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Reactivity of Functional Groups on the Protein Surface: Development of Epoxide Probes for Protein Labeling

Gong Chen, Alexander Heim, Doris Riether, Dominic Yee, Yelena Milgrom, Mary Ann Gawinowicz, and Dalibor Sames*

Contribution from the Department of Chemistry, Columbia University, 3000 Broadway, New York, New York 10027, and Protein Core Facility, College of Physicians & Surgeons, Columbia University, New York, New York 10032

Received January 22, 2003; E-mail: sames@chem.columbia.edu

Abstract: We present the development of new affinity probes for protein labeling based on an epoxide reactive group. Systematic screening revealed that an epoxide functionality possesses the special combination of stability and reactivity which renders it stable toward proteins in solution but reactive on the protein surface outside the active site (proximity-induced reactivity). Highly efficient and selective labeling of purified HCA II (human carbonic anhydrase II) was achieved. For instance, 2 equiv of epoxide probe **9** was sufficient for nearly quantitative labeling of HCA II (>90% yield, 20 h reaction time). MS analysis of the labeled protein revealed that 1 equiv of the probe was attached and that labeling occurred at a single residue (His 64) outside the active site. Importantly, epoxide probe **9** selectively labeled HCA II both in simple protein mixtures and in cellular extracts. In addition to the chemical insight and its relevance to many epoxide-containing natural products, this study generated a promising lead in the development of new affinity probes for protein labeling.

Introduction

Covalent modification of proteins either in vivo (posttranslational modification) or ex vivo (postsynthetic modification) is a process of fundamental importance, allowing for alteration of protein properties, immobilization, cross-linking, and tagging. A need for selective labeling probes spans a broad terrain of disciplines, including life sciences and biomaterial sciences.

From the standpoint of chemical reactivity, there are two types of protein surfaces: first, an area of special reactivity or a catalytic site, and second, a general protein surface (Figure 1). High reactivity of active sites has been utilized in the development of irreversible inhibitors, usually electrophiles which covalently label one of the active site residues.[1,2] Recently, this approach has been applied for profiling the reactivity of entire proteomes.[3] In contrast, a general protein surface has not evolved to exert special reactivity characteristics.

Usually, selective labeling of a general surface, as opposed to unselective multiple labeling, requires the presence of a distinctly nucleophilic residue, such as cysteine, and a highly electrophilic probe (iodoacetamide, maleimide).[4] Although this methodology has frequently been used, it lacks generality as it is difficult to achieve site-selective labeling of a protein with none or multiple reactive cysteines. For similar reasons, this approach is unsuitable for selective labeling of protein mixtures.

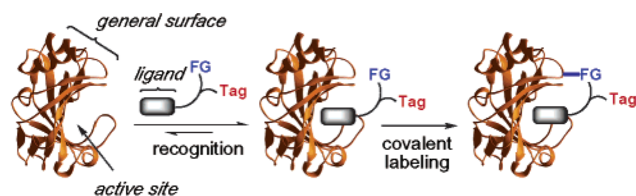

Figure 1. A concept of an affinity labeling probe. Labeling may occur either at the active site (active site affinity label) or outside the active site (general affinity label). FG = functional group.

With the view of developing selective protein labels, we set out to explore the reactivity of an array of functional groups (electrophiles) with general protein surfaces in the context of ligand-guided (affinity) labeling (Figure 1). The selectivity of such probes relies on two key elements: the protein ligand and the reactive functional group. The role of the ligand is to bind the protein of interest with high selectivity and affinity, escorting the functional group into proximity of the protein surface. The reactive functionality, however, should not react with proteins or other molecules (nucleophiles) in an intermolecular fashion, yet it should undergo reaction on the protein surface (proximity-induced reactivity). Importantly, such affinity probes do not require genetic engineering of the target protein to achieve selective labeling.[5−7]

