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Structure and Substrate Specificity of the Pyrococcal Coenzyme A Disulfide Reductases/Polysulfide Reductases (CoADR/Psr): Implications for S^0-Based Respiration and a Sulfur-Dependent Antioxidant System in *Pyrococcus*

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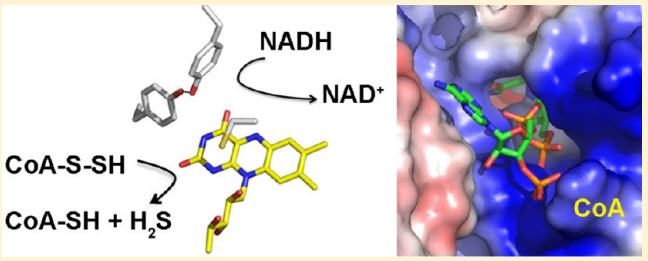
Supporting Information

ABSTRACT: FAD and NAD(P)H-dependent coenzyme A disulfide reductases/polysulfide reductases (CoADR/Psr) have been proposed to be important for the reduction of sulfur and disulfides in the sulfur-reducing anaerobic hyperthermophiles *Pyrococcus horikoshii* and *Pyrococcus furiosus*; however, the form(s) of sulfur that the enzyme actually reduces are not clear. Here we determined the structure for the FAD- and coenzyme A-containing holoenzyme from *P. horikoshii* to 2.7 Å resolution and characterized its substrate specificity. The enzyme is relatively promiscuous and reduces a range of disulfide, persulfide, and polysulfide compounds. These results indicate that the likely *in vivo* substrates are NAD(P)H and di-, poly-, and persulfide derivatives of coenzyme A, although polysulfide itself is also efficiently reduced. The role of the enzyme in the reduction of elemental sulfur (S_8) *in situ* is not, however, ruled out by these results, and the possible roles of this substrate are discussed. During aerobic persulfide reduction, rapid recycling of the persulfide substrate was observed, which is proposed to occur via sulfide oxidation by O_2 and/or H_2O_2. As expected, this reaction disappears under anaerobic conditions and may explain observations by others that CoADR is not essential for S^0 respiration in *Pyrococcus* or *Thermococcus* but appears to participate in oxidative defense in the presence of S^0. When compared to the homologous Npsr enzyme from *Shewanella loihica* PV-4 and homologous enzymes known to reduce CoA disulfide, the *ph*CoADR structure shows a relatively restricted substrate channel leading into the sulfur-reducing side of the FAD isoalloxazine ring, suggesting how this enzyme class may select for specific disulfide substrates.



The redox versatility of sulfur makes it unique among the elements involved in Earth's geochemical cycles. No other element is stable in such a wide range of redox states under normal conditions; sulfur can easily interchange between oxidation states ranging from −2 to +6 in the presence of proper catalysts.[1] Sulfur-based respiration ($S^0 + 2e^- \rightarrow S^{2-}$) is a common metabolic strategy among prokaryotes, and it is almost ubiquitous in hyperthermophilic archaea. Species in the genus *Pyrococcus* are among the best-studied hyperthermophiles, and the basis of their ability to use S^0-based respiration as a strategy for energy conservation has been attributed to three enzymes — two sulfide dehydrogenases (SudH I, an FAD, NADPH, and Fe−S-dependent enzyme,[2] and SudH II, an FAD, NADPH, Fe−S, and Ni-dependent enzyme[3]) and an FAD and NAD(P)H-dependent enzyme originally isolated as a coenzyme A disulfide reductase (CoADR).[4,5] *Pyrococcus* does not contain the molybdopterin-dependent PsrABC complex commonly found in sulfur-reducing mesophilic bacteria,[6] which is absent in many of the hyperthermophilic sulfur-reducing archaea.

While the three pyrococcal enzymes above display *in vitro* activities consistent with their involvement in the reduction of sulfur or sulfur species, the relative *in vivo* role played by each is much less clear. It has been proposed that all three enzymes use S^0 (or a sulfur derivative) as an electron acceptor in the regeneration of the $NAD(P)^+$ pool that accepts electrons from glycolysis and passes them to the membrane-bound oxidoreductase complex (MBX) that is believed to generate the proton-motive force for ATP synthesis.[7] Two recent studies, one on a CoADR (or NSR) from *Pyrococcus furiosus*[7] and another on a CoADR homologue from *Thermococcus*,[8] indicate that the enzymes are not essential for growth on sulfur.

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However, both the pyrococcal and thermococcal CoADRs have been shown to be highly up-regulated during growth on sulfur,[8,9] and the thermococcal deletion mutant of CoADR exhibited enhanced oxygen susceptibility in the presence of S^0, an observation that at first glance was quite puzzling.

Pyrococcal CoADR has been shown to be reactive toward coenzyme A disulfide (CoAD) and S^0, although in the later case it is not clear which form of sulfur reacts with the enzyme, as S^0 would appear to be relatively insoluble.[10] The hot, reducing environment inhabited by *Pyrococcus* and other members of the Thermococcales will solubilize a range of sulfur compounds, including sulfide, polysulfides, and persulfides. Coenzyme A is one of the most stable thiol compounds to auto-oxidation and has been shown to be the main intracellular thiol of several hyperthermophiles, including *Pyrococcus* and *Thermococcus*.[11]

In the studies described below, we have characterized the reactivity of the CoADR from *P. horikoshii* to a range of S-containing substrates to assess the physiological function of the enzyme. During the reduction of persulfide substrates under aerobic conditions, it was noted that recycling of the persulfide substrate occurred, a reaction that results in the consumption of O_2. This reaction, which disappears under anaerobic conditions, may account for the observation that the *Thermococcus* CoADR is able to play an antioxidant role in the presence of S^0.[8] We have also determined the X-ray crystal structure of the enzyme to 2.7 Å resolution. From these studies we were able to identify the likely *in vivo* substrates for CoADR and to comment on the ways in which the pyrococcal enzyme differs from the homologue present in some mesophilic bacteria (for example, the CoADRs of *Staphylococcus aureus* and *Bacillus anthracis*) and how these structural differences affect substrate preference.

