

# Nitric Oxide Inhibition of Glycyl Radical Enzymes and Their Activases

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**ABSTRACT:** Innate immune response cells produce high concentrations of the free radical nitric oxide (NO) in response to pathogen infection. The antimicrobial properties of NO include non-specific damage to essential biomolecules and specific inactivation of enzymes central to aerobic metabolism. However, the molecular targets of NO in anaerobic metabolism are less understood. Here, we demonstrate that the *Escherichia coli* glycyl radical enzyme pyruvate formate lyase (PFL), which catalyzes the anaerobic metabolism of pyruvate, is irreversibly inhibited by NO. Using electron paramagnetic resonance and site-directed mutagenesis we show that NO destroys the glycyl radical of PFL. The activation of PFL by its cognate radical S-adenosyl-L-methionine-dependent activating enzyme (PFL-AE) is also inhibited by NO, resulting in the conversion of the essential iron-sulfur cluster to dinitrosyl iron complexes. Whole-cell EPR and metabolic flux analyses of anaerobically growing *Escherichia coli* show that PFL and PFL-AE are inhibited by physiologically relevant levels of NO in bacterial cell cultures, resulting in diminished growth and a metabolic shift to lactate fermentation. The class III ribonucleotide reductase (RNR) glycyl radical enzyme and its corresponding RNR-AE are also inhibited by NO in a mechanism analogous to those observed in PFL and PFL-AE, which likely contributes to the bacteriostatic effect of NO. Based on the similarities in reactivity of the PFL/RNR and PFL-AE/RNR-AE enzymes with NO, the mechanism of inactivation by NO appears to be general to the respective enzyme classes. The results implicate an immunological role of NO in inhibiting glycyl radical enzyme chemistry in the gut.

## INTRODUCTION

Opportunistic pathogens infecting the gastrointestinal and respiratory tracks, such as *Shigella dysenteriae*, *Salmonella enterica*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, are facultative anaerobes.<sup>1-5</sup> This metabolic flexibility allows for aerobic pathogen transmission and subsequent establishment and proliferation in the anaerobic or microaerobic environment of the host mucosal epithelium-microbiome interface.<sup>6,7</sup> Dysregulation of the microbiome at this interface induces a host innate immune response, resulting in the release of reactive oxygen species (ROS, *e.g.* H<sub>2</sub>O<sub>2</sub>)<sup>8,9</sup> and reactive nitrogen species (RNS, *e.g.* NO),<sup>10-12</sup> both of which have pleiotropic and hormetic, yet still undefined roles in pathophysiology.<sup>13,14</sup> For microorganisms adapted to anaerobic metabolism, ROS disrupt redox homeostasis and damage biomolecules and essential cofactors.<sup>15</sup> The effect(s) of RNS, such as NO, on anaerobic metabolism are less defined and may have practical implications in therapeutic developments to manage or treat diseases.<sup>15-18</sup>

During anaerobic glycolysis, many bacteria and archaea in the human gut, at least partially, metabolize pyruvate to acetyl-coenzyme A (acetyl-CoA) and formate through the action of pyruvate formate lyase (PFL).<sup>19-21</sup> PFL is a member of the glycyl radical enzyme (GRE) family, requiring a post-translationally installed glycyl radical for activity.<sup>22-24</sup> This essential radical is installed by a specific activating enzyme,

PFL-AE, a member of the radical S-adenosyl-L-methionine (SAM)-dependent enzyme superfamily.<sup>25-27</sup> The acetyl-CoA product can be used for substrate level phosphorylation to form adenosine triphosphate (ATP) and serve as an electron sink to recycle nicotinamide adenine dinucleotide (NAD<sup>+</sup>), or provide carbon in anabolic biosynthesis.<sup>28</sup> The formate product can be expelled as a waste product, either as formate or carbon dioxide and hydrogen, or used as an electron and carbon source.<sup>29-31</sup> Anaerobic growth in organisms that utilize PFL is often possible in the absence of PFL, provided a suitable electron acceptor is present, such as nitrate, or acetate is available for acetyl-CoA biosynthesis.

During infection, PFL is up-regulated in many opportunistic pathogens. The deletion of *pflA* (PFL-AE) and *pflB* (PFL) genes results in a loss of virulence in some pathogens, further suggesting PFL provides a virulent fitness advantage.<sup>32-36</sup> Given its role in central metabolism and pathogenesis, and the inherent reactivity of amino acid radicals, we hypothesize that PFL is a molecular target of the immune response. The glycyl radical of PFL is extremely sensitive to O<sub>2</sub>, cleaving the enzyme polypeptide chain at the glycyl  $\alpha$ -carbon, but to our knowledge, no other ROS have been investigated.<sup>37,38</sup> The radical SAM PFL-AE contains an essential [Fe<sub>4</sub>S<sub>4</sub>] cofactor and is also sensitive to inactivation by O<sub>2</sub>, suggesting that both PFL and PFL-AE are susceptible to inactivation by O<sub>2</sub>, and potentially other ROS.<sup>19</sup> Evidence for the reactivity of the RNS NO with PFL or PFL-AE,

however, is indirect. In a study of *S. aureus*, exposure to NO under anaerobic conditions results in the diversion of metabolism from ethanol fermentation via PFL to lactate production, and in a murine infection model, *S. aureus* was non-pathogenic in the absence of an NO-inducible lactate dehydrogenase.<sup>39</sup> These results suggest that NO inhibits PFL *in vivo*, with implications in pathogenic disease progression.

Here, we report the reactivity of NO with active glycy radical-containing PFL (aPFL) from *E. coli*, PFL-AE, and additional representative members of the GRE and radical SAM enzyme families. *In vitro*, the addition of an NO donor to aPFL results in the complete loss of the glycy radical and enzyme activity. The reaction appears to be diffusion-limited and specific to the PFL glycy radical. We observe similar reactivity with the *E. coli* class III ribonucleotide reductase (RNR), suggesting the mechanism of irreversible inhibition is common to the GRE family. We also demonstrate that PFL-AE and RNR-AE are irreversibly inhibited by NO, although through a slower process, involving dinitrosyl iron products, known inhibition products of [Fe<sub>4</sub>S<sub>4</sub>] cluster-containing enzymes associated with aerobic metabolism such as aconitase and succinate dehydrogenase.<sup>40–42</sup> The inhibition of PFL and PFL-AE was also observed *in vivo* by whole-cell electron paramagnetic resonance (EPR) spectroscopy, revealing the quenching of the glycy radical signal and the concomitant formation of dinitrosyl iron complexes (DNICs) in PFL and PFL-AE overexpressing cells. This inhibition was accompanied by a shift in the metabolic products of anaerobically growing *E. coli* from acetate, formate, and ethanol to lactate production. These findings suggest that NO inhibits anaerobic microbial metabolism through multiple mechanisms, contributing to the antimicrobial activity of NO in the host immune response against pathogenic infections in anaerobic or microaerobic environments.

## EXPERIMENTAL

**Materials.** Electrocompetent DH5 $\alpha$  and BL21(DE3) *E. coli* and NEBuilder® HiFi DNA Assembly Master Mix were purchased from New England Biolabs. Carbenicillin and L-arabinose were purchased from GoldBio. Chloramphenicol, kanamycin and agarose were purchased from Apex BioResearch Products. LB Miller broth, citrate synthase (porcine heart), malate dehydrogenase (porcine heart), bovine serum albumin, myoglobin (equine heart), alcohol dehydrogenase (*Saccharomyces cerevisiae*), tris(hydroxymethyl)aminomethane base (Tris),  $\beta$ -nicotinamide adenine dinucleotide (NAD<sup>+</sup>), S-(5'-adenosyl)-L-methionine (SAM) iodide salt, KH<sub>2</sub>PO<sub>4</sub>, Triton X-100, glycerol, MgCl<sub>2</sub>·6H<sub>2</sub>O, oxamic acid, sodium pyruvate, sodium formate, sodium lactate, ethanol, malic acid, iodoacetamide, LC-MS grade trifluoroacetic acid (TFA), dithiothreitol (DTT), L-cysteine, (NH<sub>4</sub>)Fe<sup>II</sup>(SO<sub>4</sub>)<sub>2</sub>, formic acid, 4-hydroxy-TEMPO, urea, sodium dithionite (NaDT), sodium borohydride (NaBH<sub>4</sub>), glycine, glucose, MgSO<sub>4</sub>, CaCl<sub>2</sub>, biotin, thiamin, Na<sub>2</sub>HPO<sub>4</sub>, NH<sub>4</sub>Cl, ethylenediaminetetraacetic acid (EDTA), FeCl<sub>3</sub>·6H<sub>2</sub>O, ZnCl<sub>2</sub>, CuCl<sub>2</sub>·2H<sub>2</sub>O, CoCl<sub>2</sub>·6H<sub>2</sub>O, H<sub>3</sub>BO<sub>3</sub>, MnCl<sub>2</sub>·6H<sub>2</sub>O, iron ICP standards (TraceCERT), 70% trace metal-free nitric acid, isopropyl- $\beta$ -D-1-thiogalactopyranoside (IPTG), cytidine triphosphate (CTP), adenosine triphosphate (ATP), cytidine, 2'-deoxycytidine (dC), and Amicon Ultra centrifugal filter units were purchased from Millipore Sigma. 5-Deazariboflavin was obtained from Santa Cruz Biotechnologies.

