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Engineered Surface-Immobilized Enzyme that Retains High Levels of Catalytic Activity in Air

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Supporting Information

ABSTRACT: In the absence of aqueous buffer, most enzymes retain little or no activity; however, "water-free" enzymes would have many diverse applications. Here, we describe the chemically precise immobilization of an enzyme on an engineered surface designed to support catalytic activity in air at ambient humidity. Covalent immobilization of haloalkane dehalogenase on a surface support displaying poly(sorbitol methacrylate) chains resulted in ~40-fold increase in activity over lyophilized enzyme powders for the gas-phase dehalogenation of 1-bromopropane. The activity of the immobilized enzyme in air approaches 25% of the activity obtained in buffer for the immobilized enzyme. Poly(sorbitol methacrylate) appears to enhance activity by replacing protein−water interactions, thereby preserving the protein structure.

There is considerable interest in adapting enzymes to work in water-free environments to allow the remarkable selectivity and efficiency of enzymatic reactions to be exploited in organic solvents, ionic liquids and gas-flow reactions that are widely used in industrial processes.[1] Numerous approaches have been taken to adapting enzymes to work in nonaqueous solvents.[2] These include colyophilizing enzymes with inert excipients such as salts,[3] saccharides,[4] polyols,[4b,5] or substrate-analogs[6] that are thought to stabilize enzyme structure and to conserve the hydration shell around the protein. Other approaches include covalent modification of surface residues with hydrophobic groups, encapsulating enzymes within reverse micelles[2i] and coformulation of enzymes with ion-paired polymer surfactants[7] allowing dispersion in organic solvents while maintaining an aqueous microenvironment around the enzyme.

A further challenge is to develop enzymes that work efficiently in the absence of any solvent. The gas-phase delivery of substrates to "dry" enzymes on solid supports has many advantages;[1b] e.g., in sensing and detoxification of nerve gases or volatile, air-borne pollutants[8] and as green catalysts in industrial gas-phase reactions in flow reactors.[9] However, although the earliest observations of gas-phase enzymatic activity date to 1969,[1a] only a small number of gas-phase enzyme reactions have been investigated in any detail.[1b,10] Studies have focused on the optimization of reactor design and reaction conditions to maximize enzyme activity and stability.[11] In favorable cases, it has even been possible to dissect individual rate constants and thermodynamic parameters for the solid-state reactions.[2g,12] These studies have highlighted the important and opposing roles that humidity (water activity) plays in enzyme activity and stability: whereas a threshold level of humidity is needed to support activity, high humidity reduces enzyme stability.

Interactions between the enzyme and the supporting matrix are also recognized to be important for enzyme activity and stability, but less attention has been paid to optimizing these interactions for solvent-free catalysis. Here, we describe the development of a solvent-free biocatalyst that retains high activity and improved stability in air. This is achieved by site-specifically covalently tethering an enzyme to a surface designed to preserve the protein structure by providing surrogate water−protein interactions.[13]

As a model enzyme, we selected haloalkane dehalogenase (HLD), which catalyzes the hydrolysis of a wide range of alkyl halides and is of interest for the sensing and bioremediation of haloalkane pollutants.[14] The high volatility of its haloalkane substrates makes this enzyme attractive for optimization as a solvent-free biocatalyst.[15] Previous studies using HLD demonstrated that low levels of dehalogenase activity could be obtained from the reaction of vapor-phase haloalkanes substrates with lyophilized enzyme powders.[15] We reasoned that by better tailoring the environment around the enzyme to stabilize the protein's structure in the absence of bulk water we should significantly improve the enzyme activity.

The lyophilization buffer has an important effect on enzyme activity in the dry state, by helping to preserve enzyme structure and maintain the correct protonation state of active site residues.[16] We therefore surveyed a range of organic buffers representing structurally diverse compounds including TAPS, Bicine, CAPS and AMPSO (Na^+ as counterion) and Tris and Lysine (Cl^- as counterion).[17] First, HLD (LinB gene product from *Sphingobium japonicum* UT26),[18] 0.2 mg/mL, was lyophilized from 20 mM buffer adjusted to pH 9.0, the optimal pH for dehalogenase activity.[15] Then the dehalogenase activity of the lyophilized enzyme powders was assayed in a sealed glass reaction vessel at 37 °C at 50% relative humidity (RH) using 1-bromopropane vapor as the substrate. The formation of 1-propanol was quantified by GC−MS analysis of the reaction headspace. The dry-state activity of HLD varied quite widely depending on the lyophilization buffer (Figure S1), with Tris

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buffer providing the highest turnover number of 0.04 s^{-1} under the conditions of the experiment.

