Characterization of the nonheme iron center of cysteamine dioxygenase and its interaction with substrates

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Cysteamine dioxygenase (ADO) has been reported to exhibit two distinct biological functions with a nonheme iron center. It catalyzes oxidation of both cysteamine in sulfur metabolism and N-terminal cysteine-containing proteins or peptides, such as regulator of G protein signaling 5 (RGSS5). It thereby preserves oxygen homeostasis in a variety of physiological processes. However, little is known about its catalytic center and how it interacts with these two types of primary substrates in addition to O₂. Here, using electron paramagnetic resonance (EPR), Mössbauer, and UV-visible spectroscopies, we explored the binding mode of cysteamine and RGSS5 to human and mouse ADO proteins in their physiologically relevant ferrous form. This characterization revealed that in the presence of nitric oxide as a spin probe and oxygen surrogate, both the small molecule and the peptide substrates coordinate the iron center with their free thiols in a monodentate binding mode, in sharp contrast to binding behaviors observed in other thiol dioxygenases. We observed a substrate-bound B-type dinitrosyl iron center complex in ADO, suggesting the possibility of dioxygen binding to the iron ion in a side-on mode. Moreover, we observed substrate-mediated reduction of the iron center from ferric to the ferrous oxidation state. Subsequent MS analysis indicated corresponding disulfide formation of the substrates, suggesting that the presence of the substrate could reactivate ADO to defend against oxidative stress. The findings of this work contribute to the understanding of the substrate interaction in ADO and fill a gap in our knowledge of the substrate specificity of thiol dioxygenases.

Thiol dioxygenases are a group of nonheme, ferrous enzymes that incorporate two oxygen atoms from molecular oxygen into the thiol groups of their corresponding substrates (1). Among this group of proteins, cysteamine dioxygenase (ADO) (2), cysteine dioxygenase (CDO) (3–6), 3-mercaptopropionate dioxygenase (3MDO) (7–9), and mercaptosuccinate dioxygenase (MSDO) (10) are found to catalyze the oxidation of small molecules, as shown in Fig. 1A. To date, ADO and CDO are the only two known thiol dioxygenases in humans, and they are directly involved in cysteine metabolism and the biosynthesis of hypotaurine and taurine (1). Dysfunction of these thiol dioxygenases is associated with oxidative stress, autoimmune and neurodegenerative diseases (11–18).

Additionally, some thiol dioxygenases oxidize N-terminal cysteine to cysteine sulfenic acid in polypeptides to regulate protein stability (Fig. 1B). For example, plant cysteine oxidase (PCO) promotes the N-terminal thiol dioxygenation of VII ethylene response factors in normoxia to precede arginylation, resulting in proteosomal degradation (19). Recently, this type of posttranslational modification mediated by thiol dioxygenases was reported in ADO (20). Analogous to PCO in plants, ADO has been proposed to function as a conserved oxygen sensor in animals, regulating the degradation of N-cysteine proteins, including regulator of G signaling 5 (RGSS5), and, thus, transduces cellular responses to hypoxia. RGSS5 belongs to the R4 subfamily of RGS proteins that are known to negatively regulate the signaling of G protein-coupled receptors (21). Enriched in cardiovascular tissues, RGSS5 is related to pericyte maturation, vascular functions, and hypoxia-induced apoptosis (22–24). As an essential modulator, RGSS5 is also one of the most effective substrates of human ADO, with kCAT of 16.9 s⁻¹ and Kₐ of 71.5 μM (20). In contrast, CDO does not possess such dioxygenation activity on N-cysteine peptides. Overall, ADO is not only part of the thiol metabolism cycle, regulating through its dioxygenase activity of cysteamine, but also has functions which extend to oxygen homeostasis and signal transduction in mammals. The biological significance of ADO is increasingly appreciated; thus, exploring its catalytic center and especially how it responds to small-molecule and protein substrates is needed.

A protein-derived Cys-Tyr cofactor is autocatalytically generated in mammalian ADO and CDO to amplify their enzymatic activities when the substrate level rises (25–27). This substrate-initiated enzyme posttranslational modification and activity amplification is likely a mechanism for maintaining the physiologically significant, free thiol-containing substances at a proper cellular level. The active-site geometry and substrate binding mode of CDO in the ternary complex with a mature Cys-Tyr cofactor is revealed by the X-ray crystal structure with nitric oxide (NO) as an O₂ surrogate (28). The Cys-Tyr cofactor is formed by Cys93 and Tyr157 through a thioether bond located ~5 Å from the iron center in CDO (Fig. 1C). Its presence increases the catalytic rate by 10-fold (25). The substrate cysteine binds to the iron center in a bidentate fashion with both its amine and thiol groups. Together with an end-on bound NO, the iron center forms octahedral coordination. This active-site architecture in the complex resembles the...
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Figure 1. Reactions catalyzed by thiol dioxygenases and superposition of human ADO and CDO structures. A, thiol dioxygenases catalyze the oxidation of thiol-bearing small molecules. Cysteamine is the only small molecule without a carboxylate group among all the substrates. B, thiol dioxygenases oxidize selective peptides containing cysteine at the N terminus. C, superposed structures of a predicted model of ADO (green) and a ternary complex of CDO (white, PDB code 6N43). The substrate of CDO, cysteine, extensively interacts with the second sphere residues, including Cys93-Tyr157 cross-link, Arg60, and Tyr58. Cys220-Tyr222 cross-link of ADO locates at a position opposite that of CDO.

Here, we conducted an EPR-centered spectroscopic study of ADO from both human and mouse origin. The two proteins share 85.6% amino acid sequence identity (Fig. S1). We have found that the substrate of the ADO, either cysteamine or the N terminus of RGS5, is capable of reducing the iron center by forming a disulfide product if the enzyme is in the ferric oxidation state. The spectroscopic data also reveal a new binding mode of the substrate, monodentate coordination through the thiol group. This study fills the gap in the substrate specificity of the thiol dioxygenases for promoting a reaction without a free carboxylate group.

