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Determination of concentration and activity of immobilized enzymes

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Abstract

Methods that directly measure the concentration of surface-immobilized biomolecules are scarce. More commonly the concentration of the soluble molecule is measured before and after immobilization, and the bound concentration is assessed by elimination, assuming that all bound molecules are active. An assay was developed for measuring the active-sites concentration, activity, and thereby the catalytic turnover rate (k_{cat}) of an immobilized dihydrofolate reductase as a model system. The new method yielded a similar first order rate constant, k_{cat} , to that of the same enzyme in solution. The findings indicate that the activity of the immobilized enzyme, when separated from the surface by the DNA spacers, has not been altered. Additionally, a new immobilization method is described that leads to solution-like activity of the enzyme on the surface. The approaches developed here for immobilization and for determining the concentration of an immobilized enzyme is general and can be extended to other enzymes, receptors, and antibodies.

Keywords

Immobilization; activity assay; radiolabeled inhibitor; dihydrofolate reductase

The analytical determination of the concentration and kinetic properties of immobilized enzymes are of great contemporary interest in biotechnology and biochemical research. Enzymes are nature's sustainable catalysts and their use in the field of biotechnology is of both industrial and academic contemporary interest. These biological catalysts exhibit a remarkable rate enhancement and are capable of speeding the chemical reactions up to 20 orders of magnitude with respect to the corresponding reaction in solution [1]. Moreover, enzymes are biocompatible, biodegradable and are derived from renewable resources which makes their use in industry more environmentally friendly, cost-effective and sustainable as compared to conventional techniques [2]. Since they also provide excellent selectivity for their target substrate and have high catalytic activity, enzymes are increasingly being used in diverse industries and hence are an important topic of research [3].

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There is currently a great demand to enhance enzyme productivity and develop novel techniques to improve their shelf life [4]. Enzyme immobilization is an approach that provides excellent platform for increasing the durability of enzymes for a variety of industrial applications. Additionally, the immobilization of these catalysts enables their use in flow-based bioreactors where the catalyst can be maintained in the reactor via mechanical or magnetic attachment to the reaction chamber (e.g., fixed catalytic beads or bio-coated magnetic beads) [5]. Moreover, several methods for basic research require immobilized enzymes (e.g., single-molecule or mono-layer studies) [5–7]. Broadly, methods of enzyme immobilization can be divided into three categories: binding to a support, entrapment, or encapsulation and cross linking [3, 4]. The method used in the current study for immobilization involves binding the enzyme to a mica chip support through dsDNA spacers. An ideal immobilization should yield a stable biocatalyst while minimizing distortions in its structure and function. However, in many cases, immobilization results in a decreased enzyme activity, and only a few examples where the activity is unaltered or even increased [8]. Importantly, in all cases it is critical to check enzyme activity after immobilization, and determine the effective concentration of active sites available after immobilization. One of the major problems faced in studying the activity of immobilized enzymes arises from the lack of accurate assays using conventional spectroscopic methods due to the presence of solid support. Another common problem is calculating active site concentrations, since the enzyme is immobilized on a solid surface and therefore inaccessible to conventional biochemical techniques. Traditionally, the estimation of immobilized enzyme on the support is calculated from the enzyme content in the solution before and after immobilization [9–11]. But because this is an indirect method, it is not likely to give the actual active site concentration. Consequently, there is a need of an assay that can directly measure concentration of the immobilized enzyme, or, ideally, the number of available active sites.

In the present study we have developed an assay to measure the concentration of active site, activity, and thereby the specific activity of an immobilized enzyme on a solid support at a sub-monolayer concentration level. We have used dihydrofolate reductase (DHFR) from *Escherichia coli* as a model enzyme system because of its small size, lack of metal cofactors or disulfide bonds, and its medicinal importance. DHFR catalyzes the transfer of the pro-R hydride from the C4 position of reduced nicotinamide adenine dinucleotide phosphate (NADPH) to the *si*-face of 5,6-dihydrofolate (DHF), forming S-5,6,7,8,-tetrahydrofolate (THF) and NADP⁺ as shown in Fig. 1a. Since this enzyme is critical in nucleotide biosynthesis, it is a target for many chemotherapeutic and antibacterial drugs [12–14].

