Direct Screening for Phosphatase Activity by Turnover-Based Capture of Protein Catalysts**

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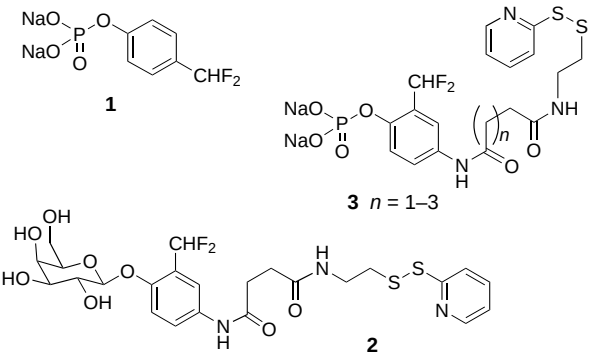
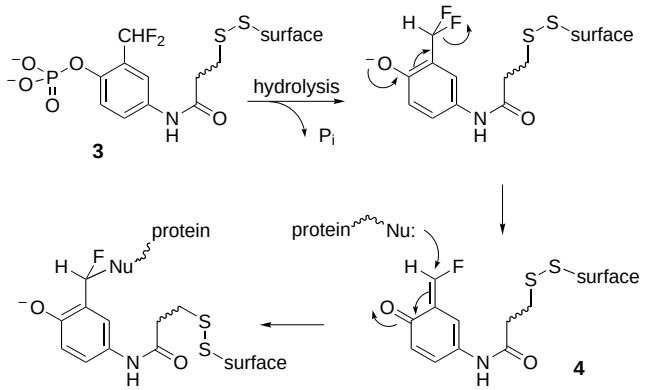
In studies towards the generation and identification of catalytic antibodies with phosphate monoesterase activity, we have developed a direct selection approach for covalent modification of catalysts founded on mechanism-based phosphatase inhibitors. It builds on the work of Halazy et al.[1] in the use of *o*- and *p*-fluoromethylphenols to generate quinone methides as suicide substrates for glucosidases. Withers and co-workers conceived the use of 4-difluoromethylphenyl phosphate **1** as a suicide substrate for a human prostatic acid



phosphatase and a phosphotyrosine phosphatase,[2] while Myers and Widlansky described the use of 4-monofluoromethylphenyl phosphate as a substrate.[3] This strategy was later adapted by Janda et al.[4] to pan a phage–Fab library of 9×10^3 members against the bovine serum albumin (BSA) conjugate of a reactive substrate **2** to select catalytic antibodies with galactosidase activity.

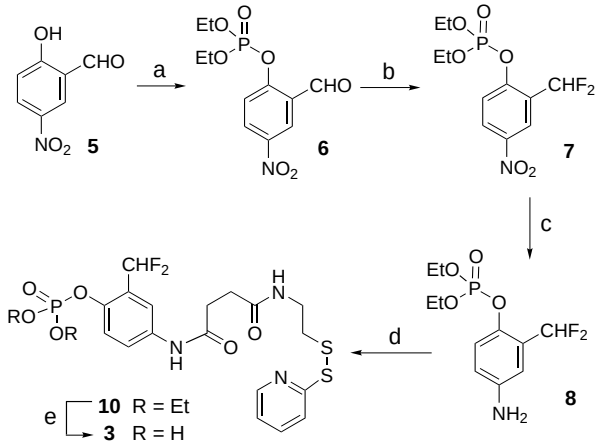
Our design of reagent **3** was developed in line with these examples (Scheme 1). The reactive 2-difluoromethylphenyl phosphate is linked by a variable spacer arm to a pyridyl-2′-disulfide. This enables the suicide substrate to be anchored to



Scheme 1. Mechanism of covalent binding of the suicide substrate **3** by formation of quinone methide **4**. Following phosphate hydrolysis, the protein catalyst becomes covalently attached to a carrier surface.

the surface of a solid support and also subsequent release of the trapped protein species by disulfide reduction with dithiothreitol. Covalent attachment to the protein results from the formation of a reactive quinone methide[5] species **4** by elimination of fluoride after protein-catalyzed cleavage of the phosphate monoester bond. The catalytic protein (antibody or enzyme) then becomes covalently trapped as a consequence of the addition of a nucleophile to the quinone methide at or near the active site.