Results
Enzyme reactivation through chemical reduction of the ferric iron center by the primary substrate

Considering that cysteamine, as well as the cysteine-containing peptide substrates, contains a free thiol, we tested if these ADO substrates can reduce the ferric form of the enzyme to the ferrous state. X-band continuous-wave EPR spectroscopy was used to monitor the change of the oxidation state of the iron center upon incubation with cysteamine or the N terminus of RGS5. After exposure to air overnight at 4 °C, the as-purified ADO became oxidized, and the EPR spectrum showed a notable shift in the high-spin (g = 4.30) feature originating from the middle doublet of a rhombic (E/D = ∼1/3), high-spin (S = 5/2) nonheme ferric iron coordinated by protein ligands (Fig. 2A, black trace). A few minor EPR signals were observed at g = 9.31, 7.25, and 5.82. The g = 9.31 feature can be attributed to the overlapping resonances within the lowest- and highest-lying doublets of the S = 5/2 spin system, whereas other minor features are likely because of inhomogeneity of the iron center that will be discussed later. After anaerobically mixing with the substrate cysteamine and incubating for 1 min, the major ferric EPR signal of the oxidized ADO was diminished, and its resonance at g = 4.30 broadened slightly (Fig. 2A). Additionally, new spectral features at g = 9.22 and 4.73 arose, concomitant with the disappearance of the minor species observed in the spectrum of FeIII-ADO. The noticeable shift of the g
value and the appearance of new resonance after cysteamine addition are tentatively attributed to substrate ligation to the ferric iron center. Similar EPR features have been observed in samples of as-isolated desulfoferredoxin (36) and neelaredoxin (37), two nonheme iron-binding proteins coordinated by cysteinyl-sulfur ligands. Hence, based on literature precedence, the thiol moiety of cysteamine is expected to participate in iron coordination, although at this stage it is not definitive. After 3- and 6-min substrate incubations, the high-spin ferric signal at $g = 4.30$ continuously decreased, along with the resonances at $g = 9.22$ and 4.73. After a 10-min incubation, only a minor fraction of $g = 4.30$ remained, and all other resonances were no longer detectable. This experiment confirmed that cysteamine can reduce ferric ADO to the EPR silent, ferrous state.

Next, the same anaerobic incubation was done with the peptide substrate. The first 14 amino acids of the Met-excised N terminus of RGS5 was chemically synthesized with a sequence of CKGLAALPHSCLER by following a previous study (20). A similar phenomenon was observed when the N terminus of RGS5 was incubated with ADO (Fig. 2B). After incubation with the peptide substrate for 1 min, the high-spin ferric signal centered at $g = 4.30$ was converted to a new signal at $g = 4.42$ and 4.26 with a decreased ferric population; small resonances at $g = 9.13$ and 5.02 were present. These new signals have different $g$ values compared with the samples incubated with cysteamine, which suggests the electronic structure of the ferric iron center differs upon cysteamine or peptide ligation, though both substrates are able to reduce Fe$^{III}$-ADO. At longer incubation times of 3 and 6 min, the primary ferric signal, as well as the minor resonances, continuously decreased. After a 10-min incubation, only a small fraction of the major ferric species remained oxidized, and the minor resonances were nearly absent. Together, the above results revealed that both the cysteamine and N-terminal cysteine-containing peptide could readily reduce the ferric center of ADO through their corresponding thiol groups, implicating thiolate ligation in both cases.

It was previously unknown that the primary substrate of ADO can reduce its ferric ion to the catalytically active ferrous form. To verify this finding, especially that the disappearance of the nonheme ferric ion EPR signal upon substrate binding was because of the change of the metal oxidation state rather than the relaxation caused spectral line broadening of the EPR signal, a separate set of EPR experiments was conducted on mouse ADO (Fig. 3A). The mouse ADO lacks 12 amino acids that are present in the N terminus of the human protein. However, this region is not directly related to the active site (Fig. S1). The mouse protein can tolerate chemical oxidation. Thus, ferricyanide was employed to oxidize as-isolated ADO for 5 h. After removing excess oxidant by desalting and buffer exchange, the oxidized ADO was made anaerobic and mixed with 50 mM cysteamine for 1.5 min (C), 5 min after cysteamine incubation (D), 20 min after cysteamine incubation (E), and addition of $^{15}$NO (F). The signal intensity is presented at an arbitrary scale to put each spectrum in approximately the same height for easy comparison. Spectra were recorded at 10 K with 1.0 mW microwave power and 0.5 mT modulation.
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Characterization of the ADO iron center by Mössbauer spectroscopy

To elaborate the substrate-induced iron reduction and analyze the iron inhomogeneity in the ligand-free ADO by an independent technique, we executed a Mössbauer spectroscopic characterization of the iron center of as-isolated mouse ADO. The concentrated, as-isolated ADO protein was analyzed by Mössbauer spectroscopy at 4.2 K. For resting ADO, the concentrated, as-isolated ADO protein was analyzed by Mössbauer spectroscopy at 4.2 K. For resting ADO, the observed zero-field Mössbauer spectrum was fitted to three species. As shown in Fig. 4A, ~23% of the total iron was assigned to a ferric doublet (red trace, \( \delta = 0.27 \text{ mm/s}; \Delta E_Q = 0.47 \text{ mm/s} \)), and the remaining 77% was in the ferrous state. The 77% ferrous iron was composed of two quadrupole doublets, \( \text{Fe}^{II}_A \) [purple trace, \( \delta_A = 1.16 \text{ mm/s}; \Delta E_Q(A) = 2.45 \text{ mm/s} \)] and \( \text{Fe}^{II}_B \) [green trace, \( \delta_B = 1.26 \text{ mm/s}; \Delta E_Q(B) = 2.93 \text{ mm/s} \)], with the ratio 1:2.2. The quadrupole doublets and isomer shift values of \( \text{Fe}^{II}_A \) and \( \text{Fe}^{II}_B \) suggest a high-spin ferrous iron coordinated by N- and O-atom ligands, forming penta- or hexa-coordination. All thiol dioxygenases known so far possess a 3-His motif as the first sphere metal coordination in a cupin fold (1), and this is also expected to be the case for ADO (27). Hence, the iron center presumably contains two or three solvent-derived ligands. The multiple species observed also suggest that the geometry of the iron center is inhomogeneous. The Mössbauer spectrum is largely in accord with the results reported for other thiol dioxygenases with the 3-His coordination motif (9, 31, 38).

