A Biochemical Nickel(I) State Supports Nucleophilic Alkyl Addition: A Roadmap for Methyl Reactivity in Acetyl Coenzyme A Synthase

A. C. Manesis
B. W. Musselman
Breena C. Keegan  
*Trinity University*

Jason M. Shearer  
*Trinity University, jshearer@trinity.edu*

N. Lehnert

See next page for additional authors

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A Biochemical Nickel(I) State Supports Nucleophilic Alkyl Addition: A Roadmap for Methyl Reactivity in Acetyl Coenzyme A Synthase

Anastasia C. Manesis, Bradley W. Musselman, Brenna C. Keegan, Jason Shearer, Nicolai Lehnert, and Hannah S. Shafaat

†Department of Chemistry and Biochemistry, The Ohio State University, 100 W. 18th Avenue, Columbus, Ohio 43210, United States
§Department of Chemistry, University of Michigan, 930 N. University Avenue, Ann Arbor, Michigan 48109, United States
⊥Department of Chemistry, Trinity University, One Trinity Place, San Antonio, Texas 78212, United States

Supporting Information

ABSTRACT: Nickel-containing enzymes such as methyl coenzyme M reductase (MCR) and carbon monoxide dehydrogenase/acetyl coenzyme A synthase (CODH/ACS) play a critical role in global energy conversion reactions, with significant contributions to carbon-centered processes. These enzymes are implied to cycle through a series of nickel-based organometallic intermediates during catalysis, though identification of these intermediates remains challenging. In this work, we have developed and characterized a nickel-containing metalloprotein that models the methyl-bound organometallic intermediates proposed in the native enzymes. Using a nickel(I)-substituted azurin mutant, we demonstrate that alkyl binding occurs via nucleophilic addition of methyl iodide as a methyl donor. The paramagnetic NiIII-CH3 species initially generated can be rapidly reduced to a high-spin NiII-CH3 species in the presence of exogenous reducing agent, following a reaction sequence analogous to that proposed for ACS. These two distinct bioorganometallic species have been characterized by optical, EPR, XAS, and MCD spectroscopy, and the overall mechanism describing methyl reactivity with nickel azurin has been quantitatively modeled using global kinetic simulations. A comparison between the nickel azurin protein system and existing ACS model compounds is presented. NiIII-CH3 Az is only the second example of two-electron addition of methyl iodide to a NiI center to give an isolable species and the first to be formed in a biologically relevant system. These results highlight the divergent reactivity of nickel across the two intermediates, with implications for likely reaction mechanisms and catalytically relevant states in the native ACS enzyme.

INTRODUCTION

Nickel enzymes are responsible for some of the most important energy conversion reactions found in nature, specifically those relevant to carbon-centered processes. Within the environment, one-carbon species such as CO, CO2, and CH4 are continually interconverted through nickel-dependent gas cycles. The selection of nickel to accomplish such challenging organic transformations within anaerobic archaea and bacteria implicates its use in primordial life processes, particularly when considering the high concentrations of nickel in the earth’s early oceans and the broad reactivity of nickel with reduced, sulfur-containing compounds. The global carbon cycle relies heavily on three enzymes. The Ni-containing F430 cofactor of methyl coenzyme M reductase (MCR) is used extensively in anaerobic methane conversion reactions, responsible for the production of over one billion tons of methane per year. This enzyme is also capable of catalyzing methane oxidation, another highly sought after reaction. The Ni-containing active sites of the carbon monoxide dehydrogenase (CODH) and acetyl coenzyme A synthase (ACS) enzymes occupy a central role in the metabolisms of anaerobic bacteria and archaea, catalyzing the reversible synthesis of acetyl-CoA from carbon dioxide and a...
methyl group.\textsuperscript{10–12} It has been suggested that these enzymes cycle through a series of nickel-based organometallic intermediates during catalysis (Figure 1); however, the reactivity of nickel varies greatly in each enzyme, with different products formed from the use of similar building blocks. This diverse chemistry is unsurprising given the distinct coordination environments around nickel in each enzyme.

A closer look at the catalytic mechanisms for both ACS and MCR reveals ambiguity regarding nickel oxidation states and unresolved intermediates. In the case of ACS, one proposed mechanism suggests a catalytically relevant \( \text{Ni}^\text{II} \) state is required for activity.\textsuperscript{15–17} Because it has been shown that the reactive methyl group in ACS is transferred as a cationic species from a cobalt corrinoid protein, this paramagnetic species is a Ni\(^{III}\)-CH\(_3\) species as a catalytic intermediate (if only transiently formed). Alternatively, it has been suggested that a Ni\(^{II}\) state could not support addition of a cationic methyl group on the basis of comparison to small molecule models in organic solvents. This mechanism requires the active state of ACS to be two electrons more reduced than that of the as-isolated, \( \text{Ni}^{\text{II}} \) \text{Aox} state and necessitates only a Ni\(^{II}\)-CH\(_3\) species.\textsuperscript{16,17} On the other hand, a square pyramidal Ni\(^{II}\)-CH\(_3\) species in MCR has been trapped and characterized using electron paramagnetic resonance (EPR) spectroscopy,\textsuperscript{18} though studies suggest this state is not relevant for catalysis.\textsuperscript{23,24} With such ongoing debate, a biochemical model system that can reproduce nickel-based organometallic reactions performed by ACS. The reactivity of M121A Ni\(^{II}\)Az with biologically relevant methyl donors has been investigated, and the elusive Ni\(^{III}\)-CH\(_3\) species has been trapped during catalysis (Figure 1); however, the elusive Ni\(^{III}\)-CH\(_3\) state has been generated and trapped, supporting the analogy of M121A Ni\(^{II}\)Az as a biochemical model for ACS. The structure and reactivity of this state have been characterized using an array of techniques, including time-resolved optical and EPR spectroscopies as well as computational analyses. Additionally, the Ni\(^{II}\)-CH\(_3\) species has been interrogated with resonance Raman, magnetic circular dichroism (MCD), and X-ray spectroscopy to refine the geometric and electronic structure, and this state has been shown to react further with CO. Methane is found to be the sole reaction product following CH\(_3\)I addition, indicating this system is incapable of supporting hydrolysis, drawing further analogies to reactions seen in methylated ACS and MCR. Together, these results have implications for the order of substrate addition and relevant oxidation states for the organometallic reactions performed by ACS.

