Characterisation of an unusual cysteine pair in the Rieske carnitine monooxygenase CntA catalytic site

Mussa Quareshy1, Muralidharan Shanmugam2, Alexander D. Cameron1, Timothy D. H. Bugg3 and Yin Chen1

1 School of Life Sciences, University of Warwick, Coventry, UK
2 Department of Chemistry and Photon Science Institute, The University of Manchester, UK
3 Department of Chemistry, University of Warwick, Coventry, UK

Keywords
carnitine; cw-EPR; cysteine residues; electron transfer; mononuclear Fe; Rieske monooxygenase

Correspondence
M. Quareshy, School of Life Sciences, University of Warwick, Gibbet Hill Road, Coventry CV4 7AL, UK
Tel: +024 7657 4251
E-mail: mussaquareshy@gmail.com
and
M. Shanmugam, Department of Chemistry and Photon Science Institute, The University of Manchester, Manchester M13 9PL, UK
Tel: +0161 275 1000
E-mail: muralidharan.shanmugam@manchester.ac.uk

(Received 21 July 2022, revised 1 December 2022, accepted 6 January 2023)
doi:10.1111/febs.16722

Introduction
Rieske monooxygenases are comprised of five subgroups [1,2]. Members of the subgroups I to IV, known to accept aromatic substrates [3–7], have been characterised extensively and their catalytic mechanisms elucidated [3,8–11]. Members of Group V have only been recently reported and associated with the oxidation/breakdown of quaternary ammonium substrates such as glycine betaine [2], carnitine [1] and benzalkoniums [12]. In general, the underlying mechanism of function for Rieske monooxygenases is driven by an electron transfer from a reductase/ferredoxin partner protein facilitated through a series of [2Fe-2S] clusters culminating at a mononuclear Fe centre [13] typically coordinated by a 2-His-1-carboxylate (His-His-Asp) triad [14]. Substrates are oriented in the active-site pocket, adjacent to the mononuclear Fe centre as well as a molecule of O2 [15] and the mechanism proceeds via an oxidative addition process [15]. In all five groups of the Rieske oxygenase family, the coordinating residues of the [2Fe-2S] and mononuclear Fe centres are highly conserved and are an identifying feature for this family [1,16]. Unique to some Group V

Abbreviations
CntA, carnitine monooxygenase; Cys, cysteine amino acid residue; DTNB, 5,5-dithiobis-(2-nitrobenzoic acid); cw-EPR, continuous-wave Electron Paramagnetic Resonance; TCEP, Tri(2-carboxyethyl)phosphine.
Rieske oxygenases is the presence of a cysteine pair Cys-X-X-Cys adjacent to the catalytic mononuclear Fe site [17,18] for which a role is yet to be reported. It is well-understood that disulphide bridges between cysteine pairs are an integral structural feature of protein secondary and tertiary structures [19] crucial to stabilise and maintain folded protein architecture [20]. In the example of insulin, disulphide bridges can be established as interchain and intrachain linkages [21] and in some cases, even vicinal disulphide bonds are known [22,23]. In the structure of cytochrome bc1, a disulphide bridge is observed in the vicinity of the [2Fe-2S] Rieske cluster and was shown to be critical to stability and function of the cluster when the disulphide bridge is disrupted [24]. Analogous to the CntA and Stc2 enzymes, in terms of a Cys-X-X-Cys motif, is the human thioredoxin TRx1 [25,26], which can oxidise sulphhydrils or reduce disulphide bonds on target proteins via its active site Cys32 and Cys35 residues. At present, there are reported structures for just two members of the group V clade; carnitine monoxygenase from Acinetobacter baumannii (CntA) [18] which catalyses carnitine to trimethylamine (TMA) and stachydrine demethylase (Stc2) [17] from Sinorhizobium meliloti (Fig. 1B). For the CntA structure, we reported the cysteine pair (Cys206-Cys209) as free thiols (Fig. 1C) whilst the corresponding (Cys202-Cys205) pair in Stc2 were assigned as a disulphide bridge (Fig. 1D). It should be noted that the Stc2 structure was obtained under anaerobic conditions but conversely aerobic conditions for CntA. Another distinguishing feature is that we observed the intact substrate in CntA; in Stc2, however, the substrate stachydrine has undergone two subsequent demethylation steps, which is observed as the end product.

