JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Communication

pubs.acs.org/JACS

Metabolic Synthesis of Clickable Glutathione for Chemoselective Detection of Glutathionylation

Kusal T. G. Samarasinghe, Dhanushka N. P. Munkanatta Godage, Garrett C. VanHecke, and Young-Hoon Ahn*

Department of Chemistry, Wayne State University, Detroit, Michigan 48202, United States

Supporting Information

ABSTRACT: Glutathionylation involves reversible protein cysteine modification that regulates the function of numerous proteins in response to redox stimuli, thereby altering cellular processes. Herein we developed a selective and versatile approach to identifying glutathionylation by using a mutant of glutathione synthetase (GS). GS wild-type catalyzes coupling of γGlu-Cys to Gly to form glutathione. We generated a GS mutant that catalyzes azido-Ala in place of Gly with high catalytic efficiency and selectivity. Transfection of this GS mutant (F152A/S151G) and incubation of azido-Ala in cells efficiently afford the azide-containing glutathione derivative, γGlu-Cys-azido-Ala. Upon H_2O_2 treatment, clickable glutathione allowed for selective and sensitive detection of glutathionylated proteins by Western blotting or fluorescence after click reaction with biotin-alkyne or rhodamine-alkyne. This approach affords the efficient metabolic tagging of intracellular glutathione with small clickable functionality, providing a versatile handle for characterizing glutathionylation.

Redox regulation is a fundamental mechanism that controls numerous cellular processes, including proliferation, inflammation, and apoptosis.[1] Reactive oxygen species (ROS), which were once thought to be toxic byproducts of respiration, are now believed to be physiological signaling molecules that are cautiously generated for regulating multiple signaling pathways.[2] Importantly, one major mechanism of action of ROS is the oxidation of protein cysteine residue into various oxoforms, such as disulfide, sulfenic, sulfinic, and sulfonic acid, by which protein function can be turned on or off.[3] The reactivity and stability of individual oxoforms appear different and their functional roles may vary accordingly. Among various oxoforms, glutathionylation is disulfide formation of a protein cysteine residue with glutathione. Glutathionylation serves as an important regulatory switch that mediates the effect of ROS in normal redox signaling and pathological diseases.[4,5]

There is a growing list of redox-sensitive proteins regulated by glutathionylation of which identifications were made possible by various biochemical approaches. Current methods include an antibody pull-down approach in which a glutathione antibody is used to detect or enrich target proteins of glutathionylation. However, existing antibodies appear to suffer from low specificity and sensitivity.[4,6] Alternatively, biotinylated glutathione is used to pull-down glutathionylated proteins,[7] but the bulky nature of biotin is likely to interfere with enzyme-mediated (de)-glutathionylation. Moreover, direct incubation of biotinylated glutathione with cells can increase cellular thiol content, thus altering the redox state. In proteomics, the biotin switch method has been used with glutaredoxin in the reducing step[8] and the similar resin-capture approach identified numerous glutathionylated proteins.[9] While promising, the complexity may arise from this indirect identification method. Recently, tagging glutathione with biotin-spermine by *E. coli* glutathionylspermidine synthetase was developed, identifying many target proteins.[10] However, this analogue of glutathione is also bulky because of the presence of biotin-spermine.

Here we report the selective and versatile approach to characterizing glutathionylation: i.e., metabolic tagging of glutathione with an azide or alkyne functional group by engineering glutathione synthetase (GS) (Figure 1). Glutathione, an abundant tripeptide made of γGlu-Cys-Gly, is biosynthesized by two concerted ATP-dependent enzymes, γ-glutamylcysteine ligase (GCL) and GS.[11] GCL, the rate-limiting enzyme of glutathione biosynthesis, ligates Glu and Cys to form γGlu-Cys. Subsequently, GS catalyzes coupling of γGlu-Cys to Gly to form glutathione. We envisioned that the active site of GS can be engineered to incorporate an azide- or alkyne-containing Gly derivative in place of Gly. In this way, mutant GS could afford clickable glutathione in cells that can undergo glutathionylation in response to ROS stimulus. The subsequent bio-orthogonal click reaction with biotin or fluorophore would provide an effective handle for detecting and enriching target proteins of glutathionylation. Notably, the limiting amino acid for glutathione synthesis is Cys;[12] thus our approach is less likely