To pinpoint how the iron center responds to the addition of cysteamine, the binary complex was characterized (Fig. 4B). The spectral fitting indicates that there was still one ferric and two ferrous species present in ADO upon substrate incubation. The quadrupole splitting and isomer shift of the ferric species (red trace) had no measurable change; however, its population decreased to 14%. The Fe\(^{II}_A\) species (purple trace) also maintained the same \( \delta \) and \( \Delta E_Q \) values but with a reduced population. Notably, the Fe\(^{II}_B\) species in the resting ADO (green trace) completely disappeared, concomitant with the appearance of a new, predominant ferrous species, termed Fe\(^{II}_C\) [blue trace, \( \delta_C = 1.19 \text{ mm/s}; \Delta E_Q(C) = 2.85 \text{ mm/s} \)] that comprises 72% of the iron content. The Mössbauer parameters are summarized in Table 1. This observation suggests that Fe\(^{II}_B\) is directly responsible for the substrate binding and reduction, and the newly evolved Fe\(^{II}_C\) is the binary ES complex. Moreover, the addition of cysteamine induces homogeneity of the iron species by converting the inactive species, Fe\(^{III}\) and Fe\(^{II}_A\), to the active form, Fe\(^{II}_B\).

By subtracting the fitted signals of Fe\(^{III}\) and Fe\(^{II}_A\) from the experimental spectra, the spectra of active enzyme alone (green line) and ES complex (blue line) are shown in Fig. 4C. Comparing the subtracted spectra reveals a change to both isomer shift and quadrupole splitting of the iron center upon substrate binding. The decrease of the \( \delta \) value, from 1.26 mm/s of the substrate-free enzyme to 1.19 mm/s of the ES complex, agrees with sulfur atom coordination to the ferrous center (9, 38, 39).

Detection of by-products in the substrate-mediated enzyme reactivation

Based on the Mössbauer and EPR analyses, the reduction of Fe\(^{III}\)-ADO occurred through the interaction between the thiol of the substrate and the iron center. The reactivation of ADO from the ferric form to the catalytically active ferrous form is at the expense of substrate oxidation. The process forming disulfide product could proceed through the following reaction (Equation 1):

\[
\text{Fe}^{III}-\text{ADO} + 2\text{R}-\text{SH} \rightarrow \text{Fe}^{II}-\text{ADO} + \text{R-S-S-R} \quad \text{(Eq. 1)}
\]

To investigate whether a fraction of the substrate forms R-S-S-R, cysteamine was anaerobically incubated with the oxidized mouse ADO at room temperature for one hour. Cysteamine is known to be readily oxidized to cystamine, which is a dimerized...
product with a disulfide bridge. Thus, commercially purchased cysteamine unavoidably contains cystamine impurity. A control sample was prepared with cysteamine in the absence of ADO. Samples were then filtered and analyzed by LC-MS. As shown in Fig. S2, although a portion of cysteamine was already dimerized and eluted as cystamine in the control, the amount of cysteamine had a noticeable increase after anaerobic incubation with the oxidized enzyme. This shows that the oxidized product is indeed the substrate dimer with a disulfide bond.

The most appealing conclusion was, however, derived from the experiment with the peptide substrate. The N-terminal peptide contains 14 amino acids (CKGLAALPHSCLER), which should have a larger steric hindrance than cysteamine. Although we did observe the N-terminal RGS5 peptide could readily reduce the iron center and reactivate the ferric form of ADO to the catalytically active form, whether or not the dimeric peptide can still be formed as the product was in question. We next executed the experiment with the RGS5 peptide and human ADO. Similarly, RGS5 (5 mM) was anaerobically incubated with oxidized ADO for one hour and analyzed using high-resolution MS. The control of peptide alone generated a spectrum of pure RGS5 peptide with peaks carrying different charges (Fig. 5A). The m/z values of 375.1975, 499.9274, and 749.3871 represent the intact peptide at +4, +3, and +2 charge states, respectively, with a mass error of less than 1.1 ppm. The heavier species shown at m/z 999.1714 corresponds to a dimerized product. It is more favored when encountering the thiol within the same peptide chain. Because the RGS5 peptide contained a disulfide bond, as we observed by intact protein MS. For such a reason, substrate or product quantitation was not further pursued to investigate the precise stoichiometry of the reduction.