DHFR immobilization

DHFR from *E. coli* was expressed, purified, and stored as described elsewhere [15, 16] and was immobilized on a mica chip surface using labeled dsDNA spacers (see sequence and more details in the enclosed Supplementary Information, SI). The 5' end of one strand of DNA was labeled with a thiol while the 5' end of the complementary strand ends with an amino group. The amino end of the dsDNA was attached to an activated mica chip surface [17, 18], while the thiol end was attached to a surface cysteine (remote from the enzyme's active site, C152) via a disulfide bond. Fig. 2 shows a schematic cartoon of the immobilized DHFR on mica chip with dsDNA spacers. The functionalized DNA can be stored for long

period of time, and it is relatively simple to chemically modify or functionalize as needed. Such spacers enable a variety of applications including the recovery and reconfiguration of biosensor surfaces and fabrication of arrays in generation of biochips [19].

Activity assay

Conventional techniques to measure enzyme activity are not accessible for the immobilized enzyme on solid surface. We therefore used a radiometric-based assay. Since DHFR is NADPH dependent enzyme, NADPH labelled with ^{14}C on the adenine ring ([Ad- ^{14}C]-NADPH) was used to follow ^{14}C -NADP $^{+}$ (product) formation as a function of time using HPLC and liquid scintillation analysis. The synthesis of [Ad- ^{14}C]-NADPH and HPLC separation methods have previously been described [20, 21]. The reaction was carried out at 25 °C with 100 μM [Ad- ^{14}C]-NADPH, 100 μM DHF in MTEN buffer (50 mM MES, 25 mM Tris, 25 mM ethanolamine, and 100 mM sodium chloride, pH 7.5) and initiated by adding the mica chip with the immobilized enzyme. Aliquots were removed from the reaction mixture at different time points to measure fraction conversions ranging from 1–30% [7, 22]. Fig. 1c. shows a typical RP-HPLC radiogram illustrating the separation of product ([Ad- ^{14}C]-NADP $^{+}$) and reactant ([Ad- ^{14}C]-NADPH). The fraction conversion (f) of was determined from Fig. 1c. using equation 1, which is the ratio of ^{14}C in products to the total ^{14}C [22, 23]:

$$f = \frac{[\text{Ad-}^{14}\text{C}]\text{NADP}^{+}}{[\text{Ad-}^{14}\text{C}]\text{NADP}^{+} + [\text{Ad-}^{14}\text{C}]\text{NADPH}} \quad (1)$$

The V_{max} (product per second under saturating substrates concentrations) was determined by a linear fit of at least three time points. To assure saturation of both substrates the rate was measured at different concentrations of NADPH and DHF ranging from 100 μM to 1 mM. The fact that the rate is independent of substrate concentration indicates that the mass flow of substrate does not affect the measured rate. Fig. 1d. shows a representative time course. The V_{max} value by itself could be characteristic of the chip if reported per surface area or per weight. However, such value is not a rate constant and cannot be used to compare the properties of the immobilized enzyme to the same enzyme in solution. Dividing V_{max} with the enzyme concentration (see assay below) gives the first order rate constant k_{cat} . The value found in this case was $6.7 \pm 0.6 \text{ s}^{-1}$, which is in good agreement with the reported value of 8–12 s^{-1} for the same enzyme in solution [14].