In optimising our CntA protein purification methodology, we found the addition of TCEP [27] in purification and crystallography buffers was crucial. The addition of this phosphine-reducing agent was key for long-term stability [28] of purified fresh and frozen CntA. Our breakthrough in obtaining protein crystals was attributed to sodium thiocyanate (NaSCN) as an additive in the crystallography buffer, co-bounding the mononuclear Fe centre. We noted that the presence of NaSCN at high concentrations impeded CntA activity. We also reported small-molecule inhibitors for CntA with a crystal structure of the MMV12 inhibitor co-bound to CntA under similar conditions where we still observe the cysteine pair as free thiols. These cysteines in CntA do not seem to coordinate the mononuclear Fe centre being ~ 5 Å away, nor do they interact with the carnitine or γ-butyrobetaine substrates in any canonical manner, although the ammonium group of the substrate is oriented towards C206 and is ~ 4.4 Å away from the substrate. With almost no reported insight into this cysteine pair and no clear role attributed yet, in this work, we sought to focus our attention on the cysteine pair. We investigated with biochemical, spectroscopic and
structural approaches to better understand these unusual residues proximal to the active site.

Results and discussion

Establishing the redox state of the Cys206 and Cys209 in CntA

In order to establish the roles of the Cys206-Cys209 cysteine pair on the oxidation state of the purified CntA protein, we expressed and purified a series of alanine and serine (both single and double) mutants for the C206 and C209 residues (Fig. 2A). Using UV–Vis spectroscopy, we studied the as-isolated proteins in the presence of dithionite or H₂O₂. (C) Quantification of free thiols with Ellman’s reagent for WT and cysteine pair alanine mutants. (Error bars represent SD, n = 3). (D) Enzymatic activity comparison of mutants to WT showing a significant reduction in activity. (Error bars represent SD, n = 3). (E) Circular dichroism measurements of cysteine mutants relative to the WT (blue trace) in each subpanel.

Fig. 2. CntA mutagenesis and characterisation of mutants. (A) An SDS/PAGE electrophoresis gel of purified WT CntA and its mutants (~44 kDa). (B) As isolated UV–Vis spectra of WT and CntA mutants compared with UV–Vis spectra in the presence of dithionite or H₂O₂. (C) Quantification of free thiols with Ellman’s reagent for WT and cysteine pair alanine mutants. (Error bars represent SD, n = 3). (D) Enzymatic activity comparison of mutants to WT showing a significant reduction in activity. (Error bars represent SD, n = 3). (E) Circular dichroism measurements of cysteine mutants relative to the WT (blue trace) in each subpanel.

Cys206 and Cys209 are crucial for CntA enzyme activity but the C209A mutation does not affect the overall protein structure

When assayed for activity, we observed a significant reduction in activity for C206A, C209A, C206S and C209S mutants (Fig. 2D). We studied the secondary structures of the WT CntA and the mutants using Ellman’s reagent [(5,5-dithiobis-(2-nitrobenzoic acid), DTNB)], expecting five solvent accessible cysteine residues in the WT: Cys12, Cys125, Cys160, Cys206 and Cys209. We only observed four free thiols in the WT, however (Fig. 2C). Given that the C206 and C209 residues are relatively close to one another (3.8 Å), both are proximal to the Fe centre, it is plausible that only one of these cysteines can bind the 2-nitro-5-thiobenzoate (TNB) at once. However, we observed between 2 and 3 free thiols in the C206A/C209A double mutants and between 3 and 4 in the single mutants. Overall, this result corroborates with the observation from CntA structure and suggests that the cysteines C206 and C209 do not appear to form a disulphide bridge in the WT protein.
structure of these variants with Circular Dichroism measurements and observed the most pronounced difference between the WT and C206A mutant, with the overall secondary structure also different for the other CntA mutational variants (Fig. 2E). Secondary structure estimation [29,30] (Table S1) of the CD data shows variations of secondary structure in all C206 and C209 mutants relative to WT. We have also included quantification for a previously reported E205A, which showed an identical CD spectra to the WT with small secondary structure differences [1,18]. We looked at the stability of these mutants with a thermal shift assay (TSA) with C206A producing a 1.42 °C decrease in melting temperature, indicating a slight destabilisation of the protein relative to WT CntA (Table S2), agreeing with the CD data and indicating some instability. Overall, we saw differences in the CD and melting points between corresponding Alanine and Serine mutations of C206 and C209 single mutants with no obvious pattern or uniform trends in instability. We conclude that within these mutations there is some, albeit small influence of the C206 and C209 residues on the secondary structure of CntA and that overall, the proteins were folded correctly.

