Activity-Based Protein Profiling of Ammonia Monoxygenase in *Nitrosomonas europaea*

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*Nitrosomonas europaea* is an aerobic nitrifying bacterium that oxidizes ammonia (NH₃) to nitrite (NO₂⁻) through the sequential activities of ammonia monoxygenase (AMO) and hydroxylamine dehydrogenase (HAO). Many alkenes are mechanism-based inactivators of AMO, and here we describe an activity-based protein profiling method for this enzyme using 1,7-octadiyne (17OD) as a probe. Inactivation of NH₄⁺-dependent O₂ uptake by *N. europaea* by 17OD was time- and concentration-dependent. The effects of 17OD were specific for ammonia-oxidizing activity, and *de novo* protein synthesis was required to reestablish this activity after cells were exposed to 17OD. Cells were reacted with Alexa Fluor 647 azide using a copper-catalyzed azide-alkyne cycloaddition (CuAAC) reaction, solubilized, and analyzed by SDS-PAGE and infrared (IR) scanning. A fluorescent 28-kDa polypeptide was observed for cells previously exposed to 17OD but not for cells treated with either allylthio-urea or acetylene prior to exposure to 17OD or for cells not previously exposed to 17OD. The fluorescent polypeptide was membrane associated and aggregated when heated with β-mercaptoethanol and SDS. The fluorescent polypeptide was also detected in cells pretreated with other diynes, but not in cells pretreated with structural homologs containing a single ethynyl functional group. The membrane fraction from 17OD-treated cells was conjugated with biotin-azide and solubilized in SDS. Streptavidin affinity-purified polypeptides were on-bead trypsin-digested, and amino acid sequences of the peptide fragments were determined by liquid chromatography-mass spectrometry (LC-MS) analysis. Peptide fragments from AmoA were the predominant peptides detected in 17OD-treated samples. In-gel digestion and matrix-assisted laser desorption ionization–tandem time of flight (MALDI-TOF/TOF) analyses also confirmed that the fluorescent 28-kDa polypeptide was AmoA.

Activity-based protein profiling (ABPP) is a well-established proteomics method used to identify catalytically active enzymes in complex mixtures (1, 2). Although many variations exist, ABPP often involves the use of bifunctional enzyme probes. One group enables the probe to act as a mechanism-based inactivator. Activation of this functional group by the target enzyme results in covalent modification and inactivation of the enzyme by the probe (Fig. 1A). The probe’s second functional group is often either an ethynyl or azide group that can then be reacted with a complementary azide- or ethynl-containing reporter molecule using a copper-catalyzed azide-alkyne cycloaddition (CuAAC) reaction (1, 3) (Fig. 1B). Depending on the reporter molecule used, the inactive enzyme-probe-reporter conjugate can then be visualized in SDS-PAGE or affinity purified, proteolytically digested, and then identified after analysis of the resulting peptide fragments by mass spectrometry. This type of ABPP has been used to study mammalian cytochrome P450s (4) and several classes of bacterial enzymes (3) but has not been previously applied to bacterial monoxygenases.

In this study, we have characterized 1,7-octadiyne (17OD) and various other diynes as ABPP probes for ammonia monooxygenase (AMO) in the ammonia-oxidizing bacterium (AOB) *Nitrosomonas europaea*. This chemolithoautotroph obtains energy for CO₂ fixation and growth from oxidizing ammonia (NH₃) to nitrite (NO₂⁻) (5). The initial oxidation of ammonia is catalyzed by membrane-bound AMO while hydroxylamine (NH₂OH), the immediate product of ammonia oxidation, is further oxidized to nitrite (NO₂⁻) by the periplasmic enzyme hydroxylamine dehydrogenase (HAO) (5). Although *N. europaea* is the most extensively studied AOB, studies of AMO in this bacterium and AOB in general have historically been hampered by the labile nature of this enzyme (6–8). However, even though AMO has not yet been obtained in a highly purified active state, considerable insights into the activities and structure of this important enzyme have been obtained from whole-cell studies of *N. europaea* using different classes of inhibitors (5, 9). For example, ammonia oxidation is often strongly but reversibly inhibited by metal-binding agents, and some of the most potent of these are copper-selective compounds such as allylthiourea (9). The selectivity of these compounds for copper as well as the fact that AMO activity can be stimulated and stabilized by copper ions in cell extracts (6, 7) suggests that AMO is a copper-dependent enzyme. Many organic compounds also reversibly inhibit ammonia oxidation through their action as alternative substrates for AMO. These compounds include diverse alkanes (10, 11), alkenes (11, 12), aromatics (13, 14), ethers (15, 16), and halogenated compounds (15, 17, 18). The simplest organic AMO substrates, such as methane and ethylene, are competitive inhibitors of ammonia oxidation (10, 12), while other substrates exhibit more complex inhibition patterns (19).
Activity-Based Protein Profiling of AMO

Insights into the structure of AMO have more often come from studies of irreversible inactivators than from those of reversible inhibitors of this enzyme. Recognized AMO inactivators include terminal and subterminal alkynes (9, 11, 20, 21), allyl sulfide (22), and some aniline and cyclopropane derivatives (21). These compounds are thought to be catalytically activated by AMO to reactive intermediates that subsequently covalently bind to and irreversibly inactivate the enzyme. The canonical mechanism-based inactivator of AMO is acetylene (C₂H₂). The potent and specific effects of acetylene on ammonia oxidation by N. europaea were first recognized by Hynes and Knowles (23, 24). A subsequent kinetic study (20) demonstrated that the effects of acetylene conform to many of the well-established criteria for mechanism-based inactivation (25). For example, the effects of acetylene on NH₄⁺-dependent O₂ uptake are time and concentration dependent, and AMO activity is not inactivated by acetylene under anoxic conditions when AMO is catalytically inactive (20). The effects of acetylene are also irreversible, and cells require de novo protein synthesis to reestablish AMO activity after exposure to this gas (26). Incubation of N. europaea with ¹⁴C₂H₂ leads to the covalent radiolabeling of a membrane-associated 28-kDa polypeptide (20, 26), and this radiolabeling is prevented if cells are exposed to a reversible inhibitor, such as thiourea, during exposure to ¹⁴C₂H₂ (20). Based on this series of observations, the ¹⁴C-labeled 28-kDa polypeptide was proposed to be a structural component of AMO (20). The binding of ¹⁴C from ¹⁴C₂H₂ on the 28-kDa polypeptide is compatible with covalent attachment of a ketene to amino acid H191, which is thought to reside in, or nearby, the active site of AMO (27). The 28-kDa polypeptide in N. europaea has also been labeled in vivo with a fluorescent derivative of propargylamine (prop-2-yn-1-amine), and the N-terminal amino acid sequence of the polypeptide was used to identify its corresponding gene (amoA) (28). Like several other genes encoding key enzymes involved in ammonia oxidation in N. europaea, identical copies of amoA occur in multiple operons (amoCAB) (5, 29), and expression of these amoA copies cannot be discriminated at the translational level. AmoA also has a predicted mass of ~32 kDa rather than 28 kDa (29). This suggests that AmoA migrates aberrantly in SDS-PAGE systems, and, like other intrinsic membrane proteins (30, 31), AmoA aggregates in SDS-PAGE sample buffer if heated at high temperature (95°C) in the presence of β-mercaptoethanol (32).

