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Nitrite reductase activity in F_{420}-dependent sulphite reductase (Fsr) from *Methanocaldococcus jannaschii*

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Abstract

Methanocaldococcus jannaschii (*Mj*), a hyperthermophilic and evolutionarily deeply rooted methanogenic archaeon from a deep-sea hydrothermal vent, produces F_{420}-dependent sulphite reductase (Fsr) in response to exposure to sulphite. This enzyme allows *Mj* to detoxify sulphite, a potent inhibitor of methyl coenzyme-M reductase (Mcr), by reducing it to sulphide with reduced coenzyme F_{420} ($F_{420}H_2$) as an electron donor; Mcr is essential for energy production for a methanogen. Fsr allows *Mj* to utilize sulphite as a sulphur source. Nitrite is another potent inhibitor of Mcr and is toxic to methanogens. It is reduced by most sulphite reductases. In this study, we report that *Mj*Fsr reduced nitrite to ammonia with $F_{420}H_2$ with physiologically relevant K_m values (nitrite, 8.9 µM; $F_{420}H_2$, 9.7 µM). The enzyme also reduced hydroxylamine with a K_m value of 112.4 µM, indicating that it was an intermediate in the reduction of nitrite to ammonia. These results open the possibility that *Mj* could use nitrite as a nitrogen source if it is provided at a low concentration of the type that occurs in its habitat.

DATA SUMMARY

The authors confirm all supporting data, code and protocols have been provided within the article.

INTRODUCTION

Nitrite is toxic to methanogens as it inhibits methyl-coenzyme M reductase (Mcr) at low micromolar concentrations [1, 2], an enzyme that is essential for energy production in these archaea [3]. Yet, several *Methanocaldococcus* species and one *Methanotorris* isolate from deep-sea hydrothermal vents and a hot spring thermophilic methanogen utilize nitrate as a nitrogen source [4, 5]. This capability requires the reduction of nitrate to ammonia, which involves nitrite and hydroxylamine as an intermediate; similar to nitrite, hydroxylamine is toxic to many organisms [6, 7]. We therefore looked for the possibility of a nitrite reductase enzyme that would not impart hydroxylamine toxicity in these organisms.

Similar to nitrite, sulphite inhibits Mcr [8, 9] and is toxic to methanogens [4, 10–14]. However, *Methanocaldococcus jannaschii* (*Mj*), an inhabitant of deep-sea hydrothermal vents, is resistant to sulphite and can use this oxyanion as a sole sulphur source [13, 15]. This capability is due to a novel sulphite reductase that uses coenzyme F_{420} as an electron carrier, and is called F_{420}-dependent sulphite reductase (Fsr) [15]. The homologues of Fsr are present in all hydrothermal vent methanogens [4, 5]. Phylogenetic and comparative structural analysis has shown that the Fsr homologues form two distinct clades, called FsrI and FsrII, and *Mj*Fsr belongs to the FsrI clade and henceforth we call it *Mj*FsrI [16].

Most sulphite reductases reduce nitrite [15, 17–22] and a preliminary study in our laboratory has shown an indication for this activity in *Mj*FsrI [23]. Also, recently an FsrII from one of the anaerobic methanotrophic archaea (ANME), which oxidize methane anaerobically, has been shown to act as an F_{420}-dependent nitrite reductase (FNiR) and lacks sulphite reductase activity [17]. Accordingly, we examined the nitrite reductase activity of *Mj*FsrI and characterized the respective kinetic properties. The ecological relevance of the findings was also explored.

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Keywords: coenzyme F_{420}; deazaflavin; F_{420}-dependent nitrite reductase (FNiR); F_{420}-dependent sulphite reductase (Fsr); $F_{420}H_2$; methanogen.
Abbreviations: ANME, anaerobic methanotrophic archaea; FAD, flavin adenine dinucleotide; FNiR, F_{420}-dependent nitrite reductase; Fsr, F_{420}-dependent sulphite reductase; Mcr, methyl coenzyme-M reductase; *Mj*, *Methanocaldococcus jannaschii*.

Impact Statement

Nitrite is highly toxic to methanogens and the deep-sea hydrothermal vent environment provides a constant supply of this oxyanion at low concentrations. This environmental situation calls for a nitrite detoxification tool in vent methanogens. Here, the occurrence of F_{420}-dependent nitrite reductase (FNiR) activity in F_{420}-dependent sulphite reductase (Fsr) opens the possibility of vent methanogens employing this enzyme to detoxify nitrite as well as to generate ammonia for cell biosynthesis.

METHODS

Purification of *Mj*FsrI

The protein was purified anaerobically from *Mj* cells grown with sulphite as a sulphur source via a previously reported procedure [15, 24] but with the following modifications. Cell extracts were fractionated via precipitation with ammonium sulphate and gravity flow-based column chromatography conducted at room temperature (~25 °C) and inside an anaerobic chamber filled with a mixture of N_2 and H_2 (96:4, v/v). The $(NH_4)_2SO_4$ and NaCl solutions were prepared in 25 mM potassium phosphate buffer, pH 7 (buffer A). All chromatography resins were obtained from Cytiva, except F_{420}-Sepharose which was prepared in the laboratory [15, 25, 26]. Recovery of the enzyme was followed by use of an assay for F_{420}-dependent sulphite reductase (Fsr) as described below. The first step of purification was treatment of an *Mj* cell extract with $(NH_4)_2SO_4$ at 60% saturation, and activity was found in the supernatant, which was fractionated over a 1×20 cm phenyl-Sepharose column with 6 ml resin. After sample application, the column was washed with five solutions of $(NH_4)_2SO_4$ at the following concentrations, and these were applied in the sequence as presented: 1, 0.75, 0.5, 0.25 and 0 M. The volume of each wash was 12 ml, except that of the first (1 M) which was 36 ml. The eluates from the 0.25 M $(NH_4)_2SO_4$ wash contained Fsr activity and these were pooled and loaded onto an F_{420}-Sepharose column [15, 25, 26] (1×10 cm; 4 ml resin). After sample application, the column was washed first with 28 ml of buffer A and then five aliquots of 8 ml NaCl solutions at the following NaCl concentrations: 0.1, 0.2, 0.3, 0.4 and 0.5 M. Most of the activity was found in the 0.3 M NaCl fractions, which were pooled. The resulting preparation was fractionated on a 1×20 cm column packed with 6 ml QAE-Sephadex resin. Then, the column was washed with 36 ml of buffer A and 12 ml aliquots of four solutions with NaCl at the following concentrations: 0.25, 0.5, 0.75 and 1 M. The 0.25 M NaCl fractions contained Fsr activity and were pooled. SDS-PAGE showed that this final preparation contained an apparently homogeneous enzyme preparation.

