Selective Biocatalysis in Bacteria Controlled by Active Membrane Transport

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Membrane reactors are attractive when retention of biocatalysts is desirable and when chemical conversion or selectivity can be enhanced by selective permeability of reactants. Unlike polymer or inorganic membranes, biological membranes have transport proteins that use cellular energy to selectively pump components against concentration gradients. This paper analyzes the transport of polynuclear aromatic hydrocarbons (PAHs) across the cell membranes of Pseudomonas fluorescens, an organism that oxidizes aromatic compounds. Experimental data for PAHs were consistent with a model for uptake and energy-dependent transport controlled by the permeability of the outer cell membrane permeability. A model for the enzymatic reaction of mixtures of PAHs showed that energy-dependent transport out of the cell increases the selectivity for the reaction of the less permeable component. Consequently, selective transport may be a useful tool in engineering the metabolism of microorganisms, especially for less hydrophobic compounds with lower permeation rates across biological membranes.

Introduction

Membranes have been frequently used in bioreactors to retain cells, as in hollow-fiber bioreactors, or to retain enzymes, as in ultrafiltration membrane bioreactors. Strategies based on membrane separation have also been used to overcome the low solubility of substrates for reaction and to control inhibition of enzyme byproducts (for example, Liese et al.2,3). Inorganic membrane reactors have attracted significant interest because of their potential to enhance the reactor performance at higher temperatures, either by increasing the yield from thermodynamically controlled reactions by selectively removing a product or by controlling undesirable secondary reactions by limiting the local concentrations of reactants. The basic concepts of membrane reactors based on inorganic membranes are provided by reviews of the subject.4–8 In all of these cases, the ceramic, metal, or polymer membranes were passive barriers to transport. Flux of each component across the membrane depended on the concentration or pressure gradient across the membrane and the permeability of the membrane. In living cells, however, transport of solutes across the cell membrane can be assisted by “pump” proteins that use metabolic energy to transport components against the local concentration gradient.1

Considerable research has recently focused on efflux pumps that remove components from the cell interior and export them to the exterior of the cell using the proton gradient across the cell membrane. One of the mechanisms for antibiotic resistance in bacteria is efflux of antibiotics from the cell interior to the surrounding medium, driven by energy-dependent protein pumps.9,10 Related proteins can efflux hydrophobic organic solvents, such as toluene, from the interior of bacterial cells.11 Energy-dependent transport of solvents can be detected by adding an inhibitor of membrane transport, such as azide or cyanide, to a cell suspension. When efflux was inhibited, the intracellular concentrations of toluene, xylene, benzene, and 1,2,4-trichlorobenzene increased in Pseudomonas putida relative to uninhibited cells.11,12 A correlation between antibiotic and organic solvent resistance has been observed in strains of Pseudomonas sp.13,14 The general trend in the study of efflux pumps in Pseudomonas sp. is that these proteins confer a generalized ability to efflux hydrophobic organic compounds.

An important exception to this trend is recent work by Bugg et al.,15 who showed the presence of selective energy-dependent efflux of polycyclic aromatic hydrocarbons (PAHs) in Pseudomonas fluorescens LP6a. This organism was able to efflux three-ring compounds (phenanthrene, anthracene, and fluoranthene) but not naphthalene or toluene. This observation suggests that selective pumping of compounds can be achieved by modifications to the efflux proteins. Insertion of genes on plasmids is a well-established technique for Pseudomonas sp.; therefore, these recent studies raise the prospect of designing bacteria as microscopic reactors, with control over both the intracellular enzyme activity toward a specific reaction of interest and the transport of reactants and products to and from the cell. In the future, the metabolic engineering of reaction pathways in bacteria may expand to include selective transport of reactants and products of desired reactions.

The theory for the design of membrane reactors with passive transport is well developed, based on a number of studies such as Agaralla and Lund16 and Moon and Park.17 In contrast, the importance of active transport of components has not been considered in selecting and developing microorganisms for conversion reactions. The objective of this paper is to analyze the interactions...
between active transport and reaction as a guide to selecting biochemical reaction systems where control of membrane transport may increase yield or selectivity. The analyses of two systems are presented: an analysis of transport without reaction in a two-membrane bioreactor (i.e., a cell) based on the experimental results of Bugg et al. This model is then extended to consider a membrane bioreactor with selective active transport of the reacting species.