Sequencing grade modified trypsin was purchased from Promega Corporation. Coenzyme A was purchased from Co-Ala Biosciences. HiTrap desalting 5 mL columns were purchased from Cytiva Life Sciences. Nickel nitrilotriacetic acid (Ni-NTA) agarose resin was purchased from Qiagen. His-Pur™ Cobalt Resin and 0.5 mL Zeba™ spin desalting columns of 7 kDa molecular weight cut-off (MWCO) were purchased from Thermo Scientific. Adenosine (A) was purchased from VWR. Diethylammonium (Z)-1-(N,N-diethylamino)diazene-1-ium-1,2-diolate (NONOate) was purchased from Cayman Chemicals. SapphireAmp® Fast PCR master mix was purchased from Takara Bio. Vivaspin 20 filtration units were purchased from Sartorius. The acetic acid assay kit (ACS Manual format) was purchased from Megazyme. Calf alkaline phosphatase was purchased from Roche. HPLC-grade water with 0.1% TFA and acetonitrile with 0.1% TFA were purchased from Honeywell. Milli-Q water (>17 M $\Omega$ ) was used for preparing all solutions. The plasmid pCAL-n-EK encoding the *pflA* gene was a gift from Dr. Joan Broderick.<sup>43</sup> The *pflB* gene (Uniprot ID P09373) and the mutants C<sub>418</sub>S and C<sub>419</sub>S were cloned into the plasmid pCm8 previously.<sup>44</sup> The plasmid pCm2 *NikJ* was available from a previous study.<sup>45</sup> The *Pseudomonas* sp. 101 formate dehydrogenase (FDH) gene (Uniprot ID P33160) was codon optimized and synthesized by Integrated DNA Technologies. The plasmids pFGET19\_Ulp1 and pHYRSF53 were a gift from Hideo Iwai (Addgene plasmid # 64697 and # 64696, respectively).<sup>46</sup> The TEVSH plasmid was a gift from Dr. Helena Berglund (Addgene plasmid # 125194).<sup>47</sup> The plasmid pDB1282 was a gift from Dr. Squire Booker and previously constructed in the laboratory of Dr. Dennis Dean.<sup>48,49</sup> The plasmid pET28a-EcNrdD and pN9-EcNrdG were a gift from Dr. JoAnne Stubbe<sup>50</sup>. The plasmid pLZ113 harboring the D176G-I177L-F178W LDH was a gift from Dr. Han Li.<sup>51</sup>

**Construction of Plasmids.** To produce and purify PFL-AE, RNR-AE and FDH, we followed similar cloning strategies. Each gene was cloned into the pYRSF53 plasmid using Gibson assembly.<sup>52</sup> These plasmids yield expressed proteins with an N-terminal 6 $\times$  polyhistidine tag, up-stream of a small ubiquitin-like modifying (SUMO) protein fusion, termed SUMO-PFL-AE, SUMO-RNR-AE and SUMO-FDH respectively. The genes were cloned into the plasmid replacing the gene of the protein of interest encoded in pYRSF53, downstream of the SUMO gene and in the same open reading frame by Gibson assembly. The DNA fragment containing the genes (fragments) and the plasmid backbone (vector) were cloned using the primers listed in the **Supporting Information Table S1**.

The PCR-amplified complementary DNA fragments were assembled using NEBuilder® HiFi DNA Assembly Master Mix following the manufacturer's instructions. Assembled reaction products were transformed into *E. coli* DH5 $\alpha$  cells and streaked onto LB-agar plates supplemented with 50  $\mu$ g/mL kanamycin. Successful transformants were identified using colony PCR with the SapphireAmp fast PCR-hot-start master mix, as directed by the manufacturer, and the fidelity of the cloning was confirmed by Sanger sequencing through UC Berkeley DNA Sequencing Facility using the T7 promoter/terminator primers.

**Protein Expression and Purification.** The expression and purification of the TEV protease and the ubiquitin-like

protease 1 (ULP1) were performed as previously described.<sup>46,47</sup> The protein expression and purification of PFL wild type (wt,  $77 \pm 3$   $\mu\text{mol}/\text{min} \times \text{mg}$  normalized to G●) and the serine (S) mutants C<sub>418</sub>S and C<sub>419</sub>S, as well as PFL-AE, were carried out as previously described without further modifications.<sup>44</sup> The enzyme NikJ ( $2.6 \pm 0.1$  Fe/protein) was expressed and purified as reported before using the pCm2 plasmid.<sup>45</sup> Lactate dehydrogenase (LDH,  $1,330 \pm 30$   $\text{s}^{-1}$ ) was expressed and purified as described before.<sup>53</sup> Expression and purification of FDH ( $2.3$   $\mu\text{mol}/\text{min} \times \text{mg}$ ), RNR ( $1,350 \pm 90$   $\text{nmol}/\text{min} \times \text{mg}$  normalized to G●), RNR-AE ( $2.2 \pm 0.1$  Fe/protein) and PFL-AE ( $2.1 \pm 0.2$  Fe/protein) are detailed in **Supporting Information Supplemental Methods**. Pure proteins concentration in water was determined using the Edelhoch method<sup>54</sup> but using the extinction coefficients for tryptophan (W) and tyrosine (Y) determined by Pace.<sup>55</sup>

**Iron Quantification.** We quantified iron by inductively coupled plasma optical emission spectroscopy (ICP-OES). Typically, 8 nmol of protein was digested by adding 86  $\mu\text{L}$  of 70% (v/v) trace metal-free nitric acid and incubated overnight at room temperature followed by 2 h incubation at 90 °C. Once samples returned to room temperature, 70  $\mu\text{L}$  of 30% (v/v) hydrogen peroxide was added and incubated at 90 °C for 1 h. Finally, water was added to a final weight of 3 g and analyzed in an Agilent 5800 ICP-OES in the following configuration: read time 5 s, RF power 1.45 kW, stabilization time 15 s, in axial viewing mode, nebulizer flow 0.7 L/min, plasma flow 12 L/min, and auxiliary flow 1 L/min. A calibration curve using iron standards in 2% nitric acid, with varying concentrations between 12.5 ppb and 1 ppm was used to quantitate iron in protein samples.

**PFL Activation.** All experiments with glycy radical enzymes or activator enzymes were performed in a VAC Atmospheres glovebox (< 2 ppm O<sub>2</sub>), unless otherwise described. We activated PFL photochemically by mixing 25  $\mu\text{M}$  PFL, 2.5  $\mu\text{M}$  PFL-AE, 2 mM SAM, 20 mM oxamate, and 100  $\mu\text{M}$  5-deazariboflavin in activation buffer composed of 100 mM Tris, 100 mM KCl, 10 mM DTT, and 8% (w/v) glycerol at pH 7.6 in a 50 mL beaker, volumes ranging from 800  $\mu\text{L}$  to 2 mL were activated each time, producing a layer of protein sample of 0.6 mm to 1.4 mm. The activation mixture was then exposed to a 1 W 405 nm LED light (Thor Labs) located approximately 5 cm above the protein sample for 1.5 h at room temperature. The lamp irradiance is 760 mW over the sample surface area. The extent of PFL activation was estimated by activity assays and EPR quantitation of the resultant G● (routinely 0.8-1.0 G●/PFL homodimer).