Covalent modification of structural proteins, a definitive feature of mechanism-based inactivation, has not been confirmed for putative AMO inactivators other than acetylene and propargylamine. This likely reflects the limited commercial availability and high cost of suitably radiolabeled forms of these compounds. In this study, we have characterized 17OD as a mechanism-based inactivator of AMO, and we have shown that after inactivation, AmoA can be specifically labeled using CuAAC reactions with azide-containing reporter molecules that can then be used to either detect or selectively purify this polypeptide. Our results are discussed in terms of the advantages and disadvantages of using ABPP for studying different AMOs and the potential applicability of this approach to other alkyne-sensitive bacterial monoxygenases in pure culture studies and environmental samples.

MATERIALS AND METHODS

Materials. N. europaea (ATCC 19178) was obtained from the American Type Culture Collection (Manassas, VA). Alexa Fluor 647 azide (99% purity) was obtained from Invitrogen (Grand Island, NY). Aminoguanidine hydrochloride (≥98% purity), 1-allyl-2-thiourea (98% purity), bovine serum albumin, 3-butyn-1-ol (97% purity), 1,4-diethylbenzene (96% purity), dipropargylamine (97% purity), 1-hexyne (97% purity), hydroxylamine hydrochloride (>99.99% purity), N-(1-naphthyl)ethylenediamine dihydrochloride (98% purity), 1,7-octadiyne (17OD; 98% purity), phenylacetylene (98% purity), propargylamine (98% purity), sulfanilamide (>99% purity), and Tris-(3-hydroxypropyltriazolymethyl) amine (THPTA; 95% purity) were obtained from Sigma-Aldrich Co.
(Milwaukee, WI). N-[(3-Azidopropyl)biotinamide (biotin-azide) (95% purity), 1-heptyne (98% purity), 1,6-heptadiyne (97% purity), 1-octyne (97% purity), 1-nonyne (99% purity), and 1,8-nonadiyne (>95% purity) were obtained from TCI America (Portland, OR). 1,5-Heptadiyne (50%, vol/vol, in pentane) was obtained from Alfa Aesar (Ward Hill, MA). Streptavidin agaro (6% beaded agarose slurred in water) and Tris-(2-carboxyethyl)phosphinehydrochloride (TCEP) (>98% purity) were obtained from Thermo Fisher Scientific (Grand Island, NY). Tris-[[1-benzyl-1H-1,2,3-triazol-4-yl]methyl]amine (TBITA) (97% purity) was obtained from AnaSpec (Fremont, CA).

**Growth and harvesting of bacteria.** Cells of *N. europaea* were grown in batch culture in mineral salts medium containing (NH₄)₂SO₄ (25 mM), harvested, and finally resuspended at ~0.2 g wet weight ml⁻¹ in buffer (30 mM sodium phosphate, pH 7.8, plus 2 mM MgCl₂) as described previously (26). Unless otherwise stated, this buffer was used throughout the experiments described in this study.

**Oxygen uptake measurements.** All measurements of O₂ uptake were made using a Clark-style oxygen electrode (Hansatech, King’s Lynn, Norfolk, United Kingdom) operated at 30°C. The reaction chamber contained buffer (2 ml), and in a typical reaction, a basal rate of O₂ uptake in the absence of cells was established for 2 to 3 min. Substrates and inactivators (~10 µl) were then added to the electrode chamber from concentrated stock solutions using microsyringes inserted through the capillary aperture in the screw-top seal of the reaction chamber. The reactions were then initiated by the addition of cells (50 µl; ~100 µg total protein), and the time course of O₂ uptake was monitored using a chart recorder. The rates of inactivation of NH₄⁺-dependent O₂ uptake by 17OD were determined by measuring the slope of the recorded O₂ uptake reactions at 30-s intervals after the addition of cells. The activity at each time point was then compared to the rate of O₂ uptake at the same time point for reactions conducted in the absence of 17OD. The differences between the slopes of the O₂ uptake reactions at each time point were then expressed as percentages of activity remaining.

**Inactivation of ammonia-oxidizing activity of resting cells by 17OD and other alkynes.** The ammonia-oxidizing activity of *N. europaea* was routinely inactivated by 17OD in reactions conducted in glass serum vials (60 ml). The reaction vials initially contained buffer (~19 ml) and were sealed with butyl rubber stoppers and aluminum crimp seals (Wheaton Scientific, Millville, NJ). NH₄Cl (200 µmol) was added from a concentrated aqueous stock solution (1 M), and 17OD (20 µmol) was added from concentrated stock solution (1.0 M) in dimethyl sulfoxide (DMSO). The reaction was initiated by the addition of a concentrated cell suspension (1 ml) to give a final reaction mixture volume of 20 ml. The reaction vials were incubated for 1 h at 30°C in a shaking water bath operated at 150 rpm. Untreated control cells were incubated as described above, except that DMSO alone (20 µl) was added to the reaction mixture rather than 17OD in DMSO. After 1 h, a sample (4 µl) was withdrawn from the reaction medium to colorimetrically determine the amount of NO₂⁻ that had been generated during incubation (33). In all cases, 17OD-treated cells had NH₄⁺-dependent NO₂⁻-generating activities that were ~1% of the activity of untreated control cells. The cells were then harvested from these reaction mixtures by centrifugation using a microcentrifuge (10,000 × g for 2 min), and the resulting cell pellets were resuspended in buffer (15 ml). This washing procedure was repeated 3 times. After the final centrifugation, the cell pellets were resuspended in buffer (1 ml) and were stored at 4°C for ≤2 h prior to use in experiments.

