Photoinduced charge separation and enzyme reactions in reversed micelles

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Proefschrift

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De vraag is genuanceerd
De vraag is ludiek
De vraag is verruimend

Het antwoord is genadeloos, bindend, conflictrijk.

FREEK DE JONGE
uit: *De Tragiek*

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CONTENTS

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Chapter 4.3. "Enzyme kinetics in reversed micelles: 3. Behaviour of 20ß-hydroxysteroid dehydrogenase." B. Tyrakowska, R.M.D. Verhaert, R. Hilhorst and C. Veeger.

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LIST OF ABBREVATIONS AND SYMBOLS

a_o	molar alcohol to surfactant ratio
$[A_{ov}]$	concentration of A expressed with respect to the total volume of the reversed micellar medium
$[A_{wph}]$	concentration of A expressed with respect to the aqueous volume in a reversed micellar medium
$[\bar{A}'_{im}]$	average concentration of A in A-filled reversed micelles
AOT	Aerosol OT, sodium di(ethylhexyl)sulphosuccinate
CTAB	Cetyltrimethylammonium bromide
ER	Enoate reductase (EC 1.3.1.31)
$[E_o]$	enzyme concentration expressed with respect to the overall volume
$^FP_1$	5-[4-N-methylpyridiniumiodide]-15-[4-[methoxy]-3-fluorophenyl]-2,8,12,18-tetraethyl-3,7,13,17-tetramethylporphyrin
$^FP_{16}$	5-[4-N-methylpyridiniumiodide]-15-[4-[hexadecyloxy]-3-fluorophenyl]-2,8,12,18-tetraethyl-3,7,13,17-tetramethylporphyrin;
HSDH	20ß-Hydroxysteroid dehydrogenase, (EC 1.1.1.53)
K_A	Michaelis constant for substrate A (NADH)
K_B	Michaelis constant for substrate B (2-methylbutenoic acid (Chapter 4.2) or progesterone (Chapter 4.3))
K_m^{app}	apparent Michaelis constant (expressed with respect to the total volume)
k_{ex}	exchange rate between reversed micellar droplets ($M^{-1}s^{-1}$)
k_{in}	rate of diffusion from a solute from the organic phase into the reversed micelle
k_{out}	rate of diffusion from a solute from waterpool to the organic phase
MV	methylviologen
[M]	concentration of reversed micelles
P_1	5-[4-N-methylpyridiniumiodide]-15-[4-[methoxy]phenyl]-2,8,12,18-tetraethyl-3,7,13,17-tetramethylporphyrin

P_{16}	5-[4-N-methylpyridiniumiodide]-15-[4-[hexadecyloxy]phenyl]-2,8,12,18-tetraethyl-3,7,13,17-tetramethylporphyrin
P^a	partition coefficient of a solute between an organic and an aqueous phase
$[S_{ov}]$	concentration of S expressed with respect to the total volume of the reversed micellar medium
$[S_{wph}]$	concentration of S expressed with respect to the aqueous volume in a reversed micellar medium
$[S_{im}]$	concentration of S expressed with respect to the volume of the reversed micelle
$[S'_{im}]$	concentration of S in a given S-filled reversed micelle, expressed with respect to the volume of the waterpool of that micelle
$[\bar{S}'_{im}]$	average concentration of S in S-filled reversed micelles
TEA	triethylamine
TBA	tributylamine
TOA	trioctylamine
U	unit of enzyme activity (μmol product formed per minute, 1 U = 16.6 nkatal)
v	velocity of the enzyme reaction (s^{-1})
v_{max}	experimentally determined maximum enzyme velocity (s^{-1})
V	(intrinsic) rate constant of the forward reaction (Ms^{-1})
w_o	molar water to surfactant ratio
ZnMDPyTrPP	Zn(II) *meso*-monc(4-N-decylpyridinium)-tri(phenyl)porphyrin
ZnTMPyP	Zn(II) *meso*-tetrakis(4-N-methylpyridinium)porphyrin
$\epsilon^s_{i,o}$	efficiency of the exchange of compound S into (i) or out of (o) an enzyme filled reversed micelle
π	probability of an event to occur
Φ	volume fraction of water (l/l)
Φ_{CS}	yield of photoinduced charge separation reaction (%)

1. INTRODUCTION

1.1 REVERSED MICELLES: STRUCTURE AND DYNAMICS.

Reversed micelles are thermodynamically stable, nanometer sized water droplets dispersed in an organic phase by a surfactant. In this optically transparant solution the surfactant molecules are located in the interphase with the polar headgroups directed to the water pool and the hydrophobic tails protruding in the organic phase [1]. The relative concentrations of the components of the medium determine whether reversed micelles are formed and, if formed, determine the structural characteristics (*e.g.* size, shape, composition, the water content and the organization of the interface) of the reversed micelle. A condition for the formation of reversed micelles is that the interfacial tension between the organic phase and the aqueous phase is reduced to almost zero [2]. The surfactant is used for this purpose.
In some cases the surfactant alone is not able to reduce the surface tension sufficiently to induce a high interfacial area. Then a biphasic system is formed [2,3]. In that case a cosurfactant is required to reduce the interfacial tension further. Aliphatic alcohols are commonly used for this purpose. In general the cosurfactant partitions between the interface and the continuous phase. The partitioning depends on the nature and quantity of the cosurfactant [4].
An important parameter affecting the stability and size of a reversed micelle is w_o. This parameter (also named R) is the molar ratio of water to surfactant. The physical characteristics of the water in the reversed micellar medium depends strongly on the value of w_o and on the nature of the surfactant: water in a reversed micellar medium with a low w_o value has the same properties as immobilized water (a low dielectric constant and a lowered freezing point). Upon increasing the amount of water in the system the gradually water starts to behave like bulk water [5,6].
In contrast to micelles formed in an aqueous solution, reversed micelles are electrically neutral, so that no Coulombic repulsion occurs and collisions are facilitated. Exchange of the contents of reversed micelles is believed to occur by a collision-fusion-fission process [7,8]. That is, upon collision of two reversed micelles one micelle is formed with a single mixed aqueous pool. Separation of the fused micelle results in two new reversed micelles in which the contents are redistributed.
The number of collisions leading to exchange depends on the thermal energy (*i.e.* the temperature relative to the surface tension) and the composition of the system. In the literature values between 10^6 and 10^8 $M^{-1}s^{-1}$ for the exchange frequency have been reported. This means that only one in every thousand collisions leads to an exchange event and that reversed micelles communicate with each other on a micro- to millisecond timescale. The cosurfactant is expected to affect this exchange rate very strongly, because the energy barrier for the fusion proces is determined to a great extent by the surface tension. According to Zana and Lang [1] the interface of the reversed micelle becomes more rigid and more organized if an alcohol with a longer alkyl chain is used. This results in both a decrease of the intramicellar water mobility [9] and a decreased rate of intermicellar exchange.
Due to the microheterogeneity of the system common expressions like concentration and pH have to be carefully redefined. For instance pH in reversed micelles is a controversial phenomenon. Bardez and coworkers [10,11] and El Seoud and Chinelatto [12] have clearly shown that the

acidity of a compound strongly depends on its location in a reversed micelle because an acidity gradient is formed from the interface to the micellar center. This observation is similar to interfacial effects of charged polymer matrices on protein distribution and protein activity [13]. Upon increasing the w_o value in a reversed micellar medium also the pH behaviour of solutes located in the aqueous pool becomes similar to that in bulk water.

1.2. PHOTOINDUCED CHARGE SEPARATION.

The use of solar energy storage as an alternative to the use of fossil energy sources has been studied extensively over the past two decades. The field can be devided into three topics. The first topic is the use of solar energy to produce electricity. In this field many applications for photovoltaic cells are known [14]. The second type of system under study uses chemical means to store energy. In this field the use of semiconductors to provoke photoinduced water splitting is studied most extensively [15,16]. The third topic, the research to biomimetic systems and biological models is currently expanding to applied science. Investigations to the mechanism and components governing photosynthesis are linked to studies of artificial systems because an understanding of the parameters determining the efficiency of the first steps in photosynthetic charge separation has been shown to be very useful to optimize any biomimetic system [17]. In the next sections general considerations with respect to the charge separation in applied and biomimetic systems will be summarized.

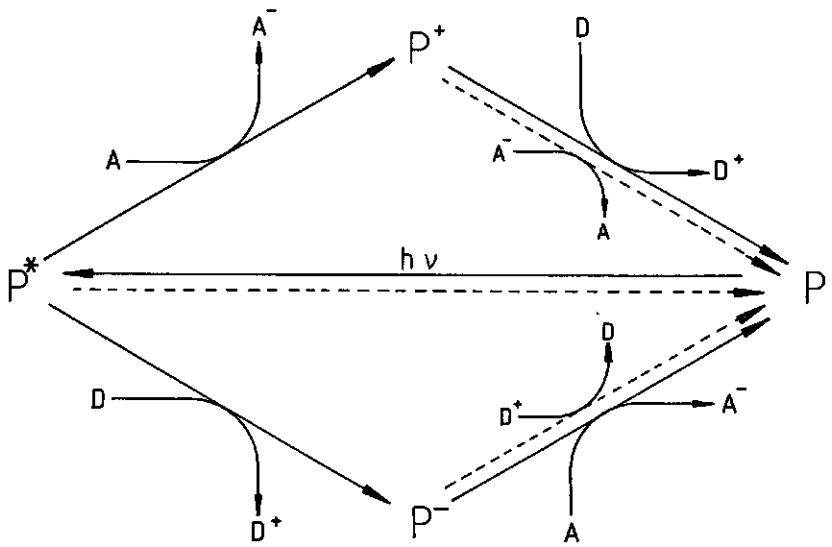

Fig. 1.1. Schematic representation of the possible reactions in a simple donor (D), photosensitizer (P) acceptor (A) system. The central reaction is the excitation of the photosensitizer. Dotted lines represent backward reactions.

1.2.1. Mechanism.

The formation of stable photo-products occurs via several steps. The first step, independent of the mechanism of the subsequent reactions, is the excitation of the sensitizer. This step is very fast (10^{-15} s). After excitation of the photosensitizer P two routes can be followed (Fig. 1.1). In the first case, transfer of one or more electrons from this sensitizer to an acceptor A followed by rereduction of the sensitizer by an electron donor D; oxidation of the electron donor by the sensitizer and subsequent transfer of an electron to the acceptor resulting in reoxidization of the porphyrin. Two different inhibitory reactions may occur, represented by dotted lines in this figure, (i) decay of the sensitizer from its excited to its ground state, without any intermolecular reactions, (ii) backward electron transfer from a charge transfer state to a charge recombined state. This state is not necessarily the ground state [18,19].
The mechanism of transfer of an electron from this excited state depends on the type of transfer reaction. Under optimum conditions, the photochemical electron transfer can compete with intramolecular reactions which occur on nanosecond timescale (fluorescence, intersystem crossing). Under less favourable conditions, the photoinduced electron transfer only competes with photoprocesses occuring at micro- and millisecond timescale (triplet decay). The latter reactions can occur only if the excited state exists sufficiently long.
From these assumptions, it can easily be concluded that the most efficient photosystem should be based on very rapid electron transfer rates. Indeed, in the bacterial reaction center the rate of the initial charge separation is higher than 10^{12} s^{-1} [20]. However, as can be deduced from Fig. 1.1 the forward electron transfer is not the only step which determines the efficiency of the overall reaction because the efficiency is the net result of the ratio of the forward and the backward reaction. The research in this field is aimed at increasing the rate of the forward reaction and decreasing the backward reaction. First the components of the photosystem will be discussed. The influence of the backward reaction will be considered in more detail in section 1.2.4.

