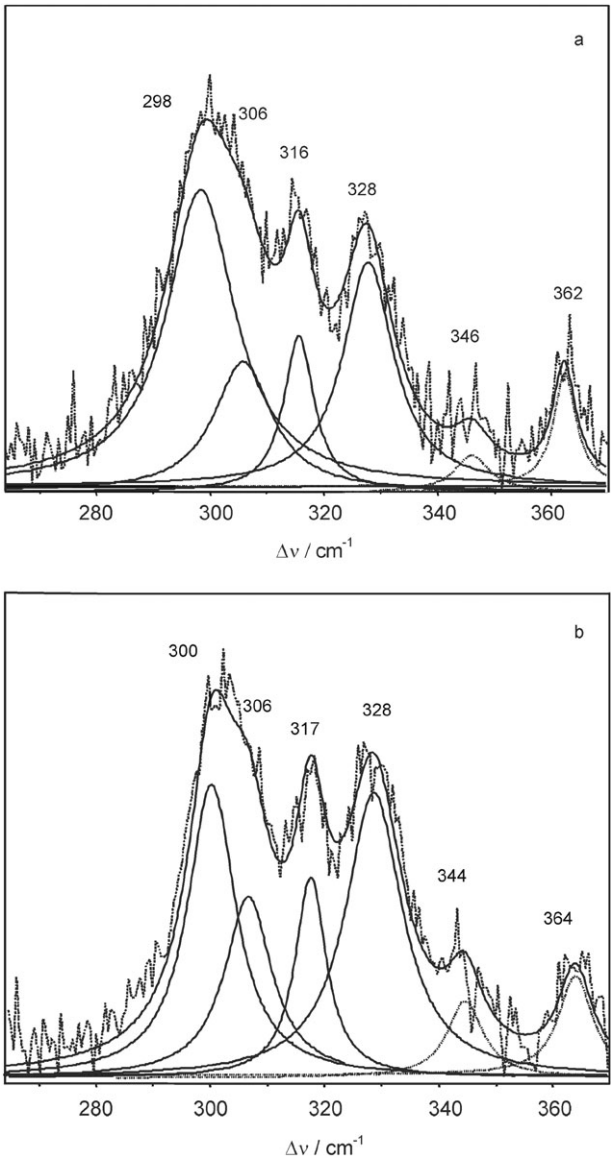


**Fig. 3** Experimental and component resonance Raman spectra of the SOR from *A. fulgidus*, (a) E12V mutant, pH 4.3, and (b) E12Q mutant, pH 5.5, measured with 647 nm excitation at 77 K.