### RESULTS

**Rapidly Mixing M121A Ni\(^{II}\)Az with CH\(_3\)I Reveals Transient Nickel-Centered Signals Using Optical Spectroscopy.** Reduction of M121A Ni\(^{II}\)Az with 50 mM Eu\(^3\)DTPA results in complete conversion to M121A Ni\(^{II}\)Az, as previously reported, which can react with CO or CH\(_3\)I.\textsuperscript{25} However, in our prior work, the presence of significant amounts of excess reducing agent in solution prevented observation of a Ni\(^{III}\)-CH\(_3\) state, which is expected to exhibit a high reduction potential and be rapidly reduced by residual Eu\(^3\)DTPA. Moreover, methyl iodide can react with nucleo-

![Figure 1. Proposed nickel methyl intermediates in ACS and NiAz.](image)

![Figure 2. Rapid mixing of M121A Ni\(^{II}\)Az with CH\(_3\)I. (A) UV–vis traces after rapidly mixing an aliquot of CH\(_3\)I to a final concentration of 1.5 mM into 300 \( \mu \)M M121A Ni\(^{II}\)Az (initial trace shown in black). Sample was prepared in 50 mM phosphate buffer, pH 8.0. Spectra were taken every 35 s after mixing. (B) CW X-band EPR spectra after rapidly mixing an aliquot of CH\(_3\)I to a final concentration of 1.5 mM into 300 \( \mu \)M M121A Ni\(^{II}\)Az, quenched at indicated time points. Samples were prepared in 50 mM phosphate buffer, pH 8.0. (C) Concentrations determined from UV–vis absorption (solid lines) and EPR (circles) experiments for M121A Ni\(^{II}\)Az (black), and the intermediate (purple) overlaid on the proposed kinetic model (dotted lines).](image)
philes through either a two-electron or a radical pathway, leading to questions about the nature of the methyl transfer and the product formed. To address these issues and resolve the capacity of a biological Ni center to support nucleophilic attack on a cationic carbon center, which remains an outstanding question for the mechanism of ACS, a hand-packed, small-volume desalting column was generated. When combined with low-speed centrifugal filter concentration, this preparation removes most of the excess EuDTPA, leaving a solution of pure M121A NiAz with only minimal residual reducing agent present (Figure S1). A spectroscopically silent methyl donor is necessary to trap and characterize the products of methyl transfer to M121A NiAz. Due to rapid hydrolysis of methyl triflate in aqueous solution,26,27 which precludes reaction with M121A NiAz (Figure S2), methyl iodide was again selected as a methylating agent for spectroscopic studies on Ni-CH$_3$ Az species, despite the potential for multiple reaction pathways.

Rapid mixing experiments with CH$_3$I revealed the formation of a transient optical signal centered at 488 nm that rises and decays on the time scale of seconds and minutes, respectively, with kinetics that are dependent on the concentration of CH$_3$I (Figure 2A). This feature decays into a signal with similar features as the M121A NiAz state, with an isosbestic point for the later transition indicating a one-to-one conversion (Figure 2A). Increasing amounts of CH$_3$I accelerate the decay of NiAz as well as rates and amount of formation of the new species (Figure S3), while addition of exogenous EuDTPA decreases the amount of transient species observed (Figure S4). This is consistent with our prior results, in which our inability to resolve any transient species after methyl addition was attributed to the presence of 10−50 mM EuDTPA. Singular value decomposition (SVD) analysis of the UV−vis spectra as a function of time, substrate, and reductant concentration reveals four distinct components, including two transient ones, that contribute to the overall spectra (Figure S5). As such, the proposed reaction model incorporated these observables. Global fitting (Figure S5) of the kinetic profiles of each of the components was used to extract the elementary constants and effective solvent kinetic isotope effects (KIEs).

No new absorption features were observed to appear upon mixing of CH$_3$I with NiSO$_4$ and EuDTPA, metal-free (apo-) Az, or NiAz in phosphate buffer at pH 8.0 (Figure S6), suggesting that the features seen in the M121A NiAz mixing experiments are due to an interaction of the methyl donor with the protein-loaded NiAz center. Additional control experiments using $^{13}$C NMR indicate that CH$_3$I is stable toward hydrolysis over at least 1 h, which is more than sufficient for the time scale of the experiments performed in this work (Figure S7).

The addition of longer, less reactive alkylating agents, such as ethyl iodide, results in the same intermediate species, albeit formed at much slower rates and in lower quantities (Figure S8). By holding the alkyl group constant and changing the leaving group from an iodide to a bromide, the decay of M121A NiAz is further slowed, and even lower concentrations of intermediate accumulate in the UV−vis (Figure S9). Recognizing that an ethyl radical is more stable than a methyl radical, these observations suggest that alkyl addition is favored to occur via nucleophilic attack rather than a radical-based mechanism.

**EPR Spectroscopy Confirms the Generation of a Bioorganometallic Ni$^{III}$-CH$_3$ Species.** To examine whether the interaction between M121A NiAz and CH$_3$I proceeds via cationic or radical methyl addition and identify the nature of the transient species, EPR spectroscopy was performed on freeze-quenched samples. An aliquot of CH$_3$I was rapidly mixed into M121A NiAz, and EPR samples were frozen using an isopentane−liquid nitrogen bath at various time points. The CW X-band EPR spectra of these samples measured at 30 K reveal the formation of a new, EPR-active species with g-values of 2.46, 2.18, and 2.04. The signal is maximized at 45 s and begins to decrease after that point (Figure 2B). Formation and decay of the EPR features correlate with the formation and decay of the UV−vis band at 488 nm, suggesting the signals derive from the same transient species (Figure 2C). The g-values and relaxation properties indicate that the EPR-active signal arises from a low-spin, nickel-centered species (Figure S10).28

Experiments using isotopically substituted CH$_3$I support a direct interaction of the methyl group with the nickel center. Samples prepared with natural abundance CH$_3$I show well-defined splitting on the high-field turning point into three peaks separated by ~40 MHz, characteristic of coupling to a single I = 1 nucleus (e.g., $^{14}$N from a histidine residue). When samples are prepared using isotopically labeled $^{13}$CH$_3$I (Figure S11), additional line broadening is seen across the spectrum (Figure 3). Specifically, substantial coupling on the high-field turning point obscures the three well-defined peaks, and the midfield feature at 310 mT is also visibly broadened (Figure S12).29−31 The low-field turning point exhibits greater line broadening in all isotopes than is typical, likely due to g-strain,

![Figure 3](https://doi.org/10.1021/acs.inorgchem.8b03546)

**Figure 3.** CW X-band EPR spectra of the M121A Ni$^{III}$-CH$_3$ Az intermediate. EPR spectra ($T = 30 K; P_{mic} = 0.2$ mW) of ~2 mM M121A NiAz rapidly mixed with 100 mM (A) CH$_3$I (red), (B) $^{13}$CH$_3$I (blue), and (C) CD$_3$I (gray) and frozen after 45 s. Samples contained 30% glycerol in 50 mM phosphate buffer, pH 8.0. Simulations overlaid on traces as dotted lines. (Left inset) Gas-phase optimized geometry of M121A Ni$^{III}$-CH$_3$ Az. (Right inset) Zoomed-in view of high-field turning point for the isotopically labeled substrates.
Table 1. Select Structural and Spectroscopic Metrics for M121A Ni^{III}-CH_3 and Ni^{II}-CH_3 Az^a

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<th>Ni-Cys_{112} (Å)</th>
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<th>Ni-Cys_{66} (Å)</th>
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<th>(\lambda_{A^{III}}) (MHz)</th>
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^a^Calculated bond lengths, Ni^{III}-CH_3 Az g-tensor values, and Ni^{II}-CH_3 Az hyperfine coupling constants compared to experimental values.