MATERIALS AND METHODS

Cloning, Overexpression and Purification of CoADR. Wild-type *P. horikoshii* CoADR (*ph*CoADR) was amplified from *P. horikoshii* genomic DNA using primers 5′-AAGGGGCATATGAAGAAGATACTGATTATTGGTGGCG-3′ (forward) and 5′-AAGGGGCTCGAGGGCCACGAAGGCCGCGG-3′ (reverse) and cloned into pET-21b+ (which introduced a C-terminal 6-histidine tag) using unique NdeI and XhoI restriction sites and standard recombinant DNA technologies. The construct was sequenced to confirm its accuracy and subsequently transformed into BL21(DE3)pLysS *Escherichia coli* cells (Novagen) for protein overexpression. Overexpression and purification were conducted identically to that of the Npsr from *Shewanella loihica* PV-4 Npsr,[12] except that cells were harvested after 5 h of growth following IPTG induction rather than 14, and the crude extract (following lysis and centrifugation) was incubated at 80 °C for 15 min in the presence of 1 mM FAD and centrifuged for 20 min at 5000*g* before application to the Ni-NTA affinity column. *ph*CoADR concentration was determined by measuring FAD concentration, using an extinction coefficient of 10 200 M^{-1} cm^{-1} at 460 nm as determined in ref 4.

Steady-State Kinetic Assays Measuring Per- and Polysulfide Reductase Activities. Kinetic assays for all substrates were conducted in 1.5 mL quartz cuvettes at 50 °C, with a total solution volume of 1 mL, by monitoring the oxidation of NAD(P)H at 340 nm, or in the case of DTNB, the appearance of the TNB product at 412 nm. All assays were conducted with 100−400 nM purified *ph*CoADR in 1 M Tris buffer at pH 8.7 (as measured during pH adjustment at room temperature). The actual pH of the buffer at assay temperature, 50 °C, was 8.1. The buffer conditions were chosen in order to stabilize polysulfides and to prevent changes in pH due to the addition of basic solutions of polysulfide and sulfide.[12,13] NAD(P)H and polysulfide or persulfide substrates were preincubated in the Tris buffer for 4 min at 50 °C before addition of enzyme. Polysulfide and persulfide were prepared as described previously[12] by adding equimolar concentrations of disulfide substrate and sodium sulfide or polysulfide to degassed 1 M Tris, pH 8.7 in a stoppered bottle to make a stock solution, except in the one case in which polysulfide alone was the oxidizing substrate.[14] The slow NADH and NADPH-dependent background oxidase activities observed were subtracted from each assay.

Protein Crystallization. *ph*CoADR was crystallized at 20 °C by using the hanging and sitting drop vapor diffusion methods. An equal amount of protein at 10 mg/mL in 25 mM MOPS, pH 7.0, 25 mM NaCl, 5% glycerol was combined with a mother liquor comprised of 100 mM Tris, pH 8.0, 2−3 M 1,6-hexanediol, and 200 mM $MgCl_2$. Large hexagonal yellow crystals with dimensions of approximately 0.3 mm × 0.3 mm × 0.5 mm appeared within 2−3 days. As 1,6-hexanediol is already a cryoprotectant, the crystals were harvested and flash frozen with liquid nitrogen in the mother liquor.

Data Collection, Structure Determination, and Refinement. The diffraction data were collected at SSRL on BL-12-2. The crystals belonged to the space group *R*32 with unit dimensions $a = b = 133.5$ Å and $c = 305.1$ Å and contained two monomers per asymmetric unit. The reflections were indexed and scaled using HKL2000.[15] Molecular replacement was performed with Phaser[16] using the *Bacillus anthracis* CoADR homologue (3CGC).[17] The model was manually built in Coot[18] and refined by using Refmac5 with TLS parameters[19] in the CCP4 suite of programs.[20] TLS parameters were generated by using the TLS Motion Determination server.[21] All data processing and model refinement statistics are listed in Table 1. Structural analysis was performed by using PROCHECK[22] and indicated 93.8% and 5.8% of residues fell into preferred and allowed regions of a Ramachandran plot, respectively.

RESULTS

Determination of phCoADR Substrate Specificity via Steady-State Kinetics. *ph*CoADR catalyzes the NAD(P)H-dependent reduction of polysulfide, CoA-polysulfides, and CoA persulfide, as well as the reduction of a range of other small persulfides, including TNB and glutathione persulfides. All reactions showed a direct dependence of observed rate on enzyme concentration. A summary of these kinetic results is shown in Table 2. These studies were conducted at 50 °C and pH 8.1 in 1 M Tris buffer in order to minimize background reactions, enhance polysulfide stability, and control pH following addition of sulfide or polysulfide to the reactions. At pHs much less than 9, polysulfide spontaneously reoxidizes to the circular S^0 form[23] making determinations of polysulfide reaction kinetics problematic at the pHs at which previous CoADR kinetic studies were carried out (pHs 7.5[4] and 7.0[5]).

A mixture of oxidized coenzyme A and polysulfide, which should consist mostly of a coenzyme A-polysulfide derivative ($CoA{-}S{-}S{-}CoA + S_n^{2-} + H^+ \rightarrow CoA{-}S_n^- + CoA{-}SH$), shows the highest activity with both NADH and NADPH cosubstrates. A definite activation of the enzyme's polysulfide reduction rate is observed with the addition of coenzyme A and is consistent with previously observed results for the *P. furiosus* CoADR.[5] The enzyme appears to saturate at low substrate

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Table 1. X-ray Data Collection, Phase Determination, and Refinement Statistics for CoADR

Data Collection	
Beamline	SSRL 12-2
wavelength (Å)	0.979
space group	*R*32
unit cell dimensions (Å)	133.5 × 133.5 × 305.1
resolution range (Å)	30−2.7
total reflections	287398
unique reflections	27620
completeness (%)[a]	99.8 (99.6)
$I/\sigma(I)$[a]	16.7 (10.5)
R_{sym} (%)[a,b]	9.0 (40.1)
Refinement	
R_{cryst} (%)[c]	26.1
R_{free} (%)[d]	30.6
average B-value (Å^2)	55.5
r.m.s.d. bond length (Å)	0.006
r.m.s.d. bond angles (°)	1.09
no. protein atoms	7370
no. non-protein atoms	164
water molecules	152
RMSD to Apo CoADR (3KD9)	0.178
PDB code	4FX9