Case 1: Active Transport with Two Membranes

Gram-negative bacteria, such as Pseudomonas sp., have two distinct cellular membranes separated by the periplasm, as illustrated in Figure 1. It is interesting to note that similar two-membrane systems have been proposed for membrane reactors with passive transport, to enhance membrane selectivity. The efflux protein complex spans the inner and outer cell membranes and serves to remove compounds either from the cell interior (cytoplasm) or from the inner boundary of the inner membrane and to export them into the medium outside the cell. In steady state, the diffusion of a compound across the membranes into the cell is opposed by the efflux process. The data of Figure 2 illustrate the time course of establishing a steady state for phenanthrene. In this case, a mutant strain of P. fluorescens LP6a was used that was deficient in the first enzyme of the pathway for degradation of naphthalene or higher PAHs. This mutant strain allowed study of the membrane transport of phenanthrene and other compounds, without enzymatic reactions. When the cells are exposed to phenanthrene at time zero, a steady state is established very rapidly. The data for the cells are from the cell pellet, where the measured concentration will be an average of the concentration in the various cell components, particularly the inner and outer membranes. When azide was added at 8 min, the efflux pump stopped exporting the phenanthrene and the cell concentrations rose toward the equilibrium level. Both the steady-state cell concentration (with efflux) and the equilibrium cell concentration (efflux inhibited) followed linear partitioning as a function of the phenanthrene concentration, as illustrated in Figure 3.

The shifts in the cell concentration of phenanthrene due to inhibition of active efflux (Figures 2 and 3) can be used to test models for transport across the cell membranes at steady state. The concentration gradients in the inner and outer cell membranes were assumed to be linear, and the resistance in the aqueous phases, the periplasm and the cytoplasm, was ignored, as illustrated in Figure 1. At steady state, the important concentrations were the concentration at the outer membrane–liquid interface \( [y_{om}(x=L)] \), the concentration at the outer membrane–periplasm interface \( [y_{om}(x=0)] \), the periplasmic concentration \( (C_p) \), the concentration at the inner membrane–periplasm interface \( [y_{im}(x=0)] \), the concentration at the inner membrane–cytoplasm interface \( [y_{im}(x=L)] \), and the cytoplasmic concentration \( (C_c) \). The thickness of each membrane was L. The concentration in the liquid phase, \( C_{liq} \), was measured.

The flux of PAH into the outer and inner membranes of the cell was given by

\[
J_{om} = P_{om}A_o[y_{om}(x=0) - y_{om}(x=L)] \quad (1)
\]

\[
J_{im} = P_{im}A_i[y_{im}(x=0) - y_{im}(x=L)] \quad (2)
\]

where \( P_{om} \) and \( P_{im} \) were the permeability coefficients for the outer and inner membranes, respectively, and \( A_o \) and \( A_i \) were the surface areas of the outer and inner membranes, respectively. The assumption of linear gradients was an approximation, because PAHs are likely to accumulate at the center of the lipid bilayer, causing a localized concentration maximum.

Figure 1. Schematic diagram of concentration gradients in bacteria cell membranes and location of important parameters.

Figure 2. Time dependence of liquid-phase and cell pellet \(^{14}\)C concentrations for the transport of phenanthrene in P. fluorescens LP6a mutant (data of Bugg et al.).

Figure 3. Cell pellet phase–liquid phase phenanthrene partitioning isotherm for steady-state and equilibrium phenanthrene concentrations in P. fluorescens LP6a mutant and wild type (data of Bugg et al.).
bacterium was assumed to be a cylinder 1 \, \mu m in diameter and 3 \, \mu m in length, with a thickness (L) of 4 \, nm for each membrane. Because the known efflux pumps in Pseudomonas sp. are made up of three proteins spanning both membranes, we assume that the volume of the periplasm will be small relative to the dimensions of the cells and that the outer wall and inner membrane are in close proximity to each other. Thus, the surface areas of the outer wall and inner membrane were approximated as being equal.