Previous studies in our lab have demonstrated that the location of the tether impacts both the activity and the stability of the enzyme.[19] Therefore, on the basis of the crystal structure (PDB: 1D07), we engineered variants of HLD that introduced cysteine residues at three surface-exposed loops: A141C, A196C and N262C (Figure 1). The surface cysteine-containing HLD

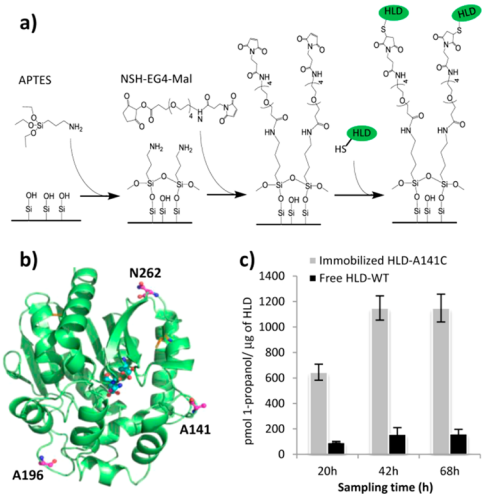

Figure 1. (a) Scheme for the preparation of EG_4-maleimide-terminated SAMs and covalent immobilization of HLD. (b) Residues selected as covalent attachment points were mutated to cysteine. (c) Comparison of the activity of lyophilized wild-type HLD with the dry-state activity of surface-immobilized HLD-A141C.

variants were covalently tethered through the engineered cysteinyl residues to glass surfaces functionalized with an $(EG)_4$-maleimide-terminated SAM (Figure 1a). The activity of HLD in aqueous buffer was unaltered by the introduction of these mutations (Figure S2).

The orientation of the immobilized enzymes with respect to the surface was examined by surface-sensitive sum frequency generation (SFG) and attenuated total reflection FTIR (ATR-FTIR) spectroscopy, as described previously (for details see SI).[19b] Each of the surface-tethered HLD variants exhibited well-defined amide-I bands in the ppp- and ssp-polarized SFG spectra, and in the s- and p-polarized ATR-FTIR spectra (Figure S4). Fitting of these spectra generated heat maps describing the distribution of protein orientations in terms of the tilt angle (θ) and twist angle (ψ) (Figure S5). In each case, the surface orientation of the tethered HLD variants deduced from the heat maps was consistent with the intended tethering point (Figure S6).

All the tethered HLD variants exhibited some reduction in specific activity relative to that in bulk solution (Figure S8), a phenomenon we have observed with other enzymes.[19b] HLD-A141C was the most active variant, with specific activity 42% of the enzyme free in solution, and was selected for further characterization. No loss of activity was observed upon prolonged storage in buffer at 4 °C.

The dry-state activity of surface-tethered HLD-A141C was measured on functionalized beads washed in 20 mM Tris/Cl buffer, pH 9.0, and then lyophilized. The beads were then introduced into a sealed reaction chamber and the dehalogenase activity assayed as described above. Surprisingly, the activity of the surface-immobilized enzyme, Figure 1c, was found to be ~7-fold higher (turnover number = 0.31 s^{-1}; Table 1) than that of lyophilized enzyme powders under the same conditions. The

Table 1. Comparison of HLD Activity with 1-Bromopropane as Substrate at 37 °C under Various Conditions[a]

	turnover number (s^{-1})	relative activity (%)
free HLD (aqueous phase)	18.5	100
immobilized HLD (aqueous phase)	7.7	42
lyophilized HLD (vapor phase)	0.044	0.24
immobilized HLD (vapor phase)	0.31	1.7
immobilized HLD+PSMA (vapor phase)	1.73	9.4

[a]For details, see main text and SI.

increased activity of the surface-tethered SAM may be ascribed to both the hydrophilicity of the EG_4-SAM and to the surface ordering of the enzyme that arises from the chemically precise tethering of the protein to the SAM. The freeze-dried HLD-functionalized beads were quite stable; no loss of activity was apparent after 1 week stored under dry nitrogen.

The HLD-catalyzed formation of 1-propanol increased linearly for ~40 h, after which little further dehalogenation of 1-bromopropane was observed (Figure 1c). The reaction likely stops due to the decrease in pH caused by the HBr produced in the reaction, although product inhibition by 1-propanol may also contribute to inhibition. Consistent with this hypothesis, a significant fraction of the enzyme activity could be regenerated by removing the beads from the reaction chamber and relyophilizing them, which presumably removes the inhibitory product molecules.

