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**Intermediate involved in the Reduction of SO₂: Insight into the mechanism of Sulfite Reductases**

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**Abstract:** Sulfite Reductase (SiR) catalyze the reduction of SO$_{3}^{2-}$ to H$_{2}$S in both biosynthetic sulfur assimilation and dissimilation of sulfate. The mechanism of the 6e/6H$^{+}$ reduction of SO$_{3}^{2-}$ at the siroheme cofactor is debated and the proposed intermediates involved in this 6e$^{-}$ reduction are yet to be spectroscopically characterized. The reaction of SO$_{3}^{2-}$ with a ferrous iron porphyrin is investigated and two intermediates are trapped and characterized; an initial Fe(III)-SO$_{4}^{2-}$ species which undergoes proton assisted S-O bond cleavage to form an Fe(III)-SO species. These species are characterized using a combination of resonance Raman (with $^{34}$S labelled SO$_{3}$), EPR and DFT calculations. The results obtained help reconcile the different proposed mechanisms for SiR.

Reduction of sulfate to sulfide is a crucial step of geochemical sulfur cycle which controls both biosynthetic sulfur assimilation and respiration of sulfate reducing bacteria.$^{1,2}$ The reduction of sulfate to sulfide is catalyzed by two key metalloenzymes, ubiquitous in microorganisms including sulfate reducing bacteria and archaea as well as in methanogens.$^{3,4}$ The reduction of sulfate (SO$_{3}^{2-}$) requires its insertion into Adenosine 5’-monophosphate to form Adenosine 5’-phosphosulfate which is then reduced to release sulfite (SO$_{3}^{2-}$). The SO$_{3}^{2-}$ is then reduced in the active site of sulfite reductase (SiR). The reduction of sulfite is catalyzed by the siroheme cofactor (Fig. 1 A) present in all SiRs.$^{5}$ The siroheme cofactor is bridged to an Fe$_{2}$S$_{4}$ cubane via one of its cysteine ligands.$^{6,7,8}$ The mechanism of the 6e$^{-}$ reduction of SO$_{3}^{2-}$ to S$^{2-}$ is debated. In its active form both the siroheme and the Fe$_{2}$S$_{4}$ clusters are reduced i.e. the iron in siroheme is in its Fe(II) state and the Fe$_{2}$S$_{4}$ cluster is reduced. Although there are crystal structures of substrate bound enzyme (Fig. 1A, left), there is no clarity on the oxidation states of the siroheme-Fe$_{2}$S$_{4}$ unit or sulfur in these structures and two different mechanisms have been proposed (Fig 1B).$^{9,10}$ Initially, based on observation of 2e$^{-}$ and 4e$^{-}$ partially reduced species, trithionate and thiosulfate, during SO$_{3}^{2-}$ reduction three consecutive 2e/2H$^{+}$ steps were proposed (Fig 1B).$^{11,12,13,14,15,16,17}$ The protons are provided by conserved arginine and lysine residues present in the active site.$^{15}$ In addition a sulfur monoxide (SO) bound siroheme intermediate was proposed based on crystal structure obtained by the oxidation of S$^{2-}$ bound siroheme-Fe$_{2}$S$_{4}$ site (Fig. 1A right).$^{9}$

Recently, the direct 6e/6H$^{+}$ reduction of SO$_{3}^{2-}$ in the active site of SiR (Fig 1B) was called into question with the identification of a trisulfide formed between the two conserved cysteine residues of another dissimilarity sulfite reductase protein C (DsrC), which is encoded by all genomes that contain the genes of the catalytic A and B domains of SiR and binds the DsrAB complex, as an end product of SO$_{3}^{2-}$ reduction. The rate of SO$_{3}^{2-}$ reduction by SiR is first order with respect to DsrC and about 15 times higher than the rate in the absence of DsrC.$^{18}$ The proposed mechanism invoked the attack on an Fe(III)-SO$_{3}$/Fe(III)-SO$_{2}$H species (Fig. 1B), formed after the initial 2e$^{-}$ reduction of SO$_{3}^{2-}$, by the two conserved cysteines of DsrC to form a trisulfide.$^{19}$ This trisulfide is then reduced to release sulfide avoiding generation of trithionate and thiosulfate and resulted in sulfide as the only product of SO$_{3}^{2-}$ reduction.