Two partition coefficients, \( K_{om} \) and \( K_{im} \), were used to describe the average partitioning in the outer and inner membranes, respectively, at equilibrium. The membrane interface concentrations were as follows:

\[
y_{om}(x=0) = K_{om}C_{liq} \tag{3}
\]

\[
y_{om}(x=L) = K_{om}C_{p} \tag{4}
\]

\[
y_{im}'(x=0) = K_{im}C_{p} \tag{5}
\]

\[
y_{im}'(x=L) = K_{im}C_{c} \tag{6}
\]

The average concentration in the cell pellet when efflux was inhibited was

\[
y_{avg} = K_{avg}C_{liq} \tag{7}
\]

where \( C_{liq} \) is the liquid-phase concentration in \( \mu mol/L \) and \( y_{avg} \) is the average cell pellet concentration in \( \mu mol/g \) of dry weight. The average partition coefficient \( K_{avg} \) is a measure of the overall partitioning between the aqueous phase and the total cell pellet concentration. Most of the PAH will reside in the membrane; therefore, \( K_{avg} \) is essentially the partition coefficient between the aqueous phase and the cell membrane phase. From the partition curve for the mutant strain in the presence of inhibitory levels of azide (Figure 3), a value of \( K_{avg} = 5.6 \, L/g \) of dry weight was calculated.

The total amount of PAH in the cell \( (V_{mem}y_{avg}) \) is equal to the sum of the average amount of PAH in each membrane (each with a volume of \( V_{mem}/2 \)) plus the amount in the periplasm \( (V_{p}C_{p}) \) and cytoplasm \( (V_{c}C_{c}) \):

\[
V_{mem}y_{avg} = \frac{V_{mem}}{2} \left( y_{om}(x=0) + y_{om}(x=L) \right) + \frac{V_{mem}}{2} \left( y_{im}'(x=0) + y_{im}'(x=L) \right) + V_{p}C_{p} + V_{c}C_{c} \tag{8}
\]

The variable \( z \) is a constant factor to convert \( y_{avg} \) from a dry cell weight basis to a membrane volume basis. A value of \( z = 2.2 \times 10^{3} \, g \) of dry weight/L (membrane volume) was obtained from the average dry weight measurement of 365.5 \( \mu g/mL \), the average cell count of 1.94 \times 10^{9} \, CFU/mL, and the bacterial dimensions. Because the aqueous concentration of PAH was much lower than the membrane concentration, the amount of PAH in the periplasm and cytoplasm was insignificant relative to that in the cell membrane. For example, from the data of Figure 2, the cell pellet concentration after azide addition was 10.97 \( \mu mol/g \) of dry weight and the liquid-phase concentration was 1.36 \( \mu mol/L \). Given the cellular volume, only about 0.006% of the phenanthrene was in the periplasm and cytoplasm of LP6a. Equation 8 then simplifies to

\[
y_{avg} = \frac{K_{om}C_{liq} + (K_{om} + K_{im})C_{p} + K_{im}C_{c}}{4} \tag{9}
\]

When active efflux was completely inhibited, PAH diffused into the cell until equilibrium was reached. At this point, there were no concentration gradients in the cell and \( y_{om} = y_{op} \), \( y_{im} = y_{ip} \), and \( C_{liq} = C_{p} = C_{c} \). Using these equalities and eq 7 for the average partitioning, eq 9 gives

\[
zK_{avg} = \frac{K_{om} + K_{im}}{2} \tag{10}
\]

The ratio of the equilibrium phenanthrene concentrations in the cell pellet of the azide-inhibited case \( (y_{avg}) \) to the steady-state pellet concentration for the no azide case \( (y_{avg}') \) was obtained from eq 9:

\[
y_{avg}' = \frac{K_{om}C_{liq} + (K_{om} + K_{im})C_{p} + K_{im}C_{c}}{K_{om}C_{liq} + (K_{om} + K_{im})C_{p} + K_{im}C_{c}} \tag{11}
\]

When cell efflux is active, the cell concentration is at steady state and the flux of PAH across the outer membrane \( (\alpha_{om}) \) is equal to the flux of PAH across the inner membrane \( (\alpha_{im}) \). From eqs 1 and 2, we obtain an expression for the periplasmic concentration in the absence of azide. Defining \( \alpha = K_{om}/K_{im} \) as the ratio of the outer membrane partition coefficient to the inner membrane partition coefficient and \( \delta = P_{om}/P_{im} \) as the ratio of permeabilities, we obtain

\[
C_{p}' = \frac{\alpha\delta C_{liq} + C_{c}}{\alpha\delta + 1} \tag{12}
\]