Cells were also treated with 17OD in the presence and absence of allylthiourea or acetylene. These incubations were conducted in glass serum vials (10 ml) that contained buffer (750 µl) and allylthiourea (100 nmol) added from a stock solution in DMSO (0.1 M), DMSO alone (1 µl), or acetylene (1%, vol/vol, gas phase). The reactions were initiated by the addition of cells (250 µl, ~25 mg total protein), and the vials were then incubated at 30°C in a shaking water bath operated at 150 rpm. After 10 min, the reaction mixtures were all supplemented with NH₄Cl (10 µmol) that was added from an aqueous stock solution (1 M); 17OD (1 µmol) was also added as a stock solution in DMSO (1 M) as needed. The reaction vials were then incubated for a further 10 min at 30°C. The cells were then harvested from these reaction mixtures by centrifugation using a microcentrifuge (10,000 × g for 2 min). The resulting cell pellets were then resuspended in 2× SDS-PAGE sample buffer (250 µl) that contained 0.125 M Tris (pH 6.8), 4% SDS, 20% glycerol, 10% β-mercaptoethanol, and 0.002% bromophenol blue. The solubilized cell extracts were subsequently conjugated with Alexa Fluor 647 azide in a CuAAC reaction as described below.

Cells were also treated with other n-alkynes and diynes in small-scale reactions conducted in glass serum vials (10 ml) that were sealed with butyl rubber stoppers and aluminum crimp seals. The reaction medium contained buffer (~750 µl) and NH₄Cl (10 µmol) added from a concentrated aqueous stock solution (1 M). The reaction mixtures also contained individual alkynes or diynes (1 µmol) added from stock solutions in DMSO (1 M). For untreated control cells, the reaction medium contained NH₄Cl (10 µmol) and DMSO (1 µl). The reactions were initiated by the addition of resting cells (250 µl; ~25 mg total protein) to give a final reaction mixture volume of 1 ml. The vials were then incubated at 30°C for a shaking water bath operated at 150 rpm. After 1 h, a sample (4 µl) was withdrawn from the reaction mixtures to determine the amount of NO₂⁻ that had been generated (33). The cells were then recovered from the remainder of the reaction medium by centrifugation (10,000 × g for 2 min), and the cell pellet was resuspended in buffer (1 ml). This procedure was repeated 3 times, and the final cell pellet was resuspended in buffer (300 µl). A portion of these cells (150 µg total protein) was subjected to CuAAC reactions with Alexa Fluor 647 azide as described below.

**Effect of NH₄⁺ concentration on the inactivation of NH₄⁺-dependent NO₂⁻ production by 17OD.** The reactions were conducted in glass serum vials (10 ml) that were sealed with butyl rubber stoppers and aluminum seals. The reaction vials contained buffer (450 µl), various concentrations of NH₄Cl (0, 0.5, 1.0, 2.5, 5.0, or 10 mM) added from an aqueous stock solution (1 M), and a fixed amount of 17OD (0.5 µmol) added from a stock solution in DMSO (1 M). The reactions were initiated with the addition of cells (50 µl, ~0.5 mg total protein), and the reaction vials were incubated at 30°C in a shaking water bath operated at 150 rpm. After incubation for 15 min, cells were recovered from the medium by centrifugation (10,000 × g for 2 min), and the resulting cell pellet was then resuspended in buffer (1 ml). This procedure was repeated 3 times, and the final cell pellet was resuspended in buffer (300 µl). The washed cells were then added to glass serum vials (10 ml) that contained buffer (700 µl) and NH₄Cl (10 µmol) that was added from a concentrated aqueous stock solution (1 M). The vials were sealed with butyl rubber stoppers and aluminum seals and were then incubated at 30°C in a shaking water bath operated at 150 rpm. After incubation for 15 min, acetylene (1 ml) was then added to the gas phase to inhibit further NH₄⁺-dependent NO₂⁻ production. Samples (4 µl) were then withdrawn from each vial to colorimetrically determine the concentration of NO₂⁻ (33).

**Recovery of NH₄⁺-dependent NO₂⁻-generating activity after exposure to 17OD.** Resting cells were treated with 17OD (1 µmol) in the presence of NH₄Cl (10 mM) and were then harvested and washed as described above. Untreated control cells were similarly treated, except neat DMSO was added to the reaction mixtures instead of 17OD dissolved in DMSO. The treated, washed cells (~15 mg total protein) were then added to glass serum vials (60 ml) that contained growth medium (~9.7 ml) (26) supplemented with either rifampin (100 µg ml⁻¹) or chloramphenicol (400 µg ml⁻¹) as required to give a final reaction mixture volume of 10 ml. The reaction vials were then incubated at 30°C in a shaking water bath operated at 150 rpm. Samples of the reaction mixtures (4 µl) were removed at intervals to determine the amount of NO₂⁻ generated from ammonia oxidation.