![Figure 1. A) active site of SO$_{3}^{2-}$ bound SiR and SO bound Sir (pdb id: 7GEP) and B) The proposed mechanisms of Sir (both direct 6e$^{-}$ reduction and trisulfide pathways are shown). The charge of the [FeS$_{4}$] cluster (excluding the cysteines) is indicated next to the cubane.](Image)

A synthetic analogue of siroheme can be useful in understanding the mechanism.$^{20}$ Unfortunately, the synthesis of this cofactor is not trivial and has not been achieved yet.$^{21,22}$ Alternatively, simpler porphyrins like iron tetraphenyl porphyrin (FeTPP), while not an exact model of siroheme-Fe$_{2}$S$_{4}$ active site, has reduction potential similar to that of siroheme.
from different enzymes (which vary between -0.19 to -0.29 V at pH 7, FeTPP is -0.20 V at pH 7) and hence can be used to gain insight into this intriguing reaction. Initial attempts to investigate the reaction of SO₂ with ferrous porphyrin in non-polar organic solvents inevitably resulted in the formation of sulfate bound ferric porphyrin. Recently, the reaction of ferrous tetrathenyl porphyrin with SO₂ was investigated in protic organic solvent at room temperatures (RT). The reaction proceeded to result in the 2e⁻ reduction of SO₂ to SO and the SO released could be trapped using 2,3-dimethylbutadiene resulting in the formation of a cyclic sulfoxide. An Fe(III)-SO intermediate was identified and was characterized using Mössbauer and EPR spectroscopy as a low spin ferric porphyrin antiferromagnetically coupled to a triplet SO, resulting in an S=1/2 species. Although, the presence of the Fe-S bond could not be established experimentally, the observed S-O vibrations for an Fe(III)-SO species was consistent with the theoretically predicted vibrations for an Fe(III)-SO species. One of the 2e⁻ needed is derived from the iron porphyrin the SO is bound to and the other from a free Fe(II)TPP. The 1:1 ratio of [Fe(III)TPP]⁺ and [Fe(III)TPP-SO]⁺ species was confirmed by Mössbauer spectroscopy. The Fe(III)-SO/Fe(III)-SO₂ species, proposed in the mechanism of SiR, however has not been observed either in the protein or in synthetic systems. 

To assess the involvement of any intermediate species prior to the [Fe(III)TPP-SO]⁺ species, the reaction of Fe(II)TPP with SO₂ is allowed to proceed at -80°C (MeOH-liq. N₂ bath) for 10 mins and then the reaction mixture frozen in liq. N₂. A new EPR signal (Fig. 2A, green) is obtained with g = 2.36, 2.27, 1.89 indicating the formation of another S=1/2 low-spin intermediate species (referred to as Int-I henceforth) prior to the formation of [Fe(III)-SO]²⁺ species (referred to as Int-II henceforth). In parallel to the Int-I signal, there is a g=6.0 signal corresponding to a high spin [Fe(III)TPP]⁺ species suggesting that Int-I results from 2e⁻ reduction of SO₂ as well, where one electron is derived from the Fe(II)TPP the SO₂ binds and the other electron is derived from a free Fe(II)TPP which gets oxidized to a high-spin [Fe(III)TPP]⁺ species with a g=6.0 EPR signal. Mossbauer data suggests an equal population of these two species in the sample (Fig. S1). When the solution is warmed up to -40°C (10 mins), the EPR signals originating from Int-I decrease and the EPR signals from Int-II emerge (Fig. 2A, yellow) indicating that Int-I decays to produce Int-II (Fig. 2A, blue) i.e. Int-I is a species formed prior to Int-II in the reaction. 

The transition of Int-I to Int-II depends on the availability of protons. When the reaction of SO₂ and Fe(II)TPP is performed at -80°C (10 mins) but in the presence of 10 eq. of xylium chloride (XylH⁺Cl⁻) as a proton source, the EPR signals from Int-I (Fig. 2B, green) is no longer observed and only those of Int-II is observed (Fig.2B, pink). Similarly, when the MeOH proton source in the solution is deuterated (1% CD₂OD instead of 1% MeOH, CD₂OD represented as MeOD) and used as the proton source instead, the EPR signal of the reaction at -40°C (Fig. 2B, red) shows Int-I and Int-II in 5:1 ratio relative to MeOH where this ratio is 1:2 indicating that there is a H/D isotope effect in the conversion of Int-I to Int-II, consistent with a protonation step being involved in the reaction. 

The Int-I trapped at -80°C is further characterized using resonance Raman spectroscopy of samples prepared with SO₂ and comparing the vibrations with those observed when ³⁴SO₂ is used. In Int-I a vibration is observed at 984 cm⁻¹ which shifts to 970 cm⁻¹ on ³⁴S substitution (Figure 3A, top). This is consistent with an S-O stretching mode from an SO₂ derived axial ligand. In the lower energy region, a vibration is observed at 340 cm⁻¹ which shifts to 337 cm⁻¹ on ³⁴S labelling (Figure 3B, bottom). The energy of this vibration and the isotope shift is consistent that it is an Fe-S stretching mode.
substitution (Figure 4B, bottom). There is also another vibration at 206 cm\(^{-1}\) which shifts to 204 cm\(^{-1}\) with \(^{34}\)S (Figure 4B, bottom) which may likely results from mixing of the Fe-S mode with a porphyrin vibration. There is some residual Fe-S vibration at 340 cm\(^{-1}\) from Int-I (Figure 4B, bottom). Thus, the Fe-S and the S-O vibrations, confirmed with \(^{34}\)S labelling, clearly indicate that both Int-I and Int-II have Fe-S and S-O bonds. Samples prepared with MeOD did not show any shift in any of the S-O or Fe-S vibrations suggesting that none of the species are likely to be protonated. Density functional theory (DFT) calculations are used to gain further insight.

