

Design, Synthesis and Application of a Hydrazide-Functionalized Isotope-Coded Affinity Tag (HICAT) for the Quantification of Oxylipid Protein Conjugates

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OVERVIEW

Purpose

Identification and quantification of carbonylated mitochondrial proteins arising from the adduction of lipid peroxidation products to the nucleophilic amino acid residues found in proteins.

Methods

- Synthesis of $^{12}\text{C}_2$ -HICAT and $^{13}\text{C}_2$ -HICAT.
- E. coli* thioredoxin (Trx) used as a model to examine the efficiency of HICAT as well as the enrichment with monomeric avidin cartridge.
- HICAT-labeling of *in vitro* HNE-modified mitochondrial proteins.
- $^{12}\text{C}_2$ - and $^{13}\text{C}_2$ -HICAT labeling of cardiac mitochondrial proteins from young and old rats.
- Mass spectrometry analysis in conjunction with MASCOT database searching.

Results

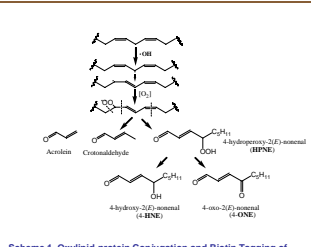
- HICAT efficacy: the expected 1:1 ratio between the m/z 2360 and m/z 2364 peptide pair from the digested $^{12}\text{C}_2$ -HICAT and $^{13}\text{C}_2$ -HICAT labeled Trx-HNE conjugate was observed in the MS survey scan.
- A total of 30 HICAT-labeled, *in vitro* HNE-conjugated peptides from 18 mitochondrial proteins identified based on the Mascot score and the assignment of b_n and y_n ions.
- The mean values and variability for the peak area ratios for isotopic peptide ions comparable to results reported for a commercially available cleavable ICAT reagents.
- In vivo* oxylipid-protein conjugates from the ADP/ATP translocase 1 (ADT1_RAT) identified and relatively quantified using the HICAT strategy.

Introduction

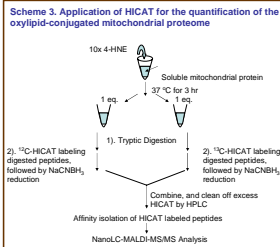
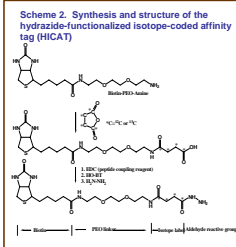
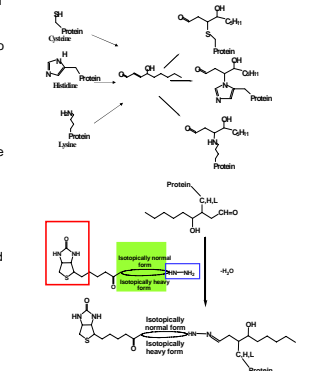
Reactive oxygen species (ROS) are the byproducts of mitochondrial energy production and ROS can cause considerable damage to the membranes of mitochondria. The important agents that give rise to the modification of proteins have been reported as being reactive aldehydic intermediates, such as keto aldehydes, 2-alkenals, and 4-hydroxy-2-alkenals. HNE-induced modification was found to occur mainly on histidine, lysine, and cysteine residues preferentially via a Michael-type addition preserving the aldehyde functionality on the targeted protein, rather than by Schiff's base formation.

While such addition reactions seem to play a role in age-related mitochondrial decay, there is a paucity of knowledge regarding the nature and the extent of oxidative protein modifications in mitochondria. To identify and quantify the oxylipids conjugated mitochondrial proteins, we are developing a mass spectrometry-based quantitative proteomics approach using a hydrazide-functionalized isotope-coded affinity tag (HICAT). Herein, we report the progress of using this probe for studying an *in vitro* model system and carbonylated mitochondrial proteins.