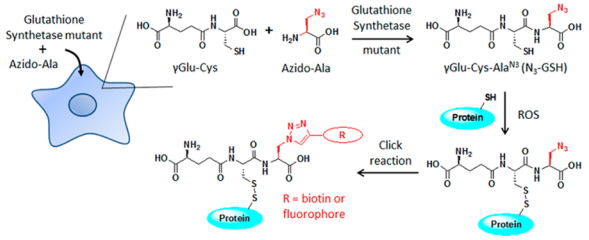

Figure 1. Identifying glutathionylation using "clickable" glutathione metabolically synthesized by glutathione synthetase mutant that catalyzes azido-Ala in place of Gly.

Received: April 20, 2014
Published: July 31, 2014

   dx.doi.org/10.1021/ja503946q | J. Am. Chem. Soc. 2014, 136, 11566−11569

to alter the total concentration of thiol or glutathione in cells. Interestingly, the major degrading enzyme of glutathione, such as γ-glutamyl transpeptidase, appears to retain the specificity to glutathione by mainly interacting with the γGlu residue of glutathione rather than the Gly residue.[13] Furthermore, there are a few structural variations of glutathione reported in plants, all of which possess the modification of Gly replaced by β-Ala, Ser, or Glu.[14] This may imply that the modification of Gly in glutathione can be tolerated for its metabolism in cells.

The crystal structure of human GS (hGS) (PDB: 2HGS) bound to glutathione and ADP predicts potential amino acids (F152 and S151) that are in close proximity to the Gly residue of glutathione (Figure 2). It appears that both F152 and S151 block

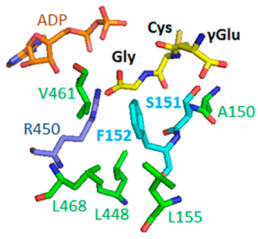

Figure 2. Active site of glutathione synthetase bound with glutathione and ADP. Note F152 and S151 (cyan) in close proximity to Gly in glutathione.

any side chain introduced in the position of Gly in glutathione. This suggests that mutation of F152 in combination with S151 is likely to provide the possibility of accommodating the azide- or alkyne-containing Gly derivative (Figure S1).

To find a GS mutant that efficiently catalyzes a clickable Gly derivative, His-tag hGS wild-type (WT) and mutants were expressed and purified in high purity (>95%) and a good yield (~6 mg/1 L culture) (Figure S2). Subsequently, the kinetic assay for monitoring ATP consumption was measured, as reported previously.[15] The results are summarized in Table 1 and Figure S3. Interestingly, GS F152G single mutant (M1) catalyzes azido-Ala most efficiently with the highest k_{cat}/K_m among all the amino acids tested. The small side chain of Gly and big side chains of Met and Val make them poor substrates with high K_m and/or low k_{cat}. This indicates that the best substrate for F152G mutant (M1) is azido-Ala. However, the k_{cat}/K_m of azido-Ala for GS F152G (M1) is still 25 times lower than the k_{cat}/K_m of Gly for GS WT. Encouragingly, this is significantly improved in the GS F152G/S151G double mutant (M2). Azido-Ala in this double mutant (M2) shows only 5-fold lower k_{cat}/K_m versus Gly for GS WT. Importantly, Gly and Ala are poorly catalyzed by this double mutant (M2), but Met is catalyzed with similar k_{cat}/K_m to that for azido-Ala. This indicates that GS F152G/S151G (M2) improved the K_m for azido-Ala, but it lost the selectivity of azido-Ala over Met. In molecular size, azido-Ala appears slightly smaller than Met.[16] Thus, we reasoned that mutation of F152G/S151G (M2) may have made a relatively large area that can accommodate not only azido-Ala but also Met, thereby giving the poor selectivity. In support of this, the GS S151G mutant (M3) shows high selectivity for azido-Ala over Met, albeit with a high K_m. We further reasoned that mutation of F152A together with S151G (M4) may have enough space for azido-Ala but not for Met. Indeed, F152A/S151G (M4) catalyzes azido-Ala the most efficiently with the highest k_{cat}/K_m (630.6 min^{-1} mM^{-1}), which is only 1.7-fold lower than the k_{cat}/K_m of Gly for GS WT (1095.0 min^{-1} mM^{-1}). In addition, GS M4 catalyzes azido-Ala with 2.6-fold higher selectivity over Met. In addition to azido-Ala, propargyl Gly (PG) and homopropargyl Gly (HPG) were catalyzed by GS M4 with a similar or lower k_{cat}/K_m to that for azido-Ala. Collectively, these kinetic data indicate that the GS M4 double mutant can selectively catalyze azido-Ala to synthesize "clickable" glutathione with similar catalytic efficiency to GS WT synthesizing "endogenous" glutathione.