1.2.2. Photosensitizers.

In artificial photosynthesis the sensitizer has to fulfil several requirements. It has to be stable, has to have a large extinction coefficient in the visible region, a long lifetime and a high yield of a reactive high energy excited state [21]. Two groups of sensitizers are widely used. The first group of ruthenium complexes ruthenium-tris-2,2'-bipyridyl has gained most attention. This compound has a strong absorption in the visible and the ultraviolet region and a relatively stable charge transfer state of 2 eV above the ground state [22].
The second group, that of the porphyrins, is more diverse. In nature chlorophyll is the compound used for harvesting solar energy in bacteria and plants. Moreover, the strong absorption of porphyrins in the visible and the ultraviolet region, their stability and the possibility to alter substituents at the porphyrin has resulted in a variety of studies of the photobiological, photochemical and photophysical properties of these compounds [25]. The basic skeleton of a porphyrin is the porphin core (Fig. 1.2) to which substituents can be attached at the methylene bridges, *i.e.* the meso position (numbered 5,10,15 and 20) or at the pyrrole rings.

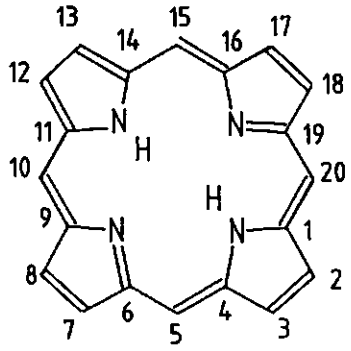

Fig. 1.2. Structure of the porphyrin core, the porphin.

Generally, information about the substituents and their link to the porphyrin moiety can be derived from the type of the absorption spectrum. Smith [23] classified porphyrins in four different groups depending on the relative intensity of the four Q-bands in a neutral solution (Fig. 1.3).

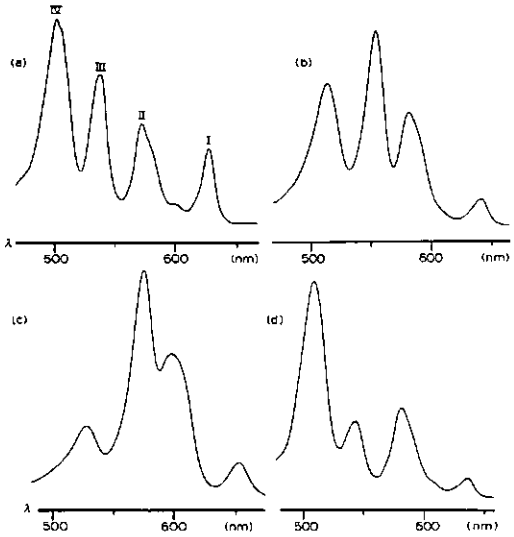

Fig. 1.3. Absorption spectra as a diagnostic tool in the determination of porphyrin structure. a) Etio type, b) Phyllo type, c) Rhodo type, d) Oxorhodo type. For further explanation: see text. From ref [23]

An etio-type spectrum is characteristic for poly pyrrole substituted porphyrins (alkyl groups or carboxylic acids), a rhodo-type spectrum for porphyrins modified with a single electron withdrawing group, an oxorhodo-type spectrum for porphyrins with an electron withdrawing group at both sides of the ring, and a phyllo-type spectrum for a highly asymmetric π electron distribution, *e.g.* originating from the presence of heterogenic substituents at the pyrrole rings or from ligands at the meso-position. The nature and the position of the substituent also affects the redox properties of the porphyrins [24-27]. The presence of a central substituent replacing the hydrogen atoms in the porphin has been studied both with respect to spectroscopic and redox features [24,25,27,28]. The most typical change in the absorption spectrum is the decrease in the number of Q-bands, because due to the increase in symmetry of the molecule the x and y axes in the molecule become indistinguishable. With respect to the redox properties it is noteworthy that especially zinc containing porphyrins turn out to be good reductants in the excited state. As a first step in charge separation electron transfer from this porphyrin to the electron acceptor occurs [24]. However, subsequent rereduction of Zinc porphyrins can be hampered (Chapter 5).

1.2.3. Donor and acceptor molecules in artificial systems.

In the search for useful acceptor and donor molecules in water splitting processes and other storage systems for solar energy, many compounds have been studied. Most studies investigating photoinduced reduction reactions focus on sacrificial electron donors. The most popular is EDTA [*e.g.* 30,31]. These compounds decompose upon oxidation. A reversible electron donor is thiophenol. This compound donates both electron and protons, and can be regenerated by hydrogenation.
As electron acceptors both compounds occuring in nature (carotene, quinones) [32] and synthetic molecules (viologens, demethylaniline) [33] are used. A group of electron acceptors which was reported to be very promising for photoinduced water splitting systems was that of cobalt complexes [34-36], but they have not widely gained attention yet.
A new area in the research to photoinduced charge transfer is the synthesis of complex molecules containing a covalently attached donor or acceptor group (or both groups). In these molecules a very fast intramolecular charge separation can be induced [19,37-40]. Unfortunately the rate constant for charge recombination for most of the investigated compounds is often of the same order of magnitude as the rate constants for charge separation [41].

1.2.4. Advantages of charge separation in heterogeneous media.

A strategy to enhance the yield of photoinduced reactions is a physical separation of the donor and the acceptor and a transfer of the captured energy into a stable form (electricity or a fuel compound) [42]. An other approach is the organisation of the components of the photosystem in a polyelectrolyte matrix. Then the backward reaction can be retarded several orders of magnitude and the efficiency of the primary charge separation can be enhanced [6]. Organisation of reactants and spatial separation of the products are the main advantages of heterogeneous media in photochemistry. As heterogeneous media vesicles [43,44], silica particles [44], micelles [28,46] and reversed micelles [46,47] have been investigated.

Photosensitized electron transfer and separation of the photo products in vesicles and reversed micellar media was reported in 1979 by the group of Calvin [48,49]. Hilhorst and coworkers [50] were the first to use a photosystem in reversed micelles to store the energy in the form of enzymatically procuced hydrogen. The effective decrease of the backward reaction in micelles and reversed micelles was demonstrated by Schmehl and Whitten [29] and by Pileni and coworkers [46]. Although many characteristics of the photoinduced charge transfer in both biological and artificial systems have been elucidated and in many reports very efficient photoinduced water splitting systems were predicted, no operative systems have been reported, yet.
In nature the rigid organization of the reaction center protein provides an environment in which both separation of the photoproducts (O_2, H^+ and electrons) occurs and the optical energy is almost instantaneously converted into chemical energy (carbohydrates). A strategy based on this principle is the *in situ* enzymatic conversion of an intermediate photoproduct in an ordered environment [50].

1.3. ENZYMATIC REACTIONS IN REVERSED MICELLES, COFACTOR REGENERATION AND KINETICS.

More than 37 years have evolved past the first report of enzyme activity in reversed micelles [51], but only in 1977 the first activity of an enzyme in a non-natural detergent in an organic solvent was reported [52]. Now the number of enzymes in reversed micelles studied, has increased to over 50 [53-55]. Many studies on the location of proteins in these surfactant aggregates, show that an interaction between the interface and the protein can exist [56-59].
For water soluble proteins the interaction seems to depend on the charges on the protein and the charges in the interphase [58-60]. For the incorporation of a water soluble protein two models have been proposed. The first one, the water shell model, assumes incorporation of the protein together with a small layer of water. In this model water that is expelled out of the reversed micelle by the protein, is redistributed over all empty reversed micelles in solution [54]. Apparently in this model incorporation of an enzyme which is larger than an empty reversed micelle results in the formation of a protein-surfactant aggregate, containing a minimal amount of hydratation water.
The second model was proposed by the group of Martinek [52,61] for small water soluble proteins, *i.e.* radius enzyme < radius empty micelle (≈1-2 nm). In this model incorporation of the enzyme in a reversed micelle is accompanied by a redistribution of the water molecules <u>within</u> that micelle. Several pieces of evidence have been reported supporting this model [62-64]. Possible effects of the incorporation of proteins larger than a reversed micelle have not been discussed in the framework of this model. However, general agreement exists about the observation that the rigidity of the protein structure in the reversed micelle is increased at low w_o and this rigidity is assumed to cause an increase of the stability of enzymes in the reversed micellar solution [65-71].
It has been shown that enzymes in reversed micelles are not only active in the conversion of water soluble compounds but also for apolar ones. Apolar substrates can diffuse through the interface from the continuous phase to the water pool. This offers the possibility to use a reversed micellar medium to convert apolar substrates [72]. In this case, besides the increased stability of the enzyme, other advantages exist. The solubility

of the substrate increases and often, due to a different solubility of the substrate and the product in the organic phase, a shift of the equilibrium concentrations occurs. Detailed rules for the optimization of such reactions have been derived in our laboratory [70,73,74].
The variation of the watercontent (w_o) of the reversed micellar system on enzyme activity has been studied extensively. Several groups observed a bell shaped dependence of the activity on the w_o value of the reversed micellar medium [68,70,75,76]. A model in which enzyme partitions over three different domains in a reversed micellar solution (*i.e.* apolar, head groups and an aqueous environment), each resulting in a specific rate constant, was presented by Bru and coworkers [77]. From recent comparison of the w_o value at which maximum activity was observed, with the size of the enzyme, it appears that the highest activity of the enzyme is observed if the reversed micelle has the same size as the protein [53 and references cited therein]. Again the specific microenvironment of the enzyme with the reversed micellar shell tightly fitting the enzyme to keep it in its optimal conformation is used to explain this observation [78].
In reversed micelles the value of the Michaelis constant, that is the concentration of substrate at which the enzyme operates at 50% of its maximum catalytic activity, has caused many discussions (see for example 55-54,79). In particular the question whether the substrate concentration of a water soluble compound is to be expressed with respect to the total volume or with respect to the aqueous phase, has gained much attention. The cause for this is that a definition designed for a homogeneous continuous solution cannot be applied in a heterogeneous medium. Similar to a new definition of acidity and H^+ concentration in reversed micelles, a reconsideration of the substrate concentration and the Michaelis constant is necessary. This phenomenon will be discussed in greater detail in Chapter 4.
Several applications have been suggested for enzymes in reversed micelles [see 72]. One of them is the use of enzymes for the conversion of apolar compounds. Most studies have been restricted to hydrolytic enzymes because many other enzymes need (expensive) cofactors like $NAD(P)^+$, NAD(P)H or ATP. For a practical use the cofactor needs to be regenerated. Such a cofactor regenerating system has to be cheap or it has to yield an economically interesting product and it should not interfere with the reaction or with the isolation of the final products.
In aqueous solutions both enzymatic systems, and systems using other means to produce reducing equivalents, *e.g.* electrochemical reduction of methylviologen, an (artifical cofactor) have been described [80]. The enzymatic methods can in principle be used in reversed micelles, but the microheterogeneous nature of the medium can be exploited for photochemical production of reducing equivalents [71,81,82]. The replacement of the cofactor by methylviologen for the enzymatic production of hydrogen and was described by Hilhorst and coworkers [50]. A photochemical method to drive enoate reductase in reversed micelles, replacing NADH as a cofactor by *in situ* recycled methylviologen is described in Chapter 2.