Sugars and polysaccharides have been shown to be effective excipients for increasing the activity of lyophilized enzyme preparations.[4] Therefore, we reasoned that a polymer such as poly(sorbitol methacrylate) (PSMA) might further increase the activity of immobilized HLD in the dry state by providing stabilizing hydrogen-bonding interactions. Sulfhydryl-terminated PSMA of M_r ~ 200 kDa was prepared using the ATRP method (Figure 2a)[20] and coimmobilized with HLD-A141C on EG_4-maleimide-terminated SAM-functionalized beads at molar ratios ranging from 0.0–0.5:1 PSMA:HLD (Figure 2b). After washing in Tris buffer and lyophilization, the specific activity of the HLD-A141C/PSMA functionalized beads was determined. Coimmobilization with PSMA resulted in up to a further 5.6-fold increase in the dry-state activity of HLD at the highest PSMA concentrations used (Figure 2c; Table 1); in contrast, in aqueous buffer the activity of HLD was unaffected by coimmobilization with PSMA.

PSMA also has a pronounced effect on the stability of the enzyme. After two rounds of catalysis and regeneration, HLD immobilized on SAMs lacking PSMA retained only ~12% of its initial activity. However, enzyme coimmobilized with 50 mol % PSMA retained ~31% of its initial activity at 37 °C (Figure 2c).

For comparison, we also examined the effect of physically coating the HLD-derivatized beads with sorbitol by adding increasing concentrations of sorbitol to the buffer prior to lyophilization (Figure S9). However, these experiments revealed no significant increase in enzyme activity due to the physically adsorbed sorbitol monomers. Interestingly, physical adsorption of sucrose, which was reported as a promising additive to enhance dry-state activity,[4c] resulted in only a ~1.5-fold increase in gas-phase dehalogenation rates by immobilized HLD (Figure S9).

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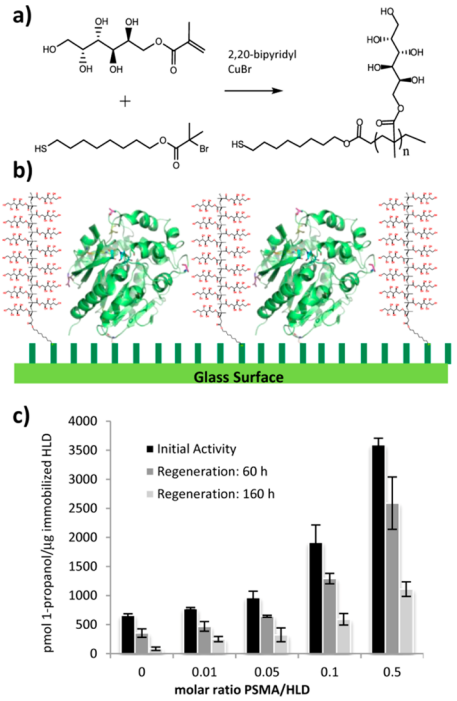

Figure 2. (a) Scheme for the synthesis of thiol-terminated PSMA. (b) Schematic representation of coimmobilized HLD-A141C and PSMA on EG_4-maleimide-terminated SAMs. (c) Dry-state activity of coimmobilized PMSA/HLD-A141C as a function of their molar ratio (all data collected after 20 h reaction at 37 °C).

To investigate the effect of PMSA on the structure of HLD in the dry state, we recorded CD and SFG spectra of surface immobilized HLD-A141C after drying. In the absence of PSMA, the intensity of the CD spectrum is dependent on the relative humidity of the air. In contrast, enzyme coimmobilized with PSMA exhibits a strong CD spectrum that is largely independent of relative humidity (Figure 3a,b). The SFG spectrum of immobilized HLD acquired in air only exhibits the amide I peak (1650 cm^{-1})[21] in the presence of PSMA (Figure 3c). These

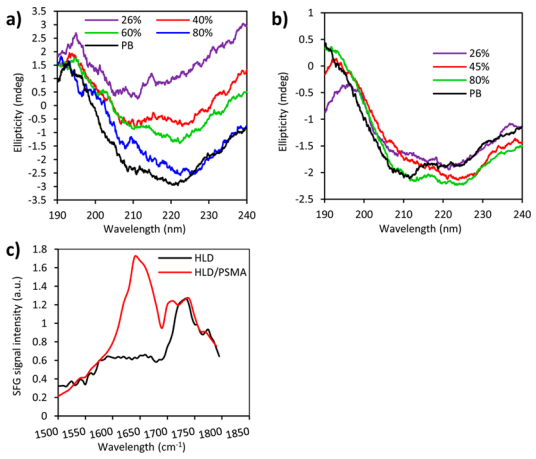

Figure 3. Effect of relative humidity (RH) on the secondary structure of surface-immobilized HLD-A141C, determined by CD spectroscopy. (a) Spectra obtained in the absence of PSMA. (b) Spectra obtained in the presence of PSMA. (PB: spectrum in phosphate buffer). (c) SFG spectra of immobilized HLD and HLD coimmobilized with PSMA, both recorded at 40% RH.

results indicate that in the absence of bulk water, the PSMA replaces the stabilizing interactions normally provided by the solvent to preserve the secondary structure of the protein.