We measured PFL activity spectrophotometrically using a multi-enzyme assay that couples the PFL-dependent formation of acetyl-CoA from pyruvate to the production of NADH by the oxidation of malate to oxaloacetate and condensation of oxaloacetate and acetyl-CoA to citrate and CoA by malate dehydrogenase and citrate synthase, respectively, as previously reported.<sup>56-58</sup> The 400  $\mu\text{L}$  assay solution contained 10 mM DTT, 1 mM NAD<sup>+</sup>, 10 mM malate, 2 U/mL citrate synthase, 30 U/mL malate dehydrogenase and 0.05 mg/mL bovine serum albumin in 100 mM Tris buffer, adjusted to pH 8.1. Reactions were initiated by adding 3 nM aPFL (based on G●) and the rate of NADH production was calculated based on the UV absorption at 340 nm using an NADH extinction coefficient of  $6.2 \text{ mM}^{-1} \text{ cm}^{-1}$  via a custom

fiber-coupled Ocean Optics QEPro spectrophotometer and a DH-2000-BAL light source.<sup>59</sup>

**Inactivation of PFL by NO.** For enzyme inactivation kinetics we buffer-exchanged aPFL into 100 mM Tris and 100 mM KCl at pH 7.6 using a Zeba Spin desalting column to a final concentration of 10  $\mu\text{M}$  aPFL and maintained the solution at 30 °C using a water bath. The NONOate was then added to the aPFL solution to an estimated final concentration of either 100  $\mu\text{M}$  or 1 mM from a stock solution in 100 mM glycine buffer pH 10.0. Aliquots of the reaction were sampled from 10 s to 26 min after mixing and immediately diluted 50-fold in buffer consisting of 100 mM glycine at pH 10.0 supplemented with 0.2 mg/mL of reduced deoxymyoglobin (deoxyMb) to stop the release of NO and bind any free NO. We then determined the remaining aPFL activity as described above.

To estimate the NO concentration during the decomposition of the NONOate we used deoxyMb as an NO indicator by measuring the conversion of the deoxyMb heme Soret peak shift from 431 nm to 421 nm upon binding NO.<sup>60</sup> First, a solution of 10 mg/mL of reduced myoglobin was prepared by mixing oxidized myoglobin, quantitated by the heme Soret absorbance at 409 nm,<sup>61</sup> with 10 mM NaDT in buffer consisting of 100 mM Tris and 100 mM KCl at pH 7.6 and then desalted to the same buffer without NaDT. The resulting deoxyMb concentration was redetermined by the heme Soret absorbance at 431 nm.<sup>62</sup> To measure NO release from NONOate, 20  $\mu\text{M}$  of deoxyMb was incubated with 100  $\mu\text{M}$  or 1 mM NONOate in the same way as for aPFL. Aliquots were sampled during the reaction from 10 s to 26 min and immediately diluted 50-fold in buffer consisting of 100 mM glycine at pH 10.0 to stop the release of NO. NO concentration was estimated using the NO-Mb Soret absorbance of the heme-NO adduct at 421 nm.<sup>63</sup>

The loss of the aPFL G● upon reaction with NO was analyzed by UV-vis absorption, monitoring the characteristic G● absorption feature at 360 nm.<sup>64</sup> In this assay, a solution of 55  $\mu\text{M}$  aPFL in 100 mM Tris and 100 mM KCl pH 7.6 was prepared in a quartz cuvette and NONOate was added to a final concentration of 500  $\mu\text{M}$  at room temperature. The reaction was followed over time by using a custom fiber-coupled Ocean Optics QEPro spectrophotometer and a DH-2000-BAL light source.

Inactivated samples were also analyzed by EPR. Similar inactivation assays were prepared using 20  $\mu\text{M}$  of aPFL wt, C<sub>418</sub>S or C<sub>419</sub>S and 200  $\mu\text{M}$  DEA NONOate. Samples (250  $\mu\text{L}$ ) were then transferred to EPR tubes and flash frozen in liquid N<sub>2</sub>-cooled isopentane (< -130 °C) at different reaction times and analyzed as described below.

**X-Band EPR Spectroscopy.** All EPR samples were prepared in a VAC Atmosphere glovebox with < 2 ppm of O<sub>2</sub> in 4 mm o.d. quartz EPR tubes and frozen in liquid N<sub>2</sub>-cooled isopentane (< -130 °C). EPR spectra of the samples were collected using a Bruker EMXplus EPR spectrometer at 100 K with a microwave frequency between 9.38-9.44 GHz, power of 20  $\mu\text{W}$  or 2 mW, modulation amplitude of 2 G, modulation frequency of 100 kHz, time constant of 0.01 ms, scan time of 20 s, and conversion time of 16 ms. All reported spectra are the average of 30 scans. Spin quantitation was computed from the double integral of the first harmonic signal and referenced to a 4-hydroxy-TEMPO standard. All EPR



spectral simulations were performed using EasySpin 6.0.0 software, and confidence intervals and standard deviations of the fitting parameters are reported.<sup>65</sup>

**Liquid Chromatography-Tandem Mass Spectrometry.** We searched for covalent modifications of the glycine radical motif tryptic peptide in reactions of aPFL treated with NO by using trypsin digestion followed by liquid chromatography and electrospray ionization quadrupole time-of-flight tandem mass spectrometry (LC-MS/MS) on a Shimadzu LCMS-9030 q-TOF system. Samples of 25  $\mu$ M aPFL were treated with 100  $\mu$ M or 1 mM NONOate and incubated at room temperature for 10 min to 1 h. Reactions were stopped by mixing 5  $\mu$ L of sample with 20  $\mu$ L of 8 M urea in 100 mM ammonium bicarbonate and incubated for 1 h at 25 °C. Alternatively, some samples were treated with 10 mM TCEP, 10 mM DTT, or 10 mM sodium borohydride to reduce cysteines or possible nitroxyl adducts. For samples in reducing conditions cysteines were alkylated with 15 mM iodoacetamide for 60 min in the dark, and the reaction was quenched by adding 10 mM DTT or TCEP and incubated for 10 min. The samples were diluted to < 2 M urea using 100 mM ammonium bicarbonate and 0.16  $\mu$ g of trypsin was added and the protein was digested at 37 °C overnight. Reactions were stopped by adding formic acid to a final concentration of 1% (v/v).

The digested samples (2-5  $\mu$ g at 0.4-1  $\mu$ g/ $\mu$ L) were injected into the LC-MS/MS. The UPLC stationary phase was a Shim-pack Arata C18 column (2.2  $\mu$ m, 150 mm  $\times$  2.0 mm). The peptides were resolved by a linear gradient composed of a solution of H<sub>2</sub>O with 0.1% (v/v) formic acid (mobile phase A) and a solution of acetonitrile with 0.1% (v/v) formic acid (mobile phase B), from 1-30% B over 233 min, with a flow rate of 0.2 mL/min at 60 °C. Eluent from the LC was injected directly into the q-ToF. The mass spectrometer interface settings were as follow: nebulizing gas flow 2 L/min, heating gas flow 10 L/min, interface temperature 100 – 300 °C, and a desolvation temperature of 526 °C. The interface voltage was set to 4.5 V and the DL temperature to 250 °C. The spectrometer was run in data-dependent acquisition mode (DDA) with positive polarity using an event time of 0.35 s (MS<sup>1</sup>). Five DDA events between 200 and 1500 m/z, with a threshold of 200 counts and ions with charges between 1 and 6, were selected for fragmentation (MS<sup>2</sup>) using a Q1 transmission window of 1 m/z. The MS<sup>2</sup> collision energy was set to 35  $\pm$  17 V and MS<sup>2</sup> ions were detected between 200 and 1,500 m/z.

The LC-MS/MS data was analyzed using the Shimadzu Lab solutions Postrun software. The MS<sup>1</sup> and MS<sup>2</sup> ions with different expected modifications on the glycy radical tryptic peptide were calculated using Skyline.<sup>66,67</sup> The DDA-selected MS<sup>1</sup> ions were fragmented and the ions matching predicted m/z for glycy radical tryptic peptides were manually compared to the calculated MS<sup>2</sup> spectra from Skyline.

**Inactivation of PFL-AE by NO.** We monitored NO inactivation of PFL-AE by measuring the effect on PFL activation (activity) and UV-vis absorbance changes associated with the essential [Fe<sub>4</sub>S<sub>4</sub>] cluster. To initiate the reaction with NO, 20  $\mu$ M PFL-AE was mixed with 100  $\mu$ M NONOate and incubated in a quartz cuvette for 30 min under continuous observation by UV-vis absorption. The reaction was then buffer exchanged to remove residual NO and NONOate by

concentrating using 50 kDa MWCO centrifugal filters and then desalting to buffer consisting of 100 mM Tris, 100 mM KCl at pH 7.6 using a Zeba desalting column. We estimate the final concentration of NO/NONOate after these steps to be <1  $\mu$ M. The resulting PFL-AE sample was then diluted 50-fold into PFL activation buffer used to activate PFL for 1 h, and PFL activity was measured using the spectrophotometric enzyme-coupled assay described above. A control sample was prepared in the same way in the absence of added NONOate.