1.4. OUTLINE OF THIS THESIS.

The main goal of the research presented in this thesis was to design a system to drive enzymatic reactions by photochemical means and, moreover, to gain understanding of the factors contributing to or adversely affecting the two partial reactions.
This was achieved by the combination of a photosystem producing reduced

methylviologen and an enzymatic conversion using the methylviologen as a source of reducing equivalents. Reversed micelles were very suitable, they provide the structured medium required for vectorial electron transport from the donor to the acceptor, and, at the same time, provide an environment where the enzyme retains its activity. The system was optimized with respect to the medium composition to yield both an efficient photoinduced methylviologen reduction, and a high enoate reductase activity and stability. The results are described in Chapter 2.
From these studies it appeared that the performance of surface active photosensitizers was strongly dependent on the composition of the medium and that the dynamics and the location of those compounds might be responsible. This assumption was investigated using ^{19}F-NMR chemical shifts to probe the environment of the porphyrin in relation to the efficiency of a photoinduced charge separation. The fluorescence behaviour of two porphyrins was investigated using both steady state and time-resolved measurements to determine the influence of the environment on the stabilization of the excited state. The results are reported in Chapter 3.
A peculiar pattern of the enzyme activity emerged when varying the composition of the reversed micellar medium. The theoretical analysis of the kinetic parameters as influenced by the incorporation of the enzyme in reversed micellar solution is elaborated in the first paragraph of Chapter 4. In this chapter also the experimental study of the kinetics of both enoate reductase and hydroxysteroid dehydrogenase are considered in two subsequent paragraphs.
In Chapter 5 the results of the preceeding Chapters are discussed with respect to both the prerequisites for efficient photoinduced charge separation and for optimizing enzymatic conversions in reversed micelles.

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2. OPTIMIZATION OF THE PHOTO-ENZYMATIC REDUCTION OF THE CARBON=CARBON DOUBLE BOND OF α-ß UNSATURATED CARBOXYLATES IN REVERSED MICELLES

2.1. SUMMARY

A system is described for the photo-induced biocatalytic reduction of enoates in reversed micelles composed of detergent, buffer and mixtures of alcohol and octane as cosurfactant and dispersant respectively. The photosystem consisted of a sacrificial electron donor (tributylamine), Zn(II) *meso*-mono(4-N-decylpyridyl)tri(phenyl)porphyrin as photosensitizer and methylviologen as electron acceptor. The enzyme enoate reductase (EC 1.3.1.31) utilizes the photoreduced methylviologen for the stereospecific reduction of enoates. The composition of the reversed micellar medium has been optimized with respect to both the photochemical and the enzymatic part of the system. The yield of the photosystem increased with increasing polarity of the reversed micellar solution. The activity and stability of enoate reductase depended strongly on the nature and concentration of the cosurfactant in the reversed micelles. Increasing the chain length of the cosurfactant stabilizes the enzyme in the presence of enoates, but it leads to a more rapid loss of activity of the reduced form of the enzyme. Reversed micelles were composed in which both operational and storage stability of enoate reductase are at least equal to that in water. The photochemical regeneration of methylviologen coupled to the enzymatic reduction of 2-methylbutenoic acid and cinnamic acid yielded a system that was active for over 100 h in the presence of a cofactor regenerating system in reversed micelles. The enzyme was as stable as in aqueous solution. Therefore this photo-enzymatic system can be used for the stereospecific reduction of apolar α-ß unsaturated carboxylates.

2.2. INTRODUCTION

A relatively new field in photochemistry and enzymology is the use of reversed micelles containing a photosystem located in the micellar boundary layer for photoinduced enzymatic reactions. Reversed micelles are water droplets of 1-10 nm dispersed in an organic solvent by a detergent. The spectroscopic and biotechnological features of reversed micelles have been reviewed recently (Luisi, 1985; Luisi and Laane, 1986; Martinek et al., 1986; Vos et al., 1987a).
The use of reversed micelles for photochemical purposes has several advantages. First, reversed micellar solutions are optically transparent in the visible light region, and secondly reactive intermediate photoproducts induced by a photochemical electron transfer reaction can be spatially separated as a result of the microheterogeneity of the system, yielding an efficient charge separation (Brochette and Pileni, 1985; Pileni et al., 1986; Degani and Willner, 1985; Brochette et al., 1987). In the absence of a micellar environment the efficiency of the charge separation reaction is generally limited by a very rapid backward reaction. This can be envisaged as follows:

$$P \xrightarrow{k_1} P^* \qquad (1)$$

$$P^* + A^- \xrightarrow{k_2} P^+ + A^- \qquad (2a)$$

$$P^+ + A^- \xrightarrow{k_{-2}} P + A \qquad (2b)$$

$$P^+ + D \xrightarrow{k_3} P + D^+ \qquad (3)$$

in which P denotes the sensitizer, A the electron acceptor and D the sacrificial donor.
It is clear that there will be a competition between the reduced electron acceptor A^- (Eq. 2b) and the donor D for the oxidized porphyrin (Eq. 3). Thus reaction 3 has to compete with the backward reaction (Eq. 2b).
In reversed micelles this backward reaction rate (k_{-2}) is retarded in favour of the reduction of the sensitizer by an electron donor D (Brochette and Pileni, 1985; Brochette et al., 1987).
Reaction 2, in the forward as well as the backward direction, depends strongly on the relative location of the sensitizer and the acceptor in reversed micelles and by changing the relative location of these reactants, the rate of the accumulation of the reduced electron acceptor can be modulated at will (Pileni et al., 1986).

In a reversed micellar solution the individual reversed micelles can be regarded as separate entities which exchange their water pools on a short timescale (less than 1 ms) and as a single continuous waterphase on a timescale of seconds (Vos et al., 1987b). This dualistic nature is advantageous to both the photochemical charge separation and the enzymatic reaction because an enzyme in a reversed micellar solution is in contact with all substrate in the aqueous phase.
The application of reversed micelles in enzymology has more advantages. The main advantage is that compounds that are poorly soluble in water, can be converted enzymatically in such solutions.
Furthermore, some enzymes in reversed micelles show enhanced catalytic activity, as compared to aqueous solutions (Klyachko et al., 1984, Levashov et al., 1986). This enzymatic activity depends on the nature of the organic solvent, the concentration of the (co)surfactant and the size of the water pool (Luisi, 1985; Martinek et al., 1986, Laane et al., 1987b; Laane, 1987). Finally, the stability of enzymes in reversed micelles often increases markedly, especially at low water content. It has been suggested that the reversed micelles invoke a reduced mobility of the protein backbone resulting in an increased stability (Martinek et al., 1981).

We studied the enzyme enoate reductase (EC 1.3.1.31) in reversed micelles. This enzyme catalyses the stereospecific reduction of the nonactivated carbon-carbon double bond in α-ß unsaturated carboxylates (Bader and Simon, 1980). Usually reductases require NAD(P)H for their action. However, the need for such an expensive cofactor limits the applicability of redox enzymes for biotechnological purposes, unless the cofactor is recycled. Several cofactor regenerating systems have been reported for aqueous media (Whitesides, 1985), but only a few for reversed micellar media (Hilhorst et al., 1982; Larsson et al., 1987). Besides NADH, enoate reductase can use reduced mediators such as methylviologen as a cofactor. The goal of this study was to couple the photochemical reduction of methylviologen to the enzymatic reduction of enoates in reversed micelles and to create the optimum operational conditions for a continuous photoinduced biocatalytic conversion. We studied the performance of the photosystem and the enzyme

system in reversed micellar solutions of various composition in order to elucidate some of the factors influencing the conversion in reversed micelles.

2.3. MATERIALS AND METHODS

2.3.1. Chemicals

Purified enoate reductase was a generous gift of Professor Dr. H. Simon (Technical University, Munich). Crotonic acid (2-butenoic acid), tiglic acid (*trans*-2-methyl-2-butenoic acid) and cinnamic acid (*trans*-3-phenylpropenoic acid) as well as tributylamine and methylviologen were purchased from Sigma Chemical Co. (St Louis, Mo, USA). NADH was from Boehringer, Mannheim (W. Germany). Cetyltrimethyl ammonium bromide (CTAB) from Serva (Heidelberg, W. Germany) was used without further purification. The various aliphatic alcohols and n-octane (Merck, Darmstad, W. Germany) were distilled before use. The porphyrins Zn(II) *meso*-mono(4-N-decylpyridyl)-tri(phenyl)porphyrin (ZnMDPyTrPP) and Zn(II) *meso*-tetrakis(4-N-methylpyridyl)porphyrin (ZnTMPyP) (Fig. 2.1) were synthesized by Mr. R. Koehorst (Department of Molecular Physics).