**CuAAC reaction conditions.** Unless otherwise stated, CuAAC reactions were conducted using whole cells of *N. europaea* that were incubated in plastic microcentrifuge tubes (500 µl) in a final reaction mixture volume of 75 µl. The concentrations of reactants and times of incubation...
FIG 2 Time courses of O\textsubscript{2} uptake were determined in an O\textsubscript{2} electrode apparatus as described in the Materials and Methods section. Trace a shows the time course for chemical reduction of dissolved O\textsubscript{2} with sodium dithionite. The traces b to g show the reaction time courses for \textit{N. europaea} incubated with 10 mM NH\textsubscript{4}Cl and the following concentrations of 17OD added from a stock solution in DMSO: (b) 0 \mu M, (c) 1.25 \mu M, (d) 2.5 \mu M, (e) 5 \mu M, (f) 10 \mu M, and (g) neat DMSO (4 \mu l). The traces h and i were for \textit{N. europaea} incubated with 1 mM NH\textsubscript{2}OH·HCl with either neat DMSO (20 \mu l) (h) or 50 \mu M 17OD (i) added from a stock solution in DMSO. In each case, the arrows indicate the point at which cells were added to the electrode chamber to initiate the reactions.

used in these reaction mixtures were based on experiments using whole cells (see Fig. S1A, S2A, S3A, and S4A in the supplemental material) and frozen, lysed cells (see Fig. S1B, S2B, S3B, and S4B in the supplemental material) as reported in the supplemental material. In a typical reaction, whole cells in buffer (150 \mu g total protein) were mixed with Alexa Fluor 647 azide (16 \mu M final concentration) that was added from a stock solution (0.6 mM) in DMSO. The reactions were initiated by the addition of CuSO\textsubscript{4} (2 mM final concentration) and sodium ascorbate (11 mM final concentration) that was added from freshly prepared aqueous stock solutions. Distilled water was added as required to obtain a final reaction mixture volume of 75 \mu l. In some reactions, either THPTA (1 mM) or aminoguanidine hydrochloride (1 mM) was also added to the reaction mixtures from concentrated aqueous stock solutions. Unless otherwise stated, the CuAAC reactions were conducted for 60 min at room temperature in darkness. The reactions were terminated by the addition of 2X SDS-PAGE sample buffer (75 \mu l). The solubilized cell samples were then centrifuged (10,000 \times g for 2 min) to remove insoluble materials. The resulting supernatant was stored in the dark at −20°C prior to analysis by SDS-PAGE.

**Cell fractionation.** Cells were fractionated in soluble and particulate fractions by repeated freezing and thawing and subsequent centrifugation as described previously (20).

**SDS-PAGE and IR scanning.** Unless otherwise stated, SDS-PAGE analyses were conducted using precast 12% discontinuous SDS-polyacrylamide gels and a Bio-Rad Mini-Protein Tetra system (Bio-Rad Laboratories, Hercules, CA). Unless otherwise stated, samples contained −25 \mu g total protein, and the gels were electrophoresed at room temperature for 30 min at a fixed current of 25 to 35 mA. To visualize fluorescently labeled polypeptides, the unfixed gel was immediately scanned with an excitation wavelength of 650 nm and a detection wavelength of 668 nm using an Odyssey 9120 infrared (IR) scanner (LI-COR Biosciences, Lincoln, NE). A near-infrared (NIR) marker protein ladder (Thermo Scientific, Waltham, MA) was used to estimate the mass of fluorescently labeled polypeptides.

**Protein determination.** Unless otherwise stated, the concentration of protein was determined with a biuret assay (34) after solubilization of cell material for 1 h at 65°C in 3 M NaOH and sedimentation of insoluble material by centrifugation (10,000 \times g for 2 min). Bovine serum albumin was used as the standard.

**RESULTS**

**Effect of 17OD on ammonia and hydroxylamine oxidation.** The effects of 17OD on the ammonia- and hydroxylamine-oxidizing activities of \textit{N. europaea} were determined by measurements of substrate-stimulated O\textsubscript{2} uptake. In the absence of 17OD, NH\textsubscript{4}Cl stimulated a high rate of O\textsubscript{2} uptake that was effectively constant until the majority (>95%) of the dissolved O\textsubscript{2} had been consumed (Fig. 2, trace b). Low concentrations of 17OD (<10 \mu M) produced a time-dependent loss of this NH\textsubscript{4}\textsuperscript{+}-dependent O\textsubscript{2} uptake, and the rate of loss of activity increased with increases in 17OD concentration (Fig. 2, traces c to f). The effects of 17OD appeared to be specific for ammonia-oxidizing activity, as there was no inhibitory effect of 50 \mu M 17OD on the rate of hydroxylamine-dependent O\textsubscript{2} uptake (Fig. 2, traces h and i).

To better characterize the irreversibility of the reaction, cells were incubated with NH\textsubscript{4}Cl with and without 17OD, washed, and then incubated in fresh growth medium. Production of NO\textsubscript{2}\textsuperscript{−} was then monitored over time in the presence and absence of protein synthesis inhibitors. Control cells that were not exposed to 17OD rapidly generated NO\textsubscript{2}\textsuperscript{−} without a lag phase (Fig. 4). The rate of NO\textsubscript{2}\textsuperscript{−} production by untreated cells was ~15% and ~30% lower in the presence of rifampin and chloramphenicol, respectively. For washed, 17OD-pre-treated cells, the rate of NO\textsubscript{2}\textsuperscript{−} production in fresh medium was close to zero over the first 1 h of the incubation. Over the subsequent 3 h, the rate of NO\textsubscript{2}\textsuperscript{−} production progressively increased and after 4 h was nearly equivalent to the rate observed with control cells that had not been pretreated with 17OD. In contrast, the rate of NO\textsubscript{2}\textsuperscript{−} production by 17OD-pre-
treated cells was strongly inhibited in incubations containing either rifampin or chloramphenicol. The maximal rate of NO$_2^-$ production for 17OD-treated cells in the presence of these inhibitors was $\approx$20% of the rate observed in their absence. Substantially similar effects were previously observed during recovery of ammonia-oxidizing activity in _N. europaea_ after its prior inactivation by acetylene (26). Our present results therefore suggest that 17OD is an irreversible inactivator of AMO and that recovery from the effects of this compound requires _de novo_ protein synthesis.

**CuAAC reaction with Alexa Fluor 647 azide.** To test whether covalent modification of proteins could be detected by IR fluorescence, 17OD-pretreated cells were subjected to CuAAC reactions using Alexa Fluor 647 azide as a reporter as described in the Materials and Methods section. The cells were then solubilized in SDS-PAGE sample buffer and analyzed by SDS-PAGE and IR scanning. A single fluorescent 28-kDa polypeptide was observed for 17OD-pretreated cells reacted with Alexa Fluor 647 azide (16 μM) in the presence of CuSO$_4$ (2 mM), sodium ascorbate (11 mM), and THPTA (1 mM) (Fig. 5A, lane 3). No fluorescence was observed for cells if they had not previously been exposed to 17OD (Fig. 5A, lane 2) or if CuSO$_4$ (Fig. 5A, lane 4), sodium ascorbate (Fig. 5A, lane 5), or Alexa Fluor 647 azide (Fig. 5A, lane 6) was individually excluded from the CuAAC reactions.