Enzyme activity and protein assays and SDS-PAGE

The assays for FNiR, Fsr and $F_{420}H_2$-dependent hydroxylamine reductase were performed anaerobically using reduced F_{420} ($F_{420}H_2$) as the electron donor and respective electron acceptors, which were nitrite, sulphite and hydroxylamine, following a previously described procedure [15, 17, 27]. Briefly, this procedure involved spectrophotometric monitoring of the oxidation of $F_{420}H_2$ at 400 nm, and an extinction coefficient value of 25 mM^{-1} cm^{-1} for F_{420} was used to calculate the reaction rate [28]. The standard assay employed a reaction mixture with the following components in a total volume of 0.8 ml, and all assays were initiated by enzyme addition: 100 mM potassium phosphate buffer, pH 7; 40 µM $F_{420}H_2$; 500 µM sodium nitrite or 1 mM sodium sulphite or 500 µM hydroxylamine. In the assays that were used to determine the kinetic constants [15], the concentration of the relevant substrate was varied.

Iron and acid-labile sulphur content determination

The contents of iron and acid-labile sulphur of *Mj*FsrI were determined as described previously [17], except a solution containing 71.8 µg ml^{-1} protein in 25 mM potassium phosphate buffer, pH 7, was used. The ammonia produced in the Fsr reaction was estimated using a glutamate dehydrogenase-based assay [15, 17, 29] employing an AA0100 Kit (Sigma-Aldrich).

UV–visible spectroscopy and HPLC analysis of the flavin component of *Mj*FsrI

The UV–visible spectrum of a 300 µl anaerobic solution of *Mj*FsrI containing 21 µg of homogeneous protein in 25 mM potassium phosphate buffer, pH 7, and 250 mM NaCl was obtained at 25 °C using a Beckman Coulter DU800 spectrophotometer as described previously [17].

The type of flavin present in *Mj*FsrI was identified and its amount was determined via non-degradative extraction followed by HPLC analysis at room temperature via an established method [17], but with modifications. A 400 µl solution of 28.7 µg purified protein in 25 mM potassium phosphate buffer, pH 7, was used and the filtered extract was concentrated by evaporation under a flow of nitrogen before analysis.

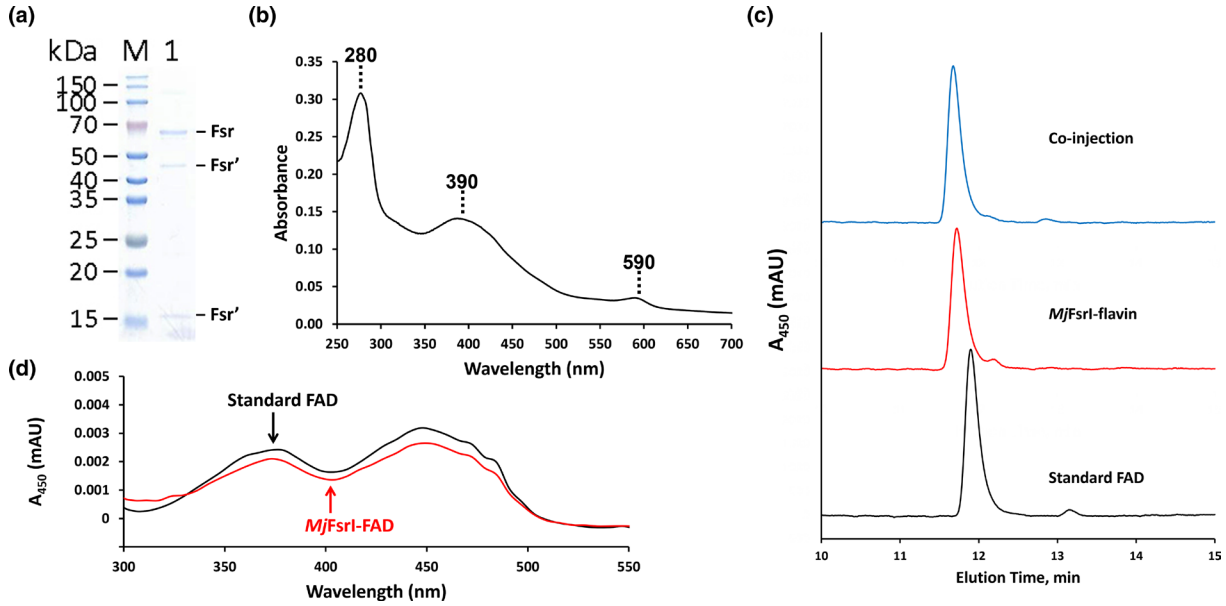

Fig. 1. Subunit size, UV–visible spectroscopic characteristics and prosthetic groups of *Mj*FsrI. (a) SDS-PAGE profile of purified *Mj*FsrI. Fsr (intact *Mj*FsrI), ~69 kDa; Fsr′ (degradation products of *Mj*FsrI), ~45 and ~16 kDa. (b) UV–visible spectrum of *Mj*FsrI obtained under anaerobic conditions. (c) Reversed-phase HPLC analysis of a methanol–methylene chloride extract of *Mj*FsrI. Standard FAD, analysis of a 100 µl volume of 0.5 µM flavin adenine dinucleotide (FAD); *Mj*FsrI-flavin, analysis of a 100 µl methanol–methylene chloride extract from 28.7 µg homogeneous protein; Co-injection, analysis of a 100 µl 1:1 (v/v) mixture of a 0.5 µM solution of FAD and methanol–methylene chloride extract of *Mj*FsrI. (d) UV–visible spectrum of *Mj*FsrI's flavin cofactor as collected by the diode array detector of an HPLC unit.

RESULTS

Before embarking on investigating new activity of *Mj*FsrI, we examined the purified *Mj*FsrI for the core properties that were established at the time of its discovery [15]. In SDS-PAGE, the protein exhibited three bands at ~16, ~45 and ~69 kDa (Fig. 1a), where the last value matched the theoretical subunit size of *Mj*FsrI, which is 69.79 kDa. The ~16 and ~45 kDa bands are known to originate from the degradation of this protein during the sample preparation for SDS-PAGE [15]. The purified enzyme displayed a UV–visible spectrum typical of sirohaem in a low-spin ferric state with peaks at 280, 390 and 590 nm (Fig. 1b) [15, 30]. Following this validation, we investigated the new properties of the protein.

Structural and spectroscopic characteristics of *Mj*FsrI

Reversed-phase HPLC identified the flavin extracted from *Mj*FsrI as FAD (Fig. 1c, d). It was estimated that *Mj*FsrI carried 0.52 mol of this molecule per mole of subunit, which suggested that a dimer of the protein assembled one FAD molecule. Chemical assays showed that *Mj*FsrI held 23.81±1.16 mol iron and 23.92±2.49 mol acid-labile sulphur per subunit, indicating that it assembled six [Fe_4-S_4] clusters. This value is consistent with the recently available crystal structure of *Mj*FsrI [31].