For the azide-inhibited case, there were no concentration gradients in the cell, so \( C_{liq} = C_{p} = C_{c} \) and the amount of phenanthrene in the cytoplasm was negligible; therefore, eqs 11 and 12 give

\[
y_{avg}' = \frac{2(\alpha + 1)C_{liq}}{\alpha + \alpha\delta(\alpha + 1)C_{liq}} \tag{13}
\]

Equation 13 can be rearranged to separate the measured concentrations from the unknown parameters:

\[
f = \frac{y_{avg}'C_{liq}}{y_{avg}C_{liq}'} = \frac{2(\alpha + 1)}{\alpha + \alpha\delta(\alpha + 1)} \tag{14}
\]

Calculated values of \( f \) for specific values of \( \alpha \) and \( \delta \) are plotted in Figure 4. Using the experimental concentrations \( C_{liq} = 2.46 \, \mu mol/L, C_{liq} = 1.38 \, \mu mol/L, y_{avg} = 2.78 \, \mu mol/g \) of dry weight, and \( y_{avg}' = 10.47 \, \mu mol/g \) of dry weight from the experiment illustrated in Figure 2), the value of \( f \) was 6.74. The data of Figure 4 indicate that only a narrow range of \( \alpha \) values (ratio of partition coefficients of the outer and inner membranes) can satisfy the experimental observations for phenanthrene transport in the absence of reaction. Given that both the inner and outer membranes will tend to accumulate phenanthrene because of their chemical composition, we would expect the value of \( \alpha \)
to be of $O(1)$. As the value of $\alpha$ approaches 0.42, then $\delta$ will approach zero. This result suggests that the permeability of the outer membrane is rate controlling for uptake of phenanthrene; therefore, $\delta \ll 1$ and the outer membrane is rate-controlling for uptake. Thanassi et al. came to the same conclusion for transport of tetracycline in Escherichia coli based on data for transient uptake, which was much slower for this more polar antibiotic.

**Case 2: Selective Active Transport with Reaction**

Following the preceding analysis and the experimental data of Figure 2 which suggest a rapid establishment of steady-state concentrations in the cells, we can analyze the role of active membrane transport on intracellular enzymatic reaction using a pseudo-steady-state analysis. During the steady-state condition, the partitioning into the cell membranes is constant. Although partitioning is extremely important in observing cell pellet concentrations, in the steady state we can express the concentrations on the basis of the aqueous phase.

In this analysis we consider parallel reactions for the biological oxidation of naphthalene and phenanthrene, in the presence of selective membrane transport as observed by Bugg et al. This analysis corresponds to the wild-type P. fluorescens LP6a, which was able to degrade both compounds. Both compounds are substrates for growth of the organism and both diffuse across the cell membrane, but only phenanthrene is effluxed from the cell by active transport. The active membrane transport across the cell membranes is approximated as first-order processes, consistent with the linear transport of phenanthrene over a range of concentrations (Figure 3). The preceding analysis suggested that the outer membrane controls permeability; therefore, the cell can be approximated as a membrane bioreactor with a single membrane that encloses the active enzymes. Because naphthalene and phenanthrene are likely degraded by a similar pathway, we assume competitive inhibition of a single set of enzymes, following Michaelis–Menten kinetics. Other types of kinetic interactions would include repression, derepression, or induction of either degradative enzymes or transport proteins by either substrate, but these cases are not considered here.

