ω-Azido fatty acids as probes to detect fatty acid biosynthesis, degradation, and modification

Alexander J. Pérez and Helge B. Bode

Abstract FAs play a central role in the metabolism of almost all known cellular life forms. Although GC-MS is regarded as a standard method for FA analysis, other methods, such as HPLC/MS, are nowadays widespread but are rarely applied to FA analysis. Here we present azido-FAs as probes that can be used to study FA biosynthesis (elongation, desaturation) or degradation (β-oxidation) upon their uptake, activation, and metabolic conversion. These azido-FAs are readily accessible by chemical synthesis and their metabolic products can be easily detected after click-chemistry based derivatization with high sensitivity by HPLC/MS, contributing a powerful tool to FA analysis, and hence, lipid analysis in general. PÉrez, A. J., and H. B. Bode. ω-Azido fatty acids as probes to detect fatty acid biosynthesis, degradation, and modification. J. Lipid Res. 2014. 55: 1897–1901.

Supplementary key words click-chemistry • β-oxidation • fatty acid metabolism • fatty acid desaturation • strained promoted cycloaddition • cyclooctyne • azido-fatty acids • S-azidoacyl-N-acetylcysteamine

FAs are found in all known living organisms, playing a vital role in cell compartmentalization, energy storage, and secondary metabolite production. In bacteria, most FAs are found in the cell membrane as part of the lipid bilayer (1). The actual FA profile of a cell can strongly vary depending on environmental and developmental conditions requiring de novo biosynthesis, degradation, and modification of the FAs involved (2–5). The monitoring of these metabolic pathways is usually conducted by GC-MS of FA methyl esters or other volatile FA derivatives (6). In spite of its merits, such as high sensibility and direct observability of especially volatile natural products, it is desirable to also have simple and effective methods of FA analysis via HPLC/MS, which today is a widespread tool in analytical chemistry as well. However, HPLC/MS-based FA analysis is difficult, mainly because of little ionizability and hence little signal strength. Small modifications introduced to certain FAs, however, can greatly increase ionizability while decreasing lipophilicity, which in turn increases signal resolution on reversed phase HPLC systems (7).

The method we developed for HPLC/MS-based detection of FA intermediates involves three steps: i) simple preparation of FAs with a terminal azido group (AFAs) that allows most FA modifications to occur; ii) use of these AFAs as metabolic probes and labeling of the in vivo formed derivatives in the corresponding organism with tetramethoxydibenzoazacyclooctyne (TDAC) (1, Fig. 1A), a cyclooctyne synthesized originally by Starke, Walther, and Pietzsch (8, 9); and iii) the detection of the clicked compounds by HPLC/MS. Although usually a reporter function such as a fluorophore is linked to the alkyne reagent, no such modifications were needed here, because the formation of an electron-rich triazole ring make it quite susceptible to protonation and thus detection by MS, especially as the molecular mass range of the clicked FAs differs strongly from other lipophilic compounds found in most cells. AFAs are bioorthogonal, do not react with other functional groups other than alkynes, are easily taken up by the cells like other FAs when externally added, and can be used to follow the fate of AFAs in a given organism in real time. Thus AFAs will add to the overall toolbox of lipid analysis in general. Similarly, alkyne-modified cholesterol has been used recently to trace cellular cholesterol metabolism and localization (10).

MATERIALS AND METHODS

General experimental procedures Solvents and reagents were obtained from Sigma-Aldrich (München, Germany). TDAC was synthesized using the procedure described by Starke, Walther, and Pietzsch (9) and can be obtained from the authors of this work. Alternative cyclooctynes,Abstract FAs play a central role in the metabolism of almost all known cellular life forms. Although GC-MS is regarded as a standard method for FA analysis, other methods, such as HPLC/MS, are nowadays widespread but are rarely applied to FA analysis. Here we present azido-FAs as probes that can be used to study FA biosynthesis (elongation, desaturation) or degradation (β-oxidation) upon their uptake, activation, and metabolic conversion. These azido-FAs are readily accessible by chemical synthesis and their metabolic products can be easily detected after click-chemistry based derivatization with high sensitivity by HPLC/MS, contributing a powerful tool to FA analysis, and hence, lipid analysis in general. Pérez, A. J., and H. B. Bode. ω-Azido fatty acids as probes to detect fatty acid biosynthesis, degradation, and modification. J. Lipid Res. 2014. 55: 1897–1901.