The inactivation of PFL-AE was also monitored by EPR spectroscopy. Samples were prepared by mixing 50  $\mu$ M of enzyme with 500  $\mu$ M of NONOate in buffer consisting of 100 mM Tris, 100 mM KCl, 10% (w/v) glycerol adjusted to pH 7.6 and incubated at room temperature. Where indicated, samples were also prepared in the same way with the addition of 2 mM SAM or 5 mM NaDT before adding NONOate. The samples were then transferred to EPR tubes and flash frozen in liquid N<sub>2</sub>-cooled isopentane (< -130 °C) at different reaction times and analyzed as previously described.

**NO Treatment of Bacterial Cell Culture.** To observe the effects of NO treatment in the metabolism of *E. coli*, we inoculated *E. coli* BL21 DE3 in 15 mL of LB medium and grew the cells overnight in 50 mL centrifuge tubes in a tube rotator at 20 rpm and 37 °C in anaerobic conditions in a vinyl glovebox at < 20 ppm O<sub>2</sub>. We used 300  $\mu$ L of these cultures to inoculate 30 mL of M9 medium with 0.4% (w/v) glucose as the only carbon source and grew the cells as previously described. We took 850  $\mu$ L samples over time and recorded the cell density by OD<sub>600</sub>. Each sample was then centrifuged at 20,000  $\times$  g for 5 min and the supernatant was used to determine the extracellular concentration of the fermentation products lactate, formate, ethanol, and acetate using spectrophotometric enzyme-coupled assays (**Supporting Information Supplemental Methods**).

The bacteriostatic and bactericidal effects of NO on anaerobically growing were assessed during growth in minimal media as previously described. At an OD<sub>600</sub> of 0.35, prior to NO treatment, an aliquot of the cell culture was sampled for cell viability, reported as colony forming units (CFUs), by rapid removal from the anaerobic chamber, centrifugation and serial dilution into sterile Milli-Q water (>17 M $\Omega$ ) before plating the cell culture on LB-agar plates. After collection of the pre-treatment control the cells were treated with 100  $\mu$ M NONOate at OD<sub>600</sub> of 0.4 and sampled again at 30 min and 4 h after NO treatment. The plated cells were then incubated at 37 °C overnight, and colonies were counted the following day, and cell viability was determined as CFU/mL. Similar experiments were performed for cultures that were not treated with NONOate.

To analyze the effect of NO over radical species *in vivo*, and in order to observe the effect of NO on PFL and PFL-AE we transformed *E. coli* BL21 DE3 with the plasmids pCm8 wt PFL or pCal-n-EK *pflA* and cultivated the cells overnight in 15 mL of LB medium supplemented with 50  $\mu$ g/mL of chloramphenicol or kanamycin respectively. We grew the cultures overnight in 50 mL centrifuge tubes in a tube rotator at 20 rpm and 37 °C in anaerobic conditions in a vinyl glovebox at < 20 ppm O<sub>2</sub>. We used 300  $\mu$ L of these cultures to inoculate 30 mL cultures of LB supplemented with the corresponding antibiotic. The cells were cultured to OD<sub>600</sub>

of 0.6 and protein expression was induced by adding 1 mM of IPTG. Induced cells were kept growing at 25 °C for 18 h, after which 100  $\mu$ M or 1 mM NONOate was added and incubated for 5 min. We proceeded to collect the cells by centrifugation at 5,000  $\times$  g for 5 min and packed the cell pellet in an EPR tube and flash froze them in liquid N<sub>2</sub>-cooled isopentane.

**RNR Activation.** We activated the class III RNR photochemically by mixing either 50 or 100  $\mu$ M RNR, 5 or 10  $\mu$ M RNR-AE, 2 mM SAM, 100  $\mu$ M 5-deazariboflavin in activation buffer composed of 100 mM Tris, 100 mM KCl, 10 mM DTT and 8% (v/v) glycerol at pH 7.6 in a 50 mL beaker, for kinetic characterization or EPR analysis and NO inhibition, respectively. The mixture was exposed to the aforementioned 1 W 405 nm LED light set up for 1 h at room temperature. The extent of activation was estimated by activity assays and EPR quantitation of the resultant G• relative to a TEMPO standard. Typical activations yielded 0.01-0.04 G• per dimer.

**RNR Activity Determined via LC MS Analysis.** We measured RNR activity via LC-MS analysis measuring conversion of cytidine triphosphate (CTP) to 2'-deoxycytidine triphosphate (dCTP). All activity measurements were performed in a VAC Atmosphere glove box (< 2 ppm O<sub>2</sub>). Following activation of 100  $\mu$ M RNR, the activated RNR (aRNR) was added to a final concentration of 2  $\mu$ M aRNR (50  $\mu$ M total RNR) in assay buffer containing 3 mM CTP, 1 mM ATP, 10 mM formate, 30 mM KCl, 10 mM MgSO<sub>4</sub>, and 30 mM Tris at pH 7.6. Aliquots were taken from 15 to 300 s and quenched by boiling in a pre-heated 1.5 mL centrifuge tube in a heat block for 2 min. Quenched samples were then brought out of the anaerobic chamber and clarified via centrifugation for 15 min at 25,000  $\times$  g. 10  $\mu$ L of the resulting supernatant was diluted 2-fold into dephosphorylation buffer with 1 U/mL calf alkaline phosphatase and digested for 2 h at 37 °C according to the manufacturer's instructions. Digestion was quenched by addition of 0.1% (v/v) TFA then diluted 20-fold with Milli-Q water and centrifuged for 30 min at 25,000  $\times$  g prior to injection on the LC-MS. Nucleoside standards of 2'-deoxycytosine (dC, 2.5–50  $\mu$ M) were prepared in Milli-Q water and adenosine (A, 12.5  $\mu$ M) was used as an internal standard.

To determine the effect of NO on aRNR activity we activated 100  $\mu$ M RNR and treated half of the assay (3.5  $\mu$ M aRNR) with 500  $\mu$ M NONOate, from a 13.6 mM stock solution in 100 mM glycine buffer pH 10.0, and incubated at room temperature for 10 min. After treatment with NO the samples were immediately diluted 5-fold in buffer consisting of 100 mM glycine at pH 10.0 to stop the release of NO. The other half of the activation assay was reserved as a control for untreated activity. The quenched NONOate-treated RNR was then diluted 2-fold into the assay buffer (10  $\mu$ M RNR) and activity was measured between 0 s and 5 min as described above. The non-treated control was diluted 5-fold into assay buffer and similarly analyzed for activity from 0 s to 5 min.

Assay samples were injected onto a Shimadzu LCMS-9030 equipped with a Luna polar C18 column (1.6  $\mu$ m, 50 mm  $\times$  2.1 mm). Nucleosides were resolved with a linear gradient composed of mobile phases 0.1% (v/v) TFA in water (C) and 0.1% TFA in acetonitrile (D) from 0-5% D in C over

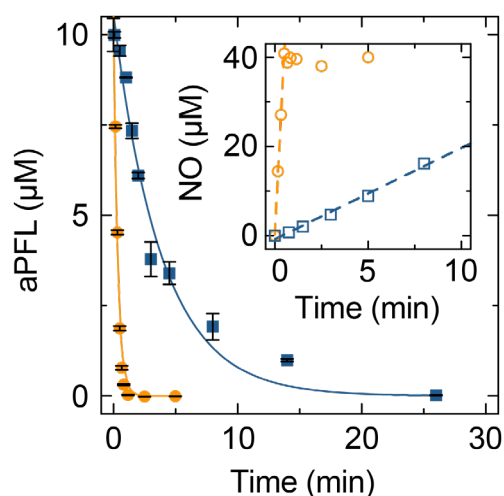
8 min with a flow rate of 0.4 mL/min and a column temperature of 40 °C. Single ion monitoring (SIM) was used to quantitate dC (228.1 m/z  $\pm$  50 ppm) and A (268.1 m/z  $\pm$  50 ppm). Time-dependent dC formation was analyzed using the Shimadzu Lab Solutions Postrun software and determined from the chromatogram SIM MS<sup>1</sup> integrated peak area normalized to the A internal standard and compared to a dC standard calibration curve.

**EPR of RNR-AE and NikJ Reacted with NO.** RNR-AE and NikJ samples for EPR spectroscopy were prepared by mixing 50  $\mu$ M of enzyme with 500  $\mu$ M of NONOate in buffer consisting of 100 mM KCl, 100 mM Tris, and 10% (w/v) glycerol adjusted to pH 7.6 and incubated at room temperature. To examine the role of SAM and reductant on the reactions with NO, samples were prepared in the same way, but with 2 mM SAM, or by preincubating the enzyme for 15 min with 5 mM NaDT before adding NONOate. Samples (250  $\mu$ L) were then transferred to EPR tubes and flash frozen in liquid N<sub>2</sub>-cooled isopentane at different reaction times and analyzed as described above.