^b^NR stands for not resolved due to unresolved broadening on the low-field turning point.

and added interactions between a \(^{13}C\) center are not well-resolved. Experiments performed using CD_{3}I (Figure S12) show very slight narrowing across the spectrum, possibly suggesting weak coupling to the methyl-derived protons (Figure 3), though high-resolution pulsed EPR studies will be necessary to resolve these small, secondary interactions.

To reproduce the observed spectral features, the CW EPR spectrum is simulated with a 45 MHz coupling constant to a nitrogen nucleus along the high-field feature and an additional, large coupling constant of 21 MHz in the \(^{13}CH_3\)I (Figure 3), though high-resolution pulsed EPR studies will be necessary to resolve these small, secondary interactions. Extra, large coupling constant of 21 MHz in the \(^{13}CH_3\)I (Figure 3), though high-resolution pulsed EPR studies will be necessary to resolve these small, secondary interactions.

The symmetry between the two histidine ligands appears to be resolved. Experiments performed using CD_{3}I (Figure S12) show a distorted trigonal pyramidal center, similar to the 18 MHz hypertrigonal (His)_{2}Cys ligand framework (Figure 3, inset; Table 1). The unique electronic axis of this system appears to be along the Ni–C bond, as the SOMO appears to be a mixed orbital with significant overlap between the hybridized sp\(^3\) orbital of the coordinating nitrogen on His_{117}, the methyl carbon, and the nickel center (Figure S14). This supports the observed large hyperfine coupling to only one nitrogen atom in the EPR spectra, along with the slightly weaker coupling to the coordinated carbon atom.

The calculated g-tensor values and hyperfine coupling constants are generally in agreement with those seen experimentally. Systematic deviation in the form of underestimated g-value shifts is commonly seen in DFT calculations owing to overestimated covalency and d–d transition energies and has been noted both for d\(^4\) and d\(^7\) compounds (e.g., Cu\(^{II}\), Co\(^{II}\)). The hyperfine constants are somewhat overestimated relative to the experimental values (Table 1), though again this is not uncommon in DFT calculations and is highly sensitive to the metal–ligand distances, covalency, and spin–orbit coupling.

The TD-DFT results on the Ni^{II}CH_3 Az species show one dominant transition in the visible region of the spectrum, at 28,606 cm\(^{-1}\) (Figure S15); this agrees well with the observed experimental band maximum at 488 nm (~20,500 cm\(^{-1}\)). Examining the electronic difference densities shows this band arises from a ligand-to-metal charge-transfer (LMCT) transition, with the primary contribution deriving from the bound methyl group (Figure S15, inset).

### Spectroscopic Characterization of Methylated NiAz in the Presence of Excess Reducing Agent Indicates the Formation of M121A Ni^{III}CH_3 Az

**Resonance Raman Spectroscopy Provides Limited Information on M121A Ni^{III}CH_3 Az Intermediates.** Having established the identity of the intermediate species as M121A Ni^{III}CH_3 Az, we sought to identify the product of the reaction upon decay of the Ni^{II}CH_3 species. The observation that the M121A Ni^{II}CH_3 Az intermediate generated using CH_3I accumulates to a much lower extent in the presence of excess reducing agent suggests that the Ni^{III}CH_3 species can be rapidly reduced, potentially to a Ni^{II}CH_3 state. While the optical absorption features following the rapid mixing experiments (green trace, Figure 2A) somewhat resemble the starting M121A Ni^{III}Az species, subtle differences in the spectra call into question the identity of this state. The intensity of the band at 416 nm is lower in the product relative to the starting Ni^{III} state, and there are broad, low-intensity features in the near-UV region of the spectrum (300–350 nm). To interrogate the structure of this species, resonance Raman (RR) spectroscopy using 407 nm excitation was used to probe the primary electronic transition. The vibrational signatures are found to be nearly identical for a sample of M121A Ni^{III}Az mixed with CH_3I in the presence of stoichiometric reducing agent and quenched after 5 min when compared to the spectrum of the resting-state, M121A Ni^{III}Az (Figure S16). Both RR profiles show an intense stretch at 343 cm\(^{-1}\) that is attributed to the Ni–S bond. This band is strongly enhanced in both samples, indicating that the electronic transition is dominated by S-Cys-to-Ni charge-transfer (CT) character. In support of this assignment, the intense band at 764 cm\(^{-1}\) is most likely due to the C–S stretching vibration of the cysteine residue, comparable to the band reported for the copper variant. The bands in the 1200–
1600 cm\(^{-1}\) region can be attributed to histidine stretching modes, which are more enhanced for the M121A Ni\(^{II}\)Az variant than WT NiAz because of the increased active site distortion.\(^{36}\) However, few changes are seen between the two samples; two bands at 360 and 1500 cm\(^{-1}\) reflect the primary differences, though they do not shift when isotopically substituted CH\(_3\)I is used and thus likely do not derive from a Ni-CH\(_3\) mode. The lack of an apparent Ni-C mode may be due to either weak enhancement from the probed electronic transition or overlap with pronounced bands from the quartz dewar used in the experimental setup, and ongoing work is aimed at developing strategies to overcome these challenges. EPR experiments, and ongoing work is aimed at developing techniques were necessary to identify the species following product of an M121A Ni\(^{IAz}\) sample mixed with 10 mM CH\(_3\)I as an agent and rapidly frozen. In both samples, the MCD spectra obtained on the resting M121A Ni\(^{IIAz}\) state as well as the putative M121A Ni\(^{II-CH_3}\) Az state are in fact two distinct species with different ground-state properties. Resonance Raman investigations on these species have demonstrated that the most intense absorption bands at 411 and 413 nm correspond to S\(_{Cys}→Ni^{III}\) CT transitions (Figure S16); therefore, these transitions must be polarized along the Ni-S\(_{Cys}\) axis.\(^{36}\) This detail enabled a comparison between experimental data and DFT-calculated zero-field splitting (ZFS) parameters (Table S2), facilitating analysis of the MCD VTVH curves. The DFT-predicted ZFS parameters were used to fit the VTVH data of both M121A Ni\(^{III}\)Az and M121A Ni\(^{II-CH_3}\) Az, considering that the 411 and 413 nm transitions, respectively, have to be polarized along the Ni-S\(_{Cys}\) bond. We note that the polarizations obtained from the VTVH data are relative to the principal axes of the ZFS tensor; these tensor orientations were taken from the DFT calculations. Using this analysis, only the ZFS parameters obtained from the B3LYP calculations were found to give reasonable results in the initial round of VTVH fits. A hallmark of the B3LYP results is that, while both M121A Ni\(^{IIAz}\) and M121A Ni\(^{II-CH_3}\) Az are calculated to exhibit large \(D\) values, in agreement with SQUID results for WT Ni\(^{III}\)Az, the species have opposite signs.\(^{40}\) This can be attributed to the sums of their individual spin–orbit contributions, which are also opposite in sign. After further refinement of \(D\), holding the \(E/D\) ratio and the \(g\)-values constant at the B3LYP-predicted value, best fits to the data were obtained, delivering \(D\) values of \(-15\) cm\(^{-1}\) and +8.7 cm\(^{-1}\) for M121A Ni\(^{III}\)Az and M121A Ni\(^{II-CH_3}\) Az, respectively (Figure 4). Moreover, the fits of the VTVH data show that in M121A Ni\(^{IIAz}\), the two optical bands at 411 and 413 nm are mostly \(y\)-polarized, where, according to the B3LYP calculations, the \(y\)-axis of the ZFS tensor aligns with the Ni–S\(_{Cys}\) bond (Figures S18–S19). Correspondingly, in M121A Ni\(^{II}\)Az.