To better understand the structural basis of these cysteines on CntA enzyme activity, we set out to solve their structures but only managed to successfully obtain the structure of the C209A mutant with carnitine co-bound which was refined at a resolution of 1.8 Å (PDB 6Y9C, Table S3). The CntA 209A + carnitine structure is a α3 homotrimer (Fig. 3A), with the mononuclear Fe centre coordinated by a 2-His-1-Asp triad, a water molecule and a [SCN]− co-factor from the protein crystallisation buffer additive sodium thiocyanate (Fig. 3B) analogous to the WT structure. The carnitine substrate was observed as expected in the electron density (Fig. 3C) and in the [2Fe-2S] Rieske centre (Fig. 3D). The carnitine substrate sits above the mononuclear Fe centre in a similar position relative to the WT (Fig. 3E). The orientation of the [SCN]− co-factor is tilted differently in the CntA C209A structure vs. the WT where the sulfur of the [SCN]− occupies the region where we would expect the thiol group of C209A to be. Overall, the structures of C209A and WT CntA are very similar with a rmsd of 0.461 Å (Fig. 3E), suggesting the mutation of C209A had minimal impact on the overall structure of CntA which contrasts the observations CD and TSA assays. We acknowledge that in the C209A structure represents a snapshot of the protein structure and that the presence of l-carnitine as well the [SCN]− [31] may have promoted increased protein stability.

Cys206 and Cys209 mutants do not impede electron transfer into the catalytic Fe Centre

To understand the inactivity and/or significantly reduced catalytic activity of the single and double mutants, C206A, C209A and C206AC209A, cw-EPR spectra were measured on these mutants and qualitatively compared with that of the CntA-WT enzyme. We previously characterised the complete electron transfer pathway in the CntA WT protein in the presence of a reductase, CntB + NADH and carnitine as the substrate [18]. We demonstrated the E205 bridging residue plays an integral role in the electron transfer pathway (Fig. 4A), with the E205A enzyme variant showing significantly reduced catalytic activity explained by a retardation of the electron transfer process from the reduced, [2Fe-2S]1+ clusters of CntB (ferredoxin/CntA(Rieske) centre into the mononuclear Fe centre. Should the cysteine pair also be part of the electron transfer pathway, we would expect to observe the same retardation. First, near-identical EPR spectra are observed for the resting state when CntA-WT and single mutant, CntA-C209A were purified without TCEP buffer, implying that TCEP has no direct role in keeping the catalytic Fe centre in its ferrous oxidation state (Fig. S1). We then measured the cw-EPR spectra of the purified single (C206A and C209A) and double (C206AC209A) mutants of CntA protein at 20 K and observed an EPR silent state similar to that of the WT protein [18] (Fig. 4B). It is consistent with the oxidised [2Fe-2S]2+ Rieske centre and a catalytic, mononuclear iron centre in its ferrous state, demonstrating these mutations have no effect on the redox properties of the [2Fe-2S]2+ and mononuclear iron centres in CntA enzyme. When the mutants were measured in the presence of CntB + NADH + Carnitine, the spectra show identical EPR traces to that of the WT (Fig. 4C). This implies that none of the C206/C209 single and double mutants impeded the electron transfer pathway, in contrast to what has been observed for the E205A mutant (Fig. 4C; blue trace). Together, these data suggest that these cysteines are unlikely to be part of the normal electron transfer pathway. The additional EPR signals observed around 3250–3300 G and 3400–3600 G in the blue trace are due to the one-electron reduced, ferredoxin [2Fe-2S]1+ due to inefficient electron transfer as we reported previously [18]. The broadening observed at 3550 G is due to the overlapping of reduced, ferredoxin/ Rieske and activated mononuclear Fe EPR signals at this field position.
Like P450 oxygenases, CntA can also perform a peroxide shunt oxidation of the substrate using \( \text{H}_2\text{O}_2 \) in the absence of NADH and the reductase CntB [18]. To determine whether C206 and C209 play a role in the \( \text{H}_2\text{O}_2 \)-mediated peroxide shunt mechanism, EPR spectra were measured on the \( Ab\text{CntA-WT} \), single (\( Ab\text{CntA-C206A} \) and \( Ab\text{CntA-C209A} \)) and double (\( Ab\text{CntA-C206AC209A} \)) mutants (Figs 5 and 6). In the absence of the substrate (Fig. 5), these spectra show no indication of high-spin, ferric EPR signals at low magnetic field, between 0 and 2000 G in the presence of an oxidising agent (4 mM \( \text{H}_2\text{O}_2 \)) at 20 K (red dotted traces) and 7 K (blue dotted traces), respectively. For a comparison purpose, all spectra were overlaid on to the EPR spectrum of the \( Ab\text{CntA-WT} + Ab\text{CntB} + \text{NADH} + \text{carnitine} \) (black traces in all four panels; Fig. 5). No differences between the