[a]Values in parentheses are for the highest resolution shell. [b]$R_{sym} = \Sigma_i\Sigma_{hkl}|I_i(hkl) - \langle I(hkl)\rangle|/\ \Sigma_{hkl} < I(hkl)>$, where $I_i(hkl)$ is the *i*th measured diffraction intensity and $\langle I(hkl)\rangle$ is the mean intensity for the Miller index (hkl). [c]$R_{cryst} = \Sigma_{hkl}||F_o(hkl)| - |F_c(hkl)||/\ \Sigma_{hkl}|F_o(hkl)|$. [d]$R_{free} = R_{cryst}$ for a test set of reflections (5% in each case).

concentrations (relative to physiological levels), with the enzymatic efficiency of most of the substrates hovering around 1×10^5 M^{-1} s^{-1} (in oxidizing substrate, with 100 μM NAD(P)H). It should be noted that these assays were performed at 50 °C, which means that the true catalytic efficiency of the enzyme under physiologically relevant conditions would be much higher (T_{opt} 100 °C[24]). The enzyme shows a preference for NADPH, although it makes efficient use of NADH as well. At the physiological concentrations of reduced pyridine nucleotides in *Pyrococcus* (140 μM NADH and 40 μM NADPH), the enzyme would be saturated with both NADH and NADPH.[25]

Evidence for "Recycling" of the Persulfide Substrate under Aerobic Conditions. During assays with low concentrations of persulfide substrate, it was noted that the reactions consumed more NAD(P)H than they should have (with a 1:1 stoichiometry of R−S−S−H/NADH consumed in the reaction) (Figure 1A). On the basis of the reaction which occurs when CoA disulfide is reacted with Na_2S (as utilized in previous methods for the preparation of CoA persulfide[26]), it appeared that the combination of the sulfide and free CoA thiol products from the persulfide reductase reaction oxidize under aerobic conditions to (re)form CoA persulfide (Scheme 1). This hypothesis was confirmed by experiments run under anaerobic conditions with NADH and NADPH, using CoA or glutathione persulfides as oxidizing substrates (Figure 1A,B). In each case, identical velocities were observed at the beginning of the run under aerobic or anaerobic conditions; however, under anaerobic conditions, the amount of NADH or NADPH consumed correlated with the amount of persulfide substrate added, and the kinetic traces showed the marked curvature that would be expected as the reaction consumed all of the substrate.

For assays with the glutathione persulfide substrate under aerobic conditions, the reaction stayed relatively linear following consumption of the initial substrate (Figure 1B). This indicated that the recycling reaction was fast relative to the enzyme-catalyzed reduction. For the CoA-persulfide substrate, however, the reaction rate slowed significantly after the initial substrate was consumed (Figure 1A), indicating that the recycling reaction was a bit slower than the enzyme-catalyzed reduction. This result is consistent with the previously seen slower rate of CoA thiol oxidation relative to glutathione.[4] In either case, however, the nonenzymatic reaction does occur relatively quickly, and it would be expected to be even faster at the physiological temperature of 100 °C, although the relatively high pH of 8.1 used for the per- and polysulfide reductase reactions in these studies likely accelerates the rate of reaction somewhat, as the spontaneous reoxidation of thiols is dependent on the pH of the medium and the pK_a of the thiol. The recycling reaction is also likely to be further

Table 2. Steady State Kinetics of *ph*CoADR

substrate (cosubstrate)	$K_{m\ app}$ (μM)	$k_{cat\ app}$ (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
CoA persulfide (NADH)[a]	20.5 ± 3.0	1.11 ± 0.04	5.4×10^4
CoA persulfide (NADPH)[a]	56.2 ± 13.2	1.50 ± 0.09	2.7×10^4
NADH (CoA persulfide)[b]	13.9 ± 3.2	1.07 ± 0.99	7.7×10^4
NADPH (CoA persulfide)[b]	12.7 ± 2.8	2.08 ± 0.22	1.6×10^5
CoA polysulfide (NADH)[a]	94.9 ± 26.5	6.56 ± 0.98	6.9×10^4
CoA polysulfide (NADPH)[a]	non-Michaelis behavior	18.3 s^{-1}[d]	
NADH (CoA polysulfide)[b]	125 ± 51	8.86 ± 2.21	7.9×10^4
NADPH (CoA polysulfide)[b]	14.1 ± 5.5	16.1 ± 2.2	1.2×10^6
polysulfide (NADH)[a]	12.30 ± 2.57	1.04 ± 0.05	8.5×10^4
CoA disulfide (NAD(P)H)[a,c]		not detected	
TNB persulfide (NADH)[a]	25.1 ± 6.7	3.37 ± 0.32	1.35×10^5
[a]DTNB (NADH)	129 ± 48.3	0.69 ± 0.07	5.35×10^3
glutathione persulfide (NADH)[a]	<1	0.35 ± 0.01	
glutathione disulfide (NADH)[a]		not detected	

[a]Conditions: 50 °C, 1 M Tris buffer pH 8.1, 100 μM NAD(P)H. [b]Conditions: 50 °C, 1 M Tris buffer pH 8.1, 100 μM CoA poly- or persulfide. [c]Activity not detected under these conditions, for CoA disulfide reductase activity see ref 4. [d]Enzyme turnover (V_0/[E]) at 150 μM CoA polysulfide, 100 μM NADPH given for comparison to other values.