Nitrite reduction activity in *Mj*FsrI and kinetics of the reaction

*Mj*FsrI catalysed the reduction of nitrite and hydroxylamine to ammonia by utilizing $F_{420}H_2$ as an electron donor and was not able to utilize NADH or NADPH for these conversions. Accordingly, we term this action as FNiR activity. At a fixed concentration of 300 µM for nitrite and a concentration range of 2–80 µM for $F_{420}H_2$, the apparent K_m for $F_{420}H_2$ was 9.72±1.7 µM and the apparent maximum velocity (V_m) value was 20.3±0.96 µmol $F_{420}H_2$ oxidized or 40.6±1.92 µmol electrons transferred per minute per milligram enzyme (Fig. 2a). Similarly, with 40 µM $F_{420}H_2$ and 5–150 µM nitrite, the apparent K_m for nitrite was estimated to be 8.9±0.9 µM and the V_m was 18.4±0.36 µmol of $F_{420}H_2$ oxidized or 36.8±0.72 µmol electrons transferred per minute per milligram enzyme (Fig. 2b). A kinetic analysis at 40 µM $F_{420}H_2$ and 25–600 µM hydroxylamine showed that the apparent K_m for hydroxylamine was 112.4±14.4 µM and the respective V_m was 45.3±1.9 µmol of $F_{420}H_2$ oxidized or 90.6±3.8 µmol electrons transferred per minute per milligram enzyme (Fig. 2c). From an average of the values for the amounts of products in three independent 30 min FNiR reactions with *Mj*FsrI, the enzyme produced 0.0427±0.0005 µmol F_{420} and 0.0133±0.0051 µmol ammonia in an assay with 0.08 µmol $F_{420}H_2$ and 0.40 µmol nitrite; in effect, 0.043 µmol $F_{420}H_2$ was consumed or 0.086 µmol electrons was made available for nitrite reduction. Since the production of 1 mol nitrite to ammonia would require 6 mol electrons or 3 mol $F_{420}H_2$, and the enzyme produced F_{420} and ammonia at a ratio of 3:1, in the FNiR reaction, about 94% of the consumed reducing equivalents was recovered in the product.

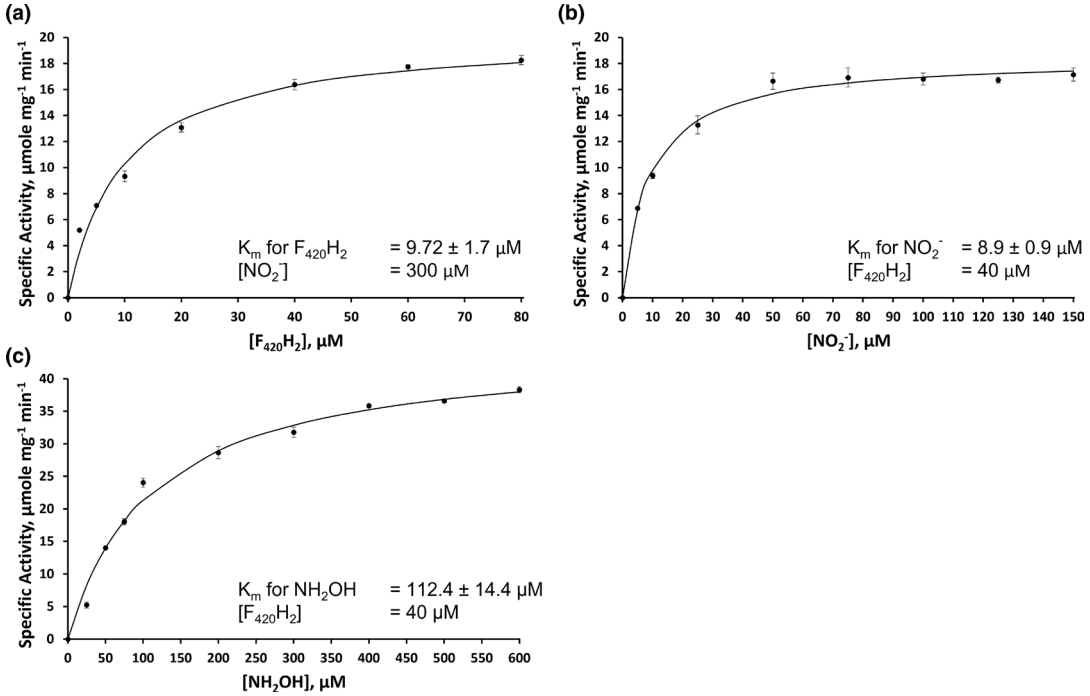

Fig. 2. Kinetic analysis of the *Mj*FsrI reaction. Specific activity is defined as the amount (µmol) of $F_{420}H_2$ oxidized per minute per milligram enzyme. Substrate for which the concentration was varied: (a) $F_{420}H_2$; (b) nitrite; (c) hydroxylamine. Each data point is an average of values from three independent assays and the respective standard deviations are shown. Each solid curve represents the best fit of the data to the Henry–Michaelis–Menten hyperbola function, $v=V_m[S]/K_m+[S]$; fitting was performed by using the add-in Solver analysis tool in Microsoft Excel [39].

DISCUSSION

Fsr of *Mj* exhibited FNiR activity. This observation could be rationalized thermodynamically and it is also consistent with the chemical mechanism established for other sulphite reductases. Since $F_{420}H_2$ with a mid-point redox potential ($E^{0\prime}$) value of −360 mV (see equation 1 below) is an effective reductant for Fsr-catalysed sulphite reduction (equations 2 and 4), thermodynamically the enzyme would be able to utilize $F_{420}H_2$ for nitrite reduction (equations 3 and 5). Studies with *Archaeoglobus fulgidus* (*Af*) dissimilatory sulphite reductase (Dsr) have shown that the transformation of sulphite to sulphide involves the formation of enzyme-bound SO_2^- and SO^- as intermediates which are stabilized by a set of basic residues (Arg^{98}, Arg^{170}, Lys^{211} and Lys^{213}) [21, 32]. The SO_2^- intermediate is structurally similar to NO_2^- and *Af*Dsr indeed reduces nitrite using the above-mentioned structural units [21, 32]. The Arg^{98}, Arg^{170}, Lys^{211} and Lys^{213} of *Af*Dsr are fully conserved in *Mj*FsrI (Arg^{355}, Arg^{423}, Lys^{460} and Lys^{462} [15, 31]) and therefore *Mj*FsrI employed a common chemical mechanism for reducing sulphite and nitrite; the recently available crystal structure of *Mj*FsrI indeed shows that Arg^{355}, Arg^{423}, Lys^{460} and Lys^{462} are involved in the binding of sulphite and two water molecules at the active site of this protein [31]. The ability of *Mj*FsrI to reduce hydroxylamine (NH_2OH) indicated that the Fsr-catalysed reduction of nitrite proceeded through the intermediate formation of hydroxylamine and this sequence is also seen with the other sulphite/nitrite reductases [17, 18, 20, 21]. The enzyme was efficient in the utilization of the $F_{420}H_2$-derived reducing equivalents as it exhibited almost 100% recovery of this resource into ammonia, the product. These data indicated that NH_2OH, a reaction intermediate, did not accumulate and this finding is consistent with the observation that the rate of hydroxylamine reduction was more than double of that for the overall nitrite reduction reaction. The higher K_m value for hydroxylamine did not pose a problem for this conversion as this intermediate was probably not released from the enzyme.