Table 1. Kinetic Data of Amino Acid Substrates for GS WT and Mutants[a]

	GS	Substrate	K_m(mM)	k_{cat}(min^{-1})	k_{cat}/K_m
	WT	Gly	0.10 ± 0.02	109.5 ± 6.3	1095.0
M1	F152G	Gly	4.60 ± 0.70	103.6 ± 9.3	22.5
		L-Ala	ND	ND	ND
		L-Azido-Ala	2.46 ± 0.33	106.1 ± 8.1	43.1
		L-Met	3.67 ± 0.46	41.0 ± 3.0	11.2
		L-Val	7.37 ± 3.29	24.4 ± 6.1	3.3
M2	F152G/S151G	Gly	4.35 ± 1.85	37.2 ± 7.6	8.6
		L-Ala	1.85 ± 0.54	13.5 ± 1.0	7.3
		L-Azido-Ala	0.26 ± 0.02	48.7 ± 1.0	187.9
		L-Met	0.09 ± 0.02	18.5 ± 1.0	198.9
		L-PG	0.65 ± 0.20	37.8 ± 3.7	57.9
		L-HPG	0.12 ± 0.05	18.2 ± 1.4	149.5
M3	S151G	L-Azido-Ala	1.33 ± 0.49	51.7 ± 8.5	38.8
		L-Met	ND	ND	ND
M4	F152A/S151G	Gly	0.65 ± 0.32	48.2 ± 7.9	74.2
		L-Ala	1.60 ± 0.60	35.0 ± 5.6	22.0
		L-Azido-Ala	0.09 ± 0.02	55.5 ± 3.4	630.6
		L-Met	0.17 ± 0.03	40.8 ± 1.6	240.0
		L-PG	0.06 ± 0.02	28.5 ± 1.8	475.0
		L-HPG	0.08 ± 0.03	49.8 ± 5.2	622.5

[a]ND: not determined due to low activity. PG: propargyl-Gly. HPG: homo-propargyl-Gly.

Next, we sought to monitor the biosynthesis of azido-glutathione, a glutathione derivative containing azido-Ala in place of Gly, in cells. Interestingly, transient transfection of FLAG-tagged GS M4 to HEK293 cells induced overexpression of the mutant about 5−10 times higher than endogenous GS WT (Figure S4). A high ratio of GS mutant (M4) over GS WT may facilitate the flux of γGlu-Cys dipeptide toward the generation of azido-glutathione. After incubation of azido-Ala (1 mM) for 20 h, cells were lysed and the lysates free from proteins were injected into LC-MS. Encouragingly, cells expressing GS M4 mutant incubated with azido-Ala showed a strong mass ion peak of m/z 363, which corresponds to the mass of azido-glutathione (Figure 3b). At the same time, a large mass ion peak of m/z 308, corresponding to endogenous glutathione, was also detected. In contrast, control cells without GS M4 and azido-Ala showed only a mass ion peak of m/z 308 but no ion peak of 363 (Figure 3a). To confirm the identity of the peaks, we added fluorescein-iodoacetamide (FL-IA) during lysis. This will conjugate all glutathione with FL-IA by thiol-alkylation. LC-MS analysis detects a mass shift to m/z 695 and 750, which are in agreement with masses of endogenous glutathione and azido-glutathione conjugated with FL-IA, respectively (Figure S5). The incubation of azido-Ala alone without GS M4 in cells showed a mass of m/z 695 only, corresponding to endogenous glutathione, demonstrating that GS M4 is absolutely necessary for azido-glutathione biosynthesis (Figure S5). These data indicate the effective biosynthesis of clickable glutathione by GS M4 in cells.