In further experiments, we examined whether PSMA stabilizes the structure of HLD by recruiting water molecules to the surface or directly interacts with the protein. We first used ellipsometry to estimate the surface coverage of HLD and PSMA, and then used a quartz crystal microbalance with dissipation monitoring (QCM-D) to measure the amount of water recruited to surfaces.

From ellipsometric thickness measurements (Figure 4a), we estimate a surface coverage of ~3 HLD molecules and ~1 PSMA

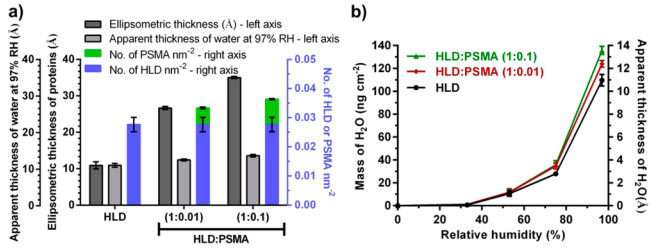

Figure 4. (a) Ellipsometric measurement of the surface thickness of HLD immobilized with and without PSMA, together with the apparent thickness of adsorbed water molecules at 97% RH. (b) Mass of water adsorbed onto immobilized HLD/PSMA surfaces as a function of RH determined by QCM-D measurements.

molecule per 1000 nm^2 when HLD and PSMA were immobilized in a 1:0.1 ratio. These estimates assume M_r values for HLD and PMSA of 34 and 200 kDa, respectively, and that presence of PSMA does not decrease the surface coverage of HLD significantly (see Figure S10 for more details).

QCM-D measurements made as a function of RH (Figure 4b and S11) demonstrate that the adsorption of water to the surfaces is little affected by PSMA. Indeed, relatively little water is recruited to either surface until RH exceeds ~75%, i.e., significantly higher RH than the activity measurements were made. These results were further substantiated by thermogravimetric analysis of HLD-functionalized beads (Figure S12), which similarly showed little effect of PSMA on water adsorption. At 50% RH, the estimated number of water molecules per enzyme molecule ranges between ~140 (by QCM-D) and ~290 (by thermogravimetric analysis) (Table S8). Notably, this is far less than the ~1400 molecules needed to solvate the enzyme with a monolayer of water or estimates for the minimum water activity necessary for enzyme activity.[22]

These results indicate that PSMA does not simply trap water but actually replaces protein−water interactions in the dry state. This observation is consistent with previous studies[23] that have ascribed the stabilization of protein structure in the solid state to the ability incipient sugar molecules to replace protein−water interactions. PMSA may play a further role as a "molecular lubricant" by preserving protein dynamics essential to catalytic activity, as has been suggested previously.[24]

In conclusion, we have shown it is possible to increase by some 40-fold the low level of dry-state activity exhibited by lyophilized HLD. The specific activity of the surface-immobilized HLD in the presence of PSMA in the dry state is ~10% and 25% of the activity of the free enzyme and immobilized enzyme in solution, respectively (Table 1). Although it is difficult to make direct comparisons between systems due to differences in how enzyme activity is measured and reported, the activity of immobilized HLD on the PSMA-modified SAM surfaces appears to be among

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the highest levels of enzyme activity achieved in a nonaqueous environment.[2g]

Significantly, PSMA also increases the stability of the enzyme: ~3-fold more activity remains after 3 rounds of reaction for HLD coimmobilized with (50 mol %) PSMA compared to HLD immobilized in its absence. Coimmobilization with PSMA may therefore provide a solution to the well-documented problem that increasing enzyme activity by raising humidity (water activity) conversely also decreases enzyme stability.[10a−c,11a] The enzymes retain activity over long periods of time so that total turnover numbers of 10^5 (mol substrate per mol enzyme) or higher can be obtained. Based on work with other enzymes,[1b,10c,11h] the total turnover number could most likely be significantly increased if reactions are run in a gas-flow apparatus, which would allow better control of substrate delivery and product removal.

We expect that the approach we have described here to reformulate enzymes to work in "water-free" environments should be generally applicable to other enzymes. Furthermore, the approach may be adaptable to different support surfaces to provide high-activity enzymes that work in organic solvents or ionic liquids.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b12174.

Experimental details (PDF)

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Notes

The authors declare no competing financial interest.

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