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Specific biotinylation of IMP dehydrogenase

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ABSTRACT

IMP dehydrogenase (IMPDH) catalyzes a critical step in guanine nucleotide biosynthesis. IMPDH also has biological roles that are distinct from its enzymatic function. We report a biotin-linked reagent that selectively labels IMPDH and is released by dithiothreitol. This reagent will be invaluable in elucidating the moonlighting functions of IMPDH.

Inosine 5′-monophosphate dehydrogenase (IMPDH) catalyzes the penultimate step of guanine nucleotide biosynthesis, the oxidation of IMP to XMP with the concomitant reduction of NAD^+[1]. IMPDH is up-regulated in rapidly dividing cells, and inhibition of IMPDH blocks proliferation and induces differentiation. IMPDH-targeted drugs are used in anticancer, antiviral, and immunosuppressive therapy, and are in development for antimicrobial applications. Some recent observations suggest additional functions for IMPDH beyond its well-established role in purine nucleotide biosynthesis. IMPDH associates with telomeric sequences, polyribosomes and actively transcribing promoters, localizes to lipid vesicles in response to insulin and aggregates in response to guanine nucleotide depletion.[2–6] Clearly many questions remain regarding the biological function of this housekeeping enzyme: is IMPDH enzymatically active in these different cellular contexts? what factors interact with IMPDH in each location? how do these interactions change in response to insulin and other signals?

A mechanism-based probe would be an ideal tool to address these questions.[7,8] The IMPDH reaction is unusual among the enzymes involved in nucleotide metabolism in that it involves the nucleophillic attack of a conserved Cys residue, and several nucleotide analogs inactivate IMPDH by modifying this Cys.[1,9–14] For example, 6-Cl-purine ribotide (6-Cl-PRT) reacts with the conserved Cys to form a stable 6-linked adduct (Fig. 1).[9,12,15] We exploited this unusual reactivity to create a reagent that specifically biotinylates IMPDH by attaching a biotin to the 2′ position of 6-Cl-PRT via an aminopropyl linker to produce 6-Cl-PRT-biotin (**1**, Fig. 1). The synthetic route started with xylofuranose and is described in the Supplementary data. Importantly, the reagents are relatively inexpensive, the products easy to purify and each step is high-yielding, and scalable.

The reactions of 6-Cl-PRT and 6-Cl-PRT-biotin were characterized with IMPDHs from various organisms already in hand (Fig. 2).[16] 6-Cl-PRT rapidly inactivates *E. coli* IMPDH, and the presence of the biotin has no significant effect on this reaction (k_{inact}/K_i = 120 M^{-1} s^{-1} and 130 M^{-1} s^{-1} for 6-Cl-PRT and 6-Cl-PRT-biotin, respectively, at 25 °C; standard errors are within 20%

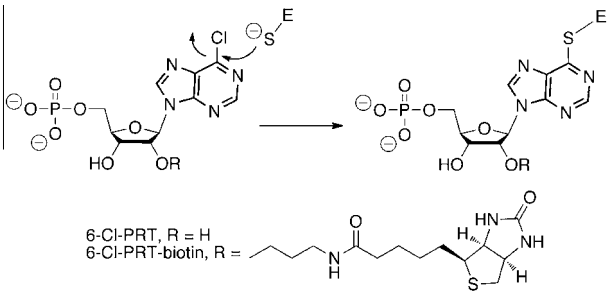

Figure 1. Reaction of 6-Cl-PRT and 6-Cl-PRT-biotin with IMPDH.

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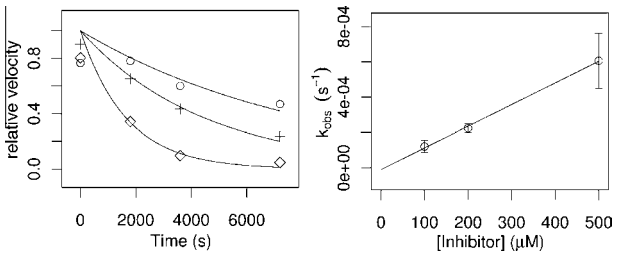

Figure 2. Inactivation of human IMPDH type 2 by 6-Cl-PRT-biotin at 37 °C. A. Loss of IMPDH activity over time incubated with 6-Cl-Purine-biotin, (○) 100 μM 6-Cl-PRT-biotin, (+) 200 μM 6-Cl-PRT-biotin, (◇) 500 μM 6-Cl-PRT-biotin. B. Rate of inactivation of IMPDH as a function of 6-Cl-PRT-biotin concentration.

for all values unless otherwise noted). 6-Cl-PRT is a less effective inactivator of human IMPDH type 2 (hIMPDH2), with k_{inact}/K_i = 0.9 M^{-1} s^{-1} at 25 °C; hIMPDH2 has a lower specific activity than *E. coli* IMPDH,[1] so this reduced activity is not surprising. The addition of the biotin reduces k_{inact}/K_i by at least a factor of 10. Inactivation of hIMPDH2 by 6-Cl-PRT-biotin is readily observed at 37 °C (k_{inact}/K_i = 1.4 M^{-1} s^{-1}), so this reagent will be useful for probing IMPDH in human cells.