The pseudo-steady-state balances for reactants naphthalene ($N$) and phenanthrene ($P$) give

$$P_N A (C_{N0} - C_N) - \frac{V_N k_P E_P C_P}{K_{M,N} + C_N + K_{M,P} C_P / K_{M,P}} = 0$$  \hspace{1cm} (15)

$$P_P A (C_{P0} - C_P) - \frac{V_P k_E E_P C_P}{K_{M,P} + C_P + K_{M,P} C_N / K_{M,N}} - A k_E C_P = 0$$  \hspace{1cm} (16)

In these equations the permeabilities, $P_N$ and $P_P$, are for the overall transport from the cell exterior to the cytoplasm, while $k_P$ and $k_E$ are the rate constants for reaction and $k_E$ is the rate constant for efflux per unit area of cell membrane. Nondimensionalizing the cytoplasmic concentrations relative to the bulk concentrations ($\psi_N = C_N / C_{N0}$, $\psi_P = C_P / C_{P0}$, and $\psi_0 = C_{P0} / C_{N0}$) gives

\[
1 - \psi_N - \phi_N^2 \frac{\psi_N}{K_{M,N} + \psi_N + \gamma \psi_P} = 0
\]

\[
\phi_N^2 = \frac{k_N E_0 V_c}{P_N A C_{N0}}
\]  \hspace{1cm} (17)

\[
\zeta_{PN}(\psi_0 - \psi_P) - \phi_P^2 \frac{\psi_P}{K_{M,P} + \psi_P + \gamma \psi_N} - \phi_E^2 \psi_P = 0
\]

\[
\phi_P^2 = \frac{k_P E_0 V_c}{P_N A C_{N0}}; \quad \phi_E^2 = k_E / P_N
\]  \hspace{1cm} (18)

where the Thiele moduli for reaction are $\phi_N$ and $\phi_P$, respectively, $\phi_E$ is the modulus for efflux, $\gamma = K_{M,P} / K_{M,N}$, $K_{M,N} = K_{M,N} / C_{N0}$, $K_{M,P} = K_{M,P} / C_{P0}$, and $\zeta_{PN} = P_P / P_N$ is the ratio of the membrane permeabilities of the phenanthrene and naphthalene. Finally, the selectivity of reaction of phenanthrene versus naphthalene can be defined as the ratio of the reaction rates:

\[
S = \frac{\phi_P^2 \psi_P (K_{M,N} + \psi_N + \gamma \psi_P)}{\phi_N \psi_N (\gamma K_{M,N} + \psi_P + \psi_N / \gamma)}
\]  \hspace{1cm} (19)

Equations 17–19 can be solved once the seven parameters are specified: $\phi_N$, $\phi_P$, $\phi_E$, $\gamma$, $K_{M,N}$, $\psi_0$, and $\zeta_{PN}$. The solutions can be simplified by considering two limiting cases: linear kinetics where $K_{M,N} \gg \psi_N$ and zero-order kinetics where $K_{M,N} \ll \psi_N$. For the limit of first-order kinetics, the solutions for the steady state are

\[
\psi_N = \frac{1}{1 + \phi_N^2 K_{M,N}}
\]  \hspace{1cm} (20)

\[
\psi_P = \frac{\zeta_{PN} \psi_0}{\zeta_{PN} + \phi_P^2 / \gamma K_{M,N}}
\]  \hspace{1cm} (21)

\[
S = \phi_P^2 \zeta_{PN} \psi_0 (1 + \phi_N^2 K_{M,N})
\]

\[
\phi_N^2 \left(\frac{\zeta_{PN} + \phi_P^2 / \gamma K_{M,N} + \phi_E^2}{\zeta_{PN} + \phi_P^2 / \gamma K_{M,N} + \phi_E^2}\right)
\]  \hspace{1cm} (22)

These equations have only five independent parameters: $\phi_N^2 / K_{M,N}$, $\phi_P^2 / \gamma K_{M,N}$, $\phi_E$, $\psi_0$, and $\zeta_{PN}$.
the case of zero-order kinetics, eqs 17–19 reduce to the following:

\[ 1 - \psi_N = \phi_N^2 \frac{\psi_N}{\psi_N + \gamma \psi_p} = 0 \quad (23) \]

\[ \zeta_{PN}(\psi_0 - \psi_p) = \phi_p^2 \frac{\psi_p}{\psi_p + \psi_N / \gamma} - \phi_E^2 \psi_p = 0 \quad (24) \]

\[ S = \frac{\phi_p^2 \psi_p / \gamma}{\phi_N^2 \psi_N} \quad (25) \]

with six independent parameters: \( \phi_N, \phi_p, \phi_E, \gamma, \psi_0, \) and \( \zeta_{PN} \).