$$F_{420} + 2e^- + 2H^+ \rightarrow F_{420}H_2 \quad E^{0\prime} = -360\,\text{mV} \tag{1}$$

$$HSO_3^- + 6e^- + 6H^+ \rightarrow HS^- + 3H_2O \quad E^{0\prime} = -116\,\text{mV} \tag{2}$$

$$NO_2^- + 6e^- + 8H^+ \rightarrow NH_4^+ + 2H_2O \quad E^{0\prime} = +440\,\text{mV} \tag{3}$$

$$HSO_3^- + 3F_{420}H_2 \rightarrow HS^- + 3H_2O + 3F_{420} \quad \Delta G^{0\prime} = -135\,\text{kJ/mol} \tag{4}$$

$$NO_2^- + 3F_{420}H_2 + H^+ \rightarrow NH_4^+ + 2H_2O + 3F_{420} \quad \Delta G^{0\prime} = -457\,\text{kJ/mol} \tag{5}$$

The K_m value of *Mj*FsrI for nitrite (~9 µM) was comparable to a value that has been reported for sulphite (12 µM) and a similar case was found for the specific activities of this enzyme (µmol electrons transferred min^{-1} mg^{-1}): 37 for nitrite reduction (this study)

and 32 for sulphite reduction [15]. Thus, the nitrite reduction activity of *Mj*FsrI could be physiologically relevant in *Mj* with *in vivo* roles in nitrite detoxification and deriving nitrogen nutrition from this oxyanion. We elaborate on these two possibilities below.

As mentioned in the Introduction, *Mj*FsrI acts as a sulphite detoxification enzyme and allows *Mj* to use this toxic compound as a sulphur source [13, 15]. Similar to sulphite, nitrite is highly toxic to methanogens as it oxidizes the Ni(I) centre of coenzyme F_{430}, a prosthetic group of Mcr [1], an essential enzyme for energy production in these organisms [3]; at a concentration of 50 µM, nitrite fully inactivates purified Mcr in 15 min [1]. In the natural habitat of *Mj*, a segment of a deep-sea hydrothermal vent, mixing of extremely hot and anaerobic vent water with oxygen-containing cold seawater brings the temperature to a level that is conducive for living organisms [33]. At this locale, the nitrite concentration is kept at a very low level due to chemical reoxidation to nitrate [34]. However, for a constant albeit low-level supply of this oxyanion and high sensitivity of Mcr towards it, it would be advantageous for a vent methanogen, such as *Mj*, to carry a nitrite detoxification tool, and with the observed high activity and low K_m value for nitrite (~ 9 µM), *Mj*FsrI could satisfy this need.

The highest nitrite concentration in a deep-sea hydrothermal vent is never more than 4 µM [34, 35]. Based on the data in Fig. 2b, at this nitrite concentration, *Mj*FsrI will provide an FNiR activity of about 6 µmol $F_{420}H_2$ oxidized per minute per milligram protein, which would translate to an ammonium production rate of 2 µmol min^{-1} mg^{-1} protein. The doubling time of *Mj* is about 26 min [36], which corresponds to a maximum growth rate of 1.6 h^{-1} or 1.6 g of daughter cells per 1 g of mother cell mass per hour. Also, nitrogen constitutes about ~15% of the cell mass [37]. Thus, to produce 1.6 g of new cells per hour, 1 g of mother cells needs to generate 0.24 g of usable nitrogen or 0.29 g or 0.017 mol ammonia per hour. As estimated from the published data (Fig. 2a of [15]), under sulphite induction, Fsr constitutes about 10% of the total cell proteins; 50% of the dry weight of a cell is due to proteins [38]. This means that 1 g of mother cells could contain up to 50 mg of Fsr protein, and consequently, could produce 0.006 mol ammonia per hour. Therefore, in a hydrothermal vent environment, the FNiR activity of *Mj*FsrI would allow *Mj* to utilize available nitrite as a sole nitrogen source to maintain about one-third of its maximal growth rate as determined in a laboratory [36] if the nitrogen is the only limiting nutrient. A similar role has also been proposed recently for FsrII in an ANME from marine methane seep sediment [17]. Thus, the Fsr group broadly protects Mcr of marine methanogens and certain ANMEs from sulphite and nitrite inhibition and provides sulphur and nitrogen nutrition to these organisms by reducing these oxyanions.

In summary, the protein that has been described as *Mj*FsrI [15] was shown to provide an FNiR function with physiologically relevant kinetic properties without causing toxicity of hydroxylamine, a reaction intermediate. A calculation based on the kinetic properties of the enzyme, growth kinetics of the organism, and known concentrations for nitrite in the hydrothermal vent fluid showed that the FNiR activity of *Mj*FsrI would support a reasonably high growth rate for *Mj* with nitrite as the sole nitrogen source if it is induced under *in situ* conditions [15]. This assessment made for a rather low concentration of nitrite, a toxic oxyanion for the methanogens, sets the stage for growth studies with *Mj* and other vent methanogens with nitrite as the sole nitrogen source in a continuous culture system. Here a steady supply of nitrite at a low concentration would provide an ecologically relevant environmental condition.

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Author contribution
C.H., E.F.J. and B.M. conceptualized the study; C.H. and E.F.J. performed the research; C.H., E.F.J. and B.M. analysed the data; C.H. and B.M. wrote the original draft; C.H. and B.M. reviewed and edited the final manuscript.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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Peer review history

VERSION 2

Editor recommendation and comments

https://doi.org/10.1099/acmi.0.000482.v2.3

John Munnoch; University of Strathclyde, SIPBS, UNITED KINGDOM, Glasgow

Date report received: 09 January 2023
Recommendation: Accept

Comments: The work presented is clear and the arguments well formed. This study would be a valuable contribution to the existing literature. This is a study that would be of interest to the field and community.