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such as dibenzocyclooctyne-amine, with comparable structures can be obtained commercially from Sigma-Aldrich and mostly share the abilities of the cyclooctyne chosen in this work. Silica chromatographic purification was performed on a Biotage SPI™ Flash purification system (Biotage, Uppsala, Sweden), using 40+M, 25+M, or 12+M KP-Sil cartridges (Biotage, Uppsala, Sweden) in combination with an UV detector, $^1$H, $^1$H-COSY, $^1$H,$^1$H-HSQC, and $^1$H-$^1$H-CMB NMR spectra for the synthesis products were recorded on a Bruker AV500 (500 MHz), AV400 (400 MHz), or AM250 (250 MHz) spectrometer using CDCl$_3$ or CD$_3$CN as solvent and internal standard, using the chemical shifts described by Gottlieb et al. (11) with the exception of the $^{13}$C shift of CDCl$_3$, which was set to 77.00 ppm. $^1$H-NMR: CHCl$_3$, $\delta$ = 7.24 ppm; CH$_3$CN, $\delta$ = 1.96 ppm; $^{13}$C-NMR: CDCl$_3$, $\delta$ = 77.00 ppm; CD$_3$CN, $\delta$ = 118.26 ppm. ESI HPLC/MS analysis was performed with a Dionex UltiMate 3000 system coupled to a Bruker AmaZon X mass spectrometer and an Acquity UPLC BEH C18 1.7 μm RP column (Waters) using a MeCN/0.1% formic acid in water gradient ranging from 5 to 95% in 22 min at a flow rate of 0.6 ml/min (12). High resolution mass spectra were obtained from a MALDI LTQ Orbitrap XL (Thermo Fisher Scientific, Inc., Waltham, MA) equipped with a laser at 337 nm. A 4-chloro-o-cyanocinnamic acid matrix was used, and the sum formulas and according masses were internally calibrated using fluorescein (monoisotopic mass = 332.068473) as a standard (13).

**Bacterial strains and culture conditions**

All *Escherichia coli* strains used in this study were grown on solid and liquid Luria-Bertani (LB) (pH 7.0) medium at 30°C and 180 rpm on a rotary shaker. The mutants and controls obtained from the Coli Genetic Stock Center were grown in the presence of kanamycin (40 μg/ml). *Psychrobacter urativorans* (ATCC 15174) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) and grown on solid and liquid Oxoid nutrient broth (DSMZ medium 948) at 16°C on a rotary shaker at 210 rpm.

**Feeding experiments for observation of FA degradation and desaturation**

The feeding experiments for the observation of the degradation of FAs were conducted with 16-azidohexadecanoic acid. The compound was fed at a final concentration of 1 mM to a culture of *fadE, fadA*, and *ilvE*, respectively. Cultures were grown in 250 ml Erlenmeyer flasks containing 30 ml of LB medium. Inoculation was performed from a preculture that was incubated overnight. The optical density was set to 0.1 and the culture was incubated at 30°C and 180 rpm for 3 h before feeding ensued and samples of 1 ml were taken. The samples were frozen in liquid nitrogen and freeze-dried before adding 1 ml 1 M NaOH and heated to 80°C for 4 h. Thereafter, the now clear solution was acidified with 190 μl of 6 M HCl and 1 ml of hexane was added. After vigorous stirring, the samples were centrifuged for 5 min at 4,000 rpm (3,220 × g) and 700 μl of the supernatant were removed, dried, and taken up in 500 μl of acetonitrile, after which 40 μl of a 10 mM solution of the prepurified raw product of 1 was added. The reaction was allowed to occur at room temperature for 24 h before HPLC/MS analysis. The experiments with *P. urativorans* were conducted in the same fashion under different incubation conditions as described above.

**Feeding experiments for FA elongation observation**

The feeding experiments for the observation of the elongation of FAs were either conducted with S-(5-azidopentanoyl)-N-acetylcysteamine or S-(6-azidohexanoyl)-N-acetylcysteamine, respectively. The compounds were fed at a final concentration of 2 mM to a culture of *fadE, fadA*, and DH10B. Cultures were grown in 100 ml Erlenmeyer flasks containing 10 ml of LB medium, including kanamycin in the concentration mentioned above in the case of *fadE* and *fadA*. Inoculation was performed from a preculture that was incubated overnight. The optical density was set to 0.1 and the culture was incubated at 30°C and 180 rpm for 2 h before feeding ensued and samples of 700 μl were taken. To these samples 200 μl 4 M NaOH were added, after which the mixture was heated to 90°C for 1 h. Thereafter, the now clear solution was acidified with 200 μl of 6 M HCl and 800 μl of hexane were added. After vigorous stirring, the samples were centrifuged for 5 min at 13,300 rpm (17,000 × g) and 600 μl of the supernatant were removed, dried, and taken up in 100 μl of acetonitrile, after which 10 μl of a 0.1 M solution of the prepurified raw product of 1 was added. The reaction was allowed to occur at 50°C for 1 h before HPLC/MS analysis.

