New Tools for Molecular Imaging of Redox Metabolism: Development of a Fluorogenic Probe for 3α-Hydroxysteroid Dehydrogenases

Dominic J. Yee, Vojtech Balsanek, and Dalibor Sames*

Department of Chemistry, Columbia University, 3000 Broadway, New York, New York 10027

Received November 25, 2003; E-mail: sames@chem.columbia.edu

The development of noninvasive methods for the visualization and quantification of molecular processes in vivo is of central interest to both basic science and medicine. Recent advances in photon detection technologies enable new opportunities in optical imaging of living matter (i.e., higher resolution and sensitivity).[1] However, molecular optical imaging requires the generation of "smart" probes or functional dyes[2] which would provide an optical signal for a particular molecular event (cf., enzymatic activity).[3] We herein communicate the key findings of a study that began with a chemical concept and resulted in the development of a selective fluorogenic probe for 3α-hydroxysteroid dehydrogenases (3α-HSD), an important class of enzymes in human physiology.

Oxidoreductases, including alcohol dehydrogenases, play essential roles in maintaining the balance of metabolic energy and regulating the concentration of critical metabolites, hormones, and xenobiotics. Redox optical probes must have a built-in mechanism for coupling the chemical redox event to a change in emission properties. However, two mechanisms frequently used for construction of fluorogenic substrates (e.g., probes for hydrolases), fluorescence energy transfer (FRET)[4] and phenol- or aniline-releasing reactions,[5] are generally not suitable for alcohol dehydrogenase probes.[6] Consequently, we have formulated a simplified electronic argument that guided the design of a ketone−alcohol redox switch. Many organic fluorophores are based on the "push−pull" structural feature wherein the electron-donating and electron-withdrawing groups are electronically connected via an extended π-conjugated system.[7] This class of fluorophores seemed particularly suitable for the design of redox probes wherein the ketone carbonyl would be a part of the "push−pull" system. Reduction of the carbonyl group to an alcohol converts an electron-withdrawing group (and often a quenching group) to an electron-donating group, resulting in a profound electronic change of the system, which in turn may lead to a change in the emission profile (Figure 1).[8]

The accurate prediction of light emission properties of organic compounds remains an underdeveloped art, and thus we undertook a semiempirical approach. An array of compounds was synthesized according to the design shown in Figure 1, founded on three aromatic core structures (Figure 2). The ketone moiety was attached to the core at two positions, either directly or via a linker. The linker (benzene, alkene, and alkyne) was introduced to explore the consequences of spatial separation of the ketone and the fluorophore while maintaining the conjugation between these two components. Specifically, we were interested in the effect of the length and nature of the π-conjugation system on both emission properties and the enzyme activity and selectivity (accessibility of the carbonyl group to the enzyme active site).

Approximately 50 compounds were synthesized (see Supporting Information) and evaluated in terms of the following physical and chemical properties in aqueous solution: (1) emission switching between the oxidized (ketone) and reduced forms (alcohol); (2) emission wavelength ($\lambda_{em} > 430$ nm) and quantum yield ($\Phi >$
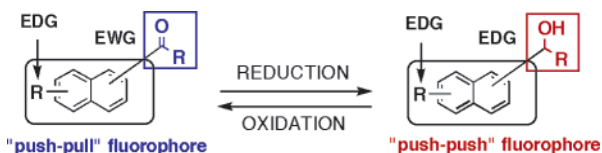
Figure 1. Design of an optical switch based on carbonyl−alcohol redox chemistry. EDG = electron-donating group, EWG = electron-withdrawing group.
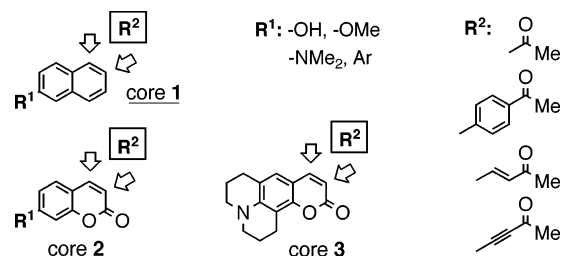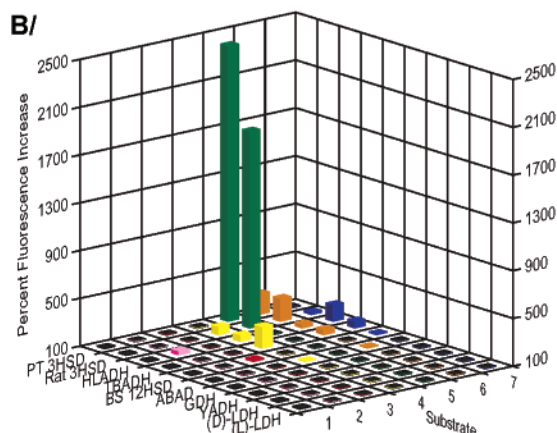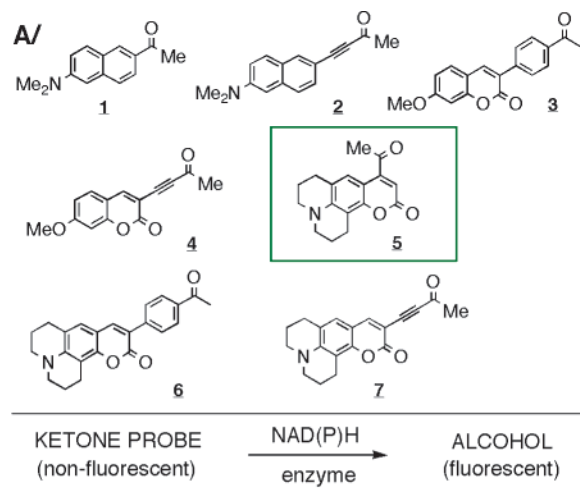