dx.doi.org/10.1021/ja503946q | J. Am. Chem. Soc. 2014, 136, 11566−11569

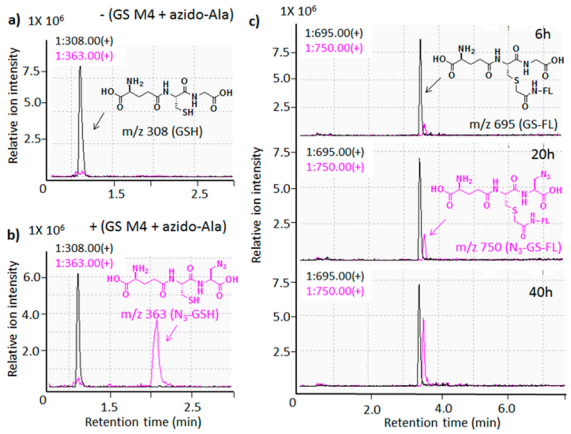

Figure 3. Analysis of azido-glutathione by LC-MS. (a) Without and (b) with transfection of GS M4 and incubation of azido-Ala (1 mM) in HEK293 cells for 20 h, or (c) after transfection of GS M4 and increasing the incubation time of azido-Ala (1 mM), lysates are collected and injected into LC-MS directly or after modification with fluorescein-iodoacetamide (FL-IA). Masses corresponding to endogenous (black) and azido-glutathione (pink) with or without modification by FL-IA are extracted and overlaid.

Our kinetic data indicate that GS M4 has a higher selectivity for azido-Ala, but it can catalyze Gly, Ala, or Met to synthesize the corresponding glutathione derivatives. As predicted, when purified GS M4 and γGlu-Cys were mixed with equal amounts of azido-Ala, Met, and Gly, in the presence of ATP, LC-MS analysis detected peaks of glutathione derivatives containing azido-Ala, Met, and Gly in a ratio of ~4:2:1, respectively (Figure S6). However, surprisingly, LC-MS analysis of cell lysates in Figure 3b did not detect any mass of modified glutathione containing Met or Ala (Figure S5). We suspect that incubation of a higher concentration of azido-Ala (1 mM) over Met (0.2 mM) or other amino acids (0.1−0.2 mM) in a typical medium may have increased the selectivity of azido-Ala over other amino acids in cells. In addition, despite the concern that the azide functionality can be reduced to an amine group in cells,[16] the mass corresponding to the reduced form of azido-glutathione was not detected in LC-MS analysis (Figure S5).

Interestingly, quantification of the mass ion peaks in Figure 3b showed an ~2:1 ratio of endogenous glutathione to azido-glutathione. But, the amount of azido-glutathione was dependent on incubation time. After transfection of GS M4 and incubation of azido-Ala for 6, 20, and 40 h, LC-MS detected a gradual increase of azido-glutathione to approximately 10%, 30%, and 60% of endogenous glutathione, respectively (Figure 3c). This gradual increase of azido-glutathione may reflect the slow rate of glutathione biosynthesis in nonstressed cells.[17] Interestingly, the measurement of the total thiol concentration in lysates by a thiol-reactive fluorogenic bromobimane showed similar thiol concentrations in samples with and without GS M4 and azido-Ala (Figure S4b). This is consistent with the fact that GCL is the rate-limiting enzyme in glutathione production and GS overexpression does not increase the total amount of glutathione.[18]