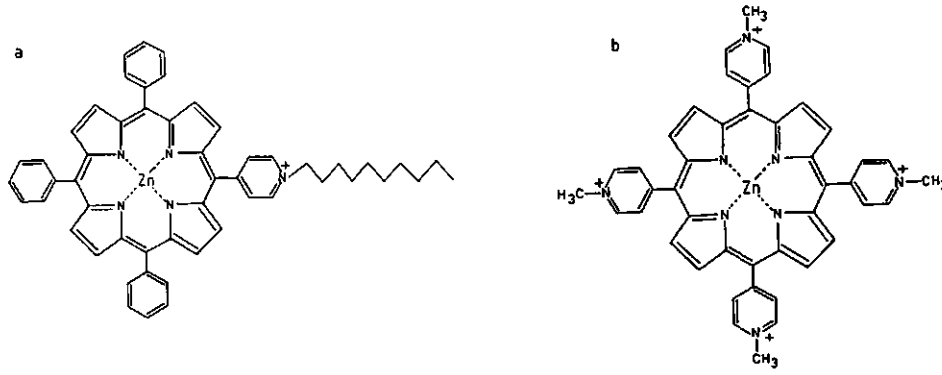

Fig. 2.1. Structure of the porphyrins a) Zn(II) meso-mono(4-N-decylpyridyl)-tri(phenyl)porphyrin (ZnMDPyTrPP) b) Zn(II) meso-tetrakis(4-N-methylpyridyl)porphyrin (ZnTMPyP)

2.3.2. Preparation of reversed micelles

Stock solutions of reversed micelles were prepared by mixing 0.22 g CTAB (0.6 mmol) in 3 ml of a cosurfactant-octane mixture and adding 54 µl of a 50 mM potassium phosphate buffer of pH 7.0. This amount of buffer corresponds to a water to detergent molar ratio (w_o) of 5. Various aliphatic alcohols were used as cosurfactant. The volume fraction of the cosurfactant in the mixture cosurfactant-octane is used to indicate the composition of the different reversed micellar media. Typically a 10 % hexanol (= hexanol:octane = 1:9 (vol/vol)) solution was chosen.
The various compounds were added to this standard micellar solution until the desired concentration was reached. Phosphate buffer of pH 7.0 was added

to obtain a final w_o value of 10. The final buffer concentration in the aqueous phase was 50 mM. All other concentrations are expressed with respect to the total volume of the medium. Experiments were carried out at 25°C.

2.3.3. Measurement of the methylviologen reduction rate

A cuvette containing the surfactant was deaerated by 3 repeated cycles of 45 seconds evacuation and 15 seconds of flushing with oxygen free argon. Organic solvents were deoxygenated separately to avoid evaporation from the reversed micellar medium, and were added anaerobically to the surfactant. Porphyrin was added to a concentration of 50 μM ($\epsilon_{426nm} \approx 10^5 M^{-1}cm^{-1}$). The final overall concentration of methylviologen was 1 mM and the concentration of tributylamine was 100 mM. Again the final phosphate buffer concentration (pH = 7.0) in the aqueous phase was 50 mM. The cuvette was placed in an Aminco DW2a spectrophotometer and the system was side-illuminated with a 150 W halogen lamp. (Fi-L151, Fiberoptic Heim AG). The incident light in the cuvette was measured by Reinecke salt actinometry (Wegner and Adamson, 1966). The intensity was 5.3×10^{-7} mol/s for white and 1.8×10^{-8} mol/s for blue (340-490 nm) light.

2.3.4. Measurement of the enoate reductase activity

The activity of the enoate reductase as such was determined by following spectrophotometrically the rate of NADH oxidation. When the enzymatic system was coupled to the photosystem (Fig. 2.2) reduced methylviologen operated as electron donor for the enzyme and the conversion of substrate into product was monitored.

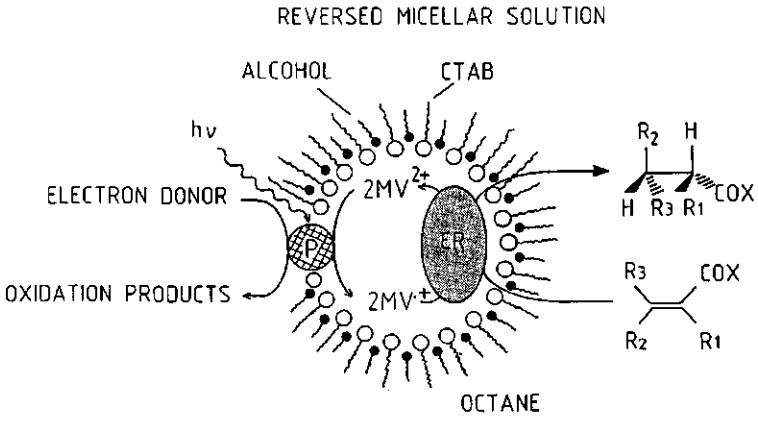

Fig. 2.2. Schematic representation of the photo-enzymatic conversion of enoates by enoate reductase in a reversed micellar medium (not to scale). The enzyme is located in the reversed micellar core. The substrate for the enzyme can be located in either phase, depending on its polarity, size and acidity. At the experimental conditions ([micel] ≈ 2 mM, [porphyrin] = 50 μM, [enoate reductase] = 5 nM) the photochemical methylviologen reduction and enzymatic oxidation are likely to take place in different reversed micelles between which rapid exchange of reactants occurs.

Cinnamic acid and 3-phenylpropanoic acid were separated on HPLC (HP 1081B Liquid Chromatograph, RP18, MeOH:H_2O 1:1 (vol/vol) pH=3, 258 nm). Stoichiometric enzymatic conversion of 2-methylbutenoic acid into 2-methylbutanoic acid was proven by GC (Packard 433) using a Chromosorb W-HP100-120, 8.9% Carbowax HP-column. The rate of enzymatic conversion was routinely determined by monitoring the disappearance of substrate from the reaction medium by HPLC (HP 1081B Liquid Chromatograph, RP18, MeOH:H_2O 2:3 (vol/vol) pH=3, 204 nm). CTAB was removed from 50 μl reversed micellar solution by precipitation with 200 μl 2-methylbutane to obtain a good resolution. After transfer of the supernatant the organic solvent was evaporated and the residue was dissolved in eluent.

2.3.5. Determination of the operational and storage stability of enoate reductase

The stability of the enoate reductase towards storage and operation was measured by following the oxidation of NADH. A 4 ml standard reversed micellar solution (w_o=8.3) or 4 ml of an aqueous solution (50 mM potassium phosphate buffer pH 7.0), containing enoate reductase (0.2 U) and 2-methylbutenoic acid (10 mM) was incubated during 24 hours at 25°C. At intervals (800 μl) were anaerobically transferred to a deaerated cuvette. Subsequently the reaction was started with 5 μl (final concentration 0.2 mM) NADH. The final w_o value was 10. When the storage stability of the enoate reductase in its reduced state was measured, the enzyme was incubated in the presence of 0.2 mM NADH and the reaction was started with 2-methylbutenoic acid (final concentration 7.5 mM).
The operational stability was determined in a similar experiment. In this case the rate of NADH oxidation was monitored in time . The quantities of enzyme and substrate were chosen in such a way that no substrate limitation occurred during the experiment.

2.3.6. Photoinduced enzymatic conversion of 2-methylbutenoic acid

To the standard reversed micellar medium 2-methylbutenoic acid, (10 mM), ZnMDPyTrPP (final concentration 50 μM), tributylamine (100 mM) and methylviologen (2 mM) were added. By illumination of the cuvette methylviologen reduction was started and immediately enoate reductase (0.2 U) was added while vortexing the solution. Samples, drawn anaerobically, were analysed as described.

2.4. RESULTS & DISCUSSION

Reports on the enzymatic conversion of apolar substrates in reversed micelles (for reviews see Luisi, 1985; Luisi and Laane, 1986; Martinek *et al.*, 1986) mostly deal with initial enzyme activity of esterases and hydrolases and, in a few cases, dehydrogenases. Following such reactions over a long period of time is hampered by the fact that in particular dehydrogenases need a cofactor. Only when this cofactor is regenerated, such reactions are feasible.
Hilhorst *et al.* (1983) described a coupled enzyme system for the reduction of steroids, in which cofactor regeneration was performed *via* hydrogenase and lipoamide dehydrogenase using hydrogen gas as the ultimate reductant. Later, cofactor regeneration was simplified by the replacement of these two enzymes by hydrogenase from *Alcaligenes eutrophus*, reducing NADH directly

with hydrogen gas (Laane and Verhaert, 1987). An alternative way for the generation of reducing equivalents is the use of a photochemical electron transport system (Hilhorst et al., 1982).
The enoate reductase can use the mediator methylviologen instead of NADH as a source of reducing equivalents for the stereospecific reduction of enoates. In Fig. 2.2 a schematic representation of our photo-enzymatic system is shown.
The photosystem consists of tributylamine as an sacrificial electrondonor, Zn(II) *meso*-mono(4-N-decylpyridyl)-tri(phenyl)porphyrin (ZnMDPyTrPP, Fig.2.1) as the photosensitizer and methylviologen as electron acceptor. The donor is preferentially located in the organic phase because of its apolar character, whereas the acceptor is confined to the aqueous phase. Electron transport between donor and acceptor is mediated by the photosensitizer and will be most effective when this compound is located between the two phases (Hilhorst et al., 1982).
This photosystem transports electrons from the organic phase to the water pool of the reversed micelles, where the enzyme enoate reductase is located. To create an effective photochemically driven enzyme system the composition of the micellar medium must be such that both the photochemical and the enzymatic reactions are tuned optimally to one another.

2.4.1. Optimization of the photosystem
Since photochemical electron transfer is most effective when a sensitizer located in the interphase is used, a porphyrin modified with a decyl chain was used, assuming that the decyl chain will anchor the porphyrin in this interphase. Irradiation of a micellar system containing this ZnMDPyTrPP and the electron donor tributylamine in the organic phase resulted in a rapid formation of reduced methylviologen in the water pools.
As a control the ZnMDPyTrPP was replaced by the water soluble porphyrin ZnTMPyP. In this case no accumulation of reduced methylviologen was observed, probably because electron donation (Eq. 3) cannot compete efficiently anymore with the back reaction (Eq. 2b) in the water pool. Brochette and Pileni (1985) showed that upon illumination of an AOT-reversed micellar system containing water soluble ZnTMPyP as the sensitizer, methylviologen was reduced only when reaction (2b) was prevented by the addition of NADH as a water soluble electron donor.

In our reversed micelles with ZnTMPyP as sensitizer and tributylamine as electrondonor, this electron donor is not able to compete with the backward reaction, implying that the donor is not in the vicinity of the oxidized porphyrin. Reductive quenching of the excited state of the porphyrin by tributylamine is very unlikely because, even at a very high concentration of tributylamine (>100 mM), no fluorescence quenching of either ZnMDPyTrPP or ZnTMPyP could be observed. Hence, the successful reduction of methylviologen using the sensitizer ZnMDPyTrPP is a strong indication that the alkylated porphyrin is located in the interphase, that is, close to both tributylamine and methylviologen.