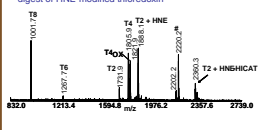
In vivo oxylipid-protein conjugates from the ADP/ATP translocase 1 (ADT1_RAT) identified and relatively quantified using the HICAT strategy.



Scheme 1. Oxylipid-protein conjugation and biotin tagging of oxylipid-protein conjugates



A. MALDI mass spectrum of the unfractionated tryptic digest of HNE-modified thioredoxin



B. The enriched fraction containing the HICAT labeled peptide



C. MALDI MS survey scan and tandem mass spectrometric identification of the HICAT-labeled HNE-modified peptide T2 from *E. coli* Trx

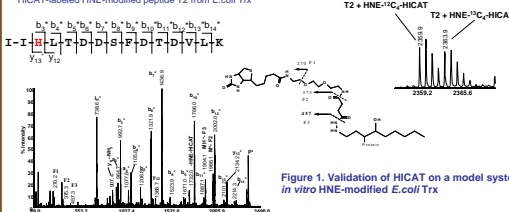


Figure 1. Validation of HICAT on a model system- *in vitro* HNE-modified *E. coli* Trx

Table 1. Selected HICAT-labeled HNE *in vitro*-modified peptides identified by Mascot search of MS/MS data.

Complex	Protein name	Labelled peptide	Labelled peptide	Labelled peptide
Complex II	ADP/ATP translocase 1	YKQKLVK	YKQKLVK	YKQKLVK
	ADP/ATP translocase 1	YKQKLVK	YKQKLVK	YKQKLVK
Complex IV	Cytochrome b	YKQKLVK	YKQKLVK	YKQKLVK
	Cytochrome b	YKQKLVK	YKQKLVK	YKQKLVK
Complex V	ADP/ATP translocase 1	YKQKLVK	YKQKLVK	YKQKLVK
	ADP/ATP translocase 1	YKQKLVK	YKQKLVK	YKQKLVK
ANT	ADP/ATP translocase 1	YKQKLVK	YKQKLVK	YKQKLVK
	ADP/ATP translocase 1	YKQKLVK	YKQKLVK	YKQKLVK
TCA	ADP/ATP translocase 1	YKQKLVK	YKQKLVK	YKQKLVK
	ADP/ATP translocase 1	YKQKLVK	YKQKLVK	YKQKLVK

* marks the HICAT-labeled HNE *in vitro*-modified residue. All MS/MS spectra were visually inspected and fragment ions annotated manually in addition to the automated annotations generated by Mascot.

Table 2. Peptide peak area ratios observed in MALDI MS survey scans of $^{12}\text{C}_2$ - and $^{13}\text{C}_2$ -HICAT-labeled HNE-peptide conjugates found in mitochondrial protein extracts treated with HNE.

Protein name	Labelled peptides	Observed ratio ($^{12}\text{C}_2$ / $^{13}\text{C}_2$)			
		sample 1	sample 2	sample 3	average \pm SD
ADP/ATP	YKQKLVK	1.06	1.11	1.00	1.06 \pm 0.06
ADP/ATP	YKQKLVK	1.15	1.14	0.90	1.06 \pm 0.14
ADP/ATP	YKQKLVK	1.20	0.98	0.95	1.05 \pm 0.06
ADP/ATP	YKQKLVK	1.16	0.99	0.97	1.04 \pm 0.10
ADP/ATP	YKQKLVK	1.21	1.06	1.07	1.12 \pm 0.10
ADP/ATP	YKQKLVK	1.11	1.09	1.09	1.09 \pm 0.04
ADP/ATP	YKQKLVK	1.21	1.15	1.01	1.13 \pm 0.11
ADP/ATP	YKQKLVK	1.16	1.23	0.95	1.13 \pm 0.15
ADP/ATP	YKQKLVK	1.00	1.25	0.96	1.07 \pm 0.16
ADP/ATP	YKQKLVK	1.16	1.12	0.95	1.08 \pm 0.11
ADP/ATP	YKQKLVK	1.03	1.00	1.08	1.04 \pm 0.04
ADP/ATP	YKQKLVK	1.16	1.12	0.95	1.08 \pm 0.11
ADP/ATP	YKQKLVK	1.07	1.20	0.98	1.07 \pm 0.17
ADP/ATP	YKQKLVK	1.16	1.00	1.10	1.05 \pm 0.10
ADP/ATP	YKQKLVK	1.08	1.10	1.16	1.11 \pm 0.04
ADP/ATP	YKQKLVK	1.12	1.09	1.00	1.07 \pm 0.06
ADP/ATP	YKQKLVK	1.15	1.09	1.11	1.09 \pm 0.09