Our clickable glutathione provides a versatile handle that can be utilized for identification of glutathionylated proteins. After transfection of GS M4 and incubation of azido-Ala, cells were treated with hydrogen peroxide (H_2O_2) (1 mM) for 15 min. The resulting cell lysates were subjected to a click reaction with biotin-alkyne and probed with streptavidin-peroxidase. Note that the click reaction proceeded with Cu(I)Br instead of Cu(II)SO_4 and a reducing agent, since a reducing agent will reduce the disulfide in glutathionylation. Azido-Ala or H_2O_2 alone without GS M4 did not produce a signal with streptavidin in Western blot (lanes 1−4, Figure 4a), indicating that azido-Ala is not

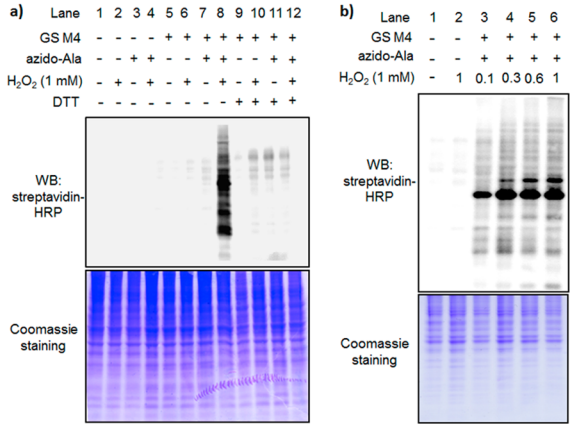

Figure 4. Azido-glutathione generated by transfection of GS M4 and incubation of azido-Ala can sensitively identify glutathionylation. (a) After transfection of GS M4, cells were incubated with azido-Ala (1 mM) for 20 h and then treated with H_2O_2 for 15 min. Lysates were subjected to click reaction with biotin-alkyne, then resolved by SDS-PAGE, and probed as shown above. Samples in lanes 9−12 were treated with DTT before loading on gel. (b) Same as (a) except with treatment of increasing concentrations of H_2O_2.

incorporated into cellular proteins (lanes 1 vs 3). Neither GS M4 alone nor GS M4 with H_2O_2 gave any signal (lanes 5−6). In contrast, H_2O_2 treatment after introducing GS M4 and azido-Ala generated numerous strong bands (lane 7 vs 8), which disappeared when treating samples with DTT (lanes 9−12). The click reaction with rhodamine-alkyne provided similar results while facilitating in-gel fluorescence detection (Figure S7a). Encouragingly, when an increasing dose of H_2O_2 (0.1−1.0 mM) was treated for 15 min, numerous bands were detected sensitively even at a low concentration of H_2O_2 (0.1 mM) (Figure 4b). Concentrations of azido-Ala higher than 1 mM did not enhance the signal of glutathionylation significantly (Figure S7b). These results demonstrate that selective and sensitive detection can be made by clickable glutathione generated by the GS M4 mutant without significant alteration of the redox state.

Azido-glutathione coupled with biotin-alkyne or fluorophore-alkyne can be used for chemoselective purification or cellular imaging of glutathionylated proteins, respectively. Indeed, the use of streptavidin-agarose enabled purification and enrichment of glutathionylated proteins, which were readily detected by silver stain (Figure S8a). In addition, the combination of immunoprecipitation, click reaction, and Western blotting allowed for detecting glutathionylation of individual proteins, such as protein tyrosine phosphatase 1B[19] and heat shock protein 90[9,10] (Figure S8b).

