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## Use of Enzymes Deactivated by Site-Directed Mutagenesis for the Preparation of Enantioselective Membranes**

Alexander Skolaut and János Rétey*

Many biologically active compounds, among them drugs, have chiral structures, of which only one stereoisomer has the desired activity. Chemical synthesis of such compounds often leads to racemic mixtures, whose resolution may be a tedious and expensive procedure. Enantioselective membranes can facilitate the transport of one enantiomer of a racemate, thus constituting a simple and fast resolution method. Lakshmi and Martin incorporated enzymes into porous polymeric membranes and prevented the undesired catalytic turnover of the substrates by depriving the enzymes of their cofactors.[1] Many enzymes do not use cofactors and thus a more general method would involve destroying the catalytic activity of the enzymes by site-specific mutagenesis, while maintaining their property to bind their substrates enantioselectively, that is, the enzymes are converted into receptors.

The enzymes histidine ammonia lyase (HAL) and phenylalanine ammonia lyase (PAL) seemed to us to be two excellent candidates for such an endeavor. Both enzymes have a catalytically essential electrophilic group, which for almost 30 years was believed to be dehydroalanine. The recently determined X-ray crystal structure of HAL revealed that the electrophilic prosthetic group is not dehydroalanine, but rather methylidene imidazolone (MIO).[2] Although there is currently no X-ray crystal structure of PAL, its MIO group has been identified by UV-difference spectroscopy.[3] We prepared a number of mutants of HAL and PAL that lack the MIO group or other catalytically essential amino acid residues.[3–6] Most of the mutants maintained their binding affinity for their substrates, but lost almost all catalytic activity. The two mutants PAL Y109F and HAL E414A were selected for incorporation into an artificial membrane.

To accelerate the transport through the membrane, the carrier molecule must be able to move freely in the whole pore volume. This movement is possible by physical entrapment between polymer films that serve as semipermeable barriers. Small molecules such as solvent or substrate molecules can diffuse through the polymer, but large molecules such as the carrier can not. The polymer film can be prepared, for example, by redox polymerization of pyrrole or its derivatives, or by polycondensation of dialkyl dichlorosilanes on the surface of a mesoporous membrane.

The quality of the synthetic polymer film can be best monitored by electron microscopy.[7] By using this method, we found that only poly(dimethylsiloxane) polymer films that were synthesized on the surface of an alumina membrane (Anodisc membrane, Figure 1a) could be obtained in a

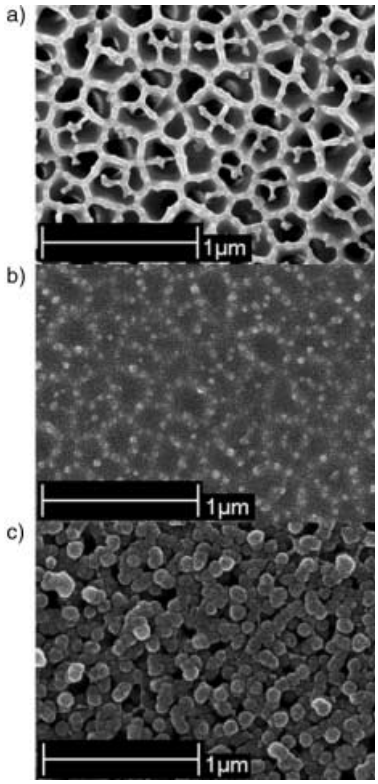


Figure 1. Electron microscope images: a) the uncoated surface of an alumina membrane (Whatman Anodisc); b) a poly(dimethylsiloxane) polymer film synthesized on the membrane surface; c) platelets of poly(pyrrole) on the surface of an alumina membrane.

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reproducibly good quality (Figure 1b). All other polymers or polymer/membrane combinations did not give polymer films; instead, only small polymer platelets were obtained (Figure 1c).

The immobilization of the enzymes was carried out as follows: a small volume ($<100\,\mu L$) of the concentrated enzyme solution ($50\,mg\,mL^{-1}$) was applied to the uncoated side of the membrane. After the enzyme solution had entered the membrane pores it was physically immobilized with a further single-coated membrane, thus forming a membrane sandwich (Figure 2a). Varying amounts of the protein (up to 10 mg) can be immobilized under very mild conditions by this procedure, thereby preventing denaturation and thus inactivation.

The transport experiments were carried out at optimal pH values and temperatures of the wildtype enzyme (Table 1). The membrane sandwich was mounted between the two halves of the test cell (Figure 2b). One side of the membrane was in contact with a solution of the racemic substrate (feed side), the other with a substrate-free buffer solution (strip side). Both chambers were stirred with a magnetic stirring bar at 250 rpm. After certain intervals, samples were withdrawn from the chambers and analyzed by reverse-phase and chiral-phase HPLC. The ratio of the transported L and D enantiomers was termed the selectivity coefficient (*SF*).