**Cysteamine alters the binding behavior of nitric oxide to the nonheme iron center of ADO**

In the resting state, nonheme iron–dependent thiol dioxygenases contain a mononuclear ferrous iron coordinated by three histidine residues in their active sites. Unlike heme-dependent iron proteins, which have unique UV-visible, EPR, and other spectroscopic features, these nonheme iron centers lack representative spectroscopic characteristics to monitor the process of substrate binding and reaction turnovers. Historically, in spectroscopic studies, using *NO as a spin probe and an oxygen surrogate to form an EPR-visible nitrosyl complex is a common

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**Figure 5. MS analysis of RGS5 peptide before (A) and after (B) incubation with oxidized human ADO.** Charge states and m/z values of RGS5 N terminus, intramolecular cross-linked peptide, and intermolecular cross-linked peptide are marked in black, red, and blue, respectively. The putative structures and theoretical molecular weights are shown on the right.
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Figure 6. EPR spectra of *NO addition to ADO in the absence (A) and presence (B and C) of cysteamine. *NO was released by 0 (black), 0.1 (gray), 0.5 (light gray), and 2.5 (red) equiv of *NO releasing agent (1 equiv of agent produces approximately 1.5 equiv of *NO). A, in the absence of substrate, changes occur in the low-field (high-spin) region with increasing *NO. Spectra were taken at 10 K with 50 mW microwave power. B, in the presence of substrate, changes occur in both high- and low-spin regions with increasing *NO. Spectra were taken at 10 K, 1 mW. C, in the presence of substrate, changes occur in the g = 2 region with increasing *NO. Spectra were taken at 50 K with 0.05 mW microwave power.

Methods to investigate how primary substrate and dioxygen interact with an EPR-silent ferrous center.

Initially, we sought to test the binding behavior of *NO to FeII-ADO. The reduced FeII-ADO protein was completely EPR silent (Fig. 6A, black trace). Addition of 0.1 equivalent (equiv) of *NO releasing agent to the ferrous enzyme resulted in the formation of a high-spin (S = 3/2) resonance with g values of g\text{max} = 4.10, g\text{mid} = 3.95, and g\text{min} = 2.00 (gray trace). The g value features correspond to an E/D value of ~0.01 based on the S = 3/2 rhombogram. This nearly axial EPR signal corresponds to the nonheme ferrous iron center bound to one equivalent of *NO generating [Fe(ONO)]7, which can also be described as an antiferromagnetic coupling between a high-spin FeIII (S = 5/2) and a nitroxylate anion (NO–, S = 1) based on the Feltham–Enemark theorem (40). This high-spin signal is similar to the [Fe(ONO)]7 species of 3MDO in the presence of its substrate (8). Additional *NO releasing agent (0.5 and 2.5 equiv) was then added to the enzyme, resulting in an increase in the signal intensity of the [Fe(ONO)]7 species (light gray and red traces). The high-spin signal reached a plateau after the addition of 2.5 equiv of *NO releasing agent, concomitant with the formation of a minor low-spin axial signal with g\text{L} = 2.039 and g\text{||} = 2.015. The line shape and g values of this new S = 1/2 signal are reminiscent of a dinitrosyl species found in synthetic and protein-based samples (41–43). However, the dinitrosyl species did not increase much, even when excess *NO was added to the sample, and its population remains insignificant, accounting for 3% of the total FeII-ADO (Fig. S3 and S4), indicating that FeII-ADO tends to bind only 1 equiv of *NO in the absence of substrate. The trace amount of dinitrosyl species only forms when excess *NO is present, and the mononitrosyl species is predominant in substrate-free ADO.

Next, we tested whether the presence of cysteamine would alter the binding behavior of *NO to the ferrous center. *NO was introduced into FeII-ADO precomplexed with cysteamine. The ES complex was completely EPR silent (Fig. 6B, black trace). Once a small amount of *NO releasing agent (0.1 equiv) was added, interestingly, no EPR signal was observed in the high-spin region; rather, a low-spin (S = 1/2) signal was observed with g values of g\text{L} = 2.040 and g\text{||} = 2.014 (Fig. 6B). This signal is very similar to the signal from dinitrosyl species in the aforementioned substrate-free ADO samples; however, the g values are slightly different. These two signals also responded very differently upon *NO titration. This new low-spin signal produced in the presence of a substrate is more sensitive to the population of *NO (Fig. 6C). Compared with similar signals reported in the literature, it is best explained as a substrate-ligated dinitrosyl iron center (DNIC), i.e. a B-type [Fe(ONO)2]9 species that has been reported in other nonheme iron-binding proteins (Table S1) (42–46). The addition of more *NO into the sample increased the DNIC signal, but no [Fe(ONO)]7 (S = 3/2) signal was observed throughout the addition of *NO to the ES complex. These experiments show that ligation of the substrate to the iron center increases the binding affinity of *NO and alters the binding stoichiometry of *NO to the Fe. The same DNIC EPR spectra were obtained from cysteamine-reduced ADO samples, as observed from the ascorbate-reduced ADO samples upon exposure to *NO and the addition of cysteamine (Fig. 3F and Fig. S5). Notably, such a dinitrosyl iron complex was not described in previous EPR studies of other thiol dioxygenases. Addition of *NO to cysteine-bound CDO mainly gave rise to a low-spin (S = 1/2), [Fe(ONO)]7 complex (47), whereas a high-spin (S = 3/2), [Fe(ONO)]7 complex was generated in the case of substrate-bound 3MDO (8).

In this work, ADO samples incubated with *NO generated either mononitrosyl (S = 3/2) or dinitrosyl (S = 1/2) complexes, depending on the presence of the substrate. These complexes responded differently to temperatures and microwave power. Therefore, EPR spectra were recorded under different conditions according to the power saturation profile (Fig. S6).