We also attempted to directly visualize glutathionylated proteins in cells by fluorescence. HEK293 cells overexpressing GS M4 were incubated with azido-Ala and treated with H_2O_2 (1 mM) for 15 min. Cells were then fixed with methanol and washed with PBS to remove excess glutathione in cells.[20] The subsequent click reaction with Alexa-Fluor 647 (AF 647)-alkyne detected a strong fluorescence signal (Figure 5e), which was widespread in cells when costained with DAPI (Figure 5d, f). In contrast, control cells without GS M4 did not produce any

dx.doi.org/10.1021/ja503946q | J. Am. Chem. Soc. 2014, 136, 11566−11569

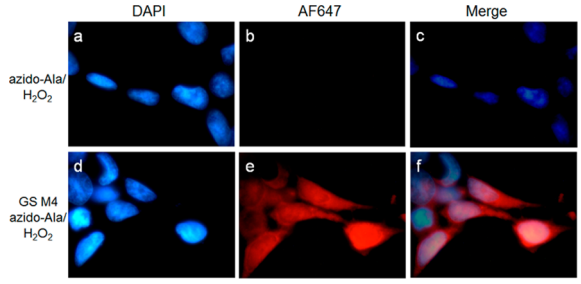

Figure 5. Azido-glutathione can selectively detect glutathionylation by fluorescence imaging. Cells without (a–c) or with (d–f) transfection of GS M4 were incubated with azido-Ala (1 mM) for 20 h and then treated with H_2O_2 (1 mM) for 15 min. After fixation, cells were subjected to click reaction with AF647-alkyne and monitored for DAPI stains (a, d) or glutathionylation (b, e). Images of DAPI stain and glutathionylation were overlaid in (c, f).

fluorescence signal after the click reaction with AF647-alkyne (Figure 5a–c). These results demonstrate that clickable glutathione can selectively visualize the localization of glutathionylated proteins in cells.

Although small, the azide functionality in azido-glutathione may interfere with redox enzyme-mediated (de)-glutathionylation. Among several redox enzymes that are implicated in glutathionylation, glutaredoxin 1 (Grx1) is a well-known enzyme that is shown to catalyze the removal of glutathionylation.[5] Lysates containing glutathionylated proteins by azido-glutathione were incubated with purified Grx1 *in vitro* (Figure S9a). Lysates without Grx1 treatment showed numerous bands. However, most signals completely disappeared when Grx1 was added for 30 min, indicating Grx1 can efficiently catalyze the removal of azido-glutathione. Similarly, when H_2O_2 was washed out after treatment in cells, a rapid deglutathionylation was observed in a time-dependent manner (Figure S9b), suggesting that glutathionylation by azido-glutathione can be efficiently reduced in cells.

Our kinetic assay showed the similar k_{cat}/K_m of PG or HPG versus azido-Ala for GS M4. Thus, we investigated the use of PG and HPG for detecting glutathionylation with transfection of GS M4, despite the concern that HPG is a good Met surrogate for *de novo* protein synthesis[16] and PG is an inhibitor of cystathionine γ-lyase. When PG or HPG was incubated in cells overexpressing GS M4, LC-MS mass analysis showed that GS M4 can effectively synthesize the clickable glutathione containing PG or HPG (Figure S10). However, the subsequent gel analysis showed that HPG was used for *de novo* protein synthesis, which prevented the selective detection of glutathionylation, and PG did not provide the sensitive detection of glutathionylation when compared to azido-Ala (Figure S11).

In summary, we developed a GS mutant for selective tagging of glutathione with clickable functionality. Azido-glutathione generated *in situ* was effective for sensitive detection of glutathionylation. A major advantage of clickable glutathione over existing approaches is the small versatile azide handle, which can couple to various applications. It not only can conjugate with biotin for purification in proteomics but also can click with fluorophores for imaging applications. Also, azido-glutathione may be used to identify small molecule substrates of glutathione S-transferase, as shown in a recent report.[21] Such efforts could significantly contribute to an enhanced understanding of the roles of glutathione and glutathionylation in redox regulation.

■ ASSOCIATED CONTENT

Supporting Information

Enzyme kinetics, LC-MS analysis, fluorescence detection of glutathionylation, and experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: yahn@chem.wayne.edu.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by Wayne State University Start-up funds and a WSU University Research Grant. We thank Dr. Miriam Greenberg laboratory for use of the fluorescence microscope.