**RESULTS AND DISCUSSION**

Prior to the application of AFAs as molecular probes for FA metabolism, a library of eight AFAs of varying chain
length was synthesized (2a–h, Fig. 1A) from the corresponding ω-bromo-FAs in a one-step reaction (supplementary Scheme I). TDAC [obtained by a simple three step synthesis (supplementary Scheme II) with good click properties and formidable solubility in a wide range of solvents, as it is common to heterocyclic methoxylated cyclooctynes (14, 15)] was then reacted with these AFAs and subsequently analyzed by HPLC/MS (Fig. 1). Because 15-bromopentadecanoic acid was not commercially available, it was synthesized from pentadecanolic acid by hydrolysis and subsequent bromination of the resulting 15-hydroxypentadecanoic acid for direct use in the corresponding AFA synthesis (supplementary Scheme III). The results showed tremendous signal amplification by a factor of about 5,000 in the extracted ion chromatogram (EIC) of the clicked product as the [M+H]+ ion in comparison to the free AFA (supplementary Fig. IA), along with clear differences in retention time according to the chain length of the corresponding AFA (Fig. 1B). Due to this strong increase in sensitivity resulting from the formation of the triazole unit, a relative quantification between different TDAC-modified AFAs also seems to be reasonable. Each signal consists of a characteristic double-peak, representing the two regioisomers formed in different amounts in accordance with the findings of Starke, Walther, and Pietzsch (9) for 3b/4b. This regioselectivity corresponds to the different steric demand of each of the transition states (9). It was observed that the more abundant isomers, 3a–h, had slightly higher retention times than the less abundant isomers, 4a–h. Analysis of the MS2 fragmentation pattern revealed distinct neutral losses for each of the two isomers. In particular, the minor isomer always features a dominant loss of water, followed by loss of the carboxylic group in the form of a dihydroxycarbene, whereas the main isomer predominantly shows only the loss of the latter group (supplementary Fig. IB). Thus, by comparing the characteristic fragmentation patterns, retention times, and peak shapes, simple identification of FA metabolites becomes possible.

In order to test whether AFAs can be used for the analysis of FA metabolism, β-oxidation was investigated initially. β-Oxidation is the primary FA degradation pathway in most organisms, consisting of desaturation, hydration, oxidation, and finally thiolyis of the resulting CoA-bound 3-keto-FA residue, resulting in the formation of acetyl-CoA and an acyl-CoA residue shortened by two carbon atoms (supplementary Fig. II) (16, 17). In most bacteria, the basic steps are performed by only three proteins: FadE (desaturation), FadB (hydratization and oxidation), and FadA (thiolyis) (18). Thus, a feeding experiment was conducted in which a long chain AFA was fed to E. coli DH10B. Hydrolysis of this culture and derivatization with TDAC allowed the combined detection of free and (previously) bound FA β-oxidation degradation products, clearly showing that ω-azido FAs are tolerated by the β-oxidation machinery. Indeed, C10ω-AFA (2h) was readily degraded in C4-steps, leading to a degradation product as short as 4-azidobutanoic acid (2a) resulting in the detection of 3a/4a (Fig. 2A). Additionally, the 3-keto and the 3-hydroxy forms were also detectable when high concentrations of the corresponding nonoxidized AFAs were reached (supplementary Fig. III). The retention times of these metabolic intermediary products proved to be similar to their saturated counterparts for long chain AFAs, while being slightly lower for short chained AFAs and clearly different from the retention times of Cn-1ω-AFAs, which have the same nominal mass as the corresponding Cn-3ω-keto-AFAs.