In CuAAC reactions, CuSO$_4$ acts as the source of Cu$^{2+}$ ions that catalyze the cycloaddition reaction. Ascorbate acts as a reductant that reduces Cu$^{2+}$ to Cu$^{1+}$ and that also reduces dissolved O$_2$. When added, THPTA acts as a ligand to stabilize Cu$^{1+}$. Along with aminoguanidine, THPTA also scavenges reaction by-products such as dehydroascorbate and reactive oxygen species that are generated during the conjugation reaction and that can react with and modify amino acids (35). Excluding THPTA from the CuAAC reactions conducted with whole cells slightly increased the level of fluorescent labeling of the 28-kDa polypeptide compared to that of reaction mixtures containing THPTA (Fig. 5A, lane 7). In the THPTA-free reaction, low levels of fluorescent labeling of an $\approx$45-kDa polypeptide were also observed. In contrast, the addition of aminoguanidine had no discernible effect on the level of labeling of the 28-kDa polypeptide (Fig. 5A, lane 8). Similar results were obtained when frozen and lysed 17OD-treated cells were used in the CuAAC reactions (Fig. 5B). However, as was also suggested by our experiments shown in Fig. S1B, S2B, S3B, and S4B in the supplemental material, the labeling of the 28-kDa polypeptide was more intense when frozen, lysed cells were used compared to whole cells, but there was no discernible effect of using THPTA on the labeling reaction. Unless otherwise stated, the remainder of the CuAAC-labeling experiments reported in this study made use of intact whole cells with THPTA and aminoguanidine excluded from the CuAAC reaction.

To determine whether catalytically active AMO was required to observe fluorescent labeling of the 28-kDa polypeptide after exposure to 17OD, resting cells were incubated with and without allylthiourea (100 μM) prior to and during exposure to 17OD. After exposure to 17OD, the two cell samples were subjected to CuAAC reactions with Alexa Fluor 647 azide, solubilized in SDS-PAGE sample buffer and then analyzed by SDS-PAGE and IR scanning. The fluorescent 28-kDa polypeptide was detected in cells treated with 17OD alone (Fig. 6A, lane 4) but not in cells treated with allylthiourea prior to and during exposure to 17OD (Fig. 6A, lane 5). A similar effect was also observed if cells were treated with acetylene prior to exposure to 17OD. The 28-kDa polypeptide was detected in cells that had been treated with 17OD
FIG 5 Intact 17OD-treated cells of *N. europaea* (A) or 17OD-treated cells that had been lysed by freezing (B) were reacted with Alexa Fluor 647 azide using a CuAAC reaction and analyzed by SDS-PAGE and IR scanning as described in the Materials and Methods section. In the two figures, the samples were as follows: lane 1, NIR markers; lane 2, cells without 17OD pretreatment reacted with CuSO₄ (2 mM), sodium ascorbate (11 mM), Alexa Fluor 647 azide (8 µM for frozen, lysed cells and 40 µM for whole cells), and THPTA (1 mM); lane 3, 17OD-pretreated cells reacted as for lane 2; lane 4, same as for lane 3 minus CuSO₄; lane 5, same as for lane 3 minus sodium ascorbate; lane 6, same as for lane 3 minus Alexa Fluor 647 azide; lane 7, same as for lane 3 minus TPHTA; lane 8, same as for lane 3 plus aminoguanidine (1 mM). SW, bottom of sample well; UA, unreacted Alexa Fluor 647 azide at gel dye front; *, 55-kDa marker protein.

FIG 6 (A) Whole cells of *N. europaea* were (or were not) pretreated with 17OD in the presence and absence of allylthiourea, reacted with Alexa Fluor 647 azide, and analyzed by SDS-PAGE and IR scanning as described in the Materials and Methods section. Lane 1, NIR markers; lane 2, untreated cells (no 17OD); lane 3, untreated cells (no 17OD) plus allylthiourea; lane 4, 17OD-treated cells; lane 5, 17OD-treated cells plus allylthiourea. (B) Lane 1, NIR markers; lane 2, untreated cells (no 17OD); lane 3, 17OD-treated cells; lane 4, untreated cells (no 17OD) plus acetylene; lane 5, 17OD-treated cells plus acetylene. (C) Lane 1, NIR markers; lane 2, whole cells (25 µg protein); lane 3, soluble fraction (25 µg protein); lane 4, membrane fraction (25 µg protein). (D) Lane 1, NIR markers; lane 2, cells solubilized at room temperature in 2× SDS-PAGE sample buffer containing β-mercaptoethanol; lane 3, cells solubilized by heating for 5 min at 95°C in 2× SDS-PAGE sample buffer without β-mercaptoethanol; lane 4, cells solubilized by heating for 5 min at 95°C in 2× SDS-PAGE sample buffer containing β-mercaptoethanol. SW, bottom of sample well; AP, aggregated polypeptides; UA, unreacted Alexa Fluor 647 azide at gel dye front; *, 55-kDa marker protein.

FIG 7 The IR fluorescence associated with the 28-kDa polypeptide in SDS-PAGE gel analyses of total protein from *N. europaea* was determined for cells pretreated with individual *n*-alkynes and diynes prior to the CuAAC-catalyzed reaction with Alexa Fluor 647 azide as described in the Materials and Methods section. (A) Cells were pretreated with the following: lane 1, 1-hexene; lane 2, 1,5-hexadiyne; lane 3, 1-heptyne; lane 4, 1,6-heptadiyne; lane 5, 1-octyne; lane 6, 1,7OD; lane 7, 1-nonyne; lane 8, 1,8-nonadiyne. (B) Cells were pretreated with the following: lane 1, 1,7OD; lane 2, phenylacetylene; lane 3, 1,4-diethylnylbenzene; lane 4, propargylamine; lane 5, dipropargylamine. In samples heated at 95°C for 5 min (Fig. 6D, lane 4) compared to that of samples kept at room temperature (Fig. 6D, lane 2). In the sample heated at 95°C, there was also an increase in fluorescence associated with high-molecular-weight polypeptides. This aggregation effect was not as pronounced if the samples were first solubilized for 5 min at 95°C in an SDS-PAGE sample buffer that lacked β-mercaptopoethanol (Fig. 6D, lane 3).