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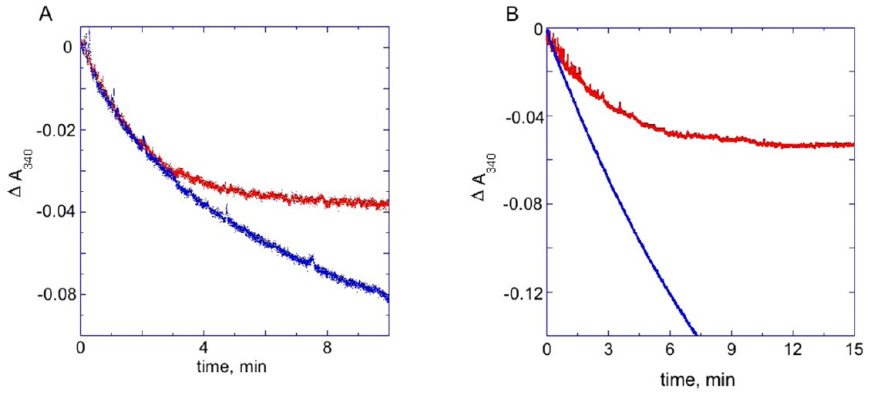
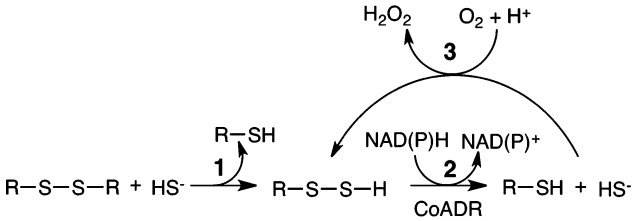


Figure 1. Coenzyme A persulfide reductase activity measured under aerobic (blue) and anaerobic (red) conditions, at 10 μM CoA persulfide (A) or 10 μM glutathione persulfide (B) and 200 μM NADH, 100 nM *ph*CoADR, 50 °C, 1 M Tris buffer, pH 8.1.

Scheme 1. (1) Nonenzymatic Generation of the Persulfide Substrate, (2) Reduction of the Substrate Catalyzed by CoADR, (3) Non-Enzymatic Regeneration of the Persulfide Substrate in the Presence of O_2, Sulfide, and Thiol Products



accelerated by the production of hydrogen peroxide early in the reaction, as thiol oxidation can occur through an H_2O_2-dependent pathway,[27] and relatively rapid oxidation of sulfides to polysulfide by H_2O_2 has been shown to occur at pHs similar to those used in this assay.[28] Additional factors, such as affinity for catalytic metals, may also strongly affect sensitivity to oxidation.[29−32]

Structure Analysis. *P. horikoshii* CoADR is a homodimer with a noncrystallographic 2-fold center of symmetry (Figure 2). The fold is similar to that of other enzymes in the pyridine nucleotide disulfide oxidoreductase (PNDOR) family, which include NADH oxidases (NOX), NADH peroxidases (Npx), and coenzyme A disulfide reductases (CoADR).[17,33] The r.m.s. deviations between the *Pyrococcus* structure and related members of this family range from 0.9 to 2.7 Å^2 (Supplemental Figure S1, Supporting Information); thus the folds are relatively well conserved.

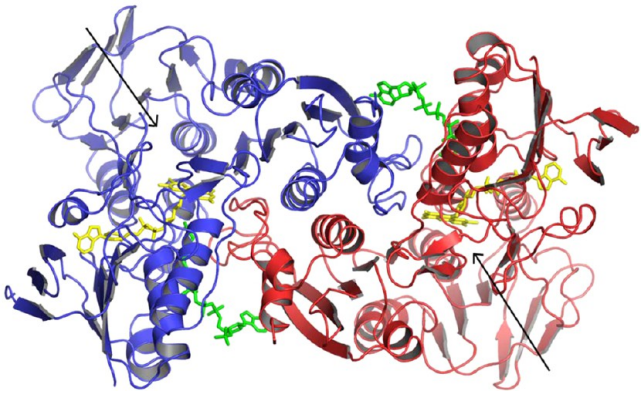


Figure 2. Global fold of *Pyrococcus horikoshii* CoADR. The α and β subunits in the homodimer are depicted as blue and red ribbons, respectively. CoA (green) and FAD (yellow) are represented as sticks. The black arrows denote the NAD(P)H binding site on each monomer.

*ph*CoADR contained bound CoA and FAD when purified, which were found in the crystal structure nestled in two distinct grooves extending from the protein surface to its core (Figure 2). Bound CoA forms a disulfide bond with a conserved catalytic cysteine, C48, which is oriented 3.2 Å above the *si* face of the FAD isoalloxazine ring (Figure 3A,B). Two additional conserved active site tyrosines, Y368 and Y425, hydrogen bond to each other at 2.5 Å and are located on the opposite protomer ~3.7−3.9 Å away from C48. Y368 also interacts with a single water molecule at 2.9 Å. This water makes additional interactions with solvent molecules that hydrogen bond to the peptide backbone near V432 and W433. CoA binding is further stabilized in this pocket by additional interactions with S20, S44, Y65, Y66 R75 and N305 from one subunit and K361 W433, and R441 from the other (Figure 3A,B). These residues are conserved among most pyrococcal and thermophilic CoADRs but less so among homologous mesophiles (Supplemental Figure S2).

The Substrate Access Channel and the Structural Basis for Substrate Specificity. Surface calculations of the CoA-binding cleft indicate this site is relatively narrow and may only be able to accommodate one molecule of CoA (Figure 4A). CoA disulfide (CoAD), which was assumed previously to be the physiological substrate for *ph*CoADR, does not appear to fit into the active site pocket.[4] The architecture of the *ph*CoADR CoA-binding pocket stands in contrast to the structures of the *Staphylococcus aureus* and *Bacillus anthracis* CoADR, both of which have wider CoA-binding clefts capable of accommodating coenzyme A disulfide substrates (Figure 4B).[17,34] Although *sa*CoADR and *ba*CoADR have 46% and 52% identity to the *Pyrococcus* enzyme, respectively, the Cα backbone of residues 56−76, which form part of the CoA binding cleft, is shifted toward the opposite subunit in *ph*CoADR by 2−5 Å in some spots, thus narrowing the CoA binding pocket (Figure 4C). In addition, this stretch of protein in *ph*CoADR contains several bulky residues, most notably Tyr 65, Tyr 66, and Arg 75, that hydrogen bond directly (or indirectly via bridging water molecules) to CoA (Figure 3A). These residues serve to narrow the channel by which a substrate approaches the active site Cys and Tyr residues, and possibly serve as a selectivity filter. Moreover, two prolines