## RESULTS

**Pyruvate Formate Lyase Inactivation by NO.** To characterize the inactivation of aPFL by NO we used the diethylamine diazeniumdiolate (NONOate) as an NO delivery agent, which is a stable aqueous solute at pH > 9, but decomposes in a pH- and temperature-dependent first order process.<sup>68,69</sup> The release of NO was quantified by monitoring the effectively diffusion-controlled and irreversible conversion of reduced myoglobin (deoxyMb) to the NO-bound myoglobin (NO-Mb) via the Soret band shift from 431 nm to 420 nm (**Supporting Information Figure S1**).<sup>70,71</sup> At 30 °C and pH 7.6 we estimated a  $t_{1/2}$  of approximately 25 min, corresponding to an NO production rate of 2.05  $\mu$ M/min and 81  $\mu$ M/min at 100  $\mu$ M or 1 mM NONOate, respectively (**Figure 1**, inset). When 10  $\mu$ M of aPFL is mixed with excess NONOate at either 100  $\mu$ M or 1 mM and aliquots were sampled for PFL activity, activity was lost concomitantly with NO release (**Figure 1**). The activity of aPFL followed an exponential decay with an apparent rate of 0.26  $\mu$ M/min at 100  $\mu$ M and at 2.96  $\mu$ M/min at 1 mM NONOate.

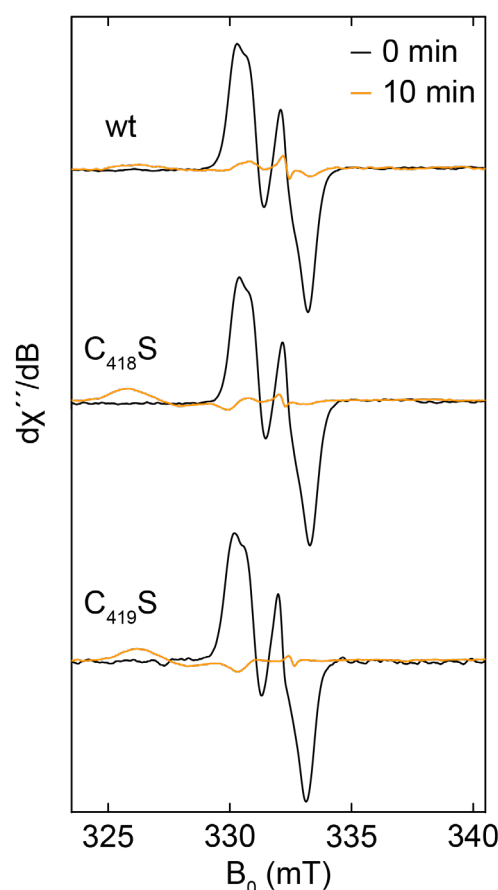
In the measurement of residual activity of PFL following reaction with NO, enzyme and NONOate were diluted 3,000-fold and free NO was removed by binding to excess deoxyMb. Despite the elimination of NO, the enzyme remained inhibited and showed no signs of recovering over the course of the NO-free activity assay, suggesting the inhibition mechanism is irreversible. For PFL, as with all GREs, elimination of the essential G• abolishes activity, but the enzyme can be re-activated by PFL-AE, assuming no other modifications have been made. To examine the nature of NO inhibition of PFL we completely inactivated aPFL with NO, buffer exchanged the inhibited PFL into NO-free reconstitution buffer, and attempted to reactivate the enzyme via the same protocol used to generate the active enzyme initially with PFL-AE. After NO inhibition, the maximal activity that could be recovered varied between 10-20% (**Supporting Information Figure S2**). We observe a similar degree of reactivation after irreversible inactivation of aPFL by exposure to O<sub>2</sub> (**Supporting Information Figure S2**), which cleaves the polypeptide chain at the glycine C $\alpha$ , rendering the protein



**Figure 1.** The inhibition of aPFL by NO. Samples of 10  $\mu$ M aPFL were mixed with either 100  $\mu$ M (blue squares) or 1 mM (orange circles) NONOate. Aliquots were taken at the indicated time points and assayed for activity. The activity decay was fit to a single exponential decay model (solid lines). Error bars represent the span of two technical replicates. Inset shows the measured NO released using deoxyMb as a NO reporter and fitted to a linear model (dashed lines).

unactivatable.<sup>37,38,72</sup> PFL is proposed to exhibit “half-of-sites” activation, with only one G• per homodimer, but diradical dimers have not been ruled out.<sup>19</sup> As such, we attribute the observed inefficient reactivation after treatment with NO to activation of a previously non-activated monomer of a former half-of-sites dimer, or to the activation of dimers that had not been activated at all previously.

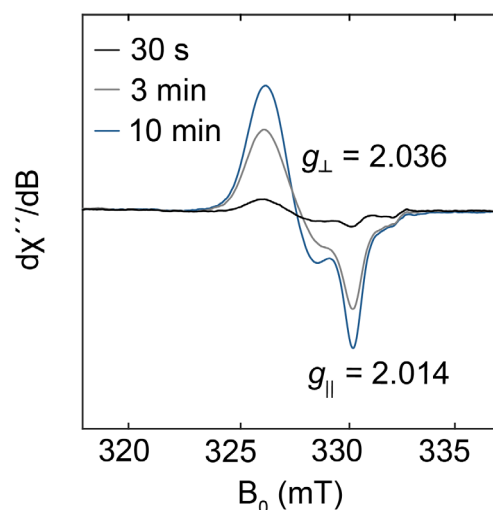
**NO Targets the Glycyl Radical of PFL.** As a free radical, we expected NO to react with one of the essential amino acid radicals associated with PFL activity, namely the stable G<sub>734</sub>• (*E. coli* numbering) or either of the transient thiyl radicals of C<sub>419</sub> or C<sub>418</sub>. The G• exhibits a characteristic UV-vis absorption feature at 365 nm that completely decays following exposure to NO (**Supporting Information Figure S3**). This observation is supported by X-band EPR spectroscopy, where samples were collected before addition of the NONOate and 10 min after (**Figure 2**). The characteristic asymmetric doublet EPR feature of G<sub>734</sub>• is completely lost after 10 min of incubation, consistent with the loss of activity.<sup>22,25</sup> The UV-vis and EPR data are consistent with a mechanism of inhibition that quenches the endogenous glycyl radical on PFL. Thiyl radicals are known to react with NO to form S-nitrosothiols,<sup>73,74</sup> but alkyl radicals also react with NO, thus the specific radical target of NO inhibition was not obvious.<sup>75</sup> We generated C<sub>419</sub>S and C<sub>418</sub>S PFL mutants which can be reconstituted to generate G<sub>734</sub>•, but are completely inactive, due to the redox-inert nature of serine. While these mutants are not active, they provide insight into the reactivity of NO with PFL. Both C<sub>419</sub>S and C<sub>418</sub>S mutants showed complete radical loss by EPR over the same time frame as the wt enzyme (**Figure 2**).



**Figure 2.** Normalized X-band EPR spectra of aPFL wt and mutants C<sub>418</sub>S and C<sub>419</sub>S before (black) and 10 mins after (orange) addition of NONOate. EPR conditions: microwave frequency, 9.3 GHz; modulation amplitude, 2 G; temperature, 100 K. 30 scans were averaged for each spectrum.

To investigate the chemical nature of the product of NO inhibition of aPFL we analyzed the product(s) of aPFL with NO by SDS-PAGE. Samples of aPFL exposed to O<sub>2</sub> are cleaved at the G<sub>734</sub> position, shifting the apparent electrophoretic mobility 3 kDa lower, however samples of aPFL treated with NO do not show changes in the total protein mass (**Supporting Information Figure S4**). The lack of change in apparent molecular weight by SDS-PAGE suggests that the chemical modification of aPFL does not involve cleavage of the polypeptide chain at G<sub>734</sub>. We also analyzed tryptic digests of the NO-inhibited PFL by LC-MS/MS for evidence of covalent modifications on peptides containing G<sub>734</sub>, C<sub>419</sub>, and C<sub>418</sub>. Despite an extensive analysis and the employment of reductants such as DTT, TCEP, and NaBH<sub>4</sub>, we observed no evidence of C<sub>418</sub> or C<sub>419</sub> nitrosothiols or G<sub>734</sub> nitrosoalkyl/oxime or reduced amine products of any of the radical transfer peptides and no major difference in the MS<sup>1</sup> chromatograms between aPFL samples and aPFL samples treated with NO, that could indicate the formation of cross-links between the