Determination of active sites concentration

The activity measured above can be used as units per chip surface or per particle weight. However, activity value without knowledge of active site concentration is not a measure of a rate constant or otherwise characteristic of the system. Hence, it is critical to measure the concentration of active sites so the measured activity can be converted to a rate constant or a meaningful specific activity value. The distribution of immobilized enzyme on mica chip is random and might consist of regions with bundles and a non-uniform distribution, which can be seen from the atomic force microscopy (see more details about imaging in the enclosed Supplementary Information, SI) image in Fig. 1b. Consequently, to the best of our

knowledge there is no good method reported in the literature to estimate the active site concentration of surface immobilized enzyme under such low number density of enzymes. Since the enzyme distribution on the chip is not uniform, imaging a section of the enzyme immobilized surface using atomic force microscopy and then calculating the number for the whole chip may not give accurate results. Furthermore, it is practically not possible to scan the whole chip to determine the number of bound enzyme molecules due to the time required for data collection and analysis. A new method that can accurately measure the active site concentration of surface immobilized enzyme was developed by utilizing radiolabeled methotrexate, a picomolar inhibitor of DHFR to determine the concentration of immobilized enzyme. After measuring the activity of the immobilized enzyme (see above), the same chip was used to measure the enzyme concentration. The chip was washed with MTEN buffer and then incubated in 0.5 mM tritium labeled methotrexate disodium salt, [3', 5', 7-³H(N)], 10 Ci/mmol (Moravek Biochemicals, Inc.), for 30 minutes. The chip was washed again several times with MTEN buffer, until no radioactivity was found in the washing liquid, to ensure that there was no unbound methotrexate remaining. A bare mica chip and the chip functionalized with DNA, but lacking DHFR was treated in the same manner and used as a control. The results showed that methotrexate does not bind either to the bare chip or to the functionalized chip lacking an enzyme. Radioactivity was measured using a liquid scintillation counter, and the specific radioactivity (10 Ci/mmol) was used to calculate the number of inhibitor bound to DHFR, which in turn gave moles of the immobilized enzyme, or more specifically its available active sites. Dividing this number (in moles) by the volume of the reaction mixture used for the activity measurements (above) yield the concentration of the enzyme, and was used to calculate the rate constant. DHFR concentration to rate relation (calibration curve) was linear as can be seen in Fig. S1.

Summary

Enzyme immobilization on a solid surface is a valuable practice to preserve catalytic activity and for a wide range of biotechnological applications (e.g., bioreactors, nanosensors, single-molecule studies, etc.). Here, we developed a novel technique to measure the concentration and activity of DHFR immobilized on a solid surface at sub-monolayer coverage. The concentration of immobilized enzyme was measured using a radiolabelled inhibitor. The k_{cat} was then calculated from the measured activity and active sites concentration, and was similar to the rate constant for the enzyme in solution. In this specific case, the finding suggests that the immobilization method used (rigid dsDNA spacer) has not modified the catalytic turnover rate (k_{cat}) of the enzyme. Most enzymes have tight-binding inhibitors that can be labeled radioactively, and thus the approach used here can be similarly extended to many other immobilized enzymes. Because of the importance of surface immobilized enzymes in multiple fields, the approach has a great potential of assisting in the analytical determination of active site concentration of a wide variety of immobilized enzymes on solid surfaces or nanoparticles.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

a) DHFR catalyzes the stereospecific transfer of hydride from C4 of NADPH to C6 of DHF, producing the product THF and oxidized cofactor NADP⁺. **b)** Atomic force microscopy image of immobilized DHFR on mica chip with dsDNA spacers **c)** Radiogram showing RP-HPLC separation of products ([Ad-¹⁴C]-NADP⁺) and reactants ([Ad-¹⁴C]-NADPH) from the kinetic assay of immobilized DHFR activity. The green line is the starting material (*i.e.* reaction mixture at 0 min); the blue line is the reaction mixture at 15 min; and the red line is the reaction mixture at 80 min; **d)** Fraction conversion versus time plot.

Fig. 2. Schematic illustration of the immobilized DHFR on mica chip with dsDNA spacers. The surface Cys residue of DHFR was attached to thiol-labeled dsDNA bound to a mica surface via a terminal amino end.