Fluorescent labeling after inactivation of AMO by *n*-alkynes and other diynes. Other potential diyne inactivators and their analogs with a single ethynyl functional group were investigated to determine whether they could be used to fluorescently label the 28-kDa polypeptide in *N. europaea*. In all cases, resting cells were first incubated with each alkyne or diyne (1 µmol) in the presence of NH₄Cl (10 mM). The amount of NO₂⁻ generated during the incubation was used to estimate the extent of inactivation of ammonia-oxidizing activity. After reaction with Alexa Fluor 647 azide in a CuAAC reaction and subsequent analysis by SDS-PAGE and IR scanning, a fluorescent 28-kDa polypeptide was observed for cells pretreated with 1,5-hexadiyne, 1,6-heptadiyne, 17OD, or 1,8-nonadiyne but not for cells pretreated with 1-hexene, 1-heptyne, 1-octyne, or 1-nonyne (Fig. 7A). In all cases, NO₂⁻ production by cells during pretreatment with *n*-alkynes and diynes was inhibited by ≥95% compared to that of untreated control cells alone but was not detected in cells treated with acetylene alone or in cells treated with acetylene prior to exposure to 17OD (Fig. 6B).

To investigate whether the fluorescent 28-kDa polypeptide was susceptible to thermal- and reductant-dependent aggregation, samples of 17OD-pretreated cells were first reacted with Alexa Fluor 647 azide and were then solubilized in SDS-PAGE sample buffer using a variety of conditions. The resulting distribution of fluorescently labeled polypeptides was then determined by SDS-PAGE and IR analysis. For cells solubilized in SDS-PAGE sample buffer containing β-mercaptoethanol, the intensity of fluorescence associated with the 28-kDa polypeptide markedly decreased.
(data not shown). Similarly, a fluorescent 28-kDa polypeptide was also observed for samples from cells pretreated with either dipropargylamine or 1,4-diethynylbenzene while little or no fluorescence was detected for cells pretreated with either propargylamine or phenylacetylene (Fig. 7B). The \( \text{NH}_4^+ \)-dependent production of \( \text{NO}_2^- \) during the pretreatment of cells with phenylacetylene, 1,4-diethynylbenzene, and dipropargylamine was inhibited by \( \geq 90\% \) compared to that of untreated control cells, while cells pretreated with propargylamine generated \( \sim 35\% \) of the amount of \( \text{NO}_2^- \) of untreated control cells (data not shown).

Mass spectral analyses of the 28-kDa polypeptide. Two approaches were used to identify the fluorescent 28-kDa polypeptide at the molecular level. In the first approach, the portion of an SDS-PAGE gel that contained the fluorescent 28-kDa polypeptide was excised and subjected to in-gel tryptic digestion followed by matrix-assisted laser desorption ionization–tandem time of flight (MALDI-TOF/TOF) analysis of the resulting peptide fragments. This analysis provided amino acid sequences for 6 distinct peptides. The most abundant of these peptides (45 hits) included 4 fragments that were all located within AmoA from \( N. \text{europaea} \) (see Fig. S5 in the supplemental material). The two remaining peptides (5 hits) were identified as fragments from the S3 30S ribosomal protein of \( N. \text{europaea} \).

In the second approach, crude membrane fractions from 17OD-treated and control untreated cells were separately conjugated with biotin-azide using a CuAAC reaction and then affinity purified using streptavidin. After on-bead trypsin digestion of the purified proteins, the resulting peptide fragments were analyzed by liquid chromatography–mass spectrometry (LC-MS). To quantify the protein targets of the probe, the accurate mass and time (AMT) tag strategy was used to analyze the MS data. The AMT tag approach quantifies the area under the curve of each MS-identified peptide for a given protein. A rollout strategy is employed to determine a protein level abundance (36), which is based on the identified peptide for a given protein. A rollout strategy is employed to approach quantifies the area under the curve of each MS-identi-

**DISCUSSION**

Our results indicate that 17OD acts as a mechanism-based inactivator of AMO in \( N. \text{europaea} \). Our results also indicate that 17OD and several other diynes can be used to detect catalytically active AMO using CuAAC conjugation and azide-containing tags suitable for fluorescent detection or affinity purification of the active-site-containing component (AmoA) of this enzyme. Many other bacterial monooxygenases are inactivated by alkynes, and the approach outlined in this study can potentially be used to detect, identify, and quantify other diyne-sensitive monooxygenases in complex samples. These main conclusions are discussed in more detail in the following sections.

**Mechanism-based inactivation of AMO by 17OD.** Establishing that a compound acts as a mechanism-based inactivator for a specific enzyme requires that the effects of the putative inactivator conform to a well-defined set of kinetic criteria (25). With purified enzymes, these criteria include, among others, an inactivator concentration-dependent, first-order loss of enzyme activity, the requirement for enzyme activity for inactivation to occur, and the irreversibility of the effects of the inactivator resulting from covalent modification of the enzyme (25). Our results obtained with whole cells illustrate that low concentrations of 17OD (\( \leq 10 \mu\text{M} \)) produced a concentration-dependent, first-order loss of \( \text{NH}_4^+ \)-dependent \( \text{O}_2 \) uptake activity (Fig. 2). Our conclusion that 17OD is an irreversible inactivator of AMO is supported by our observation that recovery of ammonia-oxidizing activity in 17OD-treated cells required de novo protein synthesis (Fig. 4).