---

**Fig. 3.** Crystal structure of the CntA C209A mutant. (A) Overview of CntA 209A Trimeric structure with head-to-tail orientation of [2Fe-2S] Rieske centre and Mononuclear Fe Centre. (B) A 2\( \text{mF}_\text{o} - \text{DF}_\text{c} \) map (Blue) at 1.0\( r \) of the mononuclear Fe centre with coordinated [SCN]\(^-\) co-factor as well as C206 and C209A residues. (C) A 2\( \text{mF}_\text{o} - \text{DF}_\text{c} \) map (Blue) at 1.5\( r \) of carnitine substrate. (D) A 2\( \text{mF}_\text{o} - \text{DF}_\text{c} \) map (Blue) at 2.0\( r \) of the [2Fe-2S] Rieske centre. (E) A cartoon representation of the CntA WT active site (Light Grey) and the C209A structure (Dark Grey) separately and overlaid, respectively, to show the substrate and mononuclear Fe coordination are unchanged whilst the orientation of the [SCN]\(^-\) and C206 are tilted differently in the C209A mutant but still [SCN]\(^-\) is coordinated to the Fe centre. Figure generated in UCSF CHIMERA 1.16 (Macintosh).
WT, single mutants or the double mutant were observed. In the presence of carnitine, however, the spectra (Fig. 6) show intense, high-spin, ferric EPR signals at ~1500 G (blue traces) when CntA-WT and AbCntA-C209A were treated with H₂O₂. This implies that AbCntA-C209A mutation does not impair the peroxide-shunt mechanism.

Cys206 and Cys209 may play a role in reactivation of the mononuclear Fe Centre

The enzymology, structure and EPR results discussed thus far do not support a role of these cysteines in electron transfer to the mononuclear Fe centre in CntA catalysis, nor for their involvement in maintaining the structure of CntA. Another hypothesis is that the reduced cysteines in CntA may be needed to reactivate the mononuclear Fe(II) centre when it is occasionally oxidised to the inactive Fe(III) centre. A similar phenomenon has been observed for prolyl hydroxylase, a 2-oxoglutarate-dependent dioxygenase that also has a mononuclear iron (II) centre, which requires the reductant, ascorbate in order to keep the iron centre in the ferrous state during catalysis [33–35]. In the absence of ascorbate, the enzyme can only carry out limited rounds of turnovers due to the oxidation of the enzyme-bound iron (II) to iron (III) caused by the so-called uncoupled reaction of decarboxylation of 2-oxoglutarate [33–35]. To investigate the involvement of C206/C209 and the reduced thiol groups in CntA
catalysis, we performed annealing of the [CntA-WT + CntB + NADH + carnitine] and [CntA-C209A + CntB + NADH + carnitine] samples prepared in the presence or absence of TCEP in the buffer. The EPR spectra of the [CntA-WT + CntB + NADH + carnitine] and [CntA-C209A + CntB + NADH + carnitine] samples purified in the presence of TCEP and flash-frozen immediately after the addition of all four components show no evidence of high-spin, ferric EPR signals at low magnetic field (Fig. 7). When the [CntA-WT + CntB + NADH + carnitine] sample was annealed at room temperature for the specified duration mentioned in the figure caption, development of the high-spin, ferric EPR signals (at ~1500 G) was observed, which reached a maximum at around 15 min of annealing at room temperature (Fig. 7B,C). A similar trend was observed for the [CntA-C209A + CntB + NADH + carnitine] single mutant sample. Further annealing of the samples led to the slow decay/disappearance of the signal as demonstrated by the ratio of the intensity of the high-spin, ferric EPR signal at ~1500 G before and after
observed the maximum in the high-spin, ferric EPR signal. For example, on the same samples purified in the absence of TCEP left and bottom-left). Identical experiments performed annealing as a function of annealing time (Fig. 8; top-left and bottom-left). Identical experiments performed on the same samples purified in the absence of TCEP show a slightly different annealing trend, for example the maximum in the high-spin, ferric EPR signal did not completely disappear (Fig. 8; top-right and bottom-right). These two comparisons clearly demonstrate that the presence of TCEP is critical for both mutant and wild-type proteins. The TCEP may either directly assist the reduction in the inactive Fe(III) centre to the catalytically active, Fe(II) or could keep the cysteine in the reduced thiol state, which in turn protects the catalytic Fe(II) centre in the ferrous state when ‘off-pathway oxidation’ occurs.