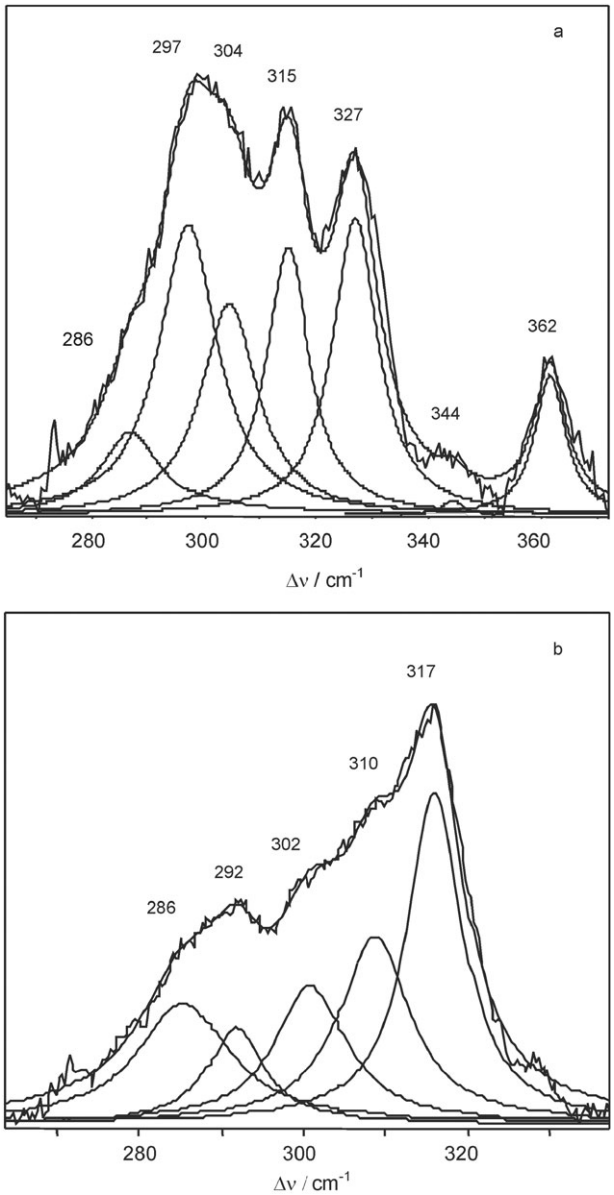


**Fig. 4** Experimental and component resonance Raman spectra of the SOR: (a) E12V from *A. fulgidus* and (b) WT from *N. equitans*, measured with 568 nm excitation, at pH 7 and 77 K.

E12Q SOR at pH 7.0, while the other bands are found at 286, 292, 302 and 310 $cm^{-1}$ (Table 1).

The $<\nu>_{Fe-S}$ value for the *N. equitans* protein obtained by deconvolution of the RR spectrum measured at pH 7 is 4 $cm^{-1}$ lower than for the "alkaline" form of WT *A. fulgidus* SOR and 2–3 $cm^{-1}$ smaller than for the alkaline forms of the corresponding E12 mutants, thus evidencing a slightly increased Fe–S bond length in the *N. equitans* enzyme. Notably, the release of the OOH-bound $H_2O_2$ intermediate is slower in SOR from *N. equitans* in comparison with the enzymes of its own family, and instead shows kinetics similar to the 2Fe-SOR.[17,19] Such differences are consistent with a distorted geometry of the active site of the *N. equitans* SOR, as suggested by the RR spectrum. This conclusion agrees with the increasing body of evidence that reveals the Fe–S(Cys) interactions as crucial for substrate reduction and product release in SORs.[1,17,20,27]

Small anionic exogenous ligands such as $CN^-$, $N_3^-$, $OH^-$ readily bind to ferric iron by displacing the glutamate residue, while $CN^-$ and $N_3^-$ bind to the vacant coordination site of the ferrous SORs.[1,17] Rodrigues *et al*. suggested that the WT and the E12 mutants of *A. fulgidus* SOR share a common sixth ligand coordinated to the ferric centre at high pH, possibly $OH^-$.[2] In order to check this hypothesis, binding of hydroxide ion to the ferric form of the studied SORs was monitored by RR spectroscopy in the presence and absence of phosphate. In addition to the low frequency envelope centred at 320 $cm^{-1}$, a band at 470 $cm^{-1}$ was observed at pH 11 in the RR spectra of E12 mutants (Fig. 5). Due to the low $pK_a$ of the alkaline transition, this band is even detectable in neutral solutions (Fig. 5D). Conversely, this band was not observed in the Raman spectrum of the alkaline buffer (Fig. 5E). Also, the

470 $cm^{-1}$ band was absent from the RR spectra of E12V and E12Q SORs in acidic conditions. A band at the same position has been observed in SOR of *D. baarsii* in alkaline solutions and attributed to the $Fe^{3+}$–OH stretching.[4] For the WT SOR of *A. fulgidus* at pH 11, no band is detected at 470 $cm^{-1}$ and, instead, we observe a broad and weak signal at 490 $cm^{-1}$. Due to a large shift of this band relative to the $Fe^{3+}$–OH stretching observed in other SORs,[4] further studies are required in order to determine its origin. Mathe *et al*. reported $OH^-$ binding to the WT SOR from *D. baarsii* and its mutants in alkaline solutions. In the case of the *P. furiosus* SOR, however, $OH^-$ ligation was only demonstrated in mutant proteins but not in the WT enzyme.[4,5,20]

Recently, Rodrigues *et al*. presented indirect evidence that the phosphate ion (commonly present in the catalytic assays) may bind to the ferric iron of the E12 mutants, affecting reaction kinetics.[2] Interestingly, we observe that the band at 470 $cm^{-1}$ in the RR spectra of the E12 mutants at pH 11 disappears upon addition of excess of phosphate to the protein solution, indicating a displacement of the $OH^-$ group by phosphate (Fig. 5C).