Substrate **3** was synthesized in six steps from 2-hydroxy-5-nitrobenzaldehyde (Scheme 2).[6] Phosphorylation of phenol **5** led to the quantitative formation of phosphate triester **6**. Fluorination of aldehyde **6**,[7] followed by quenching with ice



Scheme 2. Synthesis of **3**: a) $(EtO)_2POCl$, Et_3N, CH_2Cl_2; b) Et_2NSF_3 (2.1 equiv), 0°C, 25 min; c) H_2, Pd/C (10%); succinic anhydride, iPr_2Et, CH_2Cl_2; d) 2-$PySSCH_2CH_2NH_2$ (**9**), EDC, CH_2Cl_2; e) Me_3SiBr, CH_2Cl_2, then MeOH.

water, and purification by chromatography on silica gel gave **7**. The nitro group of **7** was catalytically hydrogenated without affecting the difluoromethyl group, and the resulting aniline **8** was treated with succinic anhydride. The succinyl hemiamide product was purified by means of silica-gel chromatography with a trace amount of acetic acid in the eluent. The thiol exchange moiety 2-$PySSCH_2CH_2NH_2$ (**9**), prepared from bis-2-aminoethyldisulfide dihydrochloride in two steps,[8] was

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coupled to **8** with EDC (*N*-ethyl-*N*′-dimethylaminopropylcarbodiimide) to give disulfide ester **10**. Finally, selective cleavage of the phosphate ethyl esters of **10** with bromotrimethylsilane[9] gave the suicide substrate **3** ($n=1$).[10] Two higher homologues of **3** were synthesized by using glutaric anhydride and adipic anhydride, respectively.[11]

BSA was modified with Traut's reagent[12] to convert some of the available ε-amino groups of surface lysine residues into thiols. Disulfide exchange with the suicide substrate **3** was undertaken immediately at 4 °C. The dephosphorylation of the BSA–**3** conjugate with alkaline phosphatase was determined by measuring the phosphate release with resorufin (P_iPer Phosphate Assay Kit, Molecular Probes). We found that all coupled substrate molecules were accessible to the enzyme and that the turnover compared well with that of the uncoupled substrate **3**. The suicide reagent **3**–BSA conjugate was immobilized onto the surface of standard Maxisorp ELISA plates by using various coating concentrations (ELISA = enzyme-linked immuno-sorbent assay). Alkaline phosphatase was added for various time periods followed by extensive washing in Tris-buffer pH 9.0 and 0.1 M glycine–HCl pH 2.2. Any enzyme that was covalently trapped through the suicide reaction with **3** was detected by using a murine anti-alkaline phosphatase antibody and an anti-mouse-HRP secondary antibody (Sigma) with TMB substrate (3,3′,5,5′-tetramethylbenzidine). Maximum linkage of alkaline phosphatase to the wells was obtained after incubation for 3 d [13] ($OD_{450}=1.5$ with 10 μg mL^{-1} BSA–**3** conjugate and 10 units of phosphatase per well).

In developing this selection tool, a major concern not fully resolved by earlier work is whether noncatalytic proteins can also be trapped. The results of Janda et al.[4] for an antibody with galactosidase activity show multiple turnovers for the covalently captured protein catalyst. This implies that proteins may be trapped at amino acid residues *not* at the active site, conceivably not even in the same protein molecule. Furthermore, Raushel and co-workers have shown that the loss of catalytic activity resulted from the precipitation of a phosphate triesterase, caused by multiple modifications by a quinone methide species.[14] To explore this possibility with **3**, alkaline phosphatase (10 units/well) was incubated with BSA–**3** for 3 d, and after appropriate washing steps, the *p*-nitrophenyl phosphate (3 mM in 10 mM diethanolamine buffer, pH 9.0, 50 μL/well) was added. The presence of trapped alkaline phosphatase was demonstrated by ELISA as described above.[15] No formation of *p*-nitrophenol was observed, even after prolonged incubation.[16] It follows that the enzyme has indeed been trapped either within the active site or sufficiently close to it, so that the bulky BSA conjugate occludes the active site. These results show that the suicide trapping of alkaline phosphatase by substrate **3** leads to the simultaneous immobilization and inactivation of the enzyme.

The ability of substrate **3** to serve as a turnover-based inhibitor for phosphatases was further investigated by means of BIAcore analysis.[17] Inhibitor **3** was covalently immobilized on a BIAcore chip in a defined orientation by disulfide exchange. Upon injection, binding of alkaline phosphatase to the inhibitor resulted in an increase in the signal (Figure 1, step c). More enzyme was bound at higher concentrations of the inhibitor on the surface. After injecting the alkaline phosphatase, a continuous flow of buffer was initiated. Only a small decrease in signal intensity was observed, which showed that little dissociation of phosphatase from the surface-bound complex had occurred. Most importantly, binding of the major part of the enzyme (>85 %) was not impaired even after extensive treatment with 6 M guanidinium chloride. Clearly, the enzyme was covalently attached to the immobilized inhibitor.