To determine if 6-Cl-PRT-biotin covalently labels IMPDH as designed, the inactivated enzymes were analyzed by denaturing SDS–PAGE, transferred to PVDF membranes and probed with avidin-horse radish peroxidase, which binds specifically to biotin.[17] Strong labeling of IMPDH was observed, demonstrating that a covalent adduct is formed (Fig. 3). No labeling occurred when IMPDH was denatured prior to treatment with 6-Cl-PRT-biotin (not shown), and labeling was reduced by a factor of >2000 when the active site Cys was replaced with Ala (Fig. 3A). These observations demonstrate that 6-Cl-PRT-biotin selectively modifies the active site Cys of IMPDH as designed.

In the course of these experiments, we noticed that labeling was less efficient in the presence of dithiothreitol. Preincubation of 6-Cl-PRT with dithiothreitol had no effect on the labeling reaction, demonstrating that the probe is stable under these conditions. In contrast, treatment of 6-Cl-PRT-modified IMPDH with dithiothreitol released the label (Fig. 3B). These experiments establish mild conditions to break the 6-S-enzyme-PRT adduct which will be very useful in isolating IMPDH complexes.

To test the specificity of labeling, an *E. coli* lysate was treated with 6-Cl-PRT-biotin and analyzed as described above (Fig. 4). One major band is observed; this band is absent in a Δ*guaB* strain that lacks endogenous IMPDH, and can therefore be assigned to IMPDH with confidence. A second very weak band is observed at

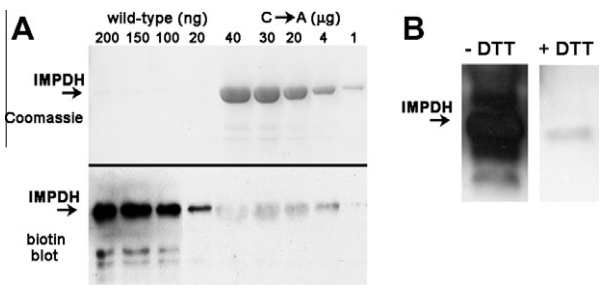

Figure 3. Labeling of IMPDH. A. Recombinant *E. coli* IMPDH was inactivated with 6-Cl-PRT-biotin and subjected to non-reducing SDS–PAGE, transferred to a PVDF membrane, and probed with avidin-HRP. B. Purified recombinant human IMPDH type 2 was treated with 200 μM 6-Cl-PRT-biotin. Half of the sample was then treated with 6 mM DTT. Samples were desalted over Sephadex G-50, denatured under non-reducing conditions, and separated by SDS–PAGE. After transfer to a PVDF membrane, the blot was probed with avidin-HRP.

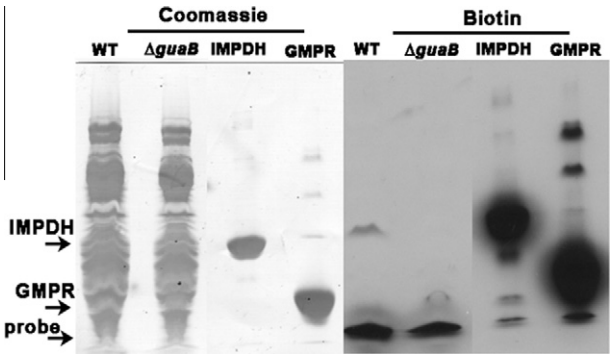

Figure 4. Selectivity of 6-Cl-PRT-biotin. Lysates of wild-type *E. coli* strain MG1655 and Δ*guaB*, which lacks endogenous IMPDH, were treated with 6-Cl-PRT-biotin and analyzed as described in Figure 3.

lower molecular weight in the Δ*guaB* strain. 6-Cl-PRT is also known to inactivate GMP reductase (GMPR),[6] an enzyme closely related to IMPDH with a similar mechanism and nucleophillic Cys. 6-Cl-PRT-biotin also labels GMPR (Fig. 4), so it is likely that the smaller band is GMPR. IMPDH and GMPR have different molecular weights and are induced under very different conditions, so this cross-reactivity should not limit the usefulness of the probe.

Biotinylation is a widely used method for labeling biological macromolecules for isolation and visualization.[7,8] Here we have described a reagent, 6-Cl-PRT-biotin, that selectively labels IMPDH and can be removed under mild conditions. This reagent will be invaluable in determining if the active site of IMPDH is accessible when the protein is associated with polyribosomes, chromatin and lipid vesicles as well as for pulldown experiments to identify interacting factors. 6-Cl-PRT-biotin and related compounds may also be useful in cellular visualization experiments, although it is likely that a prodrug approach will be required to enable probe uptake. We believe that 6-Cl-PRT-biotin will be an invaluable tool for investigating the biological functions of this 'enzyme of consequence' in virtually all organisms.

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Supplementary data

Supplementary data (synthesis of 6-Cl-PRT and detailed experimental procedures) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.01.042.

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16. *Conditions*: IMPDH was incubated in 50 mM Tris–HCl, pH 8, containing 100 mM KCl and 0.5 mM TCEP (assay buffer) with increasing concentrations of the inhibitor. The reaction was quenched by the addition of 2 mM IMP and assayed.
17. Denaturing SDS–PAGE was performed using standard methods except that TCEP was substituted for dithiothreitol to preserve the thiol linkage. Samples were denatured by heating at 100 °C for 3 min or by adding urea buffer.