The permeability of biological membranes to diffusing solutes depends on the partition coefficient of the solute in the membrane and the molecular weight of the solute.\(^2\) This relationship was measured for transport across mammalian membranes, which are much more permeable than the outer membranes of bacteria.\(^9\) If we assume that the ratio of the permeabilities in bacteria follows the same relationship as that in mammalian cells, then from Stein\(^2\) and Thanassi et al.\(^9\)

\[ \zeta_{PN} = \frac{P_p}{P_N} = \frac{K_{ow,p}/M_{W_N}}{K_{ow,N} / M_{W_p}} \quad (26) \]

From Mackay and Shiu,\(^{24}\) the octanol–water partition coefficient for naphtalene is 2.34 \( \times \) 10\(^3\) and the value for phenanthrene is 3.72 \( \times \) 10\(^4\). The resulting value for \( \zeta_{PN} \) is 14.6 from eq 26.

The data of Figure 5 show the results for linear kinetics for the simplest case of equimolar concentrations of the two reactants (\( \psi_0 = 1 \)) and equal reactivity of both species (\( \phi_{N^2}/K_{MN,N} = \phi_{P^2}/K_{MN,N} \)). The addition of selective efflux of phenanthrene (\( \phi_E \gg 1 \)) reduced the selectivity for phenanthrene from O(1) to O(0.1). The selectivity for conversion of phenanthrene, relative to conversion of naphtalene, increased with increasing rate of reaction due to the higher permeability of phenanthrene across the membranes. This permeability advantage gave higher intracellular concentrations, and higher conversions, of phenanthrene except when active efflux was increased. The results for zero-order kinetics were very similar, as illustrated in Figure 6 for the same simplified case of equimolar concentrations of the two reactants (\( \psi_0 = 1 \)) and equal reactivity of both species (\( \phi_{N^2} = \phi_{P^2}/\gamma \)). The difference from first-order kinetics arose at high conversion rates with high efflux, where the lower rate of permeation of naphtalene became limiting and selectivity for phenanthrene conversion increased sharply.

**Discussion**

Efflux pump systems have been identified for hydrophobic compounds with octanol–water partition coefficients from ca. 0.03 for tetracycline\(^9\) up to 1.8 \( \times \) 10\(^4\) for fluoranthene. With selective efflux of components, the selectivity for enzymatic reaction of one hydrophobic compound over another can be significantly increased, as illustrated in Figures 5 and 6. The selective efflux must be efficient, however, relative to the permeability of the membrane (\( \phi_E \gg 1 \)); otherwise, the selectivity for reaction is dominated by permeation into the cell and not energy-driven efflux. The analysis of the data of Bugg et al.\(^{15}\) showed that the outer membrane was the main permeability barrier in P. fluorescens; however, uptake was very rapid as indicated by the rapid achievement of steady-state concentrations in the cells (Figure 2). Consequently, the efflux of hydrophobic compounds must be very effective in order to achieve selectivity (\( \phi_E \gg 1 \)). In contrast, a less hydrophobic compound such as toluene with a K\(_{ow}\) of 200 will permeate the membrane at only 2% of the rate of phenanthrene, from eq 26, and a proportionally smaller value of the Thiele modulus for efflux would be effective in giving high selectivity between compounds.

The example considered in this paper was passive uptake of components by diffusion into the cell, with selective energy-driven efflux. A more effective approach to metabolic engineering of cells for bioconversion, from the viewpoint of the effectiveness of intracellular enzymes, would be selective energy-driven uptake of the desired substrate. This strategy is used by many bacteria for low-permeability compounds, such as sugars and amino acids, giving effectiveness factors of greater than unity. For more permeable compounds, a combination of active uptake of the desirable reactant and active efflux of undesirable compounds would give a high effectiveness factor and high selectivity.
Conclusions

The partitioning of phenanthrene between cells and the aqueous phase in the absence of metabolism was significantly altered in P. fluorescens by active energy-driven membrane transport, or efflux. A model for partitioning and transport across the inner and outer membranes showed that the permeability of the outer membrane was rate controlling. A model for membrane transport and reaction showed that selective efflux of one compound versus another controls the selectivity of the reaction by intracellular enzymes provided that the rate of efflux is significantly greater than the rate of permeation across the cell membranes.

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Literature Cited