DFT calculations are used to compute hypothetical structure of the possible intermediates in the reduction of SO\(_2\) to SO by Fe(II)TPP.\(^{33,34}\) DFT method being used was reported to reproduce the Mössbauer and vibrational spectroscopy data of the [Fe(II)TPP-SO\(_2\)]\(^{2-}\) species quite well.\(^{28}\) The EPR data indicates that both Int-I and Int-II are S=1/2 Fe(III) species and the presence of free Fe(II)TPP+ indicate that the SO\(_2\) is reduced by 2e\(^-\) to its formal +2 oxidation state in both these species. Additionally, conversion of Int-I to Int-II requires a proton as indicated by EPR data obtained with MeOD and XylH\(^+\)Cl\(^-\). The most likely description of Int-I is [Fe(III)TPP-SO\(_2\)]\(^{2-}\) and Int-II has already been proposed to be [Fe(III)TPP-SO\(_2\)]\(^{2-}\). The possibility of formation of Fe(II)TPP-SO\(_2\)H is rescinded by the lack of H/D isotope effect in the S-O region. 413.1 nm excitation and 10 mW laser power, prepared with MeOD and XylH\(^+\)Cl\(^-\) (blue traces) and \(^{34}\)SO\(_2\) (red traces). The \(^{34}\)SO\(_2\) sample contains some unreacted Fe(II)TPP (green). *Indicates a solvent peak.

The mechanism of SO\(_2\) reduction by Fe(II)TPP (Scheme 1, A) thus involves a 2e\(^-\) step resulting in a SO\(_2\)\(^{2-}\) species bound to [Fe(II)TPP]+ to form a [Fe(II)TPP-SO\(_2\)]\(^{2-}\): Int-I like the mechanism proposed for SiR by Pereira and co-workers.\(^{18}\) No 1e\(^-\) reduced intermediate could be observed even at -80°C rather an additional electron is derived from a free Fe(II)TPP to result in a 2e\(^-\) reduction which surrogates for the reduced [Fe\(_2\)S\(_4\)]\(^+\) cluster at the SiR active site. Protonation of this

Figure 3. Resonance Raman data on a frozen solution of Int-I (A) S-O region and (B) Fe-S region. 413.1 nm excitation and 10 mW laser power, prepared with \(^{34}\)SO\(_2\) (blue traces) and \(^{34}\)SO\(_2\) (red traces). The \(^{34}\)SO\(_2\) sample contains some unreacted Fe(II)TPP (green). *Indicates a solvent peak.

Figure 4. Resonance Raman data on a frozen solution of Int-II (A) S-O region and (B) Fe-S region. 413.1 nm excitation and 10 mW laser power, prepared with \(^{34}\)SO\(_2\) (blue traces) and \(^{34}\)SO\(_2\) (red traces). The \(^{34}\)SO\(_2\) sample contains some unreacted Fe(II)TPP (green). *Indicates a solvent peak and ** represents residual Int-I.
species leads to the cleavage of the S-O bond and formation of a [Fe(III)TPP-SO$_2^-$] species, Int-II. The reaction can proceed even at 40°C with a weak proton source like MeOH, albeit accelerated in the presence of XiHCl. This indicates that the pK$_a$ of the [Fe(III)TPP-SO$_2^-$] is higher than that of MeOH in THF. So the 2e$^-$ reduced intermediate in the active site of SiR, which has several arginine and lysine residues, is most likely to be Fe(III)-SO$_2$H. The reaction mechanism observed here suggests that the most likely mechanism of SiR (Scheme 1, B) involves an initial formation of Fe(III)-SO$_2$H species (scheme 2B) after a 2e$^-$ reduction of SO$_2$. The structure of DsrC bound to SiR shows that the cysteiny1 sulfur from DsrC is less than 2 Å away from the siroheme and the faster step is likely the attack of cysteines of the DsrC protein resulting in the trisulfide.

**Conflicts of interest**

There are no conflicts to declare.

**Supporting information**

Experimental methods and additional experimental and computation details are available free of charge in the supporting information.

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**Notes and references**