The reversed micellar solution used in this investigation, is composed of the surfactant CTAB, the organic solvent n-octane and an aliphatic alcohol as cosurfactant. The aliphatic alcohols partition between the continuous organic phase and the interphase. Varying the chain length and/or the quantity of alcohol induces rather large changes in the polarity of the interphase and the organic phase. The log P value as defined by Rekker (1977) was used as polarity indicator. Log P has been proven to be a useful

parameter for the polarity of organic solvents in relation to biocatalytic activity (Laane, 1987). Since the exact composition of the interphase and the continuous phase in the reversed micellar media used can only be determined at a phase boundary, we have related the quantum yield of the photosystem to the composition of the continuous phase and the interphase. Increasing the percentage of alcohol in the system leads to an increase in polarity of both the interphase and continuous phase. The overall log P value of the mixture was approached using the equation:

$$\log P = \Sigma_i X_i \log P_i \qquad (4)$$

in which X_i and $\log P_i$ are the molar fraction and the log P value for component i (Rekker, 1977; Hilhorst et al., 1984).
For octane a log P value of 4.5 was used, for CTAB -0.66 and for the alcohols butanol, pentanol, hexanol, heptanol and octanol, log P values of 0.8, 1.3, 1.8, 2.4 and 2.9 respectively.

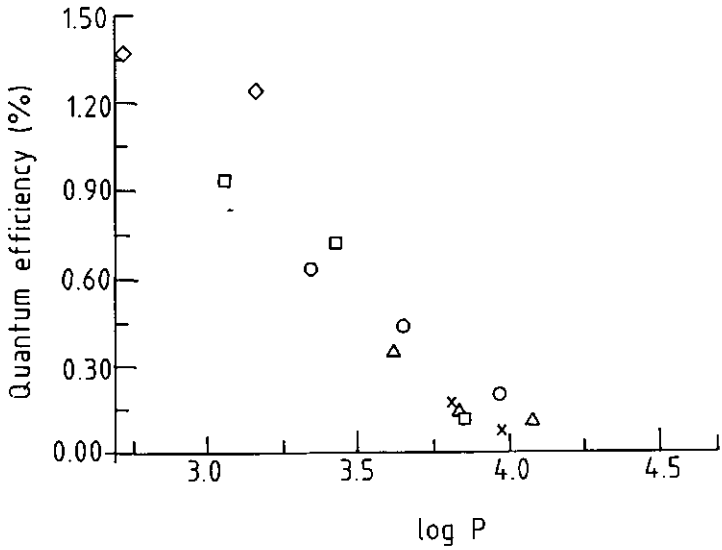

Fig. 2.3. Quantum efficiency of the photochemical methylviologen reduction in reversed micelles of differing composition.
Both the chain length and the percentage of the n-alkylalcohols were varied. The volume fractions of all alcohols with respect to the total amount of organic solvent were 10, 20 and 30 %, for butanol only 20 % and 30 % were used. For each composition the log P value of the medium has been calculated using Eq.4. High log P values correspond to the lower alcohol concentrations. In all cases the concentration of methylviologen was 1.0 mM, [ZnMDPyTrPP]=50 μM, [tributylamine] = 100 mM. For further experimental details: see section Materials and Methods.
◇--◇ *butanol*, □--□ *pentanol*, ○--○ *hexanol*, △--△ *heptanol*, ×--× *octanol*

The initial rate of methylviologen reduction was measured in reversed micellar media of different alcohol content. Varying the methylviologen concentration from 1 to 10 mM caused no significant difference in rates of reduction, thus the presence of methylviologen was not rate limiting. To study the effect of the polarity of the medium on the electron transport either butanol, pentanol, hexanol, heptanol or octanol was used as cosurfactant.
The rates of methylviologen reduction in several micellar media were converted to quantum yields, using Reinecke salt actinometry (Wegner and Adamson, 1966) and are shown in Fig. 2.3.

The yield of the photosystem with ZnMDPyTrPP as sensitizer is directly related to the polarity of the reversed micellar solution: The more polar the organic phase and the interphase (the lower the log P value), the higher the rate of electron transfer to methylviologen. Apparently only the polarity and not the chain length of the alcohol present determine the quantum yield of the system.

From Fig. 2.3 it can be concluded that the highest yield is obtained in a reversed micellar medium containing 30 % butanol. In this solution the quantum efficiency is 1.4 %.
In a slightly different system Hilhorst and coworkers (1982) obtained a quantumefficiency of 1.3 % for hydrogen production using a surfactant ruthenium complex as sensitizer, but using an apolar porphyrin the efficiency dropped to 0.26 %. Aono et al. (1986) reported a quantum yield of 1.1 % in aqueous (CTAB) micelles using hematoporphyrin as photosensitizer.

2.4.2. Optimization of the enoate reductase activity

Enoate reductase has a molecular mass of 960 (± 20) kDa and is composed of at least 12 subunits of 73 kDa; it can dissociate into subunits of 230 kDa (Kuno et al., 1985), but these subunits retain at most 10 % of the activity of the native enzyme.
Since the specific activity of enoate reductase and the turnover rates in both micellar and aqueous media are not altered, we conclude that the intact enzyme complex is encapsulated, making enoate reductase one of the largest enzymes that has been successfully incorporated in reversed micelles.
Enoate reductase in reversed micelles is capable of converting both polar and apolar substrates, using either methylviologen or NADH as a source of reducing equivalents. For an efficient photo-enzymatic system, both the light driven system and the enzyme must function well and be stable over a longer period of time. We studied these properties of enoate reductase using different compositions of the reversed micellar system, by varying the chain length and percentage of the cosurfactant in the reversed micelles. The effect on the activity of enoate reductase is given in Fig. 2.4.

There is a marked change in activity due to both the nature and the concentration of the cosurfactant. Although there is a clear dependence of the enzyme activity on the composition of the reversed micelles, no straightforward relation with a change in polarity (log P) of the system was observed.

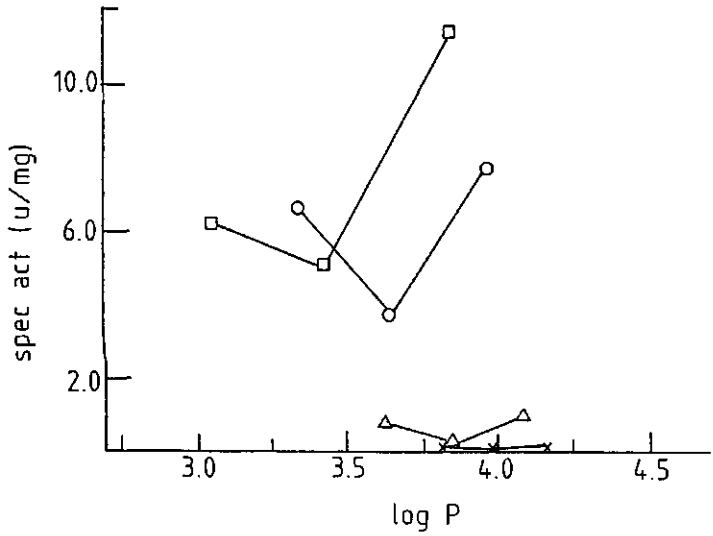

Fig. 2.4. Dependence of enoate reductase activity on the polarity of the reversed micellar medium.
The initial specific activity of the enoate reductase in reversed micelles of different cosurfactant composition is shown. The volume fractions were the same as for Fig. 2.3. [NADH]=0.2 mM, [2-butenoic acid]= 10 mM.

□--□ *pentanol*, O--O *hexanol*, Δ--Δ *heptanol*, ×--× *octanol*

However, two striking facts emerge: (a) the enzymatic activity decreases with increasing chain length of the cosurfactant. (b) the activity curve exhibits a minimum for all alcohols. In all experiments, the concentration of the cosurfactant to octane was 1:4 (vol/vol) for this minimum.
The substrate 2-butenoic acid is confined to the water pool, so, in contrast to the conversion of apolar compounds, not substrate transport across the interphase, but the exchange between the water supplies the enzyme with substrate. Therefore the dynamics of the reversed micellar medium, which are influenced by the type and concentration of cosurfactant, govern the rate of the reaction, and not the log P value.

Concerning the enzyme activity it is noteworthy that enoate reductase showed no activity at all in a reversed micellar medium containing butanol as cosurfactant. When pentanol was used, the half life was less than one minute. Also, in aqueous solutions the addition of butanol and pentanol led to immediate inactivation whereas hexanol had hardly any effect. This is in accordance with the theory formulated by Laane and coworkers (1987a).
The effect of the concentration of alcohol in the system on the enzyme activity was investigated in detail using hexanol. We prepared enoate reductase solutions in different reversed micellar environments, mixed these enzyme solutions with reversed micelles of a different composition in a 1:1 ratio and immediately, (thereby excluding long term stability differences) measured the enoate reductase activity.

Table 2.1. Effect of the hexanol concentration in reversed micelles on the enoate reductase activity.

Hexanol during assay (%)(vol/vol)	*Hexanol in enzyme stock (%)(vol/vol)*	*Hexanol in second solution (%)(vol/vol)*	*Enzyme activity (U/mg)*
10	*10*	*10*	*6.8*
15	*15*	*15*	*3.4*
	20	*10*	*3.4*
20	*20*	*20*	*3.0*
	25	*15*	*2.6*
	30	*10*	*2.5*
25	*25*	*25*	*3.7*
	30	*20*	*3.4*
30	*30*	*30*	*8.9*

Enoate reductase was introduced in a reversed micellar solution containing the indicated % of the hexanol, wo = 10 and immediately transferred to a second solution containing a different % of hexanol and 2-butenoic acid and NADH (0.6 mM and 0.2 mM respectively). Standard deviation 10%.

The enzyme activity in the reversed micelles follows the same pattern as given in Fig. 2.4 (Table 2.1); the enzyme also exhibits a minimum activity at a hexanol:octane ratio of 1:4 (vol/vol).
Furthermore, the enzyme activity depended only on the hexanol content during the assay, and was not affected by the concentration of hexanol at the moment of incorporation. (E.g. the activity of enoate reductase prepared in hexanol:octane = 1:4 (vol/vol) and mixed with an equal volume of a solution of hexanol:octane = 1:9 (vol/vol) is the same as the activity of the enzyme directly incorporated in a hexanol:octane = 15:85 (vol/vol) solution.) So no immediate irreversible inhibition of the enzyme in reversed micelles by hexanol occurs, but the possibility of reversible inhibition cannot be excluded. It seems, however, that a change in properties of the micellar system itself is responsible for the change in enzyme activity.

2.4.3. Stability

For a high yield of products it is not only necessary that the enzyme exhibits a high initial activity, but also an activity that is preserved over a long period of time. Thus, in optimizing the system not only the initial activity but also the stability of the enzyme during storage and operation has to be taken into account. The enzyme was stored anaerobically in a reversed micellar solution or in an aqueous solution both containing phosphate buffer pH=7.0 to investigate the stability of the enzyme during storage. In both solutions the substrate 2-methylbutenoic acid was present but the cofactor NADH was not added. After storage for 3, 6 and 24 h at 25°C, the residual activity was measured by adding NADH.
The best results (cf. Table 2.2) were obtained with a reversed micellar solutions with heptanol or octanol as cosurfactant, where no activity was lost after 24 hours of storage at ambient temperature.
For hexanol the activity depended on the concentration of the cosurfactant. At hexanol:octane = 1:9 and 1:4 (vol/vol) only a slight decline in activity

was observed, whereas at higher concentration of the cosurfactant (3:7), 50 % of the activity disappeared during the first 24 h. Hence the half life of the enzyme in reversed micelles is at least 24 h. It must be emphasized that the enoate reductase in the aqueous, buffered solution also lost 50 % of its activity in 24 h. So, at room temperature the storage stability of the enoate reductase in reversed micelles in the presence of substrate is at least equal to that in aqueous solution.