Reviewer 2 recommendation and comments

https://doi.org/10.1099/acmi.0.000482.v2.2

Julius Campeciño; Michigan State University, Biochemistry and Molecular Biology, SPAIN
https://orcid.org/0000-0003-0502-0177

Date report received: 06 January 2023
Recommendation: Accept

Comments: Please accept without further revision.

Please rate the manuscript for methodological rigour
Good

Please rate the quality of the presentation and structure of the manuscript
Good

To what extent are the conclusions supported by the data?
Strongly support

Do you have any concerns of possible image manipulation, plagiarism or any other unethical practices?
No

Is there a potential financial or other conflict of interest between yourself and the author(s)?
No

If this manuscript involves human and/or animal work, have the subjects been treated in an ethical manner and the authors complied with the appropriate guidelines?
Yes

Reviewer 1 recommendation and comments

https://doi.org/10.1099/acmi.0.000482.v2.1

Anonymous.

Date report received: 03 January 2023
Recommendation: Accept

Comments: In the future, I would recommend at least putting in a sentence that a bioinformatic search for other nitrite reductases returned nothing rather then not mentioning it at all. Overall glad that all comments and suggestions were addressed.

Please rate the manuscript for methodological rigour
Very good

Please rate the quality of the presentation and structure of the manuscript
Very good

To what extent are the conclusions supported by the data?
Strongly support

Do you have any concerns of possible image manipulation, plagiarism or any other unethical practices?
No

Is there a potential financial or other conflict of interest between yourself and the author(s)?
No

If this manuscript involves human and/or animal work, have the subjects been treated in an ethical manner and the authors complied with the appropriate guidelines?
Yes

Author response to reviewers to Version 1

Response to Reviews

Access of Microbiology

Manuscript Number:ACMI-D-22-00119

Manuscript Title: Nitrite reductase activity in F_{420}-dependent sulfite reductase (Fsr) from *Methanocaldococcus jannaschii*

Authors: Christian Heryakusuma, Eric F. Johnson, Endang Purwantini, Biswarup Mukhopadhyay

We thank the reviewers for thoughtful and detailed reviews of our manuscript and for offering valuable suggestions. These have helped us to improve the manuscript. We present below our responses to each of the comments.

The cited line numbers for the revised manuscript refer to the file without tracks.

Responses to the comments from Reviewer 1

Comment 1. The manuscript by Heryakusuma et al. describes the ability of an F_{420}-dependent sulfite reductase (Fsr) from *M. jannaschii*to reduce nitrite to ammonium, which was supported by a nitrite reductase activity assay. Based on this finding, the authors predict that the *M. jannaschii*Fsr may be involved in nitrite detoxification given that the environment where the organism thrives has an infinite availability of this oxyanion. It is widely known that sulfite reductases, as well as nitrite reductases, catalyze both sulfite and nitrite, and this is primarily due to the physicochemical similarities of the two substrates. Given this fact, it does not necessarily follow that a sulfite reductase may be involved in nitrite detoxification or that a nitrite reductase is involved in sulfite detoxification. Therefore, the claim that *M. jannaschii*Fsr may be involved in nitrite detoxification must be sufficiently substantiated. One way to do this is to perform RT-qPCR to determine the level of transcription of the *fsr*gene in the presence vs absence of nitrite or prove by using bioinformatics that *M. jannaschii*does not possess a homolog of any known nitrite reductases. I recommend accepting this manuscript if one of the two suggested experiments is accomplished.

Response to Comment 1: We thank the reviewer for this comment, and we have performed one of the suggested experiments and it is a bioinformatics analysis of the *M.jannaschii*genome. We found that*M. jannaschii*does not possess a homolog of known nitrite reductases. Two proteins that show homologies to siroheme nitrite reductases are *Mj*Fsr or *Mj*FNiR and a dissimilatory sulfite reductase type protein, and the latter does not allow *M. jannaschii*to reduce sulfite or nitrite(reference 24in our manuscript and our unpublished data). Since our manuscript is focused on the properties of a purified enzyme, we have not included the results of our bioinformatic analysis.We plan to perform a detailed physiological analysis of the nitrite metabolism of *M. jannaschii*in the near future.

Comment 2. The degradation seen in SDS-PAGE is likely from proteolysis given that the authors did not use any protease inhibitors during enzyme purification. The addition of a protease inhibitor (e.g., Roche Complete, Mini Protease Inhibitor Cocktail) especially during cell lysis may prevent this degradation.

Response to Comment 2: Our previously published study on the sulfite reductase activity of the same protein (reference 15) has clearly shown that thedegradation seen in SDS-PAGE is not due to a protease, as the native protein was intact. The observation in Fig. 1A is clearly an artifact of sample preparation for SDS-PAGE. We have included this description in lines 115-116 of the revised manuscript.We thank the reviewer for the suggestion that we use a protease inhibitor cocktail, and we will follow this in our future studies with *Mj*FNiR.

Comment 3. Colorimetric methods that are used to quantify proteins are not accurate. This is because the binding behavior of the dyes used in these assays towards the protein of interest may be different from that of the standard protein. Since *M. jannaschii*Fsr contains Fe ions, an ICP-OES experiment is more appropriate for this quantification - Virginia Tech ICP facility can be found here: https://www.soiltest.vt.edu/Files/labinfo.html. For enzymes that do not harbor metal ions, amino acid analysis is more appropriate.

Response to Comment 3: We thank the reviewer for this suggestion, and we will follow this in our detailed study of the enzyme in the future.

Comment 4.I would like to encourage the authors to provide the full details of the procedure within the manuscript instead of redirecting the readers to the reference articles from which the procedures were followed. This practice will be more convenient for the readers.

Response to Comment 4: We thank the reviewer for the suggestion. We have added the details for the enzyme purification, activity and protein assays, SDS-PAGE, and UV-visible spectroscopyin the Methods section of the revised manuscript (lines 64-110). For the rest, we have referred in most parts to previously published details. The papers that we refer to (references 15 and 17) are openly accessed, and for this reason, we have avoided adding the rest of the information.

Comment 5. In Figure 1D, the spectrum for *M. jannaschii*FAD should be labeled as MjFsrI FAD.

Response to Comment 5: We thank the reviewer for identifying this error and we have changed the label in Figure 1D accordingly.

Comment 6. The sentence, "From an average of the data from the estimation of products in three150 independent 30 minutes long FNiR reactions with MjFsrI" appears to be out of place.

Response to Comment 6: We regret leaving this extra sentence here. We thank the reviewer for catching it and we have deleted this sentence.