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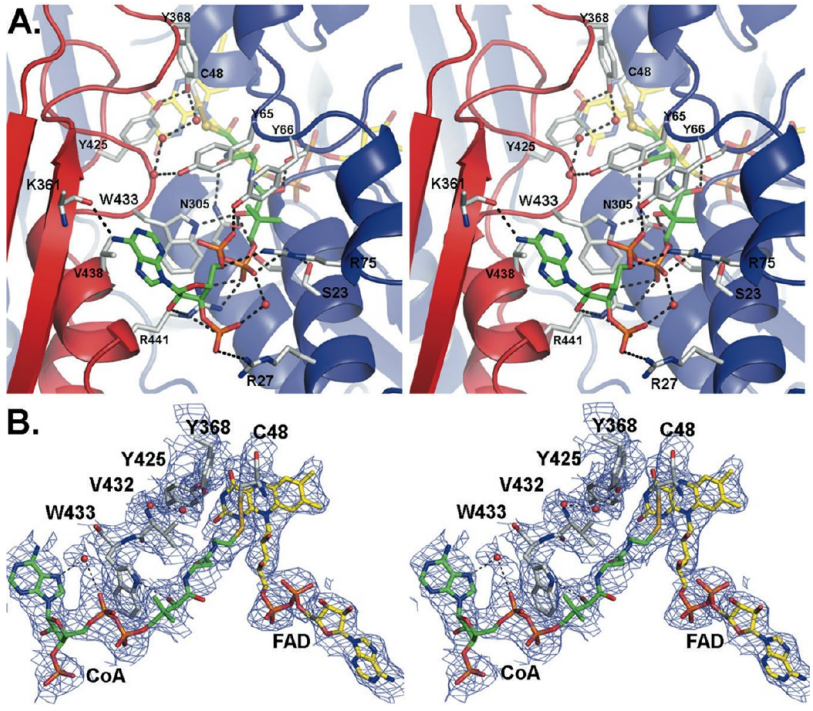

Figure 3. (A) Stereoview of the CoA binding pocket between the α (blue) and β (red) subunits. Shown as sticks are CoA (green) and residues making hydrogen bonding contacts. Atoms are colored such that nitrogen is blue, oxygen red, phosphate orange, and sulfur yellow. (B) Stereoview of the NPSR active site with a simulated annealing omit map contoured to 1σ around the catalytic residues (gray sticks), water molecules (red spheres), CoA (green), and FAD (yellow).

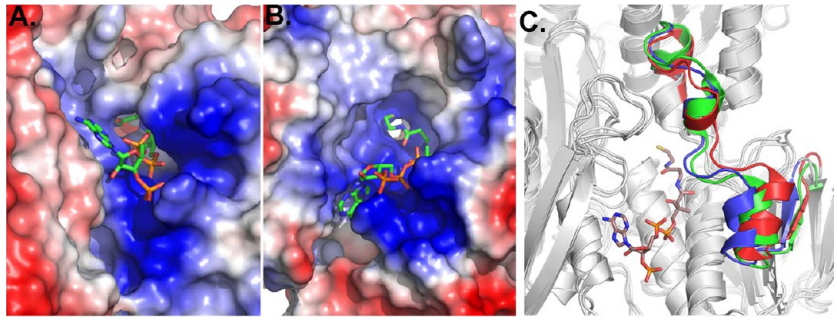

Figure 4. Electrostatic surface maps of the (A) *ph*CoADR and (B) *sa*CoADR CoA binding clefts. CoA is shown as green sticks and colored by atom type. (C) Overlap of *ph*CoADR (blue), *sa*CoADR (red), and *ba*CoADR (green) structures highlighting the orientation of residues 57−77 in the CoA-binding cleft. CoA from *ph*CoADR is depicted in sticks and colored by atom type.

localizing to a loop on this segment (Pro 67 and Pro 68) appear to be conserved in pyrococcal sequences. These may act to rigidify this region of protein and prevent the enzyme from accommodating larger disulfide substrates. By contrast, the analogous residues in *sa*CoADR and *ba*CoADR are much smaller (Supplemental Figure 2), resulting in a wider groove for CoA disulfide binding. For example, Tyr 65, which is conserved as a bulky residue in the *P. horikoshii* CoADR and the Npsr-type proteins from *S. loihica* PV-4 and *B. anthracis* (the latter of which have C-terminal rhodanase domains and do not reduce CoA disulfide) corresponds to Ala residues 61, 60 and Ser 58 in the *S. aureus*, *B. anthracis*, and *Borrelia burgerdorferi* CoADRs, respectively. This difference is consistent with a widening of the substrate channel and the greater reactivity of the bacterial CoADR enzymes toward the CoA-disulfide substrate.

Conversely, the restricted CoA-binding cleft in *Pyrococcus* suggests that the natural substrates are likely much smaller persulfides or polysulfides. An extra sulfur atom linked to the end of CoA can easily be accommodated in the space occupied by the water molecule hydrogen-bonding to Tyr 368 (Figure 3). The crystal structure is also consistent with the kinetic evidence presented in which the rate of CoA persulfide and CoA polysulfide reduction is much faster than those for CoA disulfide and DTNB (Table 2). In addition, a persulfide substrate is consistent with findings for the substrate specificity of the *Pyrococcus furiosus* homologue.[5] Because *ph*CoADR can reduce some CoA disulfide, it is possible that residues 56−76 undergo structural changes to accommodate larger substrates, especially at higher temperatures. The X-ray crystal structure of cofactorless *ph*CoADR provides some insight into the flexibility of this loop.