A more detailed analysis of the transitions using variable-temperature variable-field (VTVH) measurements, which probe the magnetization saturation behavior of C-term transitions, ultimately revealed that M121A Ni\(^{III}\)Az and M121A Ni\(^{II-CH_3}\) Az are uniquely suited to provide electronic structure information on high-spin Ni\(^{II}\) species under aqueous conditions, even for short-lived, trapped intermediates.\(^{36,39}\) MCD spectroscopy is especially interesting for studying systems containing three temperature-dependent transitions, therefore exhibiting MCD C-term intensity (Figure 4). The MCD spectra of M121A Ni\(^{III}\)Az exhibit two predominant transitions at 345 and 411 nm as well as a weak, broad transition at 480 nm. Similarly, the MCD spectra of the putative M121A Ni\(^{II}\) Az species contain two predominant transitions at 354 and 413 nm, each of which is less intense than the corresponding transition in M121A Ni\(^{III}\)Az.

Figure 4. MCD spectra of M121A NiAz. MCD spectra and VTVH curves at 2 K (red), 5 K (blue), and 10 K (black) of (A–C) ~1 mM M121A Ni\(^{III}\)Az and (D–F) ~1 mM M121A Ni\(^{IIAz}\) Az rapidly mixed with 25 mM CH\(_3\)I in the presence of 25 mM Ti\(^{III}\)(citrate). Samples were prepared in 50 mM phosphate buffer, pH 8.0, containing 50% glycerol. (B, C) VTVH intensities and fitted curves for M121A Ni\(^{III}\)Az. \(D = -15\) cm\(^{-1}\), \(E/D = 0.206\), \(g_x = 2.143\), \(g_y = 2.159\), \(g_z = 2.171\). (E, F) VTVH intensities and fitted curves for M121A Ni\(^{II-CH_3}\) Az. \(D = 8.7\) cm\(^{-1}\), \(E/D = 0.235\), \(g_x = 2.09\), \(g_y = 2.12\), \(g_z = 2.17\). For fitting the VTVH data of both species, the \(E/D\) ratios and \(g\)-tensor values were taken from the B3LYP calculations (Table S2).
CH₃Az, the two main optical bands at 413 and 354 nm are z-polarized, with the z-component of the ZFS tensor pointing along the Ni–S₈C₂ bond (Figures S18–S19). Using the RR assignments as a constraint, the VTVH data indicate a clear difference in the magnetic properties of the ground states of M121A NiIIAz and M121A NiII-CH₃ Az.

X-ray Spectroscopy Indicates Coordination of an Additional Light Atom to a NiII Center. To better define the coordination environment about the nickel site of M121A NiIIAz and M121A NiII-CH₃ Az, nickel K-edge X-ray absorption spectroscopy was employed. The EXAFS regions of the Ni K-edge X-ray absorption spectrum for M121A NiIIAz and M121A NiII-CH₃ Az were best modeled as a three coordinate Ni center with two imidazole ligands at 1.94 Å and a short sulfur ligand at 2.17 Å (Figure S5). Owing to the strong multiple scattering pathways originating from the imidazole ligands, the predicted longer Ni−N (2.02 Å) and one Ni−S (2.24 Å) bond lengths dominated by the LUMO of M121A NiIIAz. This increase in Ni(4p) character results in a significantly more intense pre-edge feature of M121A NiII-CH₃ Az relative to M121A NiIIAz. The XANES regions of the Ni K-edge X-ray absorption spectrum of M121A NiII-CH₃ Az is fully consistent with the origin of the observed increase in intensity. For both M121A NiIIAz and M121A NiII-CH₃ Az, the pre-edge feature is comprised of two nominal Ni(1s → 3d) transitions. The lowest energy transition is into an acceptor state comprised of Ni(3dₓ²) character (dominated by the LUMO), while the next lowest energy transition is into an acceptor state comprised primarily of S(3pₓ)−Ni(3dₓ) antibonding character (dominated by the LUMO+1). Conversion of M121A NiIIAz to NiII-CH₃ Az does not have a major impact on the degree of Ni(4p) character in the LUMO+1 wave function; elongation of the Ni−S bond causes only a slight decrease in the degree of Ni(4p) composition to the LUMO+1 from 1.7% to 1.4%. However, upon ligation of the −CH₃ ligand to the nickel center, the degree of Ni(4p) character in the LUMO increases significantly. The now Ni(3dₓ²)−C(2p) σ* LUMO of M121A NiII-CH₃ Az possesses 3.4% Ni(4p) character relative to <1% Ni(4p) character, contributing to the essentially nonbonding Ni(3dₓ²) LUMO of M121A NiIIAz. This increase in Ni(4p) character results in a significantly more intense pre-edge feature of M121A NiII-CH₃ Az relative to M121A NiIIAz. Thus, both the XANES and EXAFS regions of the Ni K-edge X-ray absorption spectra of M121A NiII-CH₃ Az are consistent with the formation of a NiII-CH₃ moiety at the metalloprotein active site.

Gas Chromatography Analysis Reveals CH₄ to be the Dominant Reaction Product Following CH₃I Addition. Gas chromatography analysis was used to identify and quantify the major reaction product following CH₃I addition to M121A NiIIAz. Gas chromatography analysis was used to identify and quantify the major reaction product following CH₃I addition to M121A NiIIAz.
the carbon-based products generated after methyl addition to M121A NiIAz under a variety of conditions. Using CH3I as the methyl donor, methane is produced as the primary product (Figure S21). In the absence of a large excess of Eu^3DTPA, approximately half an equivalent of CH4 is observed relative to the initial concentration of M121A NiIAz. As the residual concentration of Eu^3DTPA is increased, methane production also increases, reaching approximately stoichiometric levels; an equivalent change in methane produced by control samples suggests that this increase is simply due to background reactions (Figure S21). Solution pH, time, and concentration of CH3I added to the solution also affect the amount of methane produced. When the pH is decreased, increasing the effective concentration of free protons in solution, methane production rises, though again to an equivalent extent as is seen in control experiments with NiSO4 (Figure S21). Lower concentrations of added CH3I also result in substantially lower amounts of CH4 produced. After approximately 20 min, the optical spectra of the reaction are no longer changing drastically, and production of CH4 ceases. The formation of methane from a nickel-methyl species generated by CH3I addition is also observed in model synthetic compounds for ACS.42 Collectively, these results implicate a persistent interaction of the nickel center with the methyl ligand in the NiIII state. This NiII-CH3 Az species is consistent with that characterized using MCD and X-ray spectroscopy.