<u>Responses to the comments from Reviewer 2</u>

Comment 1. The authors set up the background that *Methanocaldococcus jannaschii*is a well studied methanogen. They also make the case that in *Mj*native habitat, there is a low level but consistent supply of oxidized nitrogen compounds, Finally, they present the premise that an abundant protein Mj-FsrI, previously shown to be a sulfitre reductase, can also act as a nitrite reductase. In fact, this multi-substrate function has been demonstrated with many habitually similar organisms. Excellent experimental setup and rationale for the methods used.

Response to Comment 1: We thank the reviewer for this supportive comment.

Comment 2. The results were laid out well. There appears to be another band that runs between 100 kDa and 150 kDa; any speculation about what this band is? It could have benefited from Mass Spec on the isolated protein to validate that was the target, if MjFsrI specific antibodies are non-existant; however, the explanation was thorough enough to be convincing.

Response to Comment 2: We thank the reviewer for the supportive comments. The band between 100 and 150 kDa corresponds to the dimer of *Mj*FsrI (~140 kDa). Our previously published study on this same protein (reference 15) has clearly established this fact.

Comment 3. The paper is organized well. It is compact and takes the reader from point to point, culminating in MjFsrI has nitrite reduction capability and it could be an advantage in its native habitat. There is no filler in this paper.

Response to Comment 3: We thank the reviewer for this supportive comment.

Comment 4. The discussion is done well and delves deeply into the estimates of how the enzymatic reduction of nitrite by MjFsrI could be essential for growth in the native habitat. The estimates of the nitrite assimilation from environmental concentrations through cell uptake and enzymatic conversion were compelling. Given how well the bulk of the discussion went, the last sentence (Line 242-243) seemed like an after thought.

Response to Comment 4: We thank the reviewer for this supportive comment We have deleted the last sentence.

Comment 5. Lines 44-50: Sets up the premise that nitrate/nitrite/hydroxylamine is broadly toxic to methanogens, then says there are pathways certain methanogens utilize to detoxify, or even benefit from, these substrates. Could the premise be that nitrate/nitrite/hydoxylamine processing occurs in a subset of methanogenic organisms which benefit from nitrite?

Response to Comment 5: Indeed, the nitrate/nitrite/hydroxylamine processing only occurs in a subset of methanogenic organisms (lines 45-47 of the original manuscriptand lines 43-45 of the revised manuscript). However, the presence of such capabilities does not mean that the host organism leveraging it for utilizing nitrite or nitrate as a nitrogen source. The nitrite reduction activity likely originally was used in *Methanocaldococcus*to deal with the toxicity of nitrite, and then it was leveraged for the assimilatory purpose. Methyl coenzyme-M reductase (Mcr), an essential enzyme for methanogens, is inhibited by nitrite at a very low micromolar concentration (50 μM) within minutes (Reference 1 in the revised manuscript).

Comment 6. Line 51-57: Similar argument to Lines 44-50.

Response to Comment 6: The answer is similar to that we presented for Comment 5, except the focus here is sulfite. Methyl coenzyme-M reductase (Mcr) is inhibited by sulfite.

Comment 7. Line 120-121: If the determination that MjFsrI-flavin is FAD, why does the HPLC elution peak in Figure 1C not line up with the purified MjFsrI-flavin ?

Response to Comment 7: The peak location in the chromatogram shifted by ~0.2 s between the analysis for the FAD standard and that for *Mj*FsrI-FAD and co-injection. Such a shift in an HPLC analysis is commonly observed, especially for a room temperature analysis. For every 1°C increase in column temperature, retention time can shift by as much as ~2%. We performed the HPLC analysis at room temperature and we have added this information in the Methods section.

Comment 8. Line 238: italicize "in situ"

Response to Comment 8: As suggested, we have italicized the word *in situ*.

Responses to the comments from Reviewer 3

Comment 1. The impact statement is not very clear. There is mention of an infinite supply of nitrite but at low concentration. I am not sure what point is being made with the 'infinite supply' statement - is there a difference here compared to any other environmental chemical component? Also, there is no mention of the toxicity of nitrite that would require a detoxification system.

Response to Comment 1: We thank the reviewer for pointing out this issue. We have modified the sentence to improve the impact statement. We meant to state that the reservoir is large (vent fluid) and the nitrite concentration is low.

Methyl coenzyme-M reductase (Mcr), an essential enzyme for methanogens, is inhibited by nitrite at a very low micromolar concentration (50 μM) within minutes (lines 44-45 of the original manuscript and lines 42-43 of the revised manuscript) (Reference 1 in the revised manuscript).

Comment 2. Although it is recognised that it's not necessary to repeat previously published methods in detail, it is also not really acceptable to repeatedly state that methods used were as previously published. The reader of this paper currently gets very little information about the methods employed without consulting previous literature. Please provide outline methods at least.

Response to Comment 2: We thank the reviewer for the suggestion. We have added the details for the enzyme purification, activity and protein assays, SDS-PAGE, and UV-visible spectroscopyin the Methods section of the revised manuscript (lines 64-110). For the rest, we have referred in most part to previously published details. The papers that we refer to (references 15 and 17) are openly accessed, and for this reason, we have avoided adding the rest of the information.

Comment 3.L115 'The purified enzyme displayed a UV-visible spectrum typical of siroheme in low-spin ferric state with peaks at 280, 390, 117 and 590 nm (Fig. 1B) (14, 30)'. The enzyme also contains 6 [4Fe-4S] clusters, which each contribute to the absorbance in the near UV/visible region. Is the Fe-S cluster contribution to the absorbance spectrum known? Presumably the absorption due to FAD is difficult to see amongst the siroheme/Fe-S cluster absorbance?

Response to Comment 3: The absorbance spectra of various types of Fe-S clusters are known, and the [Fe_4-S_4] cluster, the type found in *Mj*FNiR, has the simplest spectrum typically with a small peak or shoulder in the range of 390-420 nm. It is tricky to detect the presence of FAD in a [Fe-S] cluster-containing protein due to the overlapping spectra of these cofactors. A careful inspection would show that a trough of absorbance around 350 nm was due to flavin and the following peak around 380-390 nm and the long slope-type spectrum that follows were due to a mixture of FAD, [Fe_4-S_4], and siroheme absorbances. Peak at 590 nm is due to siroheme.

Comment 4. Figure 1C Perhaps I misunderstand what this is, but why is the FAD standard elution time shifted from that of the co-injection sample (and sample extract)?

Response to Comment 4: The peak location in the chromatogram shifted by ~0.2 s between the analysis for the FAD standard and that for *Mj*FsrI-FAD and co-injection. Such a shift in an HPLC analysis is commonly observed, especially for a room temperature analysis. For every 1°C increase in column temperature, retention time can shift by as much as ~2%. We performed the HPLC analysis at room temperature and we have added this information in the Methods section.