Table 2.2. Stability of enoate reductase in various media.

Medium	*Storage stability activity after 24 h (%)*	*Operational stability $t_{\frac{1}{2}}$ (min)*
10% Hexanol	*95*	*45*
20% Hexanol	*95*	*45*
30% Hexanol	*50*	*30*
10% Heptanol	*100*	*30*
20% Heptanol	*100*	*30*
30% Heptanol	*100*	*30*
10% Octanol	*100*	*15*
20% Octanol	*100*	*15*
30% Octanol	*100*	*15*
aqueous buffer	*50*	*30*

Storage stability: Enoate Reductase was stored in reversed micelles with varying cosurfactant content, containing 10 mM 2-methylbutenoic acid. The initial activity of the enzyme at time zero and after 24 h at 25 °C were compared using NADH as cofactor. Operational activity: 2-Methylbutenoic acid (final concentration 10 mM) and NADH (0.2 mM) were added to a standard reversed micellar solution prior to addition of enoate reductase. The rate of oxidation of NADH was followed in time spectrophotometrically. For further experimental details: see section 2.3, Materials and Methods.

The operational stability of the enzyme is expressed as the half life of the enzyme in operation, i.e. in the presence of 2-methylbutenoic acid and NADH. In reversed micellar solutions of differerent composition and in aqueous buffer the pattern of the NAD^+ formation was recorded. The operational stability of the enzyme in aqueous solution and in reversed micelles prepared with hexanol as cosurfactant was the same ($t_{\frac{1}{2}} \approx 45$ min.). The activity in heptanol and octanol reversed micelles decreased more rapidly as compared to aqueous solution and hexanol reversed micelles ($t_{\frac{1}{2}} \approx$ 30 and 15 min. respectively). Thus for an optimal operational stability a hexanol containing reversed micellar solution proved as good as buffered aqueous solution.
Whereas an increasing chain length of the cosurfactant leads to a more rapid loss of enzyme activity under operational conditions, it stabilizes the enzyme under storage. As it is well known that enzymes in their reduced

state are more labile, the influence of NADH on storage stability was investigated. Indeed it was observed that the reduced enzyme was more susceptible to inactivation in the reversed micellar medium. These results explain the striking difference between the medium in which high storage stability (in the presence of substrate but without NADH) was observed, and the medium showing the highest operational stability.
The operational stability, therefore, will be enhanced using a medium in which the enzyme is present in the oxidized state i.e. an excess of substrate and a limited amount of cofactor

In summary: for enoate reductase the conditions most suitable for the enzymatic reaction are a reversed micellar with a hexanol to octane ratio of 1:9 (vol/vol). Although a system containing 30 % (vol/vol) hexanol also gave a high activity, it is less suitable because of the lower operational stability of enoate reductase in this medium.

2.4.4. Photo-enzymatic enoate reduction

The aim of this study was to devise a photo-enzymatic system for the reduction of enoates. Optimization of the separate parts of the system indicated that the photosystem functioned best in reversed micelles containing a high percentage of a short chain alcohol. These results are in conflict with the optimum composition for enoate reductase, as the stability of this enzyme was adversely affected by butanol, pentanol and high percentages of hexanol. The use of a reversed micellar solution containing
10 % (vol/vol) hexanol is a good compromise, provided that the enzyme activity is not affected by the components of the photosystem.
No inhibition of the NADH dependent reduction of 2-methylbutenoic acid by enoate reductase in reversed micelles was observed in the presence of components of the photosystem.
Studying the photoinduced biocatalytic conversion of both cinnamic acid and 2-methylbutenoic acid in this medium showed that 100 % conversion could be obtained. No conversion at all was observed when enoate reductase was omitted (see also Fig. 2.5).

The conversion of the more polar substrate 2-methylbutenoic acid was studied in more detail. Figure 2.5 shows the course of the reduction reaction of 2-methylbutenoic acid.
As a control experiment, 2-methylbutenoic acid was enzymatically reduced in an aqueous solution using NADH as a cofactor (10 mM). 2-Methylbutenoic acid cannot be reduced by such a quantity of NADH in reversed micelles, due to the limited solubility of the cofactor in this medium. Photoinduced enzymatic conversion is prohibited in aqueous solution since no net charge separation occurs (Fig. 2.5).
The enoate reductase remained active for over 100 h. This indicates that the enzyme in reversed micelles is dominantly present in the oxidized state. The turnover number for the enzyme both in reversed micelles and in aqueous solution was over 1.1×10^6. Thus the operational stability of the enzyme in aqueous solution and in this reversed micellar medium is the same.

In this investigation we have shown that the enzyme enoate reductase is operational in reversed micelles. The use of a photochemical reduction reaction of methyl viologen provides an efficient way to drive the

reaction. Furthermore it was demonstrated that a photochemical system can be designed and exploited without any harmful effect on the operational stability of the enzyme enoate reductase. This technique opens the possibility to use such a photoenzymatic system for the stereospecific reduction of a great variety of enoates.

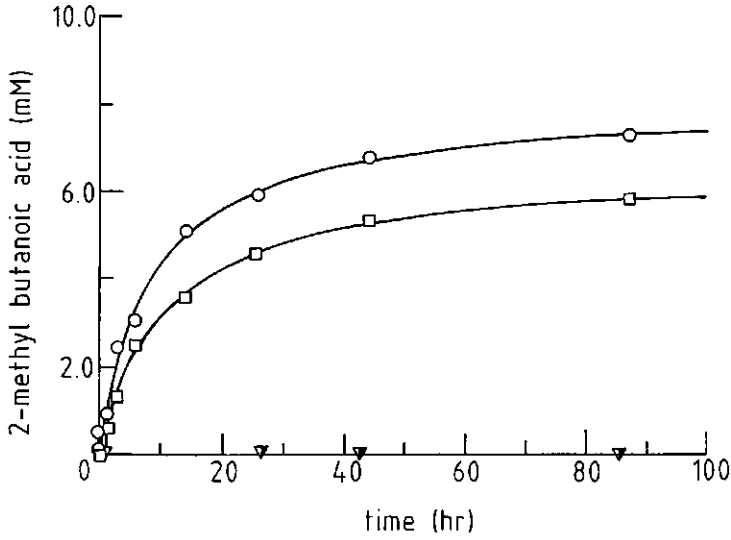

Fig. 2.5. Time course of the methylviologen dependent photoinduced enzymatic reduction of 2-methylbutenoic acid in reversed micelles and in aqueous solution as compared to the NADH-driven reaction in aqueous solution. a) O--O NADH dependent reduction, [NADH]=10 mM, [2-methylbutenoic acid] = 10 mM; b) □--□ enzymatic reduction of 2-methylbutenoic acid in reversed micelles using photochemically regenerated methylviologen [MV]=2mM, [2-methylbutenoic acid] = 10 mM, [tributylamine] = 100 mM, [porphyrin] = 50 μM. c) ∇--∇ photo-enzymatic reaction in aqueous solution. d) ▼--▼ as b) but no enoate reductase present.

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3. PHOTOINDUCED CHARGE SEPARATION AND SPECTROSCOPIC PROPERTIES OF PORPHYRINS IN REVERSED MICELLES

In this Chapter two porphyrins and their role in photoinduced charge separation in reversed micelles are investigated. In Chapter 3.1 the effect of a hexadecyl chain as a porphyrin tail is studied in relation to its efficiency in photoinduced charge separation. In Chapter 3.2 the fluorescence properties of these porphyrins are considered in detail.

3.1. EFFICIENCY OF PHOTOINDUCED CHARGE SEPARATION IN RELATION TO THE PORPHYRIN ENVIRONMENT IN REVERSED MICELLES

3.1.1. SUMMARY

The efficiency of photoinduced charge separation of a photosystem incorporated in reversed micelles is related to the composition of the micellar solution. The reversed micellar medium was composed of cetyltrimethylammonium bromide, n-alcohol, octane and water. In the photosystem trialkylamines were used as electron donor, methylviologen as electron acceptor and porphyrin as sensitizer.
The environment of the porphyrin has been studied by ^{19}F-NMR spectroscopy. In reversed micelles, the porphyrins are localized in the interphase. A porphyrin extended with a hexadecyl chain is tightly anchored in this interphase and only probes polarity changes in the hydrophobic regions of the interphase of the boundary layer. An untailed porphyrin has an increased mobility and probes a more polar environment, indicating a location close to the polar surfactant head groups in the interphase. The polarity of the environment and the mobility of the porphyrins as well as the efficiency of the charge separation increase upon increasing the concentration and/or the polarity of the alcohol in the micellar medium. No effect on the photo efficiency was observed on anchoring the porphyrin in the interphase. These findings indicate that the flexibility of the reversed micellar system and the polarity of the interphase are more effective in determining the rate of the photoinduced charge separation reaction than either the mobility or the interfacial location of a porphyrin in the interphase of the reversed micelle.

3.1.2. INTRODUCTION

In the past years there have been numerous photochemical studies on the use of porphyrins in solar energy conversion and storage, because they are stable, have a high extinction coefficient in the visible region and a high yield of a reactive high energy excited state. Their redox, photochemical and solubility properties can be manipulated relatively easy by chemical modification [1]. For an overview see Dolphin [2].
The first step in photoinduced energy conversion is charge separation, *i.e.* transfer of an electron from an excited donor to an acceptor, followed by a slower charge recombination.
The charge separation can be described by the following reactions:

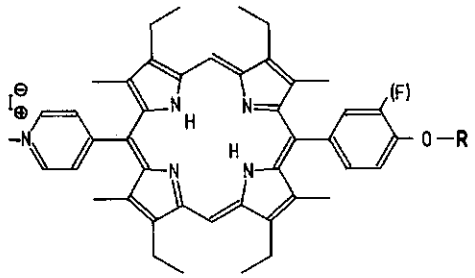
$$P \xrightarrow{h\nu} P^* \qquad (1)$$

$$P^* + A \xrightarrow{k_{CS}} P^+ + A^- \qquad (2)$$

$$P^+ + A^- \xrightarrow{k_{CR}} P + A \qquad (3)$$

$$P^+ + D \xrightarrow{k_{PR}} P + D^+ \qquad (4)$$

in which k_{cs}, k_{cr} and k_{PR} are the rates of charge separation, charge recombination and porphyrin rereduction, respectively.
The advantages of micro-heterogeneous media, *e.g.* reversed micelles (1-10 nm sized water droplets dispersed in an organic phase by a detergent) for efficient charge separation reactions have been demonstrated by several groups [3-7].
In reversed micelles not only stabilization of the charge transfer complex but also spatial separation of the products by intermicellar exchange inhibits the backward, charge recombination, reaction. In such a case subsequent reduction of the primary donor by a second donor is favoured [8,9]. Therefore the net photoinduced charge transfer is more efficient, that is the yield, Φ_{cs} is higher using a photosystem in which the photosensitizer, the electron donor and the electron acceptor are in a different phase. In the most efficient systems the sensitizer is located in interphase. [3,10,11]. Reversed micelles have the advantage that they are optically transparent allowing spectroscopic studies of the effects of the composition of the aqueous phase [8,12,13], interphase [13] and organic phase [12] on the photophysics of the system.
In a previous paper we reported that the polarity of the reversed micellar medium (expressed as logP) affects the net efficiency of the photoinduced charge separation [14], using an apolar donor (tributylamine), a surface active zinc porphyrin and methylviologen as an electron acceptor. A variety of reversed micelles were used, differing in the quantity and structure of the cosurfactant used to stabilize the reversed micelle. A more polar cosurfactant or a higher concentration of cosurfactant resulted into a higher rate of methylviologen reduction.