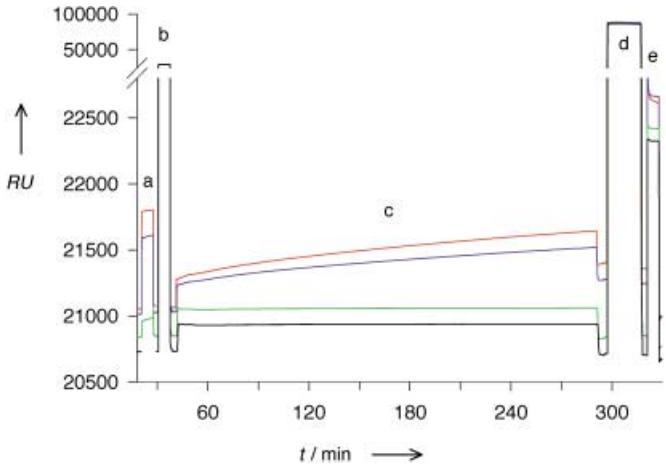

Figure 1. Plot showing turnover-based covalent interaction with enzyme at three concentrations of suicide substrate **3** ($n=2$). The sensorgram shows change of biosensor response units (*RU*) with time. Substrate **3** was immobilized on the thiol-activated chip surface by disulfide exchange (step a) with intensities of 5, 11, and 20 *RU* corresponding (according to its molecular mass) to about 0.1, 0.2, and 0.4 mM surface concentrations on flow cells 2 (green line), 3 (blue line) and 4 (red line), respectively. Flow cell 1 (black line) was an underivatized reference. Excess thiol was deactivated in all flow cells (step b) and alkaline phosphatase (0.1 μM) was injected for 4 h (step c). The enzyme was bound at intensities of 249 *RU* and 336 *RU* in flow cells 3 and 4, respectively. Noncovalently attached protein was finally washed off with guanidinium chloride (6 M)(step d). The remaining signal intensities of 215 *RU* and 295 *RU* correspond to covalently trapped enzyme, which make up 86 % and 88 % of the entire bound phosphatase in flow cells 3 and 4, respectively. Enzyme binding at the lowest inhibitor concentration was very weak (11 *RU*). No binding of alkaline phosphatase to the reference surface in flow cell 1 was observed. The enzyme–inhibitor complex was finally released by disulfide bond cleavage (step e).

The slow but progressive binding of alkaline phosphatase upon prolonged incubation for several hours in ELISA experiments or after prolonged flow of enzyme over the surface with immobilized inhibitor indicates a turnover-based interaction and is inconsistent with the rapid formation of a Michaelis complex of enzyme and substrate. Such binding would not be expected to give kinetically resolvable binding or dissociation traces in a BIAcore experiment, and at the protein concentrations used (small relative to K_M) its plateau height would be extremely small.[18] The proportion of irreversibly bound enzyme was found to be dependent on the duration of the injection of alkaline phosphatase, which is consistent with the time dependence observed on microtitre plates. Unrelated proteins (e.g. BSA) did not activate the mechanistic trap nor did they bind at all to the suicide inhibitor. These results clearly show that inhibitor **3** traps the

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phosphatase efficiently and selectively, thereby displaying the fundamental requirements for turnover-based enzyme inactivation.

We wondered whether a catalyst with low turnover rates, which would be expected to initially arise from selection experiments with a "first-generation" non-enzyme protein library (e.g. catalytic antibodies),[19] can also be identified by means of this screening procedure. As a model for such a case, we investigated a mutant of alkaline phosphatase, S102A, which lacks the primary nucleophile at the active site. Even though the turnover rate for the S102A enzyme is about four orders of magnitude lower than for the wild-type, it is still substantially faster than the uncatalyzed reaction ($k_{cat}/k_{uncat} \cong 10^5$).[20] Briefly, the mutant enzyme was injected in the BIAcore instrument in the same way as the wild-type enzyme to bind to the suicide substrate, and noncovalent bound protein was washed off with guanidinium chloride. More than 30% of the total bound enzyme remained after this step, demonstrating significant covalent coupling. The covalently coupled protein could be removed by reductive cleavage of the disulfide-containing linker that connects the suicide substrate to the surface. Thus, poorly active protein can still be identified by means of this turnover-based screening procedure. This strongly suggests that the rate of covalent inactivation depends on the successful reaction of a second nucleophile with the quinone methide rather than on the primary hydrolysis. However, under the same conditions, less enzyme is trapped than in the case of wildtype enzyme, thus suggesting that the suicide inhibitor may be potentially selective for catalytic efficiency. BSA does not show any covalent binding when used as a control in these reactions, excluding the possibility that the covalent binding might simply be a result of random reactivity of surface nucleophiles on the protein.

The synthesis and successful deployment of **3** is now being applied to the selection of catalysts from large protein libraries. Moreover, BIAcore analysis is demonstrated herein to represent a useful approach for the direct screening of library members and enables real-time analysis of the sequence of steps necessary for catalyst selection from a library of proteins. The relative advantages of *o*-trifluoro, *o*-difluoro, and *o*-monofluoromethylphenyl phosphate suicide substrates[11] are currently under investigation.

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The Inhibiting Influence of Aromatic Solvents on the Activity of Asymmetric Hydrogenations**

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Dedicated to Franz Hein (1892–1976)

Complexes of ruthenium, iridium, and especially rhodium have been used in the homogeneously catalyzed asymmetric hydrogenation of prochiral olefins, ketones, and imines.[1] Hydrogenations are usually carried out in simple alcohols, but aromatic solvents, water, or alcohol/aromatic solvent mixtures can also be used. It has been reported that aromatic solvents such as benzene can inhibit asymmetric hydrogena-

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