Analysis of Cofactorless phCoADR from Structural Genomics. A structure of *Pyrococcus horikoshii* CoADR was recently determined at pH 5.5 to 2.75 Å resolution by the New York SGX Research Center using different crystallization conditions than ours (3KD9).[35] The deposited model lacks

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both the FAD and CoA cofactors and has a nearly identical fold to that of the holo protein (r.m.s.d. 0.54 Å^2), except that residues 45−68, which are responsible for binding CoA, are disordered (Figure 5). To confirm the structural differences and

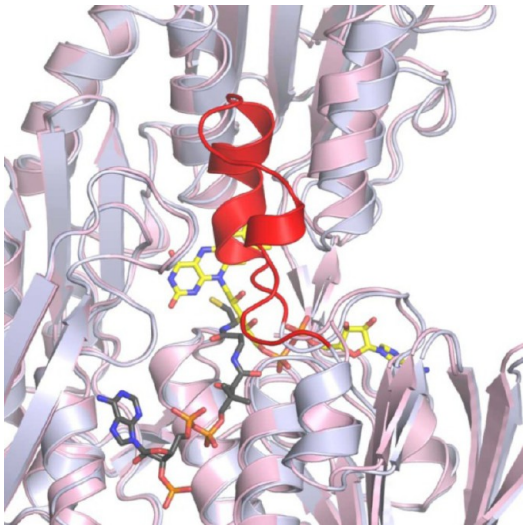

Figure 5. Superposition of the holo (pink) and cofactorless (gray) *ph*CoADR structures. The disordered region in CoA binding cleft of the apo structure are denoted in red on the holo structure. FAD and CoA are depicted as sticks and colored by atom type: oxygen (red), nitrogen (blue), sulfur (yellow), phosphate (orange), FAD carbons (yellow), CoA carbons (gray).

the possible significance of this disordering, electron density maps of the unpublished deposited model were inspected. $2F_o - F_c$ and $F_o - F_c$ electron density maps contoured at low signal-to-noise, 0.5σ and 2.0σ, respectively, indicated that some FAD may be bound at very low occupancy. In addition, electron density could be observed for some but not all residues between amino acids 45−68. Density for coenzyme A, however, was noticeably absent. To confirm these observations, FAD, coenzyme A, and residues 42−68 were modeled into 3KD9 using our structure and a single round of refinement conducted. The electron density in these regions did not improve substantially in both subunits. Weak electron density was observed for FAD at 0.8σ, mostly around the electron-rich phosphates and adenine, confirming the presence of FAD in this structure at low occupancy. In the disordered region between amino acids 45−68, $2F_o - F_c$ maps contoured to 0.8 σ continued to show no density for residues 46−51 and 57−62 in subunit A and residues 46−49 and 58−61 in subunit B. Likewise, no density was observed in $2F_o - F_c$ and $F_o - F_c$ maps around coenzyme A. Given that the region around the FAD binding site is ordered and that the region around the coenzyme A binding site is not, these observations suggest that the binding of coenzyme A substrates induce ordering of residues 45−68 and formation of the *ph*CoADR active site.

Structural Basis of NAD(P)H Specificity. A third groove on the surface near FAD is the presumed NAD(P)H binding site (Figure 2). A model of *ph*CoADR with bound NADH was generated by using the existing crystal structure of NADH-bound *ba*CoADR and indicates the substrate readily accommodates this space (Supplemental Figure 3). *ph*CoADR uses both NADH and NADPH efficiently (Table 2), and while NADPH shows greater activity and does not show Michaelis-type saturation behavior at physiologically reasonable NADPH concentrations with the CoA polysulfide substrate, the enzyme turns over at roughly similar rates (within the same order of magnitude) for the two reduced nucleotides (Table 2 and ref 4). As described earlier,[4] for the CoA disulfide reductase activity, NADPH has a K_m at least 10× lower than NADH (<9 μM vs 73 μM), although the two reduced nucleotides have nearly identical k_{cat}'s (NADPH = 7.2 s^{-1}, NADPH = 8.1 s^{-1}). In the *ph*CoADR-NADH model, the 2′-OH of the NADH ribose ring is adjacent to Arg182 and Arg188, both of which can easily support ionic interactions with the ribose-bound 2′-phosphate on NADPH and thus enhance the overall affinity of *ph*CoADR for NADPH over NADH.

DISCUSSION

Kinetic Behavior and Substrate Specificity for phCoADR. FAD and NAD(P)H-dependent enzymes in the PNDOR class are found throughout the prokaryotic world, and specific homologues to the *ph*CoADR discussed in these studies are found throughout the archaeal and bacterial thermo- and hyperthermophiles. Homologues of these proteins containing additional domains have also been found and characterized.[12,36] Despite their ubiquity and several studies of their substrate specificities and mechanisms, the specific *in vivo* function of this enzyme class is still not clear.

We have characterized the reactivity of the pyrococcal CoADR from *P. horikoshii* toward a range of sulfur-containing substrates. From these studies it is clear that persulfide and polysulfide derivatives of CoA are likely *in vivo* oxidizing substrates, and the enzyme is also reactive toward disulfide substrates such as CoA disulfide (as shown previously[4]) and DTNB. CoA is the major low molecular weight thiol of *Pyrococcus* and is present at an intracellular concentration of 860 μM in cells grown on S^0.[11] This concentration suggests that under physiological conditions, CoADR reacting with NADH (at a cellular concentration of 140 μM) would be saturated with CoA di-, per-, or polysulfide substrates *in vivo*.[25] Reactions with NADPH would either be saturated, in the case of the CoA persulfide or disulfide (results from ref 4), or turning over at a high rate, in the case of the CoA-polysulfide derivative, which does not show Michaelis−Menten (saturation) kinetics but rather a linear dependence on CoA-polysulfide concentration, likely due to a high K_m for this substrate.