Cysteamine monodentate coordinates to ADO through thiolate ligation

To further investigate the observation that cysteamine alters the binding behavior of *NO to the iron center, cysteamine was titrated into ADO preincubated with excess *NO. As shown in Fig. 7, A and B, titration of cysteamine gradually converted the high-spin [Fe(ONO)]7 complex into the low-spin [Fe(ONO)2]9, which is consistent with the stoichiometric change of *NO from one to two. Additionally, the newly produced DNIC EPR signal at g\text{L} = 2.040, g\text{||} = 2.014 continuously increased upon
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RG55 peptide coordinates to ADO through the N-terminal thiol

Like cysteamine, the reported N-terminal cysteine-containing protein substrates of ADO have a free thiol and amine. However, we wondered if the peptide chain binds to the iron in the same monodentate manner. Thus, the N terminus of RG55 peptide was titrated into the enzyme-nitrosyl complex, and the changes were monitored by EPR spectroscopy. As shown in Fig. 8A, with no substrate present, the enzyme-nitrosyl complex had a pronounced high-spin (S = 3/2) [Fe(NO)]7 signal (black trace). Under the experimental conditions (10 K, microwave power of 50 mW), an additional positive absorptive peak was observed at g = 1.98, which was assigned to excess free *NO in solution (49). Upon titration of the peptide substrate, the high-spin signal gradually decreased, consistent with the observation from the samples that underwent cysteamine addition (Fig. 8A). Additionally, the EPR signal of free *NO decreased and completely disappeared upon substrate addition at 5 mM concentration (Fig. 8A, red trace). The decrease of free *NO signal suggests that the free *NO in solution was consumed to form other nitrosyl species.

Next, the low-spin region of the above samples was analyzed at 50 K. The initial enzyme-nitrosyl complex had a small dinitrosyl signal at g⊥ = 2.039, g∥ = 2.015 (Fig. 8B, black trace). As
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Figure 8. EPR and absorbance spectra of RGS5 peptide addition to the ferrous form of human ADO precomplexed with excess *NO. A, ADO-nitrosyl complex after addition of 0 (black), 0.2 (gray), 1 (light gray), and 5 (red) mM RGS5 peptide. Spectra were taken at 10 K with 50 mW microwave power. B, the g = 2 region of enzyme-nitrosyl complexes after addition of 0 (black), 0.2 (gray), 1 (light gray) and 5 (red) mM RGS5 peptide. An axial signal (*, amplified 9 times in the inset) and a broad signal (#) were observed. Spectra were taken at 30 K with 0.05 mW microwave power. C, UV-visible absorption spectra of Fe2⁺-ADO (black), ADO-nitrosyl complex (green), ADO complexed with *NO and cysteamine (blue), and ADO complexed with *NO and RGS5 peptide (red).

expected, after titrating the peptide substrate, the major resonance of the low-spin \( (S = 3/2) \), \( \text{[Fe(NO)₂]}_9 \) complex shifted to \( g_\perp = 2.040, g_\parallel = 2.014 \) and intensified with increasing concentration of the peptide. In the presence of 5 mM RGS5 peptide, the majority of the iron center was converted to DNIC, which accounts for 61% of the total iron content (Fig. S4). This observation mirrors the phenomenon caused by cysteamine, which suggests the iron center was coordinated by a thiol group and two *NO molecules, i.e. the peptide monodentate coordinates to the iron center via thiolateigation. The peptide substrate alters the binding stoichiometry of *NO from one to two equivalents, as cysteamine does. Based on the EPR spectra, the DNIC \( \text{[Fe(NO)₂]}_9 \) signal was more responsive upon peptide addition than cysteamine addition, especially at lower concentrations (<1 mM) (Fig. S4). This suggests the iron center of ADO has a higher affinity to the peptide substrate than cysteamine, so that the peptide substrate converts the mononitrosyl complex to the ternary catalytic mimic, thiol-bound \( \text{[Fe(NO)₂]}_9 \) complex more efficiently. This interpretation is consistent with the reported \( K_m \) values of 71.5 and 3400 \( \mu \)M for the peptide and cysteamine, respectively (20, 27).

Other than the predominant dinitrosyl resonances at \( g_\perp = 2.040, g_\parallel = 2.014 \), two new signals were seen in the low-spin region of peptide samples that were absent from the samples of cysteamine. An axial signal arose at \( g_\perp = 2.244, g_\parallel = 2.131 \) (Fig. 8B, marked by an asterisk), and a broad signal centered at \( g = 2.032 \) (marked by the pound sign) overlapped the major resonance of thiol monodentate-bound, \( \text{[Fe(NO)₂]}_9 \) complex. Both signals were very similar to the signals shown in CDO nitrosyl studies, and the \( g = 2.032 \) signal corresponds to a characteristic, cysteine bidentate-bound, ferrous-nitrosyl \( \text{[Fe(NO)]}_7 \) complex (28, 47). Such an observation suggests that at high concentrations of a peptide substrate (at mM level), the amine of the peptide interacts with the iron center, forming bidentate coordination, but its population is smaller than that of the predominant, thiol monodentate coordination.

As a nonheme iron-dependent enzyme, the ferrous form of human ADO at the resting state is expected to be colorless and uncharacteristic above 280 nm by UV-visible spectroscopy. However, the concentrated protein (200 \( \mu \)M) showed a pale green color, and its absorbance spectrum exhibited a small peak with a \( \lambda_{max} \) at 405 nm (Fig. 8C, black trace). A similar spectral feature is also present in mouse ADO, with a \( \lambda_{max} \) at 409 nm (Fig. S6). This absorbance feature was only observable in concentrated protein samples and consistently showed in samples after multistep purification and from both human and mouse ADO expression systems. The origin of this absorbance feature remains unresolved. It may result from a small molecule tightly bound to a small fraction of protein during protein expression and cell lysis. Anaerobic addition of *NO gave rise to new, broad peaks centered at 340 and 406 nm (green trace), which resemble the features observed in the nitrosyl samples of other nonheme iron enzymes, including isopenicillin N synthase (IPNS) which catalyzes oxidative thioether formation of a thiol-containing substrate (39). Such absorption bands are attributed to charge transfer transitions associated with the \( \text{[Fe(NO)]}_7 \) complex. The addition of cysteamine resulted in a distinct peak with a \( \lambda_{max} \) at 397 nm, but the peaks observed in the enzyme-nitrosyl complex were no longer present (blue trace). The addition of RGS5 peptide also generated a new peak but with a different \( \lambda_{max} \) at 390 nm and more intensity than that of the cysteamine-bound nitrosyl complex (red trace). The absorbance bands centered at about 400 nm are suggestive of thiol-containing mononuclear DNICs that have been previously documented (50, 51). Therefore, our UV-visible spectroscopic results again confirm the formation of ADO-based dinitrosyl complexes in the presence of substrates. The substrate-bound nitrosyl complex of IPNS had absorbance features at 508 and 720 nm that are quite different from the ADO case, because DNIC was absent in the former. Together with the EPR and absorption spectra, the peptide substrate exhibits binding behavior similar to that of ADO as cysteamine but with higher affinity. Moreover, the thiol-bound, mononuclear DNIC has been independently characterized by both spectroscopies.