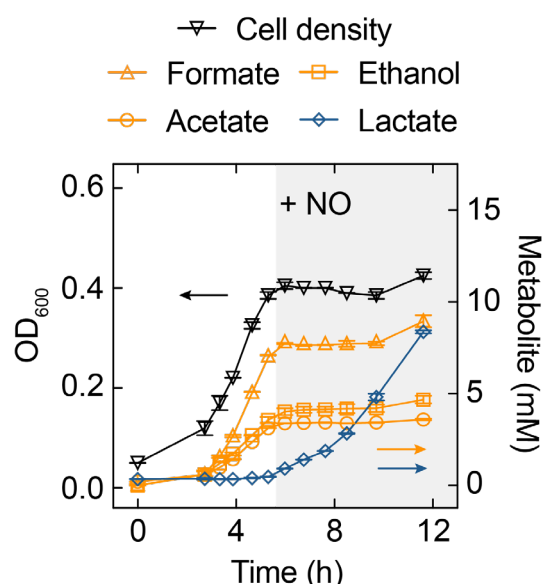




**Figure 3.** X-band EPR spectra of 50  $\mu\text{M}$  PFL-AE treated with 500  $\mu\text{M}$  NONOate freeze-quenched after 30 s (black), 3 mins (gray), and 10 mins (blue) of incubation at room temperature. The 10 min spectrum is plotted vs. magnetic field ( $B_0$ , lower axis) and  $g$ -values are indicated. For comparison, the 30 s, 3 min and 10 min spectra are reported vs.  $g$  (upper axis, unlabeled). EPR conditions: microwave frequency, 9.3 GHz; modulation amplitude, 2 G; power, 2 mW; temperature, 100 K. 30 scans were averaged for each spectrum.

modified amino acids and proximal amino acids (**Supporting Information Figure S5**). Furthermore, we observed no evidence of a C-terminal oxalylated peptide corresponding to a cleavage of the polypeptide backbone, as seen in the reaction of aPFL with  $\text{O}_2$ .<sup>22</sup> In all the samples we produced, only the unmodified  $\text{NH}_3^+-\text{V}_{732}\text{SGYAVR}_{738}-\text{CO}_2\text{H}$  tryptic peptide was observed.

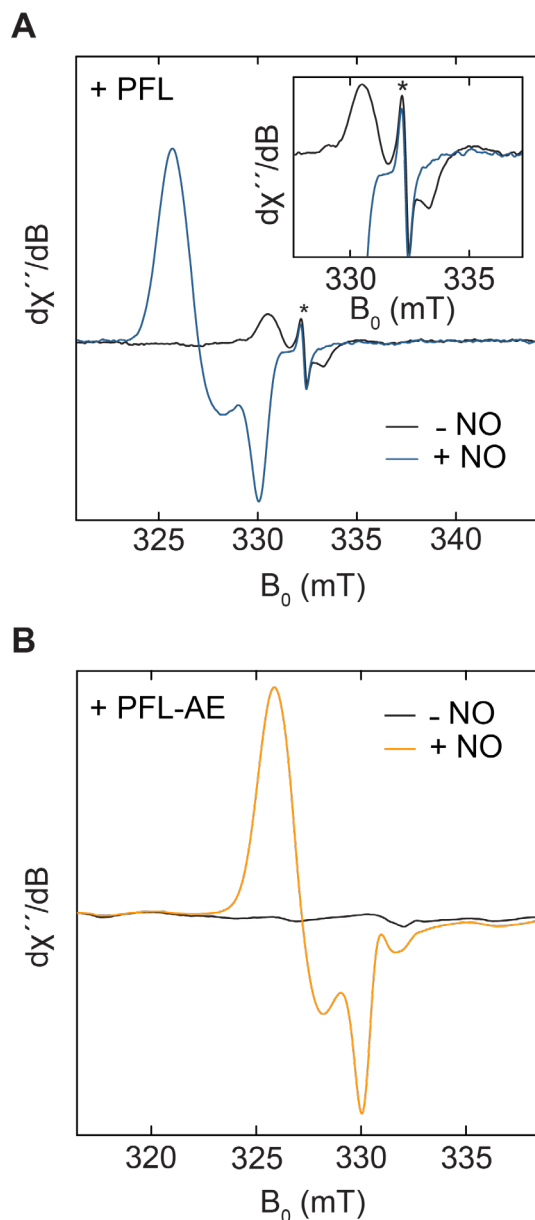
**NO inhibits PFL-AE.** The EPR spectrum of the NO-inhibited PFL was not completely devoid of EPR-active signals (**Figure 2**). We suspected the observed residual signal originated from the reaction of NO with PFL-AE, present at 10-fold lower concentration relative to PFL in the activation reaction and carried over into the NO inhibition assay. To investigate this, we analyzed the EPR spectrum of PFL-AE in isolation before and after reacting with NO (**Figure 3**). The untreated  $[\text{Fe}_4\text{S}_4]^{2+}$  cluster of PFL-AE is EPR silent, whereas addition of NONOate revealed the formation of an axial EPR signal with prominent features at  $g_{\perp} = 2.036$  and  $g_{\parallel} = 2.014$  (**Supporting Information Figure S6A and Table S2**). This EPR signature is consistent with previously characterized protein-bound monomeric dinitrosyl iron complexes (DNIC,  $\{\text{Fe}(\text{NO})_2\}^9$  in Enemark-Feltham notation) derived from iron-sulfur proteins.<sup>76–80</sup> Spin quantification allowed to estimate a rate of DNIC formation of 8.7  $\mu\text{M}/\text{min}$ , the reaction is completed in approximately 5 min with an stoichiometry of 0.8 spins/mol of PFL-AE (**Supporting Information Figure S6B and Table S2**). The DNIC signal did not change when PFL-AE was provided SAM, suggesting SAM binding does not protect the  $[\text{Fe}_4\text{S}_4]$  cluster from



**Figure 4.** The metabolic impact of NO on anaerobically growing *E. coli*. Anaerobic cultures of *E. coli* were subjected to treatment with 100  $\mu\text{M}$  NONOate at mid-exponential phase (gray shaded area). Cell density was estimated from the  $\text{OD}_{600}$  (black triangles) and extracellular concentration of the fermentation products lactate (blue diamonds), acetate (orange circles), ethanol (orange squares), and formate (orange triangles) were determined using enzymatic coupled assays. Error bars represent the span between two biological replicates.

decomposition (**Supporting Information Figure S7 and Table S2**). The reaction of PFL-AE and NO was also accompanied by changes in the UV-vis spectrum (**Supporting Information Figure S8A**). We observed a shift in the broad signal associated with the  $[\text{Fe}_4\text{S}_4]$  cluster with a peak at 410 nm to a new broad signal with a peak at 380 nm, also consistent with the monomeric DNIC.<sup>81</sup> No further reaction was observed after 15 min (**Supporting Information Figure S8B**). When in the presence of the reductant NaDT, a different axial paramagnetic species was formed, with  $g_{\perp} = 2.009$  and  $g_{\parallel} = 1.970$ , characteristic of reduced Roussin's red esters (**Supporting Information Figure S9A and Table S2**).<sup>78,79</sup> As for the DNIC formation, the Roussin's red ester signal was not affected by the presence of the substrate SAM (**Supporting Information Figure S9B and Table S2**). In either the presence or absence of SAM, the reduction was not complete and roughly 30% of the signal corresponds to a small signal at  $g = 2.036$  remained that we attributed to non-reduced DNIC (**Supporting Information Table S2**).

The  $[\text{Fe}_4\text{S}_4]$  cluster of PFL-AE is essential for activity, and thus, reaction with NO to form DNICs is expected to inactivate the enzyme. Indeed, reacting PFL-AE with NONOate for 30 min inhibits PFL-AE activation of PFL by 99% (**Supporting Information Figure S10**). Collectively, the results support a role of NO in degrading the essential  $[\text{Fe}_4\text{S}_4]$  cluster of PFL-AE via a protein-bound DNIC intermediate that renders PFL-AE inactive.



**Figure 5.** X-band EPR spectra of anaerobic *E. coli* cultures over-expressing **A** PFL or **B** PFL-AE before (black) and after (blue and orange, respectively) exposure to 100  $\mu$ M NONOate for 10 min. Inset in A shows an expanded view of the G• signal. The asterisk “\*” indicates a cavity artifact. EPR conditions: microwave frequency, 9.3 GHz; modulation amplitude, 2 G; power, 20  $\mu$ W (A) or 2 mW (B), temperature, 100 K. 30 scans were averaged for each spectrum.