The first transport experiments were performed with the immobilized PAL mutant Y109F, which was about 75000 times less active than the wildtype.[6] Figure 3a shows both the concentration dependence and time dependence of the selectivity. At low concentrations (0.1 mM) L-phenylalanine is transported faster and the selectivity coefficient reaches a maximum (2.5) at 30 min. Thereafter the selectivity decreases, which can be explained by the assumption that the facilitated transport takes place in both directions. It is known that PAL also accepts D-phenylalanine as substrate, albeit with lower affinity and turnover number. Simultaneously and independ-

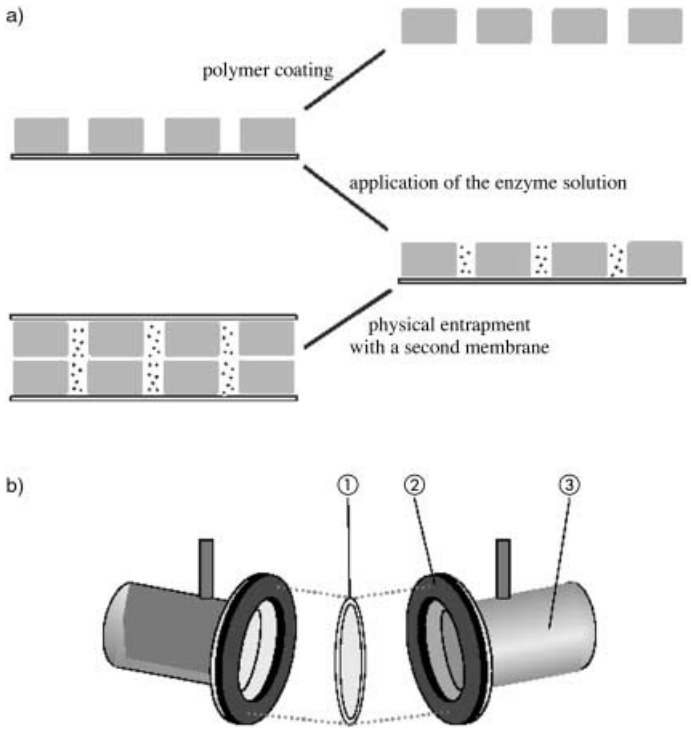


Figure 2. a) Principle of the membrane coating and immobilization process of an alumina membrane with poly(dimethylsiloxane); b) scheme of the test cell (1 = membrane, 2 = seal, 3 = chamber).

Table 1. Characteristic data of the wildtype enzyme and the inactive mutants.

| Enzyme | $K_m$ [mM] | $V_{max}$ [$U\,mg^{-1}$] | Optimum pH | Optimum $t$ [°C] |
|---|---|---|---|---|
| wildtype PAL | 0.12 | 2.6 | 8.8 | 30 |
| PAL Y109F[a] | n.d.[b] | 0.00035 | 8.8 | 30 |
| wildtype HAL | 3.9 | 24.3 | 9.3 | 25 |
| HAL E414A | 6.1 | 0.00024 | 9.3 | 25 |

[a] Y109F means the amino acid at position 109 was changed from tyrosine (Y in one letter code) to phenylalanine (F). [b] not detectable.