Discussion

Among the thiol dioxygenases, CDO has been unambiguously characterized to bind its substrate with bidentate coordination. Without an ES complex structure, the substrate-binding mode of 3MDO is controversial because of the inconsistency between spectroscopic data and a computational study.
Substrate incubation with oxidized ADO leads to the finding that the active site of ADO could be readily reduced by the substrate thiol group and, hence, reactivate the enzyme if it were to become oxidized. As a result, substrates form disulfide by-products, for example, cysteamine generates cystamine and RG55 peptide produces either intra- or intermolecular disulfide cross-links. Other peptides whose dioxygenation may be catalyzed by ADO could have different disulfide products based on the location of cysteine, because the formation of a disulfide bond is a nonenzymatic outcome that occurs after the activated thiol is liberated from the active site. Importantly, the substrate-mediated reduction could have a profound impact on oxygen homeostasis. ADO regulates N-terminal cysteine-containing proteins with an extremely high $K_m$ for oxygen (>500 μM) (20), which implies that the activity of ADO keeps a linear relationship with the oxygen concentration within the physiological range. The reduction effect of the primary substrates keeps the enzyme ready in an active form to respond as an oxygen sensor, even when facing oxidative stress. Such a substrate-induced reactivation was also reported in a life-essential enzyme, tryptophan 2,3-dioxygenase, that generates the tryptophan dimer and mono-oxygenated tryptophan as the by-products (61). The finding from ADO is another example of how metalloenzymes maintain their catalytic activity to defend against an oxidizing environment, such as that in cancer cells. We have previously shown that when the primary substrate and $O_2$ are both available, ADO autocatalytically generates a protein-bound Cys-Tyr cofactor by the iron center that functions as a catalytic amplifier to more rapidly reduce the level of the free-thiol-bearing substrate (27). Thus, the elevated level of the substrate can reactivate ADO and bring its catalysis up to speed to respond to the rise of thiol levels.

EPR results with nitrosyl complexes of ADO have striking differences from other nonheme iron-dependent enzymes that oxidize thiol-containing substrates, including CDO, 3MDO, and IPNS (Table 2). In the absence of substrate cysteine, the CDO-nitrosyl complex exhibited an $S = 3/2$ and mononitrosyl $[\text{Fe(NO)}]^+$ EPR spectrum with features similar to those of the enzyme-nitrosyl complex of ADO. The substrate bidentate coordination of CDO promotes the generation of a broad $S = 1/2$, substrate-bound $[\text{Fe(NO)}]^+$ signal. A DNIC was also seen in CDO as a minor species (~5% of the total iron), which was not a substrate-bound enzyme-nitrosyl complex (47). In the absence of a substrate, 3MDO was unable to bind *NO, and its substrate coordination mode is unclear. However, the substrate-bound nitrosyl complex gave rise to an $S = 3/2$, $[\text{Fe(NO)}]^+$ species. This high-spin species showed an EPR spectrum very similar to that of the substrate-free nitrosyl complexes of ADO and CDO, which implies a unique substrate binding mode for 3MDO that differs from both ADO and CDO. IPNS is a well-documented enzyme that catalyzes double-ring closure of a tripeptide substrate that monodentate binds to the iron center via a thiol group (62). Its nitrosyl complex in the absence of the substrate was reported to have EPR features similar to those of ADO and most nonheme iron-dependent proteins (39). In contrast to the low-spin ($S = 1/2$, $[\text{Fe(NO)}]^+$ species of ADO, the ES-nitrosyl complex of IPNS became more rhombic than substrate-free nitrosyl complex but remained as a high-spin ($S = 3/2$...
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Table 2

| Protein | Protein ligand | Substrate coordination | Enzyme-nitrosyl complex | ES-nitrosyl complex |
|---------|----------------|------------------------|-------------------------|---------------------|
| ADO     | 3-His          | Monodentate, via thiol | [Fe(NO)]\(_7\), S = 3/2, g = 4.10, 3.95, 2.00; 340, 406 nm | [Fe(NO)]\(_7\), S = 1/2, g = 2.04, 2.01; 390 or 397 nm |
| CDO     | 3-His          | Bidentate, via thiol and amine | [Fe(NO)]\(_7\), S = 3/2, g ~ 4, 4, 2 | [Fe(NO)]\(_7\), S = 1/2, g = 2.07, 2.02, 1.98 |
| 3MDO\(_{c}\) | 3-His | Unclear, thiol involved | Not applicable | [Fe(NO)]\(_7\), S = 3/2, g = 4.06, 3.96, 2.00 |
| IPNS\(_{d}\) | 2-His-2-carboxylate | Monodentate, via thiol | [Fe(NO)]\(_7\), S = 3/2, g = 4.09, 3.95, 2.00; 340, 430, 600 nm | [Fe(NO)]\(_7\), S = 3/2, g = 4.22, 3.81, 1.99; 508, 720 nm |

\(\text{a}\) Results from this work.
\(\text{b}\) Results from references 28 and 47.
\(\text{c}\) Results from references 8 and 52.
\(\text{d}\) Results from references 39 and 62.