**Metabolic Consequences of Anaerobic *E. coli* NO Exposure.** Having established the *in vitro* irreversible inactivation of PFL and PFL-AE, we sought to investigate the *in vivo* consequences of NO treatment on the metabolism of *E. coli*. We cultivated *E. coli* cells anaerobically in minimal media with glucose as the unique carbon source, directing glucose metabolism to anaerobic fermentation. Cells in mid-

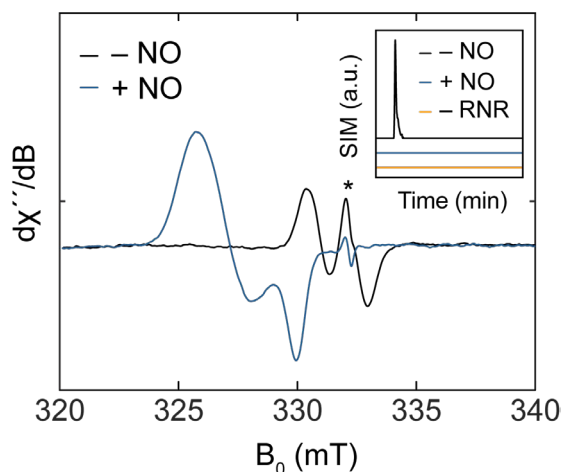
exponential phase were treated with 100  $\mu$ M NONOate or left unperturbed as a control (**Figure 4** and **Supporting Information Figure S11**). The introduction of NO immediately inhibited cell growth for a period of more than 7 h, demonstrating a bacteriostatic effect of NO in anaerobic conditions. NO also displayed bactericidal effects; cells in the mid-exponential phase displayed a significant decrease in cell viability by 65% within 30 min and 77% after 4 h of NO treatment (**Supporting Information Figure S12**). We also measured the extracellular concentration of the fermentation products lactate, formate, acetate, and ethanol using enzyme-coupled assays (**Supporting Information Figure S13**). Non-treated cultures produce principally formate and lower concentrations of ethanol and acetate, however the addition of NO causes a complete inhibition of the production of ethanol, formate, and acetate and the cell culture accumulated lactate at higher rates and concentrations than in the non-treated cultures. This metabolic shift suggests the *in vivo* inhibition of PFL and PFL-AE by NO.

To compare the mechanism of PFL and PFL-AE inhibition *in vitro* with the potentially more complex chemistry *in vivo*, we attempted whole-cell EPR of anaerobically grown *E. coli* overexpressing either PFL or PFL-AE (**Figure 5**). PFL and PFL-AE can be overexpressed in anaerobic conditions and represent >10% of the total cell protein content, as observed by SDS-PAGE (**Supporting Information Figure S14**). After protein induction and subsequent treatment with NO, we harvested the cells and analyzed them by EPR. *E. coli* over-expressing PFL can reconstitute the PFL glycy radical, which can readily be observe by X-band EPR (**Figure 5A**, inset). The addition of NO quenches the PFL glycy radical on a timescale similar to the one observed *in vitro*. We also observed significant DNIC formation in these samples, with characteristic features at  $g_{\perp} = 2.037$  and  $g_{\parallel} = 2.015$ , despite no over-expression of PFL-AE (**Figure 5A**, **Supporting Information Figure S15**, and **Table S3**).

In *E. coli* cultures over-expressing PFL-AE, a glycy radical signal can also be observed at lower intensity than the signal observed in samples of the PFL over-expressing cultures (**Supporting Information Figure S16A**). This signal can be attributed to the genomic PFL expression that can be as high as 20  $\mu$ M in the cytoplasm of anaerobically growing *E. coli*<sup>20,23</sup> However, this glycy radical signal could correspond to other glycy radical enzymes in the *E. coli* proteome, such as the class III RNR, ketobutyrate formate lyase, YbiW, or PflD.<sup>24,82</sup> The  $[\text{Fe}_4\text{S}_4]^{2+}$  of PFL-AE is EPR silent before treatment with NO *in vivo*. Following treatment with 100  $\mu$ M NONOate, a signal consistent with the formation of DNICs appears with the disappearance of the low-intensity glycy radical signal (**Figure 5B** and **Supporting Information Figure S16**), and treatment of the cultures with 1 mM of NONOate produced a composite signal of DNIC and Roussin’s red ester (**Supporting Information Figure S16B** and **Supporting Information Table S3**). Non-transformed *E. coli* cells exposed to NO produce the same signal, although at lower intensity but with similar  $g$ -values (**Supporting Information Figure S17** and **Table S3**). We attribute this signal to the formation of DNICs in other iron-sulfur proteins present in the *E. coli* proteome.

**NO Inhibits a Broad Range of GREs and Their Activases.** Our observation of the NO effect on *E. coli*





**Figure 6.** X-band EPR spectra of 100  $\mu\text{M}$  reconstituted *E. coli* class III RNR before (black) and after (blue) treatment with 500  $\mu\text{M}$  NONOate for 10 min. The asterisk “\*” indicates a cavity artifact. EPR conditions: microwave frequency, 9.3 GHz; modulation amplitude, 2 G; power, 20  $\mu\text{W}$ ; temperature, 100 K. 30 scans were averaged for each spectrum. Inset shows dC production by aRNR over 5 min. Single ion monitoring (SIM) LC-MS for dC was performed for aRNR (black), aRNR treated with 500  $\mu\text{M}$  NONOate for 10 min (blue), or assay buffer without aRNR (orange).

metabolism and loss of the whole-cell glycy radical EPR signal suggests the *in vivo* inhibition of PFL. Several enzymes in the glycy radical enzyme family are associated with primary metabolism in *E. coli*. Therefore, we sought to investigate the reactivity of NO with the *E. coli* class III RNR and its radical SAM activator enzyme, RNR-AE. The class III RNR is a glycy radical enzyme found in obligate and facultative anaerobic bacteria and archaea, including *E. coli*, and is essential for the anaerobic reduction of nucleotides to deoxynucleotides for *de novo* DNA synthesis and repair.<sup>83,84</sup>

As with PFL, we examined whether the glycy radical of RNR reacts with exogenous NO, inhibiting the enzyme. We exposed activated RNR (aRNR) to NO, analogous to our prior experiments with aPFL, and followed the reaction by EPR. After 10 min of treatment with 500  $\mu\text{M}$  NONOate we observed a complete loss of the aRNR glycy radical signature, as well as the formation of a DNIC signal we attribute to the reaction of remnant RNR-AE with NO from the RNR activation mixture (Figure 6). To investigate the effect of glycy radical loss on the activity of aRNR we utilized LC-MS to quantitate the dephosphorylated reaction product, dC, of CTP reduction (Supporting Information Figure S18 and S19). Non-treated aRNR produced dCTP with a linear rate over 1 min which ultimately slowed as the reaction approached completion; displaying a specific activity of  $1,500 \pm 100 \text{ nmol/min} \times \text{mg}$ , consistent with literature values (Supporting Information Figure S19).<sup>50</sup> On the other hand, aRNR treated with NONOate for 10 min showed no detectable dC formation over 5 min in the activity assay ( $<3.5 \text{ nmol/min} \times \text{mg}$ , Figure 6 inset and Supporting Information Figure S19).

To examine the reaction of NO with the RNR-AE in greater detail, we also reacted RNR-AE with NO in isolation and followed the reaction by EPR. As expected, upon reaction with NO, RNR-AE produced a similar DNIC EPR signal to that observed for PFL-AE (Supporting Information Figure S20A), with similar  $g_{\perp} = 2.035$  and  $g_{\parallel} = 2.014$  (Supporting Information Figure S20B and Table S4) to PFL-AE, and an estimated rate of formation of 16.3  $\mu\text{M/min}$ , again similar to the one observed for PFL-AE (Supporting Information Figure S20C). As for PFL-AE, the DNIC signal of RNR-AE and its  $g$ -values were not affected by the presence of the substrate SAM (Supporting Information Figure S21 and Table S4). In the presence of NO and reducing conditions, RNR-AE formed the characteristic axial paramagnetic signal of Roussin’s red ester, with  $g_{\perp} = 2.010$  and  $g_{\parallel} = 1.971$ , and 35% of the signal corresponding to non-reduced DNIC (Supporting Information Figure S22 and Table S4). The results of both NO inhibition of the class III RNR and the associated RNR-AE suggest a general mechanism of glycy radical and activator enzyme inhibition by NO.