P_1 R=CH_3 P_{16} R=$C_{16}H_{33}$

Fig. 3.1.1 The structure of the porphyrins. $^{(F)}P_1$*: 5-[4-N-methylpyridiniumiodide]-15-[4-[methoxy]-(3-fluoro)phenyl]-2,8,12,18-tetraethyl-3,7,13,17-tetramethylporphyrin,* $^{(F)}P_{16}$*: 5-[4-N-methylpyridyl]-15-[4-[hexadecyloxy]-(3-fluoro)phenyl]-2,8,12,18-tetraethyl-3,7,13,17-tetramethylporphyrin*

In order to understand this phenomenon, the rate of methylviologen production was investigated in a variety of reversed micellar solutions was investigated. Two porphyrins, P_1, and P_{16}, containing a methyl or hexadecyl chain respectively (Fig. 3.1.1), were used. ^{19}F-NMR was used to study the chemical shift of fluorinated porphyrins yielding time-averaged information about the environment of the porphyrins. The chemical shifts of two

fluorinated aldehydes 3-fluoro-4-methoxybenzaldehyde and 3-fluoro-4-hexadecyloxybenzaldehyde were compared to those of two fluorinated porphyrins containing either a methoxy group or a hexadecyloxy-tail in order to derive a general relation between the polarity of the environment and the fluorine chemical shift. The chemical shifts were related to the polarity changes of the environment of the porphyrins in reversed micelles. Furthermore the chemical shifts were measured in reference solutions: Aqueous micelles, aqueous micelles containing a small quantity of organic solvent and a solution of detergent in alcohol. In this paper the chemical shifts are referred to using the expressions higher and lower frequency, for downfield and upfield shifts respectively. The efficiency of the photosystem was determined by measuring the rate of photoinduced reduction of methylviologen in a system containing porphyrin, electron donor and electron acceptor.
The aim of the study was to determine the influence of the composition of the reversed micelle on the location and mobility of porphyrins in relation to the yield of photoinduced charge separation.

3.1.3. MATERIALS AND METHODS

3.1.3.1. Porphyrins

5-[4-N-methylpyridiniumiodide]-15-[4-[alkoxy]-(3-fluoro)phenyl]-2,8,12,18-tetraethyl-3,7,13,17-tetramethylporphyrins (Fig. 3.1.1) were synthesized by means of a mixed condensation of 3,3'-dimethyl-4,4'-dipyrrolylmethane with 4-pyridinecarboxaldehyde and 4-alkoxybenzaldehyde in methanol [15,16]. The reaction was carried out using an appropriate amount of p-toluenesulphonic acid as catalyst. Treatment of the reaction mixture with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone yielded a mixture of porphyrins which was separated using silicagel column chromatography. Finally the porphyrin was methylated using methyliodide.
The fluorinated aldehydes were synthesized following published methods [17].

3.1.3.2. Other chemicals

Methylviologen was purchased from Sigma Chemical Co. (St Louis, Mo, USA). Cetyltrimethylammonium bromide (CTAB) from Serva (Heidelberg, W. Germany) was used without further purification. Chloroform, all alcohols and *n*-octane were from Merck, Darmstad, W. Germany. Alcohols and octane were distilled before use. Triethylamine was from EGA-chemie, Albruch, W. Germany, tributylamine and 2,2,2-trifluoroethanol from Aldrich Chemie, Steinheim, W. Germany and trioctylamine from Janssen Chimica, Beerse, Belgium. Tetraethylammonium tetrafluoroborate was purchased from Fluka AG, Buchs, Switserland.

3.1.3.3. Preparation of reversed micellar media

Stock solutions of reversed micelles were prepared by mixing 2.2 g CTAB (6 mmol) in 30 ml of a cosurfactant-octane mixture and adding 540 μl of a 50 mM potassium phosphate buffer of pH 7.0. This amount of buffer corresponds to a water to detergent molar ratio (w_o) of 5. A series of aliphatic alcohols (butanol to octanol) was used as cosurfactant. The volume fraction of the cosurfactant in the mixture cosurfactant-octane is used to indicate the composition of the different reversed micellar media. A reversed micelle system containing 20% pentanol (= pentanol:octane = 1:4 (vol/vol)) was used as a standard reversed micellar solution.
Unless stated otherwise, all concentrations are expressed with respect to

the total volume of the reversed micellar medium and not with respect to either the organic or the aqueous phase.

3.1.3.4. Preparation of reference solutions
Reference solutions of CTAB in aqueous solvent were prepared by dissolving 10 mM CTAB in 50 mM phosphate buffer pH=7.0. Pentanol and octane swollen aqueous micelles were prepared by adding 50 mM of pentanol or octane to this solution.
The reference solution of CTAB in pentanol contained 0.1 M of the detergent.

3.1.3.5. Determination of redox potentials
Cyclic voltametry was performed on a computer controlled Autolab 110 potentiostat (Eco-Chemie, Utrecht, The Netherlands) using a glassy carbon measuring electrode, a platinum counter electrode and an Ag/AgCl reference electrode embedded in a salt bridge of 0.1 M KCl in agar. The porphyrins were dissolved in methanol containing 0.1 M tetraethylammonium tetrafluoroborate as a conduction salt. The oxidation potentials were measured on the anodic wave.
The oxidation potentials of triethylamine, tributylamine and trioctylamine were measured in aqueous solution containing 0.1 M tetraethylammonium tetrafluoroborate. All potentials were recalculated relative to the standard hydrogen electrode (NHE).

3.1.3.6. ^{19}F-NMR
^{19}F-NMR measurements were carried out on a Bruker CXP 300 spectrometer equipped with a 10 mm Bruker ^{19}F observe channel, a proton decoupling probe and a Z20A preamplifier. Measurements were performed at 282 MHz on 2.2 ml samples in Wilmad 10 mm precision NMR tubes. A (glass) insert containing D_2O was used to lock the magnetic field. Proton decoupling was achieved with the Waltz-16 pulse sequence [18] at -16 dB from 20 W with a home built hardware pulse generator circuit [18]. The spectra were recorded using 30 degree pulses of ±200 W (6 μs), a 20 kHz spectral width, a repetition time of 1 s, quadrature phase detection and quadrature phase cycling. For each measurement of the resonance position of the porphyrin at least 10,000 scans were recorded. The free induction decays were multiplied by an exponential decay resulting in an increase of the linewidth of 5 Hz.
In all P_1 porphyrin samples the concentration was 50 μM, in samples containing P_{16} the concentration of the probe was 100 μM. All chemical shifts were determined relative to 2,2,2-trifluoroethanol.

3.1.3.7. Photoinduced methylviologen reduction
Porphyrin was dissolved in chloroform and the desired quantity was pipetted into a cuvette. After evaporation of the chloroform 3 ml of a stock solution of reversed micelles was added. Methylviologen (in phosphate buffer) and electron donor were added till the desired concentration. The final overall concentrations were for porphyrin 50 μM, for methylviologen 4 mM and for the electron donor 50 mM. Phosphate buffer (pH = 7.0, 50 mM) was added till a final w_o of 10. Subsequently the cuvette was deaerated by 3 repeated cycles of 45 seconds evacuation and 15 seconds of flushing with oxygen free argon, and placed in an Aminco DW2a spectrophotometer. The system was side-illuminated with a 150 W halogen lamp (Fi-L151, Fiberoptic Heim AG). The formation of reduced methylviologen ($d[MV^{+\cdot}]/dt$) was determined by measuring the increase in absorption at 602 nm, using an extinction coefficient of 13.6×10^3 $M^{-1}cm^{-1}$ [19]. At the conditions used ($\epsilon cl>5$):

$$\Phi_{cs} = \frac{d[MV^{+\cdot}]/dt}{I_A} \qquad (5)$$

In which I_A, the intensity of the absorbed light, was 5.3×10^{-7} (M s^{-1}), as determined by Reinecke salt actinometry [20].
Experiments were carried out in duplicate at 25°C.

3.1.4. RESULTS

3.1.4.1. ^{19}F-NMR of aldehydes and porphyrins

A convenient way to probe the environment of a porphyrin is the measurement of the chemical shifts of NMR-sensitive nuclei of the porphyrin. We have introduced a fluorine atom close to the tail of the porphyrin (see Fig. 3.1.1) to determine both the effect of the tail and the effect of the composition of the medium on the location of the porphyrin. The polar moiety of the molecule is preferentially located at the polar side of the reversed micelle [12].
The effect of a long alkyl chain on the molecule was studied measuring the chemical shift of two model compounds: 3-fluoro-4-methoxybenzaldehyde and 3-fluoro-4-hexadecyloxybenzaldehyde in homogeneous solutions of chloroform, methanol, pentanol and octanol. The positions of the resonances are summarized in Table 3.1.1.
In chloroform the resonance of 3-fluoro-4-methoxybenzaldehyde was observed at lower frequency than that of 3-fluoro-4-hexadecyloxybenzaldehyde. This pattern was found in all measurements: The presence of an hydrophobic alkyl chain induced a shift to a higher frequency.
A decreasing polarity of the alcohol solutions resulted in a decreased resonance frequency for both aldehydes. The difference between the methyl and the cetyl aldehyde became slightly smaller (Fig. 3.1.2A).