Comment 5.L150 From an average of the data from the estimation of products in three independent 30 minutes long FNiR reactions with MjFsrI. This sentence does not make sense; it is lacking a verb.

Response to Comment 5: We regret leaving this extra sentence here. We thank the reviewer for catching it and we have deleted it.

Comment 6.L151 From the data presented, the 3:1 ratio for FADH2 consumed to ammonia produced is what would be expected for the six-electron reduction of nitrite. How was the ammonia production determined (I did not see this in the methods)?

Response to Comment 6: The methods are describedin lines 98-100 of the original manuscript and lines 99-101 of the revised manuscript.

Comment 7. Also, a rough calculation indicated that the enzyme should be capable of turning over all of the substrate added in 30 min, but it doesn't - only about half of the FADH2 is consumed (limiting substrate). Why is this? I note that the authors in the conclusion section suggest that ~100% of the FADH2 is utilised in the reaction, but 0.043/0.08 is ~53%.

Response to Comment 7: We have added more information in the revised manuscript (lines 151-152)to make the calculations easier.

Lines 153-156 of the original manuscript indicated that 0.0427 micromol $F_{420}$was produced, which meant that only 0.0427 micromol $F_{420}H_2$was consumed (although the assay provided 0.08 micromol $F_{420}H_2$). The thermodynamics of the reaction did not allow full consumption of the available substrates.

Thus, a calculation of the recovery of electrons needs to be based on 0.0427 micromol $F_{420}H_2$(0.0854 micromol electrons consumed) and 0.0133 micromol ammonia produced.

Comment 8. The discussion section argues that the nitrite reductase activity of MjFsrI could be physiologically relevant, both for detoxification of nitrite and as a source of nitrogen. There is a rough calculation that supports this based on levels of nitrite and enzyme. In the introduction it is mentioned that several Methanocaldococcus species utilize nitrate as nitrogen source. Does *M. jannaschii*have this capability?

Response to Comment 8:*M. jannaschii*has never been reported to utilize nitrate as a nitrogen source and our attempts did not show this capability in this organism.

Comment 9. The first step in this process is the reduction of nitrate to nitrite, so nitrite is generated endogenously in such organisms. If this occurs here, then there is all the more reason to need a nitrite reductase. On that note, the authors need to provide further information - what other potential nitrite reductases are present in *M. jannaschii*?

Response to Comment 9: Our bioinformatic analysis did not identify a potential nitrate reductase homolog in *M. jannaschii*. Thus, *M. jannaschii*is not expected to generate nitriteendogenously and *Mj*FNiR is likely used toprotectthe organism from the toxicity of nitrite that is present in the deep-sea hydrothermal vent environment at low concentrations. Methyl coenzyme-M reductase (Mcr), an essential enzyme for methanogens, is inhibited by nitrite at a very low micromolar concentration (50 μM) within minutes (Reference 1 in the revised manuscript). These aspects have been describedin lines 44-45 of the original manuscript and lines 42-43 of the revised manuscript.

VERSION 1

Editor recommendation and comments

https://doi.org/10.1099/acmi.0.000482.v1.6

John Munnoch; University of Strathclyde, SIPBS, UNITED KINGDOM, Glasgow

Date report received: 31 October 2022
Recommendation: Minor Amendment

Comments: This study would be a valuable contribution to the existing literature. This is a study that would be of interest to the field and community. The reviewers have highlighted minor concerns with the work presented. Please ensure that you address their comments. Dear Dr Biswarup Mukhopadhyay, Thank you for your submission, i would like to apologies on the delay, it took quite some time to acquire appropriate reviews for the manuscript. The reviews have generally positive views for the manuscript and I've selected minor amendments. Please address the reviewers comments, there is only one call for additional experimentation and one of the options requested is only bioinformatic. Best wishes, John.

Reviewer 3 recommendation and comments

https://doi.org/10.1099/acmi.0.000482.v1.5

Anonymous.

Date report received: 28 October 2022
Recommendation: Minor Amendment

Comments: The manuscript by Heryakusuma et al describes a kinetic analysis of nitrite reductase activity of the F420-dependent sulfite reductase from Methanocaldococcus jannaschii. The report is brief, involving confirmation that the enzyme behaves as previously reported, along with the determination of Km/Vmax values for nitrite and FADH2. The likely intermediate, hydroxylamine, is also studied. The data indicate that the enzyme is as effective in reducing nitrite as it is in reducing sulfite, leading the authors to suggest that this may be physiologically relevant. The study is scientifically sound. There are some points (detailed below) that should be addressed by the authors in preparing a revised version. The impact statement is not very clear. There is mention of an infinite supply of nitrite but at low concentration. I am not sure what point is being made with the 'infinite supply' statement - is there a difference here compared to any other environmental chemical component? Also, there is no mention of the toxicity of nitrite that would require a detoxification system. Methods Although it is recognised that it's not necessary to repeat previously published methods in detail, it is also not really acceptable to repeatedly state that methods used were as previously published. The reader of this paper currently gets very little information about the methods employed without consulting previous literature. Please provide outline methods at least. Results L115 'The purified enzyme displayed a UV-visible spectrum typical of siroheme in low-spin ferric state with peaks at 280, 390, 117 and 590 nm (Fig. 1B) (14, 30)'. The enzyme also contains 6 [4Fe-4S] clusters, which each contribute to the absorbance in the near UV/visible region. Is the Fe-S cluster contribution to the absorbance spectrum known? Presumably the absorption due to FAD is difficult to see amongst the siroheme/Fe-S cluster absorbance? Figure 1C Perhaps I misunderstand what this is, but why is the FAD standard elution time shifted from that of the co-injection sample (and sample extract)? L150 From an average of the data from the estimation of products in three independent 30 minutes long FNiR reactions with MjFsrI. This sentence does not make sense; it is lacking a verb. L151 From the data presented, the 3:1 ratio for FADH2 consumed to ammonia produced is what would be expected for the six electron reduction of nitrite. How was the ammonia production determined (I did not see this in the methods)? Also, a rough calculation indicated that the enzyme should be capable of turning over all of the substrate added in 30 min, but it doesn't - only about half of the FADH2 is consumed (limiting substrate). Why is this? I note that the authors in the conclusion section suggest that ~100% of the FADH2 is utilised in the reaction, but 0.043/0.08 is ~53%. The discussion section argues that the nitrite reductase activity of MjFsrI could be physiologically relevant, both for detoxification of nitrite and as a source of nitrogen. There is a rough calculation that supports this based on levels of nitrite and enzyme. In the introduction it is mentioned that several Methanocaldococcus species utilize nitrate as nitrogen source. Does M. jannaschii have this capability? The first step in this process is the reduction of nitrate to nitrite, so nitrite is generated endogenously in such organisms. If this occurs here, then there is all the more reason to need a nitrite reductase. On that note, the authors need to provide further information - what other potential nitrite reductases are present in M. jannaschii?