Conclusions

In elucidating a crystal structure of CntA [18], we were afforded an insight into how quaternary amine substrates are perceived in the active site. A unique feature observed from the CntA structures is the presence of two reduced Cys in the close vicinity of the active centre. Appearance of such Cys near the catalytic mononuclear iron centre is rare in Rieske oxygenases. While in the homologous Stc2, the Cysteines form a disulphide [17], the data we collected in this study do not appear to support a structural role of the Cysteines in CntA. First, these two Cys residues were present in the reduced state in the CntA crystal structure (Fig. 1) consistent with an analysis of reduced thiols in CntA using Ellman’s Reagent (Fig. 2). Second, the crystal structure of the CntA C209A mutant showed no obvious impact to the substrate binding nor to the overall active site (Fig. 3D). Admittedly, the mutation of C206/C209 does appear to interfere in the catalytic active site oxidation’ occurs.
which displayed retarded electron transfer from the Rieske centre to the catalytic mononuclear Fe centre (Fig. 4). Furthermore, using H₂O₂ as the electron donor to bypass the need for NADH and the reductase CntB, we showed that these Cys residues do not appear to be required for the substrate-dependent activation of the mononuclear Fe centre and the formation of the high-spin S = 5/2 species (Figs 5 and 6), a mechanism that is consistent with the peroxide shunt. We therefore postulate that these Cys residues are likely to be involved in maintaining Fe(II) in its reduced state in catalysis. Data from EPR experiments shown in Figs 7–9 appear to suggest that these Cys residues are indeed involved in nonstoichiometric reactivation of the catalytic Fe (II) centre. Occasional oxidation of Fe(II) to Fe(III) is a problem encountered in other nonheme iron-dependent oxygenases, which in prolyl hydroxylase is solved by nonstoichiometric reduction by ascorbate [35], and in Pseudomonas putida catechol 2,3-dioxygenase by the use of a dedicated [2Fe-2S] ferredoxin [36]. To the best of our knowledge, the use of two active site Cys residues to achieve this role is a novel mechanism to solve this problem. EPR experiments, however, only provided a
static view of unpaired electrons at a given time. As such, the data presented here using EPR cannot provide a resolution of Fe on the single turnover timescale and the impact of Cys mutations on the EPR-active Rieske centre and the catalytic Fe centre. Clearly future experiments on single turnover enzyme kinetics will provide further insight into the intriguing role of these unique Cysteine residues in CntA catalysis. It also remains to be seen whether such a mechanism operates in other group V Rieske oxygenases, where a homologous Cys pair appears common.