Studies of the CoADR homologue from *P. furiosus* (*pf*CoADR, referred to as NSR in ref 5) obtained somewhat similar results to those discussed here. Kinetic experiments for *pf*CoADR were done under very different conditions, and only K_ms and specific activities were reported; therefore it is difficult to make a detailed comparison between the two studies. On the basis of the *in vivo* concentrations of NADH and NADPH in *Pyrococcus*, which have been measured at 140 and 40 μM, respectively, and the K_ms reported for the *P. furiosus* homologue (3.3 mM and 8.5 mM for NADH and NADPH, respectively), it would appear that the enzyme would turn over exceptionally slowly under physiological conditions. The kinetic data presented by Schut et al. in the Supporting Information suggest the *P. furiosus* enzyme has a turnover of <1 s^{-1} at physiological NAD(P)H concentrations and at the 85 °C temperature of the assay.[5]

This result is significantly lower than those reported here for the *P. horikoshii* enzyme, with a turnover number of 16.1 s^{-1} at 100 μM CoA-polysulfide and saturating NADPH at 50 °C (Table 2). These values would correspond to a turnover number of roughly 99 s^{-1} at 85 °C and 195 s^{-1} at 100 °C

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(using the Arrhenius equation and an estimated energy of activation of 50 kJ/mol to calculate the approximate increase in rate with temperature). Although the 50 °C temperature and somewhat elevated pH used for these studies are less than optimal (and were chosen for their ability to produce stable polysulfide/persulfide solutions and reproducible kinetics), they do demonstrate the reactivity of this enzyme toward the polysulfide and persulfide derivatives of CoA and other small thiols. While we did not see CoA disulfide reductase activity under the conditions of the assays described here, we previously observed an NADH-dependent CoA-disulfide reductase activity with a k_{cat} of 8.1 s^{-1} at 75 °C and pH 7.5.[4] Even though it is difficult to make a meaningful comparison given the difference in conditions, the CoA disulfide reductase seems to be somewhat slower on relative terms, given the higher temperature of that assay.

The difference in these results may be due to the differences between the *P. furiosus* and *P. horikoshii* homologues (85% identity, 95% similarity), the use of solid S^0 as a substrate in the *P. furiosus* study, the ionic strength, the assay methodology utilized in these two studies, or the pH of the assays. In the studies presented here, the persulfide and polysulfide substrates were prepared at room temperature in 1 M Tris at pH 8.7, and the measured pH of the persulfide and polysulfide reductase assays, in the same buffer at 50 °C, was 8.1 (Table 2), vs pH 7.0 in the studies of the *P. furiosus* enzyme.[5] The methodology used in ref 5 allows for only a single time point per assay, while the methodology used in this work (and in refs 12 and 13) allows for continuous assay.

While the magnitude of K_ms and the activity of the enzyme vary from the previous study, there are many consistencies between the two enzymes. The preference for NADPH is seen in both cases, and the data presented in the previous study predicted the non-Michaelis behavior we observed when varying CoA polysulfide with NADPH as the reducing substrate, although Michaelis behavior was observed when NADH and NADPH were varied at a constant CoA-polysulfide concentration.

Is Persulfide the in Vivo Substrate for phCoADR? Although the precise speciation of sulfur/polysulfide/persulfides substrates under the *in situ* conditions of *Pyrococcus* have not been determined, polysulfides would tend to be unstable at pHs 6−7, as they autooxidize even under relatively reducing conditions.[23,37] Persulfide (H_2S_2/HS_2^-) is, however, stable at pH 6−7 and has been shown to be present at high concentrations in anaerobic sediments.[23] Although we have not directly measured hydrogen persulfide (H_2S_2) reduction kinetics in these studies, based on the promiscuity of the *ph*CoADR and its ability to reduce an array of small S−S-containing substrates, it seems quite likely that H_2S_2/HS_2^- would make an effective oxidizing substrate for the enzyme. On the basis of a comparison of the CoA persulfide, CoA polysulfide, and polysulfide kinetics, however, it seems most likely that CoA−S−S−H (formed by reaction of CoA−SH + H_2S_2 → CoA−S−SH + H_2S) or CoA−S−S−S−H (formed by CoA−SH + H_2S_2 + O_2 → CoA−S−S−SH + H_2O_2) would be the persulfide derivatives that would serve as the major *in vivo* substrates.

Considerations Regarding S^0 as a Substrate for the Pyrococcal CoADR. Whether elemental sulfur, which was used as the substrate in the previous characterization of the *P. furiosus* CoADR/NSR,[5] is a reasonable *in vivo* and *in situ* substrate remains an important question. While at low temperatures S_8 is relatively insoluble (6.1 nM in pure H_2O at 4 °C), at higher temperatures dissolved elemental sulfur concentrations increase dramatically, reaching 471 nM at 80 °C and 1.14 μM at 95 °C in pure water.[38] This solubility, however, is highly dependent on solution conditions: in seawater elemental sulfur solubility decreases to 61% of its value in pure H_2O, while proteins or surfactants can result in an increase in sulfur solubility.[38−40] An additional factor to consider is the kinetics of solubilization — while a 0.5 nM−1 μM concentration of a soluble electron acceptor might be reasonable if the $S_8(s) \leftrightarrow S_8(aq)$ equilibration were rapid, a slow solubilization rate might make microbial use of $S_8(aq)$ as an electron acceptor impractical on a fast time scale (such as the time scale of experiments involving short-term growth of *Pyrococcus* cultures), although slow *in situ* growth should be supported in such a case. From a biochemical standpoint, equilibration of the $S_8(s) \leftrightarrow S_8(aq)$ reaction appears to be relatively slow, occurring on the time scale of days,[38] although it is certain that these kinetics would be highly sensitive to the presence of proteins, lipids, and other surfactants expected to be present in biological systems. Given the much more rapid equilibration of $S^0 + S^{2-} \leftrightarrow S_n^{2-}$,[40] and the high concentrations of S^{2-} likely to be found in the environs of *Pyrococcus*, it seems likely that per- or polysulfides, rather than S_8^0, are the most available substrates. Detailed studies to determine the speciation of zerovalent and reduced sulfur compounds in solution under the *in situ* conditions of *Pyrococcus* growth and during catalysis by *ph*CoADR are currently underway in this laboratory and will provide information regarding the sulfur species most likely to be available to anaerobic hyperthermophiles involved in sulfur respiration.