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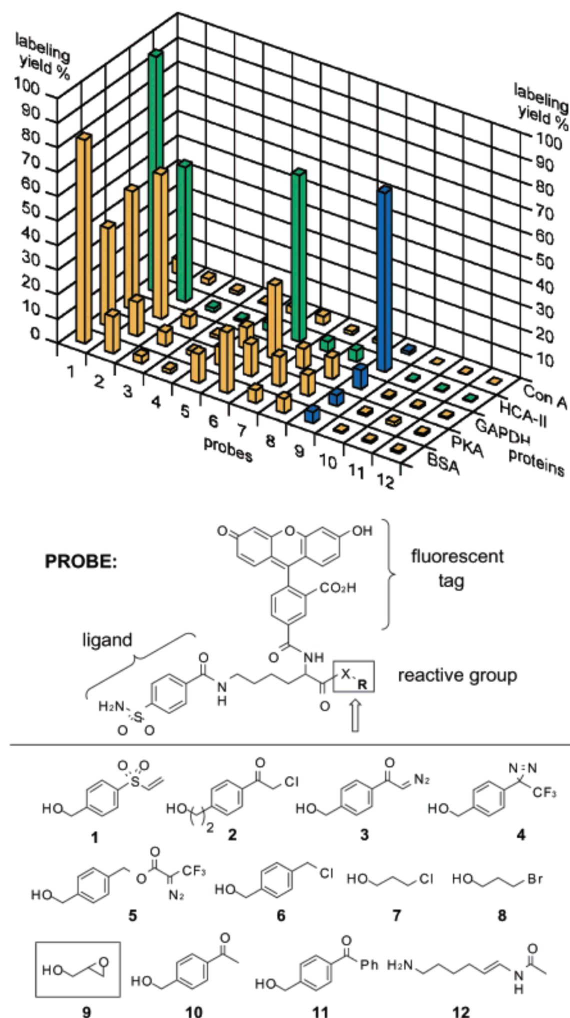
Chart 1. Reactivity of Functional Groups with Selected Proteins[a]



[a] Conditions: protein (5 μM, Tris buffer, pH 7.4), probe **1**−**12** (2 equiv), 10 h, room temperature (rt). BSA = bovine serum albumin, Con A = concanavalin A, GAPDH = glyceraldehyde-3-phosphate dehydrogenase, HCA II = human carbonic anhydrase II, and PKA = protein kinase A (catalytic subunit).

Results and Discussion

Reactivity of Functional Groups: Systematic Screening. As our first model protein, we selected human carbonic anhydrase II (HCA II) due to its availability, stability, and structural characterization. Furthermore, a simple benzenesulfonamide ligand binds to the active site with high affinity (K_d = 60−100 nM, Supporting Information).[8] Consequently, we synthesized an array of probes based on a common core, consisting of the benzenesulfonamide ligand and the fluorescein tag. To this core we attached various functional groups, affording probes **1**−**12** (Chart 1).

In the next stage of this study, individual probes were studied in a systematic screen against selected proteins. Thus, each probe (2 equiv) was added to a buffer solution of the protein (5 μM in Tris buffer, pH 7.4) and the reaction mixture incubated for 10 h at room temperature. Subsequently, the protein was denatured and analyzed on SDS−PAGE. Since only covalently labeled protein remained fluorescent, the chemical yield of labeling was determined by a quantitative gel-imaging assay (Chart 1, Supporting Information).

As expected, reactive probes such as vinyl sulfone **1**, α-chloroketone **2**, and benzyl chloride **6** afforded high yields of labeled HCA II (>50%), however with little or no selectivity. The high reactivity of these functional groups led to labeling of four out of five tested proteins, irrespective of molecular recognition. In contrast, photoaffinity labels such as diazoketone **3**, diazirin **4**, and diazoester **5** showed low reactivity toward the studied proteins in darkness. Diazoketone **3** gave only low yields (<6%) of labeling with highly reactive proteins, namely, GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and PKA (protein kinase A catalytic subunit), while diazirin **4** was inert. Both ketones **10** and **11** and enamide **12** also proved unreactive (Chart 1). Alkyl halides such as chloride **7** and bromide **8** showed relatively low reactivity as well as poor selectivity (4% labeling of HCA II, 10% labeling of GAPDH).

Thus, the functionalities discussed above were either reactive but unselective or unreactive. We were delighted to find an exciting exception, which clearly stood out in the array of experiments. Epoxide probe **9** demonstrated high selectivity for HCA II, affording a 70% yield of the labeled HCA II and only low level labeling of other proteins (<8%). It appears that *the epoxide, in contrast to other electrophilic functionalities tested herein, possesses the desired combination of stability and reactivity which enabled the proximity-induced coupling between the epoxide and nucleophilic protein surface*.[9−11]

Reactivity Profile of Epoxide Probe 9 with HCA II. Subsequently, we examined the labeling reaction between HCA II and the epoxide probe in detail with respect to stoichiometry, kinetics, and protein product analysis. Stoichiometric studies revealed that addition of a small excess of probe **9** led to high labeling yields. For instance, only 2 equiv of **9** was sufficient for nearly quantitative labeling of HCA II (>90% yield, 20 h reaction time, Chart 2; for kinetic data, see the Supporting Information). Most importantly, the use of an excess of the probe did not result in multiple labeling of the protein according to fluorescence gel imaging (Chart 2A). This result suggested that the epoxide probe was highly selective and that labeling was dependent on the molecular recognition between the probe and the protein. This hypothesis was supported by the following series of experiments (Chart 2B). First, the labeling was unaffected by the addition of 10 equiv of glutathione, showing

(6) Introduction of a nonnative or abiotic group at the desired site may provide an orthogonal handle for selective tagging: (a) Cornish, V. W.; Hahn, K. M.; Schultz, P. G. *J. Am. Chem. Soc*. **1996**, *118*, 8150−8151. (c) Muir, T. W.; Sondhi, D.; Cole, P. C. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 6705−6710. Kiick, K. L.; Saxon, E.; Tirrell, D. A.; Bertozzi, C. R. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 19−24.