Fig. 8. Monitoring the change in intensity of the high-spin/low-spin EPR signals of the AbCntA-WT and AbCntA-C209A mutant after annealing the samples at room temperature. Normalised (the plotted EPR signals in each spectrum is normalised to the maximum of the signals from the EPR spectrum before annealing of the sample; zero time) Intensity of the EPR signals at ~ 3300 G (see Fig. 7B) ~ 1500 G (see Fig. 7C) has been monitored as a function of annealing for AbCntA-WT + AbCntB + NADH (5 mM) + carnitine and AbCntA-C209A + AbCntB + NADH + carnitine in the absence (top-right and bottom-right) and presence (top-left and bottom-left) of TCEP in the buffer. Please refer to Fig. 7B,C for the EPR signals at ~ 1500 G and 3300 G, respectively. The black arrows on top-right and bottom-right panels show that the oxidised, ferric signal is not completely decayed/reduced to EPR silent or catalytically active ferrous centre. Conditions as described in Fig. 4B.

Materials and methods
Protein expression, purification, UV–visible enzyme assays and biochemical enzyme assays work were performed as reported previously [18].

UV–visible spectra characterisation
CntA WT and mutant protein material were freshly purified as reported previously [18] and prepared as 4 mg·mL⁻¹ solutions in final volume of 160 μL, referred to ‘as isolated’. For
each protein (WT and mutant), additional samples were prepared: (a) ‘+Dithionite’ with excess Dithionite reducing agent added at 2 mM final concentration and (b) ‘+H₂O₂’ hydrogen peroxide oxidising agent added at 2 mM final concentration. Samples were loaded onto a 96-Well UV Transparent Plate (Thermo Fisher Scientific, Loughborough, UK, catalogue no. 8404) and read on a BMG FLOUstar Omega 96-well plate reader scanning between wavelengths 300 and 800 nm recording absorbance and sampling in 1 nm intervals.

Circular dichroism

All proteins were buffer exchanged into a pH 7.0, 0.2 M sodium phosphate buffer with 0.01 M NaCl on a PD-10 column and concentrated to 0.1 mg/mL final concentration. Using a 0.1 mm path length quartz cuvette, the samples were analysed on a JASCO J-1500 at 20°C and data were collected between 260 and 180 nm with 8 scans per sample. Secondary structure estimation was performed using the BESTSEL server [29,30].

Thermal shift assay

The assay was adapted from reported protocols [37,38]. The CntA WT and mutant proteins were assayed at 2.5 μM with 1× SYPRO orange on a Bio-Rad CFX Connect™ Real-Time PCR instrument in a sample volume of 50 μL in triplicates for each sample. An initial 3-min equilibration at 20 °C followed by 0.5 °C increments to 90 °C every 30 s recording the FRET signal at each stage. The data were auto-processed in the BIO-RAD software (Watford, UK) from which melting temperature was obtained and reported.
Understanding unusual cysteine pair in CntA active site

EPR spectroscopy

All EPR samples were prepared in a 10 mM HPEPS buffer with 250 mM NaCl, 0.5 mM TCEP and 10% glycerol (v/v) (pH 7.6) in an aerobic condition. Samples containing ~200 μM AbCntA, 75 mM nicotinamide adenine dinucleotide (NADH) were transferred into 4-mm Suprasil quartz EPR tubes (Wilmad LabGlass, Vineland, NJ, USA) and frozen in liquid N2. Annealing of the samples was performed at room temperature for the specified time-duration in the figure caption. All EPR samples were measured on a Bruker EMX-Plus EPR spectrometer (Coventry, UK) equipped with a Bruker ER 4112SHQ X-band resonator as reported previously [39]. Sample cooling was achieved using a Bruker Stinger [40] cryogen-free system mated to an Oxford Instruments ESR900 cryostat, and temperature was controlled using an Oxford Instruments MercuryITC (Abingdon, UK). The optimum conditions used for recording the spectra are given below: microwave power 30 dB (0.2 mW), modulation amplitude 5 G, time constant 82 ms, conversion time 12 ms, sweep time 120 s, receiver gain 82 ms, conversion time 12 ms, sweep time 120 s, receiver gain 250 mM NaCl, 0.5 mM TCEP and 10% glycerol (v/v). The optimum conditions used for recording the spectra are given below; microwave power 30 dB (0.2 mW), modulation amplitude 5 G, time constant 82 ms, conversion time 12 ms, sweep time 120 s, receiver gain 30 dB and an average microwave frequency of 9.383 GHz, temperature 20 K. cw-EPR spectra for the AbCntA-WT, single (C206A and C209A) and double (C206AC209A) mutants of CntA were recorded in the presence and absence of CntB + NADH + carnitine, as reported previously [18,41].