With mechanism-based inactivators, true substrates for the target enzyme are expected to decrease the rate of inactivation due to competitive interactions between the substrate and the inactivator at the enzyme’s active site (25). However, with some AMO inactivators studied in whole cells, \( \text{NH}_4^+ \) stimulates rather than decreases the rate of inactivation (21). This effect may reflect the need for concurrent ammonia oxidation to supply the reductant needed to support continued in vivo AMO activity. In this study, our experiments did not resolve an inhibitory or stimulating effect of 17OD on the inactivation of \( \text{NH}_4^+ \)-dependent \( \text{NO}_2^- \) production by 17OD, and a more detailed kinetic analysis will be required to clarify the role, if any, of \( \text{NH}_4^+ \) on this inactivation reaction. However, our measurements of \( \text{NO}_2^- \) production indicate that in all cases complete inactivation (\( \geq 99\% \)) of AMO activity was observed for cells incubated with 17OD (1 \( \mu\)mol) and \( \text{NH}_4\text{Cl} \) (10 \( \text{mM} \)) under standardized conditions. Any variations in the subsequent CuAAC-dependent fluorescent labeling reported in this study therefore likely reflect the effects of variables associated with the CuAAC reaction itself rather than with the degree of prior inactivation of AMO.

**Alexa Fluor 647 conjugation using CuAAC reactions.** The results presented in this study consistently demonstrated that a CuAAC reaction using Alexa Fluor 647 azide and cells treated with either 17OD (Fig. 5, and 6) or a range of other diynes (Fig. 7) resulted in the fluorescent labeling of a membrane-associated 28-kDa polypeptide. Based on the precedent of \( ^{13}\text{C} \) labeling of AmoA in \( N. \text{europaea} \) after exposure to \( ^{14}\text{C} \) ethylene (20, 26), we conclude that 17OD and other diynes target and inactivate AMO through catalytic activation of a terminal ethynyl group that results in the formation of a catalytically inactive, covalent enzyme-inactivator adduct (Fig. 1A). With diyne inactivators, this adduct retains a second unreacted terminal ethynyl group that can subsequently be conjugated with a fluorescent azide-containing reporter molecule, such as Alexa Fluor 647 azide (Fig. 1B). Conversely, the use of inactivators with only a single ethynyl group also resulted in inactivation of AMO but did not result in generation of a fluorescent product after CuAAC reactions (Fig. 7). This is presumably due to the lack of a second unreacted ethynyl group in the enzyme-inactivator adduct, which prevented subsequent CuAAC-dependent conjugation with Alexa Fluor 647 azide. Our observations that
TABLE 1 Quantitative proteomic analysis of probe (17OD) labeling of crude membrane fractions from *N. europaea*

<table>
<thead>
<tr>
<th>Locus tag(s)</th>
<th>Protein description</th>
<th>Probe mean (a)</th>
<th>Probe SD (a)</th>
<th>No probe mean (a)</th>
<th>No probe SD (a)</th>
<th>Fold difference (a)</th>
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<tbody>
<tr>
<td>ALW85_RS050490, ALW85_RS10750</td>
<td>AmoA, ammonia monoxygenase (b)</td>
<td>30.42</td>
<td>0.67</td>
<td>25.36</td>
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<td>ALW85_RS050493, ALW85_RS10745</td>
<td>AmoB, ammonia monoxygenase (b)</td>
<td>29.75</td>
<td>0.57</td>
<td>22.79</td>
<td>0.12</td>
<td>124.37</td>
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<tr>
<td>ALW85_RS050495, ALW85_RS10735</td>
<td>AmoC, ammonia monoxygenase (b)</td>
<td>28.80</td>
<td>0.23</td>
<td>21.45</td>
<td>0.44</td>
<td>163.05</td>
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<td>ALW85_RS05030</td>
<td>CoxB, cytochrome c oxidase polypeptide II precursor transmembrane protein</td>
<td>27.17</td>
<td>0.56</td>
<td>21.06</td>
<td>0.49</td>
<td>69.27</td>
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<td>ALW85_RS10005</td>
<td>Chbl, ribulose bisphosphate carboxylase, large chain</td>
<td>26.75</td>
<td>0.47</td>
<td>20.53</td>
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<td>Hypothetical protein</td>
<td>26.37</td>
<td>0.28</td>
<td>20.14</td>
<td>0.09</td>
<td>69.78</td>
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<td>ALW85_RS09655</td>
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<td>0.45</td>
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<td>ALW85_RS04925, ALW85_RS10735</td>
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<td>0.20</td>
<td>19.33</td>
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<td>ALW85_RS10075</td>
<td>Inorganic H(^+) pyrophosphatase</td>
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<td>0.56</td>
<td>19.83</td>
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<td>ALW85_RS09995</td>
<td>CbbQ, nitric oxide reductase NorQ protein</td>
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<tr>
<td>ALW85_RS03415</td>
<td>AccB1, biotin carboxyl carrier protein of acetyl-CoA carboxylase</td>
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<td>21.64</td>
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<tr>
<td>ALW85_RS13365</td>
<td>General diffusion Gram-negative porins</td>
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<td>ALW85_RS04225</td>
<td>Rieske iron-sulfur protein 2Fe-2S subunit</td>
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<td>19.45</td>
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<td>ALW85_RS01135</td>
<td>Pal, bacterial outer membrane protein</td>
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<td>0.11</td>
<td>19.76</td>
<td>0.74</td>
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<tr>
<td>ALW85_RS02050</td>
<td>60-kDa inner membrane protein</td>
<td>24.27</td>
<td>0.24</td>
<td>17.94</td>
<td>0.41</td>
<td>80.28</td>
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</tbody>
</table>

\(a\) See Table S1 in the supplemental material for additional data metrics and replicate data values.

\(b\) AMT tag data (log2 values) presented are the mean of three biological replicates of samples labeled with 17OD.

\(c\) Standard deviation (SD) of AMT tag quantitative data for samples labeled with 17OD.

\(d\) AMT tag data (log2 values) presented are the mean of two biological replicates of control samples treated with DMSO and no 17OD.

\(e\) SD of control samples treated with DMSO and no 17OD.

\(f\) Data presented are the magnitude of fold differences of protein abundances measured between 17OD-labeled and untreated samples. This shows clear protein labeling by 17OD.

**Activity-Based Protein Profiling of AMO**

pretreatment of cells with either allylthiourea (Fig. 6A) or C\(_2\)H\(_2\) (Fig. 6B) prior to exposure to 17OD subsequently prevented the fluorescent labeling of the 28-kDa polypeptide provide clear evidence that catalytic activity of AMO is required for 17OD to be effective as an ABPP probe for this enzyme.