Conclusion

- Demonstrating HICAT's potentials as an analytical tool for the identification and relative quantification of mitochondrial *in vitro* protein targets of HNE.
- The successful application of the HICAT strategy on *in vivo* oxylipid protein conjugates suggested that Cys²²⁸ from ADT1 is an *in vivo* target site for oxidative damage.
- Developing and optimizing new tagging strategy on *in vivo* oxylipid protein conjugates from young and old rat cardiac mitochondria.

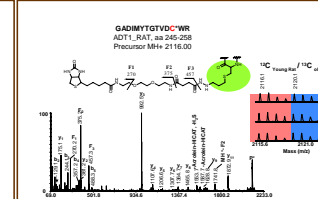
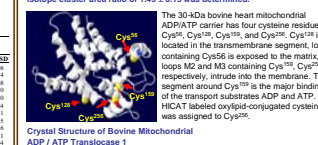


Figure 2. MALDI MS analysis of a HICAT-labeled *in vivo* acrolein-peptide conjugate from ADP/ATP translocase 1 (ADT1_RAT, aa 245-258) found in rat heart mitochondria. A, tandem mass spectrum of the $^{12}\text{C}_2$ -HICAT-labeled peptide ion with m/z 2116.0. B, MS survey scans of three independently performed HICAT labeling experiments showing the ion traces of the $^{12}\text{C}_2$ -HICAT labeled peptide ion at m/z 2116.0 (from young animals) and the $^{13}\text{C}_2$ -HICAT labeled peptide ion at m/z 2120.0 (from old animals). An average isotope cluster area ratio of 1.45 \pm 0.19 was determined.



The 30-kDa bovine heart mitochondrial ADP/ATP carrier has four cysteine residues, Cys²²⁸, Cys²³⁸, Cys²⁴⁸, and Cys²⁵⁸. Cys²²⁸ is located in the transmembrane segment, loop I containing Cys²²⁸ is exposed to the matrix, and loops M2 and M3 containing Cys²³⁸, Cys²⁴⁸, respectively, intrude into the membrane. The segment around Cys²²⁸ is the major binding site of the transport substrates ADP and ATP. The HICAT labeled oxylipid-conjugated cysteine was assigned to Cys²²⁸.

Acknowledgements

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References

- Esterbauer, H., Schaur, R. J., and Zollner, H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic Biol Med*, 11: 81-128, 1991.
- Grace, J.M., MacDonald, T.L., Roberts, R.J., and Kinter, M. Determination of site-specific modifications of glucose-6-phosphate dehydrogenase by 4-hydroxy-2-nonenal using matrix assisted laser desorption time-of-flight mass spectrometry. *Free Radic Res*, 25: 23-29, 1996.
- Ogyl, S.P., B. Gerber, S.A., Turecek, F., Gelb, M.H., and Aebersold, R. Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat Biotechnol*, 17: 994-999, 1999.
- Suh, J.H., Health, S.H. & Hagen, T.M. Two subpopulations of mitochondria in the aging rat heart display heterogeneous levels of oxidative stress. *Free Radic Biol Med*, 35: 1064-72, 2003.
- Dennehy, M.K., Richards, K.A.M., Wernke, G.R., Shyr, Y., and Liebler, D.C. Cytosolic and Nuclear Protein Targets of Thiol-Reactive Electrophiles. *Chem Res Toxicol*, 19: 20-29, 2006.