**DISCUSSION**

**Proposed Reaction Pathways for Methyl Addition to M121A NiIAz.** The work presented here demonstrates that methyl addition to NiI from methyl iodide occurs via a two-electron, nucleophilic attack on the nickel center, resulting in a transient, low-spin d7, NiII-CH3 species. Within the Az model protein scaffold, this nonporphyrinoid NiII-CH3 state features a distorted trigonal pyramidal or seesaw-like geometry with visible optical bands and a distinct EPR signature that definitively indicates direct coordination of carbon to the nickel center. However, methyl iodide can react via two-electron methyl cation addition, a radical pathway, or both (Figure 6A). Given that the reaction of M121A NiIAz with methyl iodide results in the apparent formation of an EPR-active, NiII-CH3 species, and reactions using ethyl iodide and ethyl bromide slow nucleophilic addition would result in formation of M121A NiIIIAz, which is indeed observed in the EPR spectra to a small extent (~10%, Figure S23). The resultant, highly oxidizing NiIII Az would then undergo reduction, likely from residual reducing agent in solution, giving the M121A NiII Az product.

The observation that the M121A NiIII-CH3 intermediate is formed but accumulates to a much lower extent in the presence of excess reducing agent suggests that the NiIII-CH3 species can also be directly reduced prior to protonolysis, a process that should be thermodynamically favorable due to the relatively high anticipated reduction potential of a NiIII-CH3 species. This suggests that a second decay pathway of M121A NiIII-CH3 Az involves initial reduction to NiII-CH3 Az, consistent with evidence provided by MCD and X-ray spectroscopy. An upper bound on the NiII/III-CH3 Az reduction potential is estimated to be ~250 mV, as experiments performed in the presence of excess ascorbate show no change in amount of NiII-CH3 formed nor the rate of decay (Figure S24).

![Figure 6. Comparison of methyl addition to nickel systems. (A) Proposed mechanism for addition of CH3I to M121A NiIAz. Global kinetic analysis and fitting gives indicated elementary rate constants. (B) Reaction of K[Ni[N(SiMe3)DIPP]2] with CH3I via oxidative addition. (C) Reaction of [Ni(tmc)]+ with CH3Co(dmgBF2)2L to yield NiII-CH3 via a radical pathway. (D) Reaction of [NiI(NS3R)-Cl]+ with CH3MgCl to form [NiI-(CH3)(NS3R)]+. (E) Radical reaction of NiII(dadtEt)NiI(SDmp)(PPh3) with CH3Co(dmgBF2)2(Py) and KSDmp to form NiII-CH3.](image)
After reduction to Ni\textsuperscript{II}-CH\textsubscript{3} Az, this species can undergo protonation to release methane and form the Ni\textsuperscript{II}Az product. However, stoichiometric or catalytic amounts of methane are not observed, even in the presence of excess Eu\textsuperscript{III}DTPA, indicating that the Ni\textsuperscript{II}-CH\textsubscript{3} state may be stable under anaerobic conditions. Upon exposure to air and exchange of the buffer, the M121A NiAz sample can be recovered and reduced to re-enter the reaction (Figure S25).

Kinetic analysis reveals that the rate of Ni\textsuperscript{II} decay does not directly correlate with the rate of Ni\textsuperscript{III}-CH\textsubscript{3} formation, suggesting that a branched reaction pathway is accessible. This side reaction is attributed to one-electron attack by the metal center to induce homolytic cleavage of CH\textsubscript{3}I, generating M121A Ni\textsuperscript{II}-CH\textsubscript{3} Az directly; use of ascorbate as a radical scavenger does not impact the rate of reaction (Figure S24), suggesting spontaneous homolytic degradation of CH\textsubscript{3}I prior to metal attack is not a significant contributor to reactivity. SVD and the corresponding amplitude profiles are consistent with this proposal, indicating biphasic formation of a species that is now assigned to the Ni\textsuperscript{II}-CH\textsubscript{3} state (Figure S5). The Ni\textsuperscript{II}-CH\textsubscript{3} species is suggested to form at a slightly faster rate than Ni\textsuperscript{III}-CH\textsubscript{3} from the kinetic modeling, and reduction occurs more rapidly than protonation. Collectively, global analysis of the kinetics and optical spectra support the mechanism and rate constants given in Figure 6 for reactions following CH\textsubscript{3}I addition to M121A NiAz.

Identification and Characterization of a Biological Ni\textsuperscript{III}-CH\textsubscript{3} Species. Nickel-based catalysts are increasingly used for cross-coupling reactions and carbon-heteroatom bond formation in organic synthesis.\textsuperscript{47-49} Often, these catalysts are suggested to proceed through organometallic nickel-carbon bonds in reactions that typically require the nickel center to cycle between the Ni\textsuperscript{II}/Ni\textsuperscript{III} or Ni\textsuperscript{II}/Ni\textsuperscript{IV} oxidation states.\textsuperscript{38,50,51}

Conversely, in nature, nickel can accommodate similar chemical reactions but, at this point, is thought to cycle through only three biologically relevant oxidation states: Ni\textsuperscript{0}, Ni\textsuperscript{II}, and Ni\textsuperscript{III}.\textsuperscript{12} In low-valent oxidation states, it has been proposed that nickel can form catalytically relevant organometallic intermediates in enzymes pertinent to the carbon cycle, though to date only one biological nickel-alkyl species, featuring nickel coordinated within the MCR hydroporphynoid ring system, has been isolated.\textsuperscript{16,21-23} The conjugated F\textsubscript{340} cofactor of MCR is sterically constrained within the protein and therefore is unable to adopt a highly planar conformation.\textsuperscript{52} As a result, the Ni\textsuperscript{II} form of F\textsubscript{340} is thought to feature a four-coordinate nickel center in a square planar geometry with relatively long Ni–N bonds.\textsuperscript{52} This nickel coordination environment is distinctly different from that found within ACS.

Conversely, although ACS is also coordinated in a mostly planar geometry, the proximal metal site features a three-coordinate nickel center with coordination to soft cysteinate ligands. This has subtle implications for methyl binding to the metal center, and both the strength of the Ni–C bond and geometry for methyl addition and loss are expected to vary significantly between the ACS and MCR systems. The identification of a four-coordinate Ni\textsuperscript{III}-CH\textsubscript{3} intermediate within azurin thus provides the first relevant model for methyl addition to ACS; notably, no model compounds exist that feature the same coordination number, oxidation, and spin state as those proposed for methylated ACS. Characterization of these methyl-bound intermediates in M121A NiAz provides important metrics for the spectroscopic properties and reactivity that analogous species in native ACS may possess.