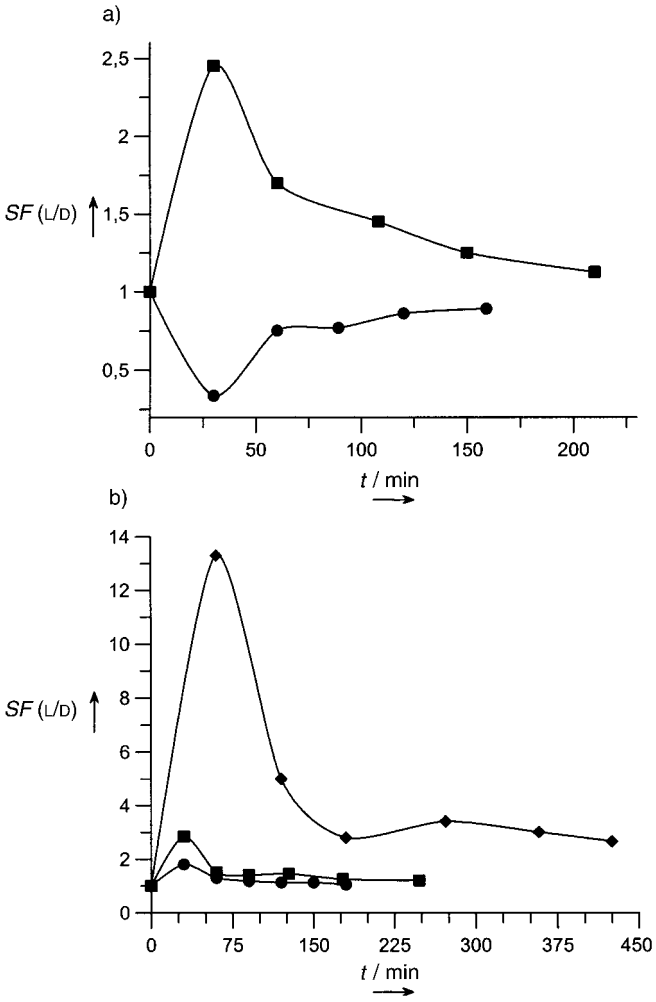


Figure 3. Time dependence of the selectivity coefficient in transport experiments: a) Anodisc membrane, 20-nm pore diameter, silane coating; PAL-Y109F at different feed concentrations of D,L-phenylalanine and protein loadings ([●] = D,L-phenylalanine (2.5 mM), protein (2.2 mg); [■] = D,L-phenylalanine (0.1 mM), protein (3.5 mg)); b) Anodisc membrane, 20-nm pore diameter, silane-coating; HAL E414A at different feed concentrations of D,L-histidine with 5-mg protein loading ([●] = D,L-histidine (2.5 mM); [■] = D,L-histidine (1.0 mM); [◆] = D,L-histidine (0.1 mM)). All substrate concentrations are those at the start of the experiments. During the first hour, 3% of the amino acids were transported to the strip side, at the end of the experiments about 15% of the amino acids had been transported.

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ently of the carrier, a slower nonselective diffusion transport also takes place. Surprisingly, at a much higher substrate concentration (2.5 mM) in the feed, D-phenylalanine is transported faster. This unexpected result can be explained by the fact that at this concentration the D enantiomer successfully competes for the binding site of PAL and occupies it for a much longer period of time, so that little free enzyme remains available for transporting the L enantiomer. The selectivity coefficient in this case also decreases after 30 min and approaches equilibrium asymptotically.

The immobilized HAL mutant E414A behaved differently. HAL is known to be strictly enantioselective for L-histidine.[8] Indeed, its mutant facilitated the transport of the L enantiomer at all three concentrations applied (Figure 3b). However, the selectivity coefficient in this case also reached the highest value (13.3) at the lowest concentration (0.1 mM). After 70 min the selectivity decreased, clearly for the same reason discussed for the PAL experiment. At high concentrations in the feed side, the maximum was much lower (2–3) and was reached faster (30 min.). As a control, the same membrane system without encapsulated enzyme has been also examined for its capacity to transport phenylalanine enantiospecifically. Analysis on a chiral column (see Experimental Section) showed that both enantiomers diffused through the membrane at the same rate.

Our results show that enzymes can be converted into enantioselective receptors by site-directed mutagenesis and used in an immobilized form to facilitate enantioselective transport across a membrane. The technique described herein differs from previously published methods in which enantioselective membranes that contain nonselective proteins, for example, serum albumin or other chiral materials.[9–11] Such membranes as well as chiral chromatography columns work by binding and retarding the transport of one enantiomer, while the other enantiomer can passively diffuse or can be eluted without retardation. In our method, the transport of one enantiomer is accelerated while the other diffuses through the membrane, but much more slowly. Continuous removal of the enriched enantiomer from the permeate (strip) side could lead to resolution on a preparative scale. Whether the present method may become competitive with other established racemate separations or not, is the subject of future research.

It is the first time that not only the concentration dependence but also the time course of the enantioselectivity of the transport through an enzyme-loaded membrane are reported. A further aspect of our results is that as a consequence of the broad substrate specificity of PAL, the present membranes have the potential to resolve the racemates of a large number of aryl alanines.[12]

*Experimental Section*

Membrane coating: The membrane was first equilibrated for 5 min with the vapor of dichlorodimethylsilane, and water was then applied to one side of the membrane for an additional 5 min. The coated membranes were washed for 1 min with water and dried at room temperature.

Analytical methods: the HPLC separations were run on a Hewlett Packard Series 1050 HPLC with the following columns and under the following conditions: D,L-phenylalanine: Grom Symbasic 125 × 4.6 mm equipped with a 10 × 2.5-mm guard column, 5 μm, isocratic elution with doubly distilled water with TFA (0.1 vol%); D,L-histidine: Grom Sil ODS-O AB, 250 × 4.0 mm, 5 μm, isocratic elution with $CH_3CN$ (20%) and potassium phosphate buffer (pH 8.0, 10 mM, 80%); Astec Chirobiotic T, 250 × 2.5 mm with a 10 × 2.5-mm guard column for chiral analysis.

Conditions according to the Chirobiotic handbook (Advanced Separation Technologies, Inc.).

All chromatograms were monitored at 210 nm.

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## Copper-Catalyzed Oxidative Heterocyclization by Atmospheric Oxygen

Martin E. Bluhm, Michael Ciesielski, Helmar Görls, and Manfred Döring*

Although molecular oxygen is the most low-priced of all known oxidants, it is applied only rarely in synthetic chemistry. This is because the $O_2$ molecule has a low reactivity at moderate temperatures due to kinetic barriers, and because of its commonly poor selectivity in the oxidation of organic substrates. However, $O_2$ can be activated in living organisms by catalytically active metalloenzymes, showing that many selective oxidations are feasible even under very mild conditions. Therefore, metal complexes possessing similarities

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