(7) For labeling mediated by genetically encoded peptide tags, see: (a) Kapanidis, A. N.; Ebright, Y. W.; Ebright, R. H. *J. Am. Chem. Soc*. **2001**, *123*, 12123−12125. (b) Amini, F.; Kodadek, T.; Brown, K. C. *Angew. Chem., Int. Ed*. **2002**, *41*, 356−359 and reference therein.

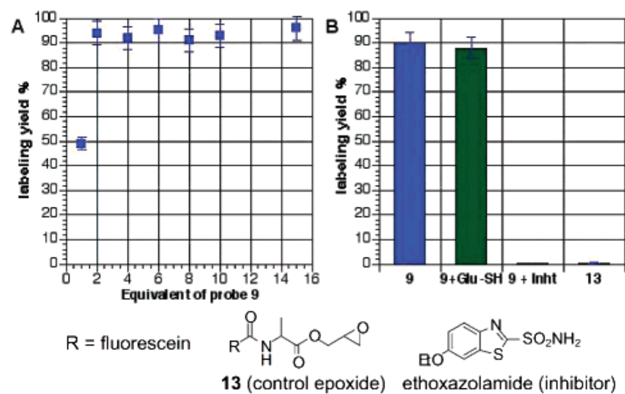
(8) Boriack, P. A. Christianson, D. W.; Kingery-Wood, J.; Whitesides, G. M. *J. Med. Chem*. **1995**, *38*, 2286−2291.

(9) For examples of the use of epoxides as active site affinity labels, see: (a) Thomas, E. W.; McKelvey, J. F.; Sharon, N. *Nature* **1969**, *222*, 485−486. (b) O'Connell, E. L.; Rose, I. A. *J. Biol. Chem*. **1973**, *248*, 2225−2231. (c) Greenbaum, D.; Medzihradszky, K. F.; Burlingame, A.; Bogyo, M. *Chem. Biol*. **2000**, *7*, 569−581. (d) Groll, M.; Kim, K. B.; Kairies, N.; Huber, R.; Crews, C. M. *J. Am. Chem. Soc*. **2000**, *122*, 1237−1238.

(10) For recent reports on the use of epoxides for protein immobilization, see: Abad, J. M.; Vélez, M.; Santamaría, C.; Guisán, M.; Matheus, P. R.; Vázquez, L.; Gazaryan, I.; Gorton, L.; Gibson, T.; Fernández, V. M. *J. Am. Chem. Soc*. **2002**, *124*, 12845−12853 and references therein.

(11) For examples of reactivity enhancement by general proximity effect, see: (a) Comens, P. G.; Simmer, R. L.; Baker, J. B. *J. Biol. Chem*. **1982**, *257*, 42−45. (b) Gartner, Z. J.; Liu, D. R. *J. Am. Chem. Soc*. **2001**, *123*, 6961−6963.

Chart 2. HCA II Labeling with Epoxide Probes **9** and **13**[a]



[a] (A) Labeling yield vs stoichiometry of epoxide probe **9**, 20 h, rt. (B) 2 equiv of **9** ± glutathione (Glu-SH, 10 equiv), inhibitor (ethoxazolamide, 10 equiv). Control epoxide **13** (2 equiv, last bar).

generally low reactivity of an epoxide with common nucleophiles (thiol, amine, carboxylate) under neutral conditions. Second, the labeling was completely abolished in the presence of ethoxazolamide, an inhibitor that competes with probe **9** for the active site of HCA II.[12] Third, control epoxide **13**, lacking the sulfonamide ligand, afforded no labeling of HCA II.

The labeled protein was purified and analyzed by MALDI-MS, which confirmed that 1 equiv of the probe was covalently attached per protein molecule. Furthermore, MS−MS analysis revealed remarkable selectivity of this reaction as labeling of a single residue (His-64) was detected (Supporting Information). This residue is situated outside the active site and thus may be considered a part of a general surface. Unusual efficiency of the epoxide labeling became particularly apparent upon comparison with the state-of-the-art photoaffinity probes.[13] The best photolabeling result was obtained with diazoketone **3** (21% yield), while probes **4**, **5**, and **11** afforded even poorer labeling yields (<10%, 30 min irradiation at 350 nm, Supporting Information).