Structure of the phCoADR and Correlation with Substrate Specificity. The smaller substrate channel for the *ph*CoADR compared to that of related enzymes like the *ba*CoADR and *sa*CoADR likely explains the preference of this enzyme for per- and polysulfide derivatives of small thiols. Given the relative differences in the size and topology of the *ph*CoADR CoA binding site versus enzymes that have a preference for CoA disulfide, it seems possible to identify the substrate preferences of related enzymes in this class and to assign putative metabolic functions to newly characterized organisms by using sequence analysis of PNDORs present in the genome. Unfortunately, because of the high sequence identity among PNDOR enzymes and the small number of confirmed CoADRs for which substrate preferences have been determined, at this point it is difficult to distinguish between CoA disulfide reductases, CoA-persulfide/CoA polysulfide reductases (and possibly other enzymes, such as NADH peroxidases and oxidases) based on sequence comparisons alone. Using simple metrics like amino acid sizes and conservation, we were unable to distinguish at this time whether sequence correlations are due to phylogeny or function of these enzymes (Supplemental Figure S2). Future work on *ph*CoADR and other enzymes in this class to determine which structural elements tune the substrate specificity of the enzyme would be useful.

Function of the phCoADR in Vivo — a Sulfur-Dependent Antioxidant System? Given the (1) high reactivity of the *ph*CoADR toward S−S bonds in small thiol poly- and persulfide compounds and (2) the up-regulation of this protein during growth on sulfur, it was surprising that deletion of the enzyme in either *Pyrococcus* or *Thermococcus* did not result in any apparent loss of ability to grow efficiently via

the dissimilatory reduction of sulfur.[7,8] While these results do not prove that the enzyme does not contribute to the dissimilatory reduction of sulfur, they certainly suggest that the enzyme serves an additional function. The observation that the thermococcal deletion mutant above resulted in a decrease in the ability of *Thermococcus* to survive exposure to oxygen while growing with S^0 (its oxygen susceptibility remains unchanged in the absence of S^0) indicates that the enzyme somehow participates in cellular oxygen detoxification. Moreover, the observed "recycling" reaction of CoADR persulfide products in the presence of oxygen, as described in Scheme 1, provides a mechanism by which oxygen detoxification might occur. In this proposed antioxidant cycle, the persulfide reduction products, a thiol and sulfide, can react in the presence of O_2 to form peroxide and a persulfide. At the 90−100 °C growth temperature of *Pyrococcus* and the high intracellular concentration of coenzyme A (860 μM), the reaction would be reasonably rapid. The peroxide could then be reduced via the proposed antioxidant systems of *Pyrococcus*[41] or may react with thiols and/or sulfides, oxidizing them to per- or disulfides, as discussed above. A similar system would be expected to operate with polysulfide substrates; however, we did not attempt to experimentally demonstrate this system due to questions regarding the precise number of sulfur atoms in the polysulfide substrate, which does not allow for an easy determination of the true substrate concentration. We are currently attempting to determine the precise identity of the sulfur species present during the reaction of *ph*CoADR, which will allow us to more accurately describe the nature of the *ph*CoADR's polysulfide reductase activity.

The correlation between sulfur and the oxidative stress response observed with *Thermococcus* is not without precedence — roles for sulfide and polysulfide in enzymatic systems involved in oxidative defense have been proposed previously. In the case of the human erythrocyte Cu/Zn superoxide dismutase, sulfide increases the rate of superoxide reduction to peroxide 2-fold, although the mechanism of this superoxide scavenging chemistry is not at all clear.[42] It has recently been shown that this superoxide dismutase is able to reversibly form an intersubunit polysulfane bridge (Cys−S−S_5−S−Cys) between Cys111 from each of the subunits of the homodimeric enzyme.[43] Additionally, a glutathione reductase homologue recently characterized from the fungus *Fusarium oxysporum* has been proposed to play a role in the detoxification of S^0, using a glutathione-polysulfide intermediate, similar to the CoA-polysulfide substrate observed with the *ph*CoADR.[44]

CONCLUSIONS

The in-depth characterization of the substrate specificity of the *ph*CoADR presented here and elsewhere provides evidence that the *in vivo* oxidizing substrates of this enzyme are likely to be di, per-, and polysulfide derivatives of coenzyme A, although the enzyme is relatively promiscuous and is able to efficiently reduce a range of small per- and polysulfide substrates. The turnover number of the enzyme with NADPH is roughly 1.5−2 times greater than with NADH (depending on the oxidizing substrate), indicating that the enzyme is able to use either of the reduced pyridine nucleotides *in vivo*. The observation that the products of the persulfide reductase reaction are able to recycle under aerobic conditions provides a mechanism that explains the ability of the thermococcal CoADR to provide protection from O_2 and/or reactive oxygen species in the presence of S^0. Comparison of the *ph*CoADR structure with the previously determined structure of the *sa*CoADR provides insight into the different substrate specificities of these two enzymes, with the access tunnel leading from the enzyme surface to the active site being more restricted in the case of the *ph*CoADR, preventing easy access to the active site by coenzyme A disulfide, which is the preferred oxidizing substrate of the *sa*CoADR. While at this point it does not seem possible to determine substrate specificity of these enzymes based solely on sequence comparisons, the characterization of a wider range of these proteins may allow for the ability to more precisely identify these enzymes in genomic studies.

ASSOCIATED CONTENT

Supporting Information

Figures with superposition of the *ph*CoADR, *sa*CoADR, and NADH peroxidase structures, a multiple sequence alignment, and a figure with NAD^+ modeled into the *ph*CoADR binding cleft. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

The coordinates and structure factors for phCoADR have been deposited in the Protein Data Bank (entry 4FX9).

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

CoADR, coenzyme A-disulfide reductase; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; Npsr, NAD(P)H-dependent polysulfide reductase; PNDOR, pyridine nucleotide disulfide oxidoreductase; Npx, NADH peroxidase; Nox, NADH oxidase; CoAD, coenzyme A-disulfide; HEPES, *N*-2(-hydroxyethyl)-piperazine-*N*′2-ethanesulfonic acid; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; r.m.s.d., root-mean-square deviation; *ba*CoADR, *Bacillus anthracis* coenzyme A-disulfide reductase;

*ph*CoADR, *Pyrococcus horikoshii* coenzyme A-disulfide reductase; *sa*CoADR, *Staphylococcus aureus* coenzyme A-disulfide reductase; EH_2, $2e^-$ reduced enzyme

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