*NO affects the N-terminal cysteine oxidation and the stability of RGS proteins (66), a process that is regulated by ADO (20). The ADO-nitrosyl complexes presented here may provide a direction for investigating how the N-degron pathway acts as an *NO sensor. Hence, even if the ES-nitrosyl complex is not directly relevant to the catalytic mechanism, the DNIC formation in ADO certainly opens a new avenue for correlating ADO with *NO-related physiological study.

Concluding remarks

Thiol dioxygenases are a family of 3-His coordinated ferrous enzymes that activate molecular oxygen and transform thiol substrates to corresponding sulfinates. Their biological significance is in regulating sulfur metabolism and thiol homeostasis in various organisms. Although they share some structural and catalytic features, those enzymes specifically handle different thiol-containing substrates, ranging from small molecules to N-terminal cysteine-containing peptides. As the only enzyme catalyzing the oxidation of substrates without a carboxylate moiety, the iron center of ADO and its interaction with cysteamine and RGS5 peptide were investigated here via biophysical and bioanalytical techniques, including EPR, Mössbauer, UV-visible spectrosopies, and MS. The results suggest that ADO preserves an inhomogeneous ferrous center in the resting state, whereas only one form is directly responsible for substrate binding and subsequent catalysis. Other forms could be readily converted to the active form in the presence of primary substrates. We also found a substrate-mediated reduction of the iron center generating disulfide by-products. These two features compose a substrate-induced reactivation mechanism that enables ADO to function as an oxygen sensor under physiological conditions. *NO, as an oxygen mimic and spin probe, was incubated anaerobically with ADO to examine further the binding behavior of primary substrates and substrate analogues. Our data suggest that substrates could alter the binding stoichiometry of *NO from one equivalent to two, generating a thiolate-bound, low-spin, B-type DNIC signal that was not previously observed in other thiol dioxygenases or oxidases. Such an observation indicates an oxygen side-on binding mode distinct from those of other thiol dioxygenases. The absence of an amine in substrate coordination also implies that the thiol dioxygenase-promoted oxidation and cofactor biogenesis do not require the involvement of an amine. Altogether, our work provides the first systematic investigation of the ferrous center
and substrate binding of ADO, and it contributes to the overall understanding of the substrate specificity in thiol dioxygenases.

Materials and methods

Chemicals and protein preparation

Cysteamine (95%), ethanolamine (99%), and 2-mercaptoethanol (99%) were purchased from Acros Organics. Peptide substrate with the sequence of CKGLAALPHSCLER (>95%) was synthesized by Biomatik, which corresponds to the first 14 amino acids of the Met-excised N terminus of RGS5 as reported previously (20).

Cloning, overexpression, and purification of His6-tagged, full-length human ADO were described previously (27). The untagged protein was obtained through the cleavage of His tag by tobacco etch virus protease. The tagged and untagged proteins showed identical activity ($k_\text{cat}$ of 1.2 s$^{-1}$ and $K_m$ of 3.4 mM cysteamine) (27) and spectral features in this study.

The mouse ADO expression plasmid was a generous gift from Dr. Martha H. Stipanuk (2). Most of the spectroscopic work, except for Mössbauer characterization, was carried out on both the mouse and human proteins. No difference was observed unless otherwise stated. The results presented were mostly from the human version of the protein. Cell culture was observed unless otherwise stated. The results presented were on both the mouse and human proteins. No difference was observed unless otherwise stated. The results presented were mostly from the human version of the protein. 

Preparation and EPR measurement of nitrosyl complexes

O$_2$-free Fe$^{III}$-ADO was freshly prepared to generate nitrosyl complexes. Sodium ascorbate (100 $\mu$M) was included to keep the protein in a reduced environment. Nitrosyl samples were prepared by the addition of *NO-releasing agent, DEA-NON-Oate (>98%, Cayman Chemical), to Fe$^{III}$-ADO (200 $\mu$M) and incubating for 20 min under anaerobic conditions. The amount of *NO in each prepared sample was estimated through titration of *NO releasing agent to the enzyme solution (1.5 mol of *NO can be liberated per mol of DEA NONOate). For substrate or analog titration, small molecules or RGS5 peptide were added to the enzyme-nitrosyl complex to a concentration of 0.2, 1, 5, or 30 mM. For *NO addition experiments, cysteamine (30 mM) was complexed with Fe$^{II}$-ADO (200 $\mu$M) prior to the addition of *NO-releasing agent. The samples were transferred to quartz EPR tubes and slowly frozen in liquid nitrogen. EPR spectra were recorded on a Bruker E500 X-band spectrometer equipped with a cryogen-free 4 K temperature system as described elsewhere (32–34), with either a dual mode (9.6 GHz, for small molecules) or an SHQ high-Q resonator (9.4 GHz, for peptide) at 100-kHz modulation frequency, 0.6-mT modulation amplitude. High-spin signals were measured at 10 K with a microwave power of 50 mW and one scan for each spectrum, and low-spin signals were measured at a higher temperature, 50 K, with a lower microwave power of 0.05 mW and an average...
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of four scans for each spectrum. To visualize both features of high- and low-spin EPR signals in the same spectrum, the data acquisition conditions were set to a temperature at 10 K and microwave power of 1.0 mW.

UV-visible absorbance spectroscopy

The preparation of nitrosyl complexes for UV-visible absorbance spectroscopic measurement was identical to the procedure described in the EPR measurement of nitrosyl complexes. 200 μM O₂-free Fe²⁺-ADO was used in each sample. Excess NO was precomplexed with enzyme prior to the addition of 10 mM cysteamine and RG5S peptide. All spectra were recorded in a 1-cm, anaerobic quartz cuvette (SpectR Ecology) using a Lambda 25 spectrometer (Perkin Elmer).