Labeling of HCA II in Protein Mixtures and Proteomes. Last, we were faced with the key question related to the applicability of the epoxide probes to protein mixtures and entire proteomes. To begin to address these issues, we examined the reactivity of probe **9** and control probe **13** in a mixture of four proteins (HCA II, BSA, GAPDH, and PKA, Chart 3). BSA, GAPDH, and PKA were selected due to their considerable reactivity as demonstrated in Chart 1. Importantly, addition of probe **9** (2 equiv) to an equimolar mixture of these four proteins led to highly selective labeling of HCA (90% yields) with only background labeling of other proteins (<2%). Encouraged by this result, we then investigated the reactivity and selectivity of probe **9** in cellular extracts (Figure 2). The soluble proteome, obtained from yeast cells (*Saccharomyces cerevisiae*), was spiked with HCA II to adjust the level of this protein to 0.05% and 0.1% (w/w) of the total protein amount. Subsequently, probe **9** (2 equiv per HCA) was added to the extract, and the resulting mixture was incubated for 20 h at room temperature (Figure 2, Supporting Information). Remarkably, a single fluorescent band corresponding to labeled HCA II was detected by gel-imaging

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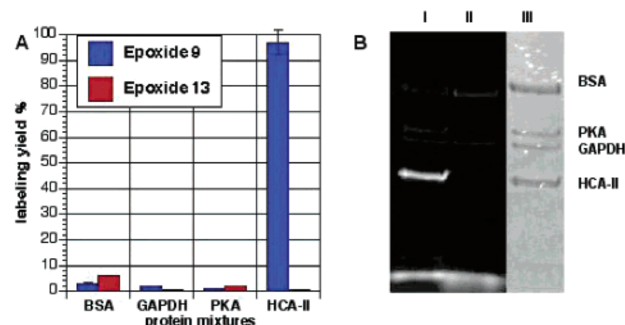
(13) (a) Chowdhry, V.; Westheimer, F. H. *Annu. Rev. Biochem.* **1979**, *48*, 293−325. (b) Bayley, H. *Photogenerated Reagents in Biochemistry and Molecular Biology*; Elsevier: Amsterdam, 1983. (c) Fleming, S. A. *Tetrahedron* **1995**, *51*, 12479−12520.

Chart 3. Selective Labeling of HCA II in Protein Mixtures[a]



[a] (A) Equimolar mixture of BSA, GAPDH, PKA, and HCA II (5 μM each, Tris buffer, pH 7.4), probe **9** or control probe **13** (2 equiv), 20 h. (B) Fluorescent gel image, lanes I (probe **9**) and II (probe **13**); Coomassie Blue stained gel, lane III (no probe).

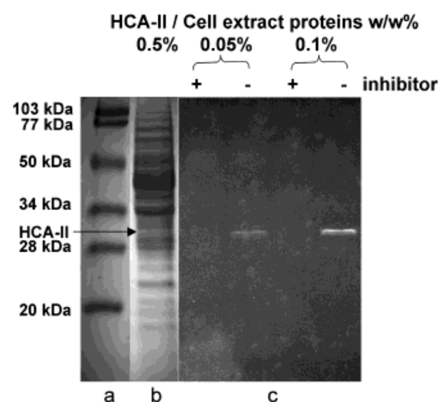


Figure 2. Selective labeling of HCA II in cellular extracts: (a) molecular weight standard, Coomassie Blue stained; (b) yeast cell extract (*S. cerevisiae*) + 0.5% (w/w) HCA II (HCA II is barely detectable at this concentration by Coomassie Blue stain); (c) yeast proteome + 0.05% and 0.1% (w/w) HCA, probe **9** (2 equiv per HCA II), ± ethoxazolamide inhibitor (10 equiv per HCA II), 20 h, rt. Fluorescence scanner image.

assay. Moreover, the labeling was completely suppressed in the presence of ethoxazolamide inhibitor (10 equiv per HCA II). It should also be noted that a high concentration of the thiol reagent present in the buffer (1 mM DTT) did not interfere with epoxide labeling, confirming the stability and proximity-induced reactivity of epoxides, however now in the context of a proteome (Figure 2).

Conclusion

In summary, a systematic study identified the epoxide as a suitable functionality for the development of affinity probes for protein labeling. This quality may stem from the special combination of stability and reactivity of epoxides. Epoxide probe **9** led to labeling of purified HCA II in high yield (>90%) and high selectivity (single residue outside the active site, His-64). Furthermore, we found that epoxide probe **9** labeled HCA II selectively in protein mixtures and cell extracts. In addition to the chemical insight and its relevance to many epoxide-containing natural products, these results represent a promising lead in the development of new affinity probes.

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Columbia University, yeast extract), and Dr. J. B. Schwarz (Pfizer, editorial assistance).

Supporting Information Available: Synthesis of probes **1**−**14**, experimental procedures for protein labeling, gel-imaging assay, and MS data for HCA II (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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