Protein crystallography

Protein crystallography was performed as previously reported. In brief, CntA C209A protein crystals were prepared at 7.5 mg·mL⁻¹ in conditions of 18% (w/v) PEG 3350, 10 mM NaCl and 0.5 mM TCEP with substrates present at 1 mM. Red hexagonal crystals developed at 22 °C between 24 and 48 h. Crystals were cryoprotected in an equivalent solution to the mother liquor supplemented with 5% glycerol and flash cooled in liquid nitrogen.

The crystals were mounted robotically on the i04 beamline (CntA C209A mutant) at the Diamond Light Source (Harwell Science and Innovation Campus, Didcot, UK). Due to the presence of ice-rings and anisotropy, the data were not ideal, and we truncated the data set with a resolution cut-off at 1.8 Å. The selected images were processed with the Dials [42] GUI in ccf4i2 [43,44] performing Indexing, refinement and integration steps, followed by scaling with Aimless [45]. We used the CntA + Carnitine structure (PDB code: 6Y8S) as a model for molecular replacement in Phaser [46]. Autobuilding in phenix.autobuild was followed by iterative rounds of manual building in COOT [47] interspersed with refinement in PHENIX [48].

Free thiol quantification using the Ellman’s reagent (DTNB)

A standard calibration range of a twofold dilution series between 3.2 and 0.1 mM of l-cysteine hydrochloride (Sigma Aldrich, Gillingham, UK) were made. All protein samples were buffer exchanged into a 100 mM sodium phosphate buffer pH 8.0 (RB) and adjusted to a concentration between 5 and 6 mg·mL⁻¹. A stock concentration of Ellman’s reagent (DTNB) was made at 4 mg·mL⁻¹. Two hundred and fifty microlitres of standard and unknowns, respectively, was added to 50 μL of the DTNB stock and 2.5 mL of RB and left for 15 min. Two hundred microlitres aliquots of this mix was assayed in triplicate in a 96-well plate in a BMG FLOUstar Omega 96-well plate reader measuring the absorbance at 412 nm. The absorbance values for the unknown samples were extrapolated from the standard curve, and the resulting molar concentration was divided by the molar concentration of protein used to yield the number of free thiol groups.

Chemical structures and protein structure depictions

Marvin was used for drawing, displaying and characterising chemical structures, substructures and reactions, MARVIN v19.10.0, 2019, CHEMAXON (http://www.chemaxon.com). Molecular graphics and analyses were performed with UCSF CHIMERA [49].

Acknowledgements

This work was supported by a Leverhulme Trust research grant (RPG-2016-307). MS acknowledges The University of Manchester and the National EPR Facility for financial supports. We thank the National EPSRC EPR service and Facility (NS/A000055/1, EP/W014521/1) for the acquisition of various EPR measurements, which are included in this manuscript. The authors acknowledge the support of the University of Warwick, School of Life Sciences Media preparation team of Cerith Harries and Caroline Stewart for preparing various reagents and culture media.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

MQ, MS and YC designed research. MQ carried out the protein expressions, protein purifications, enzyme assays, data analysis, protein crystal preparation and EPR sample preparation. MS carried out all EPR experiment measurements and analysis. MQ and ADC collected crystallographic data, processed, solved and refined the structure. MQ, MS and YC wrote the manuscript with contributions from ADC and TDHB.
YC, ADC and TDHB sourced funding and formulated the original research idea.

**Peer review**

The peer review history for this article is available at [https://publons.com/publon/10.1111/febs.16722](https://publons.com/publon/10.1111/febs.16722).

**Data availability statement**

The CntA C209A + Carnitine structure is deposited in the PDB repository with accession code PDB Code: 6Y9C.

**References**


Understanding unusual cysteine pair in CntA active site

M. Quareshy et al.


Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. cw-EPR spectra of CntA WT and C209A mutant with and without TCEP.

Fig. S2. Key figures from main manuscript to illustrate off-pathway oxidation.

Table S1. Estimated CD secondary structure content.

Table S2. Thermal Shift assay summary of temperature shifts relative to CntA WT.

Table S3. Crystallographic statistics.