To further establish the generality of inhibition of radical SAM enzyme superfamily members by exogenous NO, we analyzed the inhibition of the enzyme NikJ, a radical SAM enzyme that catalyzes the C5’ extension from enoylpyruvyl-uridine monophosphate (EP-UMP) to octosyl acid in the biosynthesis pathway of nikkomycins.<sup>85,86</sup> PFL-AE and RNR-AE are homologs and share similar function and sequence (sequence identity 28%), while NikJ does not share significant similarity with either PFL-AE or RNR-AE, representing a distant homolog from the same superfamily. The reaction of NikJ with NO resulted in the formation of an EPR-active DNIC species and followed slower kinetics relative to PFL-AE and RNR-AE (Supporting Information Figure S23 and Table S5). As observed for PFL-AE and RNR-AE the presence of the substrate SAM does not change the  $g$ -values of the observed signal (Supporting Information Figure S24 and Table S5). NikJ reduced with NaDT produced EPR signals consistent with both a DNIC and Roussin’s red ester (Supporting Information Figure S25A and Table S5), while in the presence of SAM, a signal consistent with Roussin’s red ester was observed with nearly identical spectral features as those of PFL-AE and RNR-AE, and showing low concentrations of a non-reduced DNIC signal (Supporting Information Figure S25B and Table S5).

## DISCUSSION

Phagocytes, including macrophages, microglia and neutrophils, along with intestinal epithelial cells, respond to proinflammatory cytokines by expressing the inducible nitric oxide synthase (iNOS).<sup>87–89</sup> iNOS catalyzes the oxidation of L-arginine into L-citrulline and NO, releasing high concentrations of NO into sites of infection. NO acts as both a bactericidal and bacteriostatic agent and its mechanisms of action have been investigated in both *in vivo*<sup>39,90,91</sup> and *in vitro*<sup>92,93</sup> models. Many molecular targets of NO toxicity have been recognized in bacteria, principally in aerobic conditions; NO inhibits DNA replication by targeting DNA-binding zinc metalloproteins<sup>94</sup> and deoxynucleotides production by reacting with cysteinyl and tyrosyl radicals of RNR.<sup>95,96</sup> Additionally, NO impairs respiration by binding to the  $\text{Cu}_B$  of cytochrome  $\text{bo}^{97}$  and central metabolism by

inhibiting many enzymes in the glycolytic pathway and the TCA cycle.<sup>80,98</sup> However, in anaerobic conditions, relevant to gastrointestinal infections, the molecular targets of NO have not been identified with molecular specificity. This knowledge gap limits our understanding of the innate immune response in anaerobic environments and hinders the potential application of NO as a therapeutic agent in treating gastrointestinal infections.<sup>17,99</sup>

In this study, we identified PFL as a direct target of NO and characterized the mechanism of inhibition. Upon exposure to NO, PFL is inhibited with an apparent diffusion-controlled rate, suggesting PFL is a kinetically important microbial metabolic target of NO. Thiyl radicals react with NO at diffusion-controlled rates,<sup>74</sup> however our EPR and site-directed mutagenesis data following radical quenching strongly suggest that NO reacts directly with the glycy radical of the active enzyme. In PFL, C● and G● exist in an equilibrium favoring the formation of G●.<sup>100</sup> The lack of evidence of nitrosothiol formation during aPFL inhibition implies a rapid rate of reaction with G● and a low concentration of C● in the  $G\bullet \rightleftharpoons C\bullet$  equilibrium. Despite our inability to directly demonstrate the nature of the NO adduct(s) to PFL by LC-MS/MS, we hypothesize a radical-radical coupling mechanism. The lack of observation of an NO adduct on Gly<sup>734</sup> may be related to instability of the modification during MS sample preparation or instability during ionization in the mass spectrometer; such effects have previously been observed for cysteinyl S-NO modified peptides, and their detection generally requires the use of indirect techniques.<sup>101–103</sup> Together, these data demonstrate that NO is a potent inhibitor of the glycy radical enzyme PFL via the quenching of the essential glycy radical cofactor.

Studies on the effect of NO on anaerobic gastrointestinal bacterial communities indicate that NO exposure influences fermentation products and community composition.<sup>104</sup> To evaluate the effect of PFL inactivation by NO on anaerobic metabolism in a model gastrointestinal microbe, we examined the effect of NO on anaerobic *E. coli* cell growth, metabolism, and enzyme post-translational modifications. Using whole-cell EPR, we show that NO reacts with glycy radicals and FeS clusters in *E. coli* cultures at a concentration of 100  $\mu$ M—comparable to NO levels measured in gastrointestinal samples from patients with inflammatory conditions such as ulcerative colitis and Crohn's disease.<sup>10,12</sup> Additionally, we observe that NO causes metabolic changes in anaerobic *E. coli* cultures, arresting the production of the metabolites formate, acetate, and ethanol and halting cell growth and viability. In the absence of PFL activity, lactate accumulates as the product of pyruvate metabolism via lactate dehydrogenase, which is not inhibited by NO.<sup>39</sup> Similar effects have been reported in anaerobic fermentation from healthy human fecal samples treated with NO, where NO produced a long-lasting impact on the metabolome.<sup>104</sup> In *E. coli*, deletion of the *pflB* gene shifts metabolism to lactate formation, similar to *E. coli* treated with NO, but does not completely impair growth.<sup>105,106</sup> Our observation that growth is effectively halted despite continued lactate production implies a substantial maintenance and repair burden associated with NO exposure. We hypothesized that the inhibition of class III RNR, another glycy radical enzyme essential to *de novo* deoxyribonucleotide synthesis, may contribute to the

bacteriostatic effect of NO while maintaining lactate fermentation. The glycy radical enzyme, RNR, was indeed inhibited by NO *in vitro*, with a reaction rate similar to PFL, suggesting DNA replication and repair are also inhibited, yet further experiments are required to determine the consequences of RNR inhibition by NO *in vivo*.

We further demonstrate that NO inactivates PFL-AE and RNR-AE. Therefore, NO acts as a general GRE-AE inhibitor, serving a two-fold mechanism of inactivating GRE chemistry, at both the level of GREs and GRE-AEs. Our EPR data evidences the NO reactivity with the enzymes [Fe<sub>4</sub>S<sub>4</sub>] cluster and the formation of DNICs, with similar spectroscopic properties to previously reported iron-sulfur cluster proteins and enzymes.<sup>76–80</sup> The DNIC formation is accompanied by a complete loss of activity for PFL-AE, as the [Fe<sub>4</sub>S<sub>4</sub>] is essential for enzyme activity. Radical SAM enzyme iron-sulfur clusters are coordinated by three cysteines and, when present, the amino and carboxy group of SAM.<sup>27</sup> The presence of SAM provided no protection from NO inhibition for either PFL-AE or RNR-AE, nor the distantly related radical SAM enzyme NikJ, demonstrating that SAM binding and coordination of the fourth iron does not protect the iron-sulfur cluster from decomposition.

The collective evidence that GREs and radical SAM enzymes, including GRE-AEs, are irreversibly inhibited by NO. This suggests that the mechanism by which NO inhibits GREs and GRE-AEs is part of the innate immune response, triggered by gastrointestinal pathogens. Many metabolic pathways in the gut microbiome involve GREs or radical SAM enzymes, indicating that NO effects may be extensive.<sup>21,24,27,107–111</sup> Additionally, the enzyme pyruvate:ferredoxin oxidoreductase is also involved in anaerobic metabolism of pyruvate and harbors an iron-sulfur cluster, and is another likely target of anaerobic metabolic inhibition by NO.<sup>112</sup> What remains to be seen is whether the NO response differentially affects the microbiome community, and what defense mechanisms may have adapted to anaerobic NO exposure that may inform therapeutic approaches aimed at enhancing the innate immune response.

## ASSOCIATED CONTENT

### Supporting Information

Supplemental methods, list of primers, UV/vis spectroscopy of myoglobin as an NO sensor, PFL reactivation, aPFL NO inactivation followed by UV/vis spectroscopy, Inactivation of aPFL followed by SDS-PAGE, peptide LC-MS/MS of aPFL inhibited with NO, EPR characterization of PFL-AE and kinetics, EPR simulations parameters for PFL-AE treated with NO, EPR characterization of PFL-AE reacted with NO in the presence of SAM, PFL-AE NO inactivation followed by UV/Vis spectroscopy, EPR characterization of reduced PFL-AE with NO, Inhibition of PFL-AE activity by NO, metabolic analysis of anaerobically growing *E. coli*, metabolites calibration curves, over-expression of PFL and PFL-AE in anaerobic conditions, EPR characterization of whole cells overexpressing PFL or PFL-AE treated with NO, EPR characterization of RNR-AE NO inactivation, EPR simulations parameters for RNR-AE treated with NO, EPR characterization of reduced RNR-AE reacted with NO, deoxycytidine calibration curve, LC-MS activity assay of aRNR and NO treated aRNR, EPR characterization of NikJ NO inactivation, EPR characterization of reduced NikJ with NO and EPR simulations parameters for NikJ treated with NO.

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### Author Contributions

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