Next, the degradation of 2h was analyzed over time in different E. coli strains with mutations in fadA (JW5578) and fadE (JW5020), with ilvE (JW5606) as a control. The fadA mutant supposedly lacked the ability to conduct the final thiolyis step, whereas the fadE mutant lacked the acyl-CoA dehydrogenase. In the control strain (with a defect in leucine degradation not influencing FA metabolism), signals correlated to C14ω and C12ω-AFA (2l and 2f, respectively) showed a maximum at 2.3 and 2.8 h after the addition of 2h, respectively, and also shorter degradation products could be observed (Fig. 2B). Notably, analysis of the fadA mutant showed no difference to the ilvE control strain (not shown), indicating the functional complementation of the fadA mutation, while the fadE mutant showed

Fig. 2. A: EICs of click-labeled degradation products of 2h 12 h after initial feeding at 1 mM. The same amount of 2h was fed again right before the sample was taken. 3h/4l = TDAC-C8ω-AFA, 3l/4f = TDAC-C14ω-AFA, 3f/4f = TDAC-C12ω-AFA, 3d/4d = TDAC-C10ω-AFA, 3i/4l = TDAC-C8ωAFA, 3c/4c = TDAC-C6ω-AFA, 3a/4a = TDAC-C4ω-AFA. B: Relative peak area (in percent relative to the amount of 3h-4b) of 2h degradation products when fed to ilvE mutant. Increasing amounts of C14ω and C12ω-AFA can be seen 1 h after feeding, followed by further degradation products.

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no degradation activity at all (supplementary Fig. IV) (19). The decrease in concentration of 2h in the latter experiment might be attributed to absorption on the glass wall of the cultivation flasks or micelle formation in the medium. Similarly, C15-AFA (2g) was also fed to the E. coli wild-type, and degradation products of uneven carbon chain length could be observed as expected (supplementary Fig. V). This also proved that AFA incorporation and degradation is not dependent on a specific chain length, but occurs with several long chain AFAs, furthermore indicating the broad applicability of AFAs for degradation studies.

Next, FA biosynthesis was studied, generally requiring the condensation of a given FA thioester with malonyl-ACP leading to an elongation in C2-steps in a fashion the condensation of a given FA thioester with malonyl-broad applicability of AFAs for degradation studies. with several long chain AFAs, furthermore indicating the main product of FA elongation. Production of C7-AFA could not be observed as expected (supplementary Fig. V).

Fig. 3: A: EICs of clicked uneven AFAs immediately after and 24 h after feeding of 5a (detected as 3b/4a) to fadE mutant. The other EICs prove the production of C17-AFA (3j), C21-AFA (3e), C23-AFA (3k/4k), and C25-AFA (3g/4g). Even after 24 h the wild type (DH10B) only shows minimal production of C17-AFA (3k) and C15-AFA (3g). B: Relative peak areas (in percent relative to the area of 3b) of various AFAs from the feeding experiment with the fadE mutant. C15-AFA (as click product 3g) can clearly be seen as emerging main product of FA elongation. Production of C15-AFA could not be detected due to signal overlap with an impurity in the TDAC reagent.

SUMMARY

The use of AFAs in combination with modern and easy to synthesize cyclooctynes, such as TDAC, has shown to be a viable method for the analysis of FA metabolism, ranging from biosynthesis to degradation and desaturation. Because no degradation products smaller than 4-azidobutanoic acid (2a) have been detected, it can be assumed that 2a cannot be degraded. This might lead to increased availability of labeled FAs, even after prolonged metabolization, as compared with completely degradable isotope labels (24). The azido label forms an easily detectable triazole ring after reacting with a cyclooctyne, and is readily introduced into the desired target compound, thus allowing a wide range of applications in a time-resolved manner at low cost and high sensitivity. Our method can also easily be applied to study FA uptake or FA activation, which can be coupled to FA β-oxidation and can be detected by our approach. Moreover, it can not only be applied to FAs, but it can also be applied to lipid metabolism in general. For the latter, simply leaving out the hydrolytic step during sample preparation would enable the detection of acylcarnitines or CoA derivatives, as well as other lipid species carrying the respective AFA, not only in bacteria but also in all habitats that possesses a simple membrane FA composition, consisting mostly of Δ9-unsaturated palmitoleate and oleate residues (20, 21). When adapting to temperature changes in the range of 0–20°C, P. urativorans changes its FA profile by varying the ratio of the two main FA residues in the membrane (22, 23). Feeding C16-AFA (2g) to a culture of P. urativorans at 16°C followed by the subsequent detection of a mono-unsaturated FA with slightly reduced retention time (3m/4m, Fig. 4) confirms that a desaturase is indeed involved in this process.

Fig. 4. Desaturase activity shown in P. urativorans. The EIC of the clicked AFAs shows that C15-AFA (shown as click products 3h and 4h) is converted to the monodesaturated C16:1-AFA (shown as click products 3m and 4m, with the regiochemistry assumed to be the same as with 3a-1 and 4a-1, respectively).
organisms, including mammals, in which lipid metabolism needs to be studied.

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