Figure 2. Synthesis of compound arrays based on three fluorophore cores (the corresponding alcohols are not shown).

0.1); and (3) photochemical stability and chemical stability (including stability to intracellular reductants). Following these strict criteria, we found seven fluorogenic probes possessing a suitable profile; in all cases (with the exception of probe **1**), the alcohols were highly fluorescent while the corresponding ketones showed only a background level of emission, thus constituting an optical redox switch (Figure 3A, for spectroscopic data see Supporting Information).[8] We were pleased that the selected candidates contained three different cores and a variety of linkers, thereby increasing the structural diversity of the set.

Probes **1**−**7** were subsequently tested against a collection of dehydrogenases in the presence of NAD(P)H; the extent of reduction was assessed by the measurement of fluorescence intensity at the emission maximum of each probe (Figure 3B). This assay included enzymes from two major oxidoreductase superfamilies, the short chain alcohol dehydrogenases (SDR) and the aldo-keto reductases (AKR), ranging from bacterial to mammalian as well as human enzymes.

Probe **5** ($\lambda_{em} = 510$ nm for the corresponding alcohol) clearly stood out as it was converted rapidly and selectively by 3α-hydroxysteroid dehydrogenases (3α-HSD), the bacterial (*Pseudomonas*) and the rat liver enzymes (Figure 3B).[9]
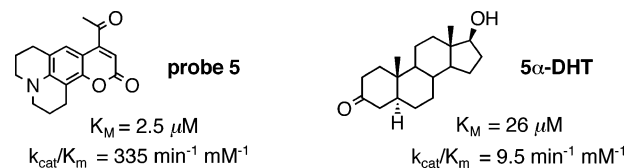
No other enzymes examined in this assay catalyzed the reduction of probe **5**. Similarly, both 3α-HSD enzymes demonstrated high selectivity for **5** among the tested probes. Compound **6** showed good conversion, however, at a significantly slower rate in comparison to that of probe **5** (Figure 3B). Surprisingly, horse liver alcohol dehydrogenase (HLAD) and *Thermoanaerobium brockii* alcohol dehydrogenase (TBAD), both well known for their substrate promiscuity,[10] were not tolerant of probe **5**. In contrast, these two latter enzymes catalyzed reduction of alkynyl-ketone probes **4** and **7**.

Figure 4. Kinetic parameters for probe **5** and the physiological substrate for human 3α-HSD (type 2, AKR1C3).

5 was in fact a far better substrate for this enzyme. This point may prove significant in future explorations of this probe in living matter.

In summary, this study addressed two key points: (1) ketone—alcohol redox optical switches with suitable emission profiles (emission wavelength, quantum yield) may be generated, and (2) these synthetic compounds may function as substrates for oxidoreductase enzymes. Specifically, a new fluorogenic probe was developed for 3α-HSD enzymes, including a human isozyme that has been implicated in important physiological functions. On the basis of its good optical properties and excellent enzyme selectivity, structure **5** represents an exciting lead for the development of a redox imaging probe. Future work will focus on determining the selectivity of probe **5** within the HSD family in vitro, followed in due course by studies in vivo.