■ REFERENCES

(1) Jacob, C., Winyard, P. G., Eds. *Redox Signaling and Regulation in Biology and Medicine*; Wiley-VCH: Weinheim, 2009.
(2) (a) Sundaresan, M.; Yu, Z. X.; Ferrans, V. J.; Irani, K.; Finkel, T. *Science* **1995**, *270*, 296. (b) Owusu-Ansah, E.; Banerjee, U. *Nature* **2009**, *461*, 537.
(3) Paulsen, C. E.; Carroll, K. S. *ACS Chem. Biol.* **2010**, *5*, 47.
(4) Grek, C. L.; Zhang, J.; Manevich, Y.; Townsend, D. M.; Tew, K. D. *J. Biol. Chem.* **2013**, *288*, 26497.
(5) Gallogly, M. M.; Mieyal, J. J. *Curr. Opin. Pharmacol.* **2007**, *7*, 381.
(6) Fratelli, M.; Gianazza, E.; Ghezzi, P. *Expert Rev. Proteomics* **2004**, *1*, 365.
(7) Sullivan, D. M.; Wehr, N. B.; Fergusson, M. M.; Levine, R. L.; Finkel, T. *Biochemistry* **2000**, *39*, 11121.
(8) Lind, C.; Gerdes, R.; Hamnell, Y.; Schuppe-Koistinen, I.; von Löwenhielm, H. B.; Holmgren, A.; Cotgreave, I. A. *Arch. Biochem. Biophys.* **2002**, *406*, 229.
(9) Su, D.; Gaffrey, M. J.; Guo, J.; Hatchell, K. E.; Chu, R. K.; Clauss, T. R.; Aldrich, J. T.; Wu, S.; Purvine, S.; Camp, D. G.; Smith, R. D.; Thrall, B. D.; Qian, W. J. *Free Radical Biol. Med.* **2014**, *67*, 460.
(10) Chiang, B. Y.; Chou, C. C.; Hsieh, F. T.; Gao, S.; Lin, J. C.; Lin, S. H.; Chen, T. C.; Khoo, K. H.; Lin, C. H. *Angew. Chem., Int. Ed.* **2012**, *51*, 5871.
(11) Dickinson, D. A.; Forman, H. J. *Biochem. Pharmacol.* **2002**, *64*, 1019.
(12) Wu, G.; Fang, Y. Z.; Yang, S.; Lupton, J. R.; Turner, N. D. *J. Nutr.* **2004**, *134*, 489.
(13) Keillor, J. W.; Castonguay, R.; Lherbet, C. *Methods Enzymol.* **2005**, *401*, 449.
(14) Skipsey, M.; Davis, B. G.; Edwards, R. *Biochem. J.* **2005**, *391*, 567.
(15) Vergauwen, B.; De Vos, D.; Van Beeumen, J. J. *J. Biol. Chem.* **2006**, *281*, 4380.
(16) Kiick, K. L.; Saxon, E.; Tirrell, D. A.; Bertozzi, C. R. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 19.
(17) Lu, S. C. *FASEB J.* **1999**, *13*, 1169.
(18) Grant, C. M.; Maclver, F. H.; Dawes, I. W. *Mol. Biol. Cell* **1997**, *8*, 1699.
(19) Barrett, W. C.; DeGnore, J. P.; König, S.; Fales, H. M.; Keng, Y. F.; Zhang, Z. Y.; Yim, M. B.; Chock, P. B. *Biochemistry* **1999**, *38*, 6699.
(20) Söderdahl, T.; Enoksson, M.; Lundberg, M.; Holmgren, A.; Ottersen, O. P.; Orrenius, S.; Bolcsfoldi, G.; Cotgreave, I. A. *FASEB J.* **2003**, *17*, 124.
(21) Feng, S.; Zhang, L.; Adilijiang, G.; Liu, J.; Luo, M.; Deng, H. *Angew. Chem., Int. Ed.* **2014**, *53*, 7149.