It is important to note that we detected low levels of fluorescent labeling of the 28-kDa polypeptide in cells treated with propargylamine (Fig. 7B, lane 4). Based on the model outlined above, this is an unexpected result, as activation of the single ethynyl group of this compound by AMO leading to covalent modification of the enzyme would not be expected to leave an unreacted ethynyl group available for subsequent CuAAC conjugation reactions. As some propargylamine syntheses also produce di- and tri-propargylamine contaminants (37), this low-level fluorescent labeling may be due to the presence of these compounds in the commercially sourced propargylamine used in this study.

Our studies of key variables in the CuAAC-labeling reactions compared the same reactions using either whole cells or frozen, lysed cells (see Fig. S1 to S4 in the supplemental material). Overall, our results suggest that the CuAAC reaction proceeded faster with frozen, lysed cells than with intact cells (see Fig. S1) and that the Alexa Fluor 647 azide concentration was more critical in reactions with intact whole cells than with frozen, lysed cells (see Fig. S3). The simplest interpretation of these results is that Alexa Fluor 647 azide has limited permeability through the cell wall and membranes of intact cells. The effect of cell walls on the permeability of CuAAC reactants should be carefully considered in future applications of ABPP to microbial cells.

**Protein analyses.** Many of our observations concerning the fluorescently labeled 28-kDa polypeptide (Fig. 5 and 6) suggest that this polypeptide is AmoA, the same polypeptide previously shown to be radiolabeled when cells of *N. europaea* are incubated with \(^{14}\)C\(_2\)H\(_2\) (20, 26). In this study, confirmation of the identity of this labeled polypeptide as AmoA was provided by two separate approaches. The most direct method involved MALDI-TOF/TOF analysis of the peptide fragments generated from an in-gel tryptic
digestion of the 28-kDa polypeptide (see Fig. S5 in the supplemental material). The less direct but potentially more versatile approach involved mass-spectral analysis of the proteolytic fragments of affinity-purified proteins from the crude membrane fraction of 17OD-treated cells (Table 1). This analysis also revealed that peptides from AmoA were the most abundant digestion fragments detected compared to control samples from cells that had not been exposed to 17OD. However, peptide fragments from other proteins were also detected, albeit at lower levels than AmoA. These peptides were either from highly abundant and metabolically important cytoplasmic (RUBISCO) or periplasmic enzymes (HAO) or other proteins directly associated with AMO (AmoB, AmoC) (5) or HAO (cytochrome c553) (5). Some of these detections may have been due to adventitious binding of these abundant proteins to the streptavidin affinity purification matrix. However, it is also likely that this nonspecific detection is caused by diffusion of an activated form of 17OD away from its site of formation within AMO (27) and subsequent covalent modification of other proteins closely located to this enzyme in intact cells. Like AmoA, these additional proteins would be expected to retain an unreacted ethynyl group that would be available for reaction with biotin-azide in CuAAC reactions. These biotinylated proteins would then copurify with AmoA during streptavidin affinity purification and produce peptide fragments that can be identified by mass spectral analysis. For example, we observed a low level of fluorescent labeling of an ~45-kDa polypeptide in some of our analyses (Fig. 5), and our on-bead proteomic analysis (Table 1) suggests that this may be due to covalent modification of the 43-kDa AmoB polypeptide. Diffusion of an activated reactive inactivator away from the active site of AMO has previously been proposed to account for radiolabeling of proteins other than AmoA following inactivation of AMO by 14C2H2 (26) and is a common feature of mechanism-based inactivators (38).

**Potential applications of ABPP.** The ABPP approach described in this study is potentially applicable to many other alkyne-sensitive bacterial monooxygenases in pure cultures and complex microbial communities. While acetylene has been used to inactivate soluble and particulate methane monooxygenase (39) as well as propane-, butane-, toluene-, and tetrahydrofuran-oxidizing monooxygenases (40, 41), longer chain n-alkynes also inactivate several bacterial monooxygenases. For example, n-alkynes up to C10 inactivate AMO in *N. europaea* (11) and toluene-2-monooxygenase in *Burkholderia vietnamiensis* G4 (42). Several n-alkynes, including 1-ocetyne, inactivate the alkene-oxidizing enzyme system of the 2-methylpropene metabolizing strain, *Mycobacterium* sp. ELW1 (43). The well-studied alkanes hydroxylase in *Pseudomonas oleovorans* GPO1 is also irreversibly inactivated by 17OD (44–46).

The sensitivity of AMO to alkyne of differing carbon chain length has been the focus of recent studies of this enzyme in ammonia-oxidizing thaumarchaea (AOA). The AMOs in AOB and AOA are structurally similar but can be discriminated on the basis of their sensitivity to the concentration of n-alkyne mechanism-based inactivators, such as 1-ocetyne (47, 48). The effects of n-alkynes on AMO in AOA conform to many of the kinetic criteria for mechanism-based inactivation. However, formation of covalent enzyme-inactivator adduct has not yet been established for n-alkyne-inactivated AMO in AOA. Although 17OD may be an effective probe for AMO in AOA, this enzyme is most sensitive to inactivation by shorter chain n-alkynes (<C6), and terminal dyynes smaller than 1,5-hexadiyne are reactive and are difficult to prepare or obtain commercially. Another potential probe suggested by this study is phenylacetylene and its diyne analog 1,4-diethylnylbenzene. Phenylacetylene is a mechanism-based inactivator for AMO in *N. europaea* (20). It would be interesting to determine whether AMO in AOA is sensitive to these inactivators and whether, like AMO in *N. europaea*, AMO in AOA can be detected using 1,4-diethylnylbenzene as an ABPP probe.

The ABPP approach described here may also potentially be applied to detect, identify, and quantify catalytically active diyne-sensitive bacterial monooxygenases in complex microbial communities and environmental samples. In particular, using ABPP to affinity purify biotin-labeled enzyme-inactivator adducts has the potential to greatly reduce the complexity of protein samples obtained from these sources. Covalent fluorescent labeling of active monooxygenases in whole cells may also be exploited in fluorescence microscopy, flow cytometry, and fluorescence-activated cell sorting. Our current research is exploring the range of bacterial monooxygenases that are susceptible to inactivation by dyynes and detection by ABPP as well as developing methods to detect these enzymes and microorganisms harboring these enzymes in environmental samples.

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