The intensity weighted frequencies suggest some modifications in the electronic configuration of the active site upon addition of phosphate to the medium at high pH. Specifically, we observe a significant increase (*ca*. 5–10 $cm^{-1}$) of the $<\nu>_{Fe-S}$ values in the presence of phosphate as compared to the phosphate-free sample at pH 11 (Table 1). In contrast, no changes of $<\nu>_{Fe-S}$ are observed for the acidic forms in spite of some minor up-shifts (*ca*. 1–2 $cm^{-1}$) of the bands with Fe–S stretching character. Thus, in principle, the results suggest phosphate binding at high pH. However, no band attributable to the $Fe^{3+}$-phosphate stretching has been identified in the RR spectra. Although this can be due to insufficient resonance enhancement of this vibration, one cannot discard the possibility that phosphate does not bind directly to the iron, but rather in its vicinity. The latter possibility is supported by the finding that all three proteins from *A. fulgidus* show the same behaviour, as judged from the RR data, although in the WT protein glutamate prevents direct binding of the phosphate.[2]

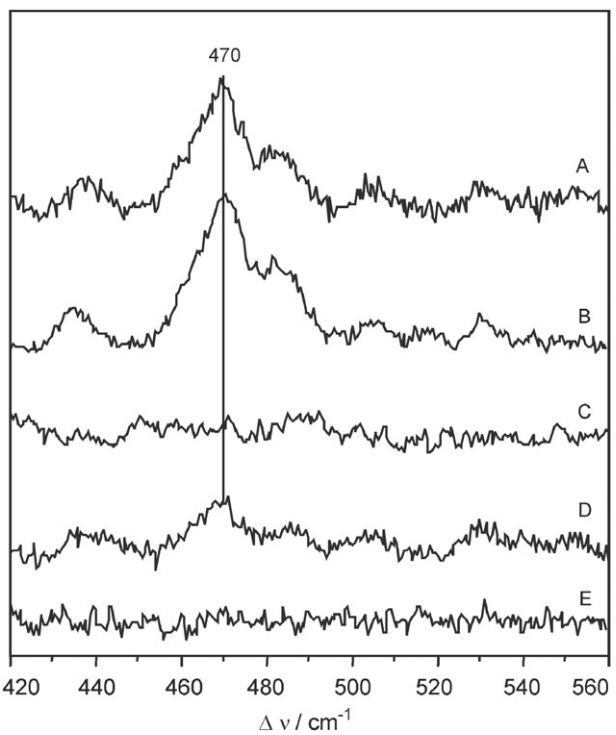


**Fig. 5** Hydroxide binding to the active centre of SOR from *A. fulgidus*. Resonance Raman spectra of: (A) E12V, pH 11, (B) E12Q, pH 11, (C) E12V, pH 11, + 50 mM sodium phosphate, (D) E12V, pH 7, and (E) glycine buffer, pH 11 + 50 mM sodium phosphate; excitation wavelength 568 nm, temperature 77 K. Spectra were taken under identical experimental conditions.

## Conclusions

The absence of the conserved glutamate residue, coordinated to the ferric iron in the WT *A. fuglidus* SOR, causes no major alterations in the active site of the E12 mutants with respect to the WT, as judged from the metal–ligand stretching region of the corresponding RR spectra. This finding supports the hypothesis that this glutamate, despite being highly conserved in the ferric active site of SORs (60 out of 67 known sequences possess the coordinating glutamate residue) and thus believed to be a selective advantage from an evolutionary perspective, is not crucial for the reaction with superoxide, under the studied conditions.[20,19] The RR spectra of the WT SOR from *N. equitans*, that also lacks the glutamate residue, however, reveal a weaker Fe-S(Cys) bond in its active site, relative to the *A. fulgidus* enzyme(s). The observed difference, although small, may well be responsible for the higher similarity of the *N. equitans* 1Fe-SOR with the 2Fe-SORs regarding kinetics and spectroscopic characteristics, than with the enzymes from its own family.[17,19]

Binding of hydroxide could be unambiguously detected only in the RR spectra of the E12 mutants. Phosphate ions, previously shown to influence the kinetics of the superoxide reduction by *A. fulgidus* WT SOR,[21] displaces the bound hydroxide and weakens the Fe–S(Cys) bond. However, the RR spectra suggest that phosphate binding may not occur at the level of the first coordination sphere.

In summary, the presented data indicate subtle differences in the active site structure of the three studied *A. fulgidus* proteins, at different pH and in the presence of phosphate ions, as reflected in the Fe–S bond length changes.

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