The work presented here demonstrates that methyl addition to Ni\textsuperscript{II} from methyl iodide can occur via a two-electron, nucleophilic attack on the methyl center, resulting in a transient, low-spin d\textsuperscript{7}, Ni\textsuperscript{III}-CH\textsubscript{3} species. Within a biological scaffold, this nonporphyrinoid Ni\textsuperscript{III}-CH\textsubscript{3} state features a distorted trigonal pyramidal or seesaw-like geometry with visible optical bands and a distinct EPR signature. The M121A NiAz model system offers insight into how the electronic structure of the nickel center contributes to this reactivity, as the Ni\textsuperscript{II}Az state exhibits marked similarity to the reduced, substrate-free A\textsubscript{red} state of ACS. However, a Ni\textsuperscript{III}-CH\textsubscript{3} state has never been observed in ACS, potentially owing to the strongly reducing conditions required to carry out the ACS reaction in vitro. It is likely that this reducing environment would facilitate rapid reduction from Ni\textsuperscript{III}-CH\textsubscript{3} to Ni\textsuperscript{II}-CH\textsubscript{3}, which, as observed here, may be necessary for persistent methylation. A Ni\textsuperscript{III}-CH\textsubscript{3} species is predicted to have a weaker Ni–C bond than the Co\textsuperscript{III}–CH\textsubscript{3} bond within a base-free corrinoid protein.\textsuperscript{53} Therefore, for ACS, it may be that only the presence of a reducing equivalent, either from within the protein or externally donated, can drive the methyl transfer reaction forward. A redox-mediated step provides a means by which to gate reactivity, retaining the methyl group on the cobalt cofactor until an appropriate reduction potential is reached for the reaction to proceed. This could prevent premature methylation of the nickel center, which, in the absence of CO and CaA, may suffer from unproductive protonolysis and release of methane. Furthermore, it appears that a biological Ni\textsuperscript{III}-CH\textsubscript{3} species may have a relatively high reduction potential, as evidenced by rapid reduction of M121A Ni\textsuperscript{III}-CH\textsubscript{3} Az in the presence of even trace amounts of excess reducing agent, complicating efforts to trap a Ni\textsuperscript{III}-CH\textsubscript{3} species within ACS.

Ni\textsuperscript{II}-CH\textsubscript{3} Az May Support Organometallic Reactivity. While both Ni\textsuperscript{II}-CH\textsubscript{3} and Ni\textsuperscript{III}-CH\textsubscript{3} intermediates are formed within the M121A NiAz scaffold, we hypothesize that the Ni\textsuperscript{II}-CH\textsubscript{3} state may be the key intermediate required for further reactivity. The direct formation of Ni\textsuperscript{II}-CH\textsubscript{3} Az via radical addition is similar to generation of traditional synthetic Ni\textsuperscript{II}-CH\textsubscript{3} compounds. These synthetic routes for alkyl-nickel compounds starting from Ni\textsuperscript{II} usually proceed through a radical pathway; two-electron chemistry is generally accomplished from a Ni\textsuperscript{II} state,\textsuperscript{42,53-55} and only one example exists of the oxidative addition of methyl iodide to Ni\textsuperscript{II}.\textsuperscript{56} Alternatively, Ni\textsuperscript{II}-CH\textsubscript{3} species can be generated from a Ni\textsuperscript{II} precursor by the use of anionic methylating agents bound to alkali or alkaline earth metals. This synthetic pathway is completely impractical under aqueous conditions. Relative to synthetic models, the M121A NiAz system is unique in that, in addition to undergoing radical methyl addition, the two-electron, nucleophilic addition of a caticonic methyl group is accessible. This is the pathway that has been proposed for native ACS.

Further differentiation between M121A NiAz and synthetic models is found in the primary coordination sphere. Like ACS, prior to substrate binding, M121A NiAz contains a 3-coordinate, trigonal planar ligand system featuring a directly coordinated thiolate ligand (Figure 6A). Most of the ACS model compounds feature square-planar, 4-coordinate nickel centers bound to either amine (e.g., 1,4,8,11-tetramethyl-1,4,8,11-tetraazacyclotetradecane, tmc),\textsuperscript{57} thioether, or phosphine (e.g., 1,2-bis(diphenylphosphino)ethane, dppe) ligands (Figure 6B–E).\textsuperscript{35,58} These nonbiological ligand scaffolds and geometries will modulate the strength of the Ni–CH\textsubscript{3} bond.
and, consequently, further reactions with CO. Therefore, we must instead examine the reactivity of a 4-coordinate Ni$^{II}$-CH$_3$ system, which more closely resembles the structure of ACS. Tatsumi has developed a dinuclear Ni$^{II}$Ni$^{II}$ compound featuring a 4-coordinate Ni$^3$ center with 2,6-dimesitylphenylmethanol (dmpS$^-$) and triphenylphosphine ligands; however, significant spectral differences between this model compound and the reduced states of ACS are suggested to arise because of differences in the coordination environment. Methyl addition to this compound results in a square planar complex (Figure 6E), with subsequent degradation upon exposure to CO. The distorted trigonal pyramidal Ni$^{II}$-CH$_3$ species formed within the M121A NiAz environment and the constraints of a large, macromolecular ligand framework thus provide a more appropriate platform to pursue reactivity similar to that seen in the ACS enzyme.

Resonance Raman experiments demonstrate that the Ni$-$S$_{Cy}$ electronic transition dominates the resonance Raman M121A NiIIAz (Figure S26). This is likely due to a decrease in for M121A NiII-CH$_3$ Az, with the d$\beta$-d$\sigma$ interaction. The potential for CO binding to the NiII-CH$_3$ state was explored through preliminary time-resolved optical studies. A population of M121A Ni$^{II}$-CH$_3$ was generated by mixing CH$_3$I into M121A NiIAz in the presence of excess reducing agent(s) from this experiment remain unresolved and a comprehensive investigation is beyond the scope of this work.

The ability of M121A NiAz to form both a Ni III-CH$_3$ and NiII-CH$_3$ species provides an intermediate, the occupied d$\sigma$-MO diagram (Figure S26) indicates that M121A NiIIAz has occupied d$_{xz}$, d$_{yz}$, and d$_{xy}$ orbitals (in decreasing energy, respectively).

Upon coordination of the methyl group, the CH$_3$ ligand forms a new $\sigma$-bond with the empty Ni $\beta$-d$_{xy}$ orbital. The energy ordering of the occupied $\beta$-d$_{xy}$ orbitals of Ni is inverted for M121A Ni$^{III}$-CH$_3$ Az, with the d$_{x-y}$, d$_{xy}$, and d$_{xy}$ orbitals occupied in decreasing energy. Additionally, in the methyl intermediate, the occupied $\beta$-d orbitals are much lower in energy and closer to the $\beta$-HOMO level when compared to M121A NiIIAz (Figure S26). This is likely due to a decrease in the effective nuclear charge of the Ni center in the presence of the strongly donating methyl ligand. The difference in the sign of $D$ originates directly from the SOC contribution, which differs between M121A Ni$^{III}$Az and M121A Ni$^{II}$CH$_3$ Az due to the discussed differences in d-orbital sequence and relative energies. Other contributions to $D$ are negligible. Interestingly, the low-lying, occupied $\beta$-d$_{xy}$ and $\beta$-d$_{xy}$ orbitals in M121A NiII-CH$_3$ Az point into the empty space trans to the His$_{17}$ residue, providing a binding site for an incoming CO ligand. Here, the CO($\pi$) orbitals would be able to form significantly stronger $\pi$-backbonding interactions with the $\beta$-d$_{xy}$ and $\beta$-d$_{xy}$ orbitals of M121A NiII-CH$_3$ Az relative to M121A Ni$^{III}$Az. Hence, whereas the latter species does not interact with CO (Figure S27), these results suggest that CO may be able to form a transient CO complex with the NiII$^{II}$-CH$_3$ intermediate, prior to possible insertion into the NiII$^{II}$-CH$_3$ bond.