Mössbauer spectroscopy

To generate ⁵⁷Fe-incorporated ADO with high metal occupancy, ⁵⁷Fe stock solution was supplied to the metal-depleted LB medium during cell culture. The stock solution was prepared by dissolving ⁵⁷Fe powder (95.4%, Science Engineering & Education Co.) in 2.5 M H₂SO₄ under anaerobic conditions, which was then anaerobically incubated at 60 °C with continuous stirring until completely dissolved. The stock solution of the pH was drop adjusted by sodium hydroxide, as described previously (35). A final concentration of 80 μM ⁵⁷Fe was added for cell growth. Expression and purification of ADO were done as aforementioned, and the protein was finally desalted into a 100 mM Tris-HCl, pH 8.0, 25 mM NaCl, and 5% glycerol buffer. The protein was then concentrated to 2–3 mM. A 500-μl aliquot of the concentrated protein was transferred into a Mössbauer cup and frozen in liquid nitrogen. Mössbauer spectra were recorded in a weak-field spectrometer equipped with a Janis 8DT variable-temperature cryostat as described previously (35). The zero velocity of the spectra refers to the centroid of a room-temperature spectrum of a metallic iron foil. For samples of the enzyme-substrate complex, cysteamine was added to a final concentration of 30 mM under anaerobic conditions. All spectra were collected at 4.2 K with 50-mT applied field parallel to γ-radiation. Isomer shifts are reported relative to Fe metal at 10 K and micro-wave power of 1.0 mW.

Conflicting of interest—The authors declare that they have no conflicts of interest with the contents of this article.

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Supporting Information

Characterization of the non-heme iron center of cysteamine dioxygenase and its interaction with substrates†

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Table S1. A summary of DNICs characterized by EPR

| Species                     | A-type *<sub>g_x, g_y, g_z</sub> | B-type <sub>g⊥, g∥ or g<sub>av</sub></sub> | Reference |
|-----------------------------|----------------------------------|------------------------------------------|-----------|
| DNIC-HoSF                   | 2.053, 2.029, 2.011              | 2.033, 2.014                             | (1)       |
| DNIC-L-cysteine             |                                   | 2.04, 2.01                               | (2)       |
| DNIC-BSA                    | 2.05, 2.04, 2.01                 |                                          | (2)       |
| DNIC-glutathione            |                                   | 2.045, 2.014                             | (3)       |
| DNIC-homocysteine           |                                   | 2.045, 2.014                             | (3)       |
| DNIC-thiosulphate           |                                   | 2.045, 2.014                             | (3)       |
| DNIC-N-acetylpencillamine   |                                   | 2.045, 2.014                             | (3)       |
| DNIC-Rat liver homogenate   |                                   | 2.03                                     | (4)       |
| DNIC-FeFur                  | 2.042, 2.032, 2.017              |                                          | (5)       |
| DNIC-Hb                     |                                   | 2.04, 2.014                             | (6)       |
| DNIC-NorA                   |                                   | 2.041, 2.018                             | (7)       |
| DNIC-Rieske cluster         | 2.09, 2.06, 2.04                 |                                          | (8)       |
| DNIC-BFR                    | 2.04, 2.015, 2.00                |                                          | (9)       |
| DNIC-aconitase              |                                   | 2.031, 2.012                             | (10)      |
| DNIC-HiPIP                  |                                   | 2.03                                     | (11)      |
| DNIC-CDO                    |                                   | 2.03, 2.01                              | (12)      |
| DNIC-ADO                    |                                   | 2.040, 2.014                             | This work |

* A-type and B-type DNICs were defined by Chasteen and colleagues (1).
Figure S1. Multiple sequence alignment of human ADO (hADO), mouse ADO (mADO), and human CDO (hCDO). hADO and mADO share 85.6 % identity and 89.3 % similarity. hADO and hCDO share 15.8 % identity and 26.5% similarity, but the second sphere residues responsible for substrate recognition in hCDO are mostly not conserved in hADO (highlighted by asterisks). The alignment was generated by Clustal Omega (13). Highly and strictly conserved residues are boxed and highlighted in black background, respectively.
Figure S2. Selected ion monitoring LC-MS spectra. (A) Sample of cysteamine alone as a control. (B) Sample of cysteamine incubated with oxidized ADO. The green, red, and blue traces represent the chromatograms monitored at m/z values of 153 for cystamine, 78 for cysteamine, and 62 for the internal standard. MS spectra of the fractions eluted at 1.29, 1.42, and 1.59 min are shown in C, D, and E, respectively.
Figure S3. The low-spin EPR signal in samples of ADO with addition of increasing \textsuperscript{·}NO releasing agent. In the absence of substrate, only a trace amount of dinitrosyl species at $g_\perp = 2.039$, $g_\parallel = 2.015$ was formed when an excess of \textsuperscript{·}NO was introduced. Spectra were collected at 50 K, microwave power of 0.05 mW with an average of four scans.
Figure S4. Population of DNIC relative to the total amount of iron content in ADO. In the presence of saturated substrate, either cysteamine (blue) or RGS5 peptide (black), DNIC accounts for the predominant species formed by Fe\textsuperscript{II}-ADO. Quantitation was achieved by double integration and comparison with a Cu(II)-EDTA standard.
Figure S5. EPR spectra of DNIC generate by (A) ascorbate-reduced and (B) cysteamine-reduced ADO samples. Spectra were recorded at 50 K with 0.05 mW microwave power.
Figure S6. The absorbance spectrum of ferrous form of mouse ADO (black trace) and after addition of \( \cdot \)NO. The inset shows the 409 nm feature of mouse ADO.
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