Supporting Information Available: Synthetic procedures, spectral data, excitation and emission profiles for compounds **1**−**7**, and enzymology assay protocol (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

Figure 3. Screening of selected probes (A) against a panel of oxidoreductases (B). Percentage fluorescence increase after 12 h incubation of 30−50 μM substrate, 100 mM phosphate buffer (pH 7), 250 μM NAD(P)H, and 100 nM enzyme. Substrates **1**, **2**, **3**, and **4** were monitored at λ_{exc} = 340 nm, λ_{em} = 440 nm. Substrates **5**, **6**, and **7** were monitored at λ_{exc} = 440 nm, λ_{em} = 510 nm. 3HSD, 3α-hydroxysteroid dehydrogenase (PT, *Pseudomonas testosteroni*); HLAD, horse liver alcohol dehydrogenase; TBAD, *Thermoanaerobium brockii* alcohol dehydrogenase; BS 12HSD, *Bacillus sp.* 12α-hydroxysteroid dehydrogenase; ABAD, amyloid-β binding alcohol dehydrogenase (human); GDH, glycerol dehydrogenase; YADH, yeast alcohol dehydrogenase; LDH, lactate dehydrogenase.

The 3α-HSD enzymes are members of the aldo-keto reductase (AKR) superfamily and play numerous physiological functions. For example, they are involved in the activation/deactivation of steroid hormones in various tissues, including prostate (androgen deactivation) and brain (neurosteroid activation).[11]

Consequently, we addressed the next key question of whether the activity of human enzymes could be imaged by probe **5**. The type 2 isozyme of 3α-HSD (AKR 1C3) was selected for this study because of its important physiological role. To our delight, probe **5** was rapidly converted by this enzyme, and subsequent quantitative measurements afforded the kinetic parameters (K_m = 2.5 μM, k_{cat} = 8.2 min^{-1}). Remarkably, when compared to 5α-dihydrotestosterone (5α-DHT, K_m = 26 μM, k_{cat} = 0.25 min^{-1}, Figure 4),[11] a likely physiological substrate in prostate, we found that synthetic probe

References

(1) Weissleder, R.; Ntziachristos, V. *Nat. Med.* **2003**, *9*, 123−128.
(2) (a) Moreira, R.; Havranek, M.; Sames, D. *J. Am. Chem. Soc.* **2001**, *123*, 3927−3931. (b) Chen, C.-A.; Yeh, R.-H.; Lawrence, D. S. *J. Am. Chem. Soc.* **2002**, *124*, 3840−3841.
(3) (a) Boonacker, E.; Van Noorden, C. J. F. *J. Histochem. Cytochem.* **2001**, *49*, 1473−1486. (b) *Handbook of Fluorescent Probes and Research Chemicals, Molecular Probes*, 9th ed.; Haugland, R. P., Ed.; Molecular Probes: Eugene, OR, 2002.
(4) Wang, G. T.; Matayoshi, E.; Huffaker, H. J.; Krafft, G. A. *Tetrahedron Lett.* **1990**, *31*, 6493−6496.
(5) (a) Rotman, B.; Zderic, J. A.; Edelstein, M. *Proc. Natl. Acad. Sci. U.S.A.* **1963**, *50*, 1−6. (b) Zimmerman, M.; Ashe, B.; Yurewicz, E.; Patel, G. *Anal. Biochem.* **1977**, *78*, 47−51.
(6) Alcohol dehydrogenase probes which require two catalytic steps (oxidation and β-elimination): Klein, G.; Reymond, J.-L. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 1113−1116.
(7) Rettig, W. *Angew. Chem., Int. Ed. Engl.* **1986**, *25*, 971−988.
(8) Previous examples of carbonyl−alcohol fluorogenic probes suffered from short excitation/emission wavelengths in the near UV region. (a) Wierzchowski, J.; Dafeldecker, W. P.; Holmquist, B.; Vallee, B. L. *Anal. Biochem.* **1989**, *178*, 57−62. (b) List, B.; Barbas, C. F., III; Lerner, R. A. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 15351−15355.
(9) Jez, J. M.; Schlegel, B. P.; Penning, T. M. *J. Biol. Chem.* **1996**, *271*, 30190−30198.
(10) Fang, J.-M.; Lin, C.-H.; Bradshaw, C. W.; Wong, C.-H. *J. Chem. Soc., Perkin Trans. 1* **1995**, 967−978.
(11) Penning, T. M.; Burczynski, M. E.; Jez, J. M.; Hung, C.-F.; Lin, H.-K.; Ma, H.; Moore, M.; Palackal, N.; Ratnam, K. *Biochem. J.* **2000**, *351*, 67−77.

JA039799F