The potential for CO binding to the NiII$^{II}$-CH$_3$ state was explored through preliminary time-resolved optical studies. A population of M121A NiII$^{II}$-CH$_3$ was generated by mixing CH$_3$I into M121A NiIIAz in the presence of excess reducing agent, conditions that were designed to mimic those used for preparation of the MCD and XAS samples. Upon injection of CO-saturated buffer, the signals attributed to M121A NiII$^{II}$-CH$_3$ quickly decayed, at a rate substantially higher than that seen in corresponding control experiments (Figure S28). While the product(s) from this experiment remain unresolved and a comprehensive investigation is beyond the scope of this work and will form the body of a subsequent publication, the rapid quenching of the M121A NiII$^{II}$CH$_3$ species by CO addition suggests great potential for downstream reactivity to be installed and characterized within the NiAz model protein system.

**Implications for the Role of Nickel-Methyl Species in the ACS Catalytic Cycles.** The ability of M121A NiAz to form both a NiIII$^{III}$CH$_3$ and NiII$^{II}$CH$_3$ species provides an opportunity to explore reactions within the context of both MCR and ACS catalysis. The lack of hydrolysis observed in either oxidation state is consistent with the reactivity seen in MCR, in which the reactive methyl species generated from methyl-CoM solely reacts to form methane. In organisms capable of methanol production or activation, the cobalt...
corrinoid-containing MtaABC complex is used instead of nickel-containing MCR.\textsuperscript{55–60} Hydrolysis of the methyl-bound state of ACS to generate methanol has also never been detected. The methylated species is suggested to be sufficiently stable to be isolated in the absence of added CO, though this is only implied by subsequent reactivity to generate acetyl-CoA and characterization of a Ni\textsuperscript{III}-CH\textsubscript{3} state remains elusive. Interestingly, minor amounts of methane have been observed as a side metabolic product of actogenetic bacteria, though to such a small extent that this cannot be a dominant decay pathway of methylated ACS. Thus, there must be additional electronic or structural influences within the ACS active site to prevent protonolysis.

The isolation of distinct Ni-CH\textsubscript{3} species within Az offers exploration of the molecular requirements for subsequent CO binding. It has been proposed that substrate binding in ACS can occur via random ordering.\textsuperscript{13} However, we hypothesize that CO will be unable to bind to an electron-deficient Ni\textsuperscript{III}-CH\textsubscript{3} center. Moreover, electronic structure analysis shows that the resulting structure of the Ni\textsuperscript{III}-CH\textsubscript{3} species following methyl binding induces a shift of the occupied β-π* and β-π orbitals to lower energy, priming them for interaction with an incoming CO ligand. This indicates that the formation of a Ni\textsuperscript{III}-CH\textsubscript{3} species may be necessary for CO binding, and that, in turn, formation of a (potentially transient) CO adduct is a prerequisite for formation of an acetyl group.

Additionally, the requirement of a redox-active buffer or low-potential conditions for ACS activity supports the idea of rapid reduction to a Ni\textsuperscript{III}-CH\textsubscript{3} state following methylation of the Ni\textsubscript{II} site but prior to CO binding.\textsuperscript{55–72} While the [4Fe-4S] cluster has been suggested to be redox-inactive for in vitro ACS turnover due to sluggish measured ET kinetics,\textsuperscript{16} we postulate that, in vivo, conformational changes may permit an electron to reversibly derive from the [4Fe-4S] cluster, another redox-active site in the protein, or even the Ni\textsubscript{II} site. This second nickel center should have a Ni\textsuperscript{III/II} couple that occurs at a lower potential than the Ni\textsuperscript{III}-CH\textsubscript{3} site owing to the bisamidinate ligation.\textsuperscript{71} Like the reactivity seen here for M121A NiAz, although the methyl group adds to ACS from the CoFeSP protein in a two-electron process, an internal electron-transfer step could rapidly reduce the transiently formed Ni\textsuperscript{III}-CH\textsubscript{3} to Ni\textsuperscript{III}-CH\textsubscript{3}, enabling CO binding and subsequent insertion into the Ni-CH\textsubscript{3} bond.\textsuperscript{55,69,72,73} Importantly, we suggest that this redox event may act as a gate for reactivity, transferring the methyl group only when a specific potential is reached. Acyl transfer to CoA would allow release of that electron from the Ni\textsubscript{II} site, potentially back to the [4Fe-4S] cluster or the Ni\textsubscript{II} center, and regeneration of the Ni\textsubscript{II} state. The ability to access this type of reactivity in the M121A NiAz model system, which has well-defined oxidation states within a simple active site, is currently being explored in our lab and will likely shed important insight into the order of substrate binding and reactions in native ACS.

\textbf{CONCLUSIONS}

Methyl binding to a protein-based model of acetyl-CoA synthase, the M121A mutant of nickel-substituted azurin, can occur via nucleophilic addition to the Ni\textsuperscript{II} oxidation state. Using methyl iodide, a transient Ni\textsuperscript{III}-CH\textsubscript{3} intermediate is generated, which has been characterized using optical and EPR spectroscopy in conjunction with DFT calculations. The Ni\textsuperscript{III}-CH\textsubscript{3} intermediate is rapidly reduced to a Ni\textsuperscript{II}-CH\textsubscript{3} species, which has been characterized using MCD and X-ray spectroscopy to support the coordination of a carbon atom to the nickel center. The reaction mechanism has been quantitatively modeled using global kinetic simulations coupled with singular value decomposition analysis. M121A Ni\textsuperscript{III}-CH\textsubscript{3} Az represents the first well-defined Ni\textsuperscript{III}-CH\textsubscript{3} species within a distorted trigonal pyramidal environment, and its characterization within a biologically derived system is of great relevance for understanding the reaction mechanism of ACS. Additionally, the ability to isolate both an EPR-active Ni\textsuperscript{III}-CH\textsubscript{3} state and an MCD-active Ni\textsuperscript{III}-CH\textsubscript{3} species provides a platform for further investigation of the reactivity requirements for both ACS and MCR enzyme systems. Overall, the metal–alkyl species characterized within the M121A NiAz system serve as models for the bioorganometallic catalytic intermediates found in multiple nickel enzymes. Ongoing efforts are aimed at characterizing potential reactivity of the methylated species upon introduction of additional ACS substrates, including CO.

\textbf{MATERIALS AND METHODS}

Solutions were prepared using deionized water (18.2 MOhm, Elga Technologies) unless otherwise noted. Reagents were purchased from Sigma-Aldrich, Alfa Aesar, or VWR Technologies, unless otherwise noted. D\textsubscript{2}O was obtained from Cambridge Isotopes.