Please rate the manuscript for methodological rigour
Good

Please rate the quality of the presentation and structure of the manuscript
Satisfactory

To what extent are the conclusions supported by the data?
Strongly support

Do you have any concerns of possible image manipulation, plagiarism or any other unethical practices?
No

Is there a potential financial or other conflict of interest between yourself and the author(s)?
No

If this manuscript involves human and/or animal work, have the subjects been treated in an ethical manner and the authors complied with the appropriate guidelines?
Yes

Reviewer 2 recommendation and comments

https://doi.org/10.1099/acmi.0.000482.v1.3

Anonymous.

Date report received: 21 October 2022
Recommendation: Minor Amendment

Comments: 1. Methodological rigour, reproducibility and availability of underlying data The authors set up the background that Methanocaldococcus jannaschii is a well studied methanogen. They also make the case that in Mj native habitat, there is a low level but consistent supply of oxidized nitrogen compounds, Finally, they present the premise that an abundant protein Mj-FsrI, previously shown to be a sulfitre reductase, can also act as a nitrite reductase. In fact, this multi- substrate function has been demonstrated with many habitually similar organisms. Excellent experimental setup and rationale for the methods used 2. Presentation of results The results were laid out well. There appears to be another band that runs between 100 kDa and 150 kDa; any speculation about what this band is? It could have benefited from Mass Spec on the isolated protein to validate that was the target, if MjFsrI specific antibodies are non-existant; however, the explanation was thorough enough to be convincing . 3. How the style and organization of the paper communicates and represents key findings The paper is organized well. It is compact and takes the reader from point to point, culminating in MjFsrI has nitrite reduction capability and it could be an advantage in its native habitat. There is no filler in this paper. 4. Literature analysis or discussion The discussion is done well and delves deeply into the estimates of how the enzymatic reduction of nitrite by MjFsrI could be essential for growth in the native habitat. The estimates of the nitrite assimilation from environmental concentrations through cell uptake and enzymatic conversion were compelling. Given how well the bulk of the discussion went, the last sentence (Line 242-243) seemed like an after thought. 5. Any other relevant comments Lines 44-50: Sets up the premise that nitrate/nitrite/hydroxylamine is broadly toxic to methanogens, then says there are pathways certain methanogens utilize to detoxify, or even benefit from, these substrates. Could the premise be that nitrate/nitrite/hydoxylamine processing occurs in a subset of methanogenic organisms which benefit from nitrite? Line 51-57: Similar argument to Lines 44-50. Line 120-121: If the determination that MjFsrI-flavin is FAD, why does the HPLC elution peak in Figure 1C not line up with the purified MjFsrI-flavin ? Line 238: italicize "in situ"

Please rate the manuscript for methodological rigour
Very good

Please rate the quality of the presentation and structure of the manuscript
Very good

To what extent are the conclusions supported by the data?
Strongly support

Do you have any concerns of possible image manipulation, plagiarism or any other unethical practices?
No

Is there a potential financial or other conflict of interest between yourself and the author(s)?
No

If this manuscript involves human and/or animal work, have the subjects been treated in an ethical manner and the authors complied with the appropriate guidelines?
Yes

Reviewer 1 recommendation and comments

https://doi.org/10.1099/acmi.0.000482.v1.4

Julius Campeciño; Michigan State University, Biochemistry and Molecular Biology, SPAIN
https://orcid.org/0000-0003-0502-0177

Date report received: 14 September 2022
Recommendation: Minor Amendment

Comments: Major: The manuscript by Heryakusuma et al. describes the ability of an F420-dependent sulfite reductase (Fsr) from M. jannaschii to reduce nitrite to ammonium, which was supported by a nitrite reductase activity assay. Based on this finding, the authors predict that the M. jannaschii Fsr may be involved in nitrite detoxification given that the environment where the organism thrives has an infinite availability of this oxyanion. It is widely known that sulfite reductases, as well as nitrite reductases, catalyze both sulfite and nitrite, and this is primarily due to the physicochemical similarities of the two substrates. Given this fact, it does not necessarily follow that a sulfite reductase may be involved in nitrite detoxification or that a nitrite reductase is involved in sulfite detoxification. Therefore, the claim that M. jannaschii Fsr may be involved in nitrite detoxification must be sufficiently substantiated. One way to do this is to perform RT-qPCR to determine the level of transcription of the fsr gene in the presence vs absence of nitrite or prove by using bioinformatics that M. jannaschii does not possess a homolog of any known nitrite reductases. I recommend accepting this manuscript if one of the two suggested experiments is accomplished. Minor: the authors may apply suggestions 1-3 in their future work. 1) The degradation seen in SDS-PAGE is likely from proteolysis given that the authors did not use any protease inhibitors during enzyme purification. The addition of a protease inhibitor (e.g., Roche Complete, Mini Protease Inhibitor Cocktail) especially during cell lysis may prevent this degradation. 2) Colorimetric methods that are used to quantify proteins are not accurate. This is because the binding behavior of the dyes used in these assays towards the protein of interest may be different from that of the standard protein. Since M. jannaschii Fsr contains Fe ions, an ICP-OES experiment is more appropriate for this quantification - Virginia Tech ICP facility can be found here: https://www.soiltest.vt.edu/Files/labinfo.html. For enzymes that do not harbor metal ions, amino acid analysis is more appropriate. 3) I would like to encourage the authors to provide the full details of the procedure within the manuscript instead of redirecting the readers to the reference articles from which the procedures were followed. This practice will be more convenient for the readers. 4) In Figure 1D, the spectrum for M. jannaschii FAD should be labeled as MjFsrI FAD. 5) The sentence, "From an average of the data from the estimation of products in three150 independent 30 minutes long FNiR reactions with MjFsrI" appears to be out of place.

Please rate the manuscript for methodological rigour
Satisfactory

Please rate the quality of the presentation and structure of the manuscript
Good

To what extent are the conclusions supported by the data?
Partially support

Do you have any concerns of possible image manipulation, plagiarism or any other unethical practices?
No

Is there a potential financial or other conflict of interest between yourself and the author(s)?
No

If this manuscript involves human and/or animal work, have the subjects been treated in an ethical manner and the authors complied with the appropriate guidelines?
Yes

SciScore report

https://doi.org/10.1099/acmi.0.000482.v1.1

iThenticate report

https://doi.org/10.1099/acmi.0.000482.v1.2