\textbf{Optical Absorption Spectroscopy.} All absorption spectra were measured on either a Shimadzu UV-2600 spectrophotometer or a Cary 60 spectrophotometer using septa-capped cuvettes. For rapid mixing experiments with methyl iodide (Sigma-Aldrich), a 50 mM or 100 mM stock suspension of CH\textsubscript{3}I was prepared in 50 mM phosphate buffer anaerobically inside a septum-capped vial. Due to low solubility and potential instability of CH\textsubscript{3}I in water, a fresh suspension of CH\textsubscript{3}I was prepared immediately prior to each experiment. Using a gastight syringe (Hamilton), aliquots of the CH\textsubscript{3}I suspension were hand-mixed into a septum-capped cuvette containing M121A NiAz and placed within the spectrophotometer within 10 s. Spectra were collected from 700 to 240 nm every 35 s over 48 min using a 0.2 cm path length and baseline-corrected using Igor Pro (WaveMetrics, Lake Oswego, OR). For experiments using both CH\textsubscript{3}I and CO, CO-saturated buffer was prepared in 50 mM phosphate buffer and added to a final concentration of 0.22 mM ~3 min after M121A NiAz reacted with 1.1 mM of CH\textsubscript{3}I.

\textbf{Kinetic Modeling.} Singular value decomposition (SVD) analysis and kinetic simulations were accomplished using the KinTek software (version 7.2) with FitSpace Explorer and SpectraFit.\textsuperscript{74,75} Baseline-corrected UV–vis data were imported to KinTek, and SVD analysis performed to decompose the absorbance spectra and isolate the representative spectral features of each component contributing to the overall absorbance. The SVD components were noted. 

### Materials and Methods

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\begin{align*}
\text{M121A Ni}^\text{II} \text{Az} + \text{CH}_3^+ & \rightarrow \text{M121A Ni}^\text{III} \text{CH}_3 \text{Az} \quad (1) \\
\text{M121A Ni}^\text{II} \text{Az} + \text{CH}_3^+ & \rightarrow \text{M121A Ni}^\text{III} \text{CH}_3 \text{Az} \quad (2) \\
\text{M121A Ni}^\text{III} \text{CH}_3 \text{Az} + e^- & \rightarrow \text{M121A Ni}^\text{III} \text{CH}_3 \text{Az} \quad (3) \\
\text{M121A Ni}^\text{III} \text{CH}_3 \text{Az} + \text{H}^+ & \rightarrow \text{M121A Ni}^\text{III} \text{Az} + \text{CH}_3 \quad (4) \\
\text{M121A Ni}^\text{III} \text{Az} + e^- & \rightarrow \text{M121A Ni}^\text{II} \text{Az} \quad (5)
\end{align*}

The concentrations could vary in the simulations by <10% to account for errors in estimated extinction coefficients and uncertainty error.

**Electron Paramagnetic Resonance Spectroscopy.** Continuous-wave X-band EPR measurements were collected at either 100 K on a Bruker EMXPlus equipped with a Bruker variable-temperature unit or at 30 K using a liquid helium cryostat using a Bruker EMX instrument equipped with an Oxford flow cryostat (ITC-500). An
aliquot of CH$_3$I, $^{13}$CH$_3$I (Sigma-Aldrich), or CD$_3$I (Sigma-Aldrich) was rapidly hand-mixed into a septum-capped EPR tube (Wilmad Lab glass, 727-SQ-230MM) containing 300 μM M121A Ni$^{II}$Az to a final concentration of 1.5 mM and quenched at varying time points in a liquid nitrogen-isopentane bath held at approximately 150 K. Spectra were collected at 30 K with a modulation frequency of 100 kHz and a modulation amplitude of 10 G at a power of 0.2 mW using 30 dB attenuation. For high-resolution spectra at 100 K, a modulation amplitude of 1 G was used, and data points were collected every 0.1 G with a time constant of 10.24 ms and a conversion time of 40 ms. Photolysis studies employed the use of a white LED light (Luxeon). A power saturation study of the trapped intermediate was collected at 5 K at the Ohio Advanced EPR Facility at Miami University. Spectra were baseline-corrected by subtraction of a spline using Igor Pro (WaveMetrics, Lake Oswego, OR) data analysis software. The baseline-corrected EPR spectra were simulated with the EasySpin toolbox within MATLAB.

Magnetic Circular Dichroism (MCD) Spectroscopy. Samples for MCD spectroscopy were prepared anaerobically inside of a glovebox. M121A Ni$^{II}$Az was diluted to a final concentration of 1 mM in 50 mM phosphate buffer, pH 8.0, containing 50% glycerol. This sample was loaded into a custom-made MCD cell and flash frozen in liquid nitrogen to generate an optical glass. Next, M121A Ni$^{II}$Az was prepared by reducing ~2 mM of M121A Ni$^{II}$Az with 25 mM titanium(III) citrate (Ti$^{III}$Cit). The reduced protein sample was diluted with glycerol to produce a 50% glycerol sample. Samples were reacted with 20 mM CH$_3$I, and the reaction allowed to proceed for ~5 min prior to freezing. Samples were loaded into aluminum sample holders between Kapton tape windows and quickly frozen in liquid nitrogen. Data were collected on the HXMA beamline (06ID-1) at the Canadian Light Source (Saskatoon, Saskatchewan, Canada) with samples maintained at 15 K throughout data collection with the use of an Oxford Instruments He(1) flow cryostat. A Si(220) double monochromator, which was detuned 40% for harmonic rejection, was used for light monochromatization. The beam (1 x 1 mm spot size) was moved after every other scan to avoid photodamage of the sample. Fluorescence data were collected with a 30-element solid-state Ge detector array (Canberra Industries) with total count rates kept under 35 kHz with a 3 μm cobalt metal filter placed between the sample and the detector. Energy calibrations were performed by simultaneously recording the spectrum of Ni foil (first inflection point set to 8333.0 eV). Data were collected in 5 eV steps from 8133–8313 eV (1 s integration time), 0.5 eV steps from 8313–8636 eV (3 s integration time), 2 eV steps from 8636–8633 eV (5 s integration time), and 5 eV steps from 8633 eV–k = 16 Å$^{-1}$ (5 s integration time). A total of 10 individual data sets were combined for each spectrum; individual channels of each data set were inspected prior to data averaging. Data workup and analysis was performed as previously described using EXAFSfit2 as previously described on unfiltered $k^2(\gamma)$ data refined over the range of k = 2.5–15.5 Å$^{-1}$. Alternative fits with statistics are presented in the Supporting Information (Tables S3–S4).

ASSOCIATED CONTENT

# Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.inorg-chem.8b03546.

Detailed materials and methods as well as supplemental optical titrations, EPR spectra and simulations, MCD molecular orbital diagrams, statistically significant alternative EXAFS fits, XANES spectra and TD-DFT calculations, DFT structures, coordinates, and a sample input file (PDF)

AUTHOR INFORMATION

Corresponding Author

*E-mail: shafaat.1@osu.edu.

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ORCID
Anastasia C. Manesis: 0000-0001-7162-3676
Jason Shearer: 0000-0001-7469-7304
Nicolai Lehner: 0000-0002-5221-5498
Hannah S. Shafaat: 0000-0003-0793-4650

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Notes
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