Subsequently the chemical shifts of the fluorinated P_1 and P_{16} (Fig. 3.1.1) were measured in homogeneous solution, using the aldehyde as reference. A single resonance was observed in all samples, implying that exchange phenomena are fast compared to the scale of the NMR measurement ($k \geq 10^3$ s^{-1}).
The presence of the porphyrin moiety induces a shift of approximately 3 ppm to a lower frequency (Table 3.1.1).
The dependence of the chemical shift on the nature of the solvent was the same for the aldehyde models and for $^FP_1$ and $^FP_{16}$ (Table 3.1.1). Clearly, the polarity of the medium is the main factor determining the position of the fluorine resonance.
The fluorine resonances of the porphyrins, $^FP_1$ and $^FP_{16}$, in reversed micelles were measured in comparison to those in homogeneous solutions. All resonances were shifted downfield, indicating a less polar environment (Table 3.1.1) (see also section 3.1.5. Discussion). At higher concentrations of alcohol in the reversed micellar medium, the polarity of the porphyrin environment increases, but the variation is relatively small (*cf.* ordinate scales of Fig. 3.1.2A and Fig. 3.1.2B). Furthermore the shift of the fluorine resonance of the porphyrins hardly changes in the presence of different cosurfactants.
The most widely studied variable in reactions in reversed micelles is the molar ratio of water to detergent (w_o). Using three different values for this ratio, no change in the fluorine shift was observed (Table 3.1.1). This effect was expected because the fluorine is located in the alkyl moiety, which is most likely positioned in the surfactant layer.

Table 3.1.1 Chemical shift of 3-fluoro-para-hexadecyloxybenzaldehyde, 3-fluoro-para-methoxybenzaldehyde and fluorinated P_1 and P_{16} porphyrin in reversed micelles and reference solutions.

The shift of the resonance is given relatively to 2,2,2-trifluoroethanol. Due to solubility problems the concentrations of the porphyrins in the aqueous CTAB micelles and in the pentanol-octane mixture were lower than 10 μM. Chemical shifts are ± 0.02 ppm. Experimental conditions are described in section Materials & Methods.

	chemical shift (ppm)	
3-FLUORO-4-ALKOXYBENZALDEHYDES	*Methoxyaldehyde*	*Hexadecyloxyaldehyde*
chloroform	-54.32	-53.87
methanol	-56.07	-55.64
pentanol	-54.17	-53.91
octanol	-53.67	-53.41
decanol	-53.49	-53.24
PORPHYRINS	P_1	P_{16}
chloroform	-57.29	-56.53
methanol	-58.22	-57.51
pentanol	-56.33	-55.75
octanol	-56.00	-55.45
decanol		-55.38
reversed micelles		
10% pentanol	-55.39	-54.88
20% pentanol	-55.63	-54.99
30% pentanol	-55.83	-55.09
40% pentanol		-55.19
50% pentanol	-56.17	-55.31
20% butanol	-55.76	-55.05
20% pentanol	-55.63	-54.99
20% hexanol		-55.00
20% octanol	-55.54	-55.01
w_o=5	-55.64	
w_o=10	-55.63	-54.99
w_o=20	-55.63	-55.01
CTAB micelles in buffer	-55.98	
pentanol swollen CTAB micelles in buffer	-55.71	
octane swollen CTAB micelles in buffer	-56.17	
0.1 M CTAB in pentanol	-56.49	-55.88
continuous phase (5% pentanol)	-54.93	

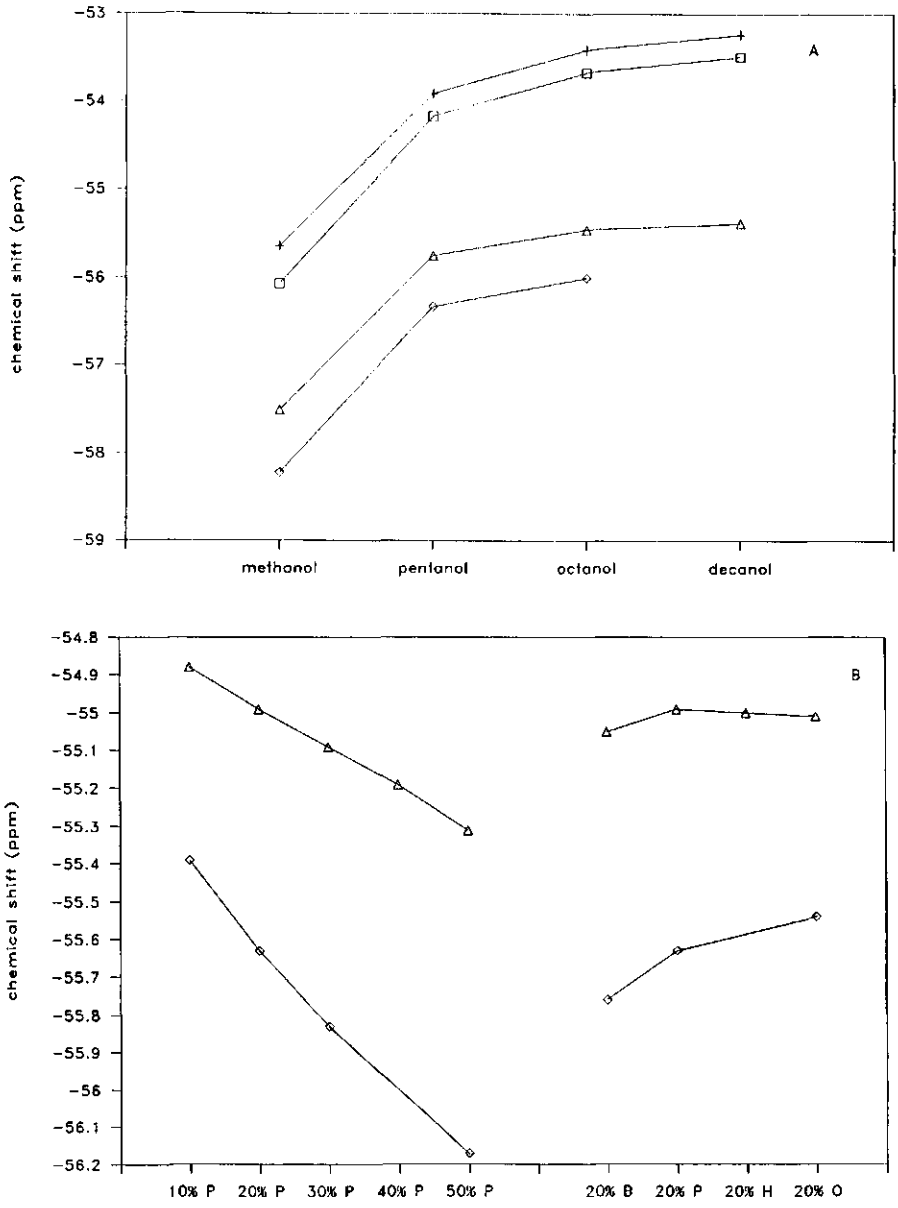


Fig. 3.1.2 A. *Chemical shift of 3-fluoro-4-methoxybenzaldehyde, 3-fluoro-4-hexadecyloxybenzaldehyde and fluorine modified porphyrins in alcohols:* □ *3-fluoro-4-methoxybenzaldehyde;* + *3-fluoro-4-hexadecyloxybenzaldehyde;* ◊ P_1 *porphyrine;* Δ P_{16} *porphyrine.*
B. *Chemical shift of porphyrins in different reversed micelles:* ◊ P_1 *porphyrine;* Δ P_{16} *porphyrine. On the horizontal axis the various reversed micellar media are represented (e.g. 10% P: reversed micelles with pentanol:octane 1:9 (vol/vol)*

The composition of the continuous phase of the reversed micellar medium was mimicked using a 5 % (vol/vol) mixture of pentanol and octane [21]. The solubility of both porphyrins in this medium is very low, but the chemical shift of $^{F}P_1$ could be measured. This value differs from the value obtained in reversed micellar solution (Table 3.1.1).
Also in four different reference CTAB solutions the fluorine chemical shift was measured: Three types of aqueous micelles and a solution of CTAB in pentanol. The NMR shifts in aqueous micelles were approximately the same as in reversed micelles.
The ^{19}F chemical shift in reversed micelles mostly resembles that in aqueous micelles with pentanol (Table 3.1.1). This indicates that those environments may be very similar (see Discussion).

3.1.4.2. Photoinduced charge separation
The photoinduced electron transfer was studied in standard (20% pentanol, w_o=10) CTAB reversed micelles from a donor (*eg.* tributylamine) in the organic phase *via* porphyrin P_{16} to methylviologen, located in the aqueous phase. The rate of the formation of reduced methylviologen was measured and the quantum efficiency (Φ_{cs}) of the photoreaction was calculated (See Materials and Methods).
Methylviologen reduction could be observed only in reversed micelles using the surface active porphyrin but not in homogeneous solution [14].

First the optimum ratio of the components of the photosystem was determined. A variation in the concentration of the porphyrin is expected to affect the electron transfer rate only slightly, because already at a concentration of porphyrin as low as 25 μM more than 95% of all incident light in the region of the Soret band is absorbed. Indeed, only a small increase in Φ_{cs} was observed upon a fourfold increase of the porphyrin concentration (Table 3.1.2). Therefore we used 50 μM in all experiments.

The dependence of the reaction on the concentration of the electron acceptor (methylviologen) was also determined. Increasing the concentration of the viologen to a value of 14 mM results in a marked increase of the efficiency of the reaction (Table 3.1.2). Obviously Φ_{cs} depends on the amount of electron acceptor available. We used a standard methylviologen concentration of 4 mM. At this concentration each reversed micelle contains on the average 2-4 viologen molecules [22]. A higher load of methylviologen molecules may affect the structure of the reversed micelle and induce dimerisation of methylviologen.

The influence of the chain length of the trialkylamine (the electrondonor) was investigated using triethylamine, tributylamine and trioctylamine. The redox behaviour of these electron donors was measured. The irreversible oxidation potential for all three compounds was 1.05±0.05 V (*vs.* NHE). Changing tributylamine to trioctylamine in the photosystem did not have noticeable effects on the photoinduced charge separation efficiency. The use of triethylamine as an electrondonor turned out to have at least twofold lower efficiency than tributylamine. The latter compound was therefore used as an electrondonor in all experiments.
The concentration of electrondonor present in the organic phase strongly affects the rate of the reaction [8]. Increasing the concentration of the donor to 100 mM showed, that the reaction rate did not reach its maximum level yet. We used a concentration of 50 mM tributylamine, since a higher concentration of the donor leads to phase separation of the micellar solution.

The value of w_o did not affect Φ_{cs} as reported earlier [14]; therefore we used a value of 10 for all experiments.

Table 3.1.2 Dependence of the yield of the photoinduced reduction of methylviologen of the composition of the photosystem

Concentration of the porphyrin	
25 μM	*0.17±0.02*
50 μM	*0.20±0.01*
100 μM	*0.20±0.04*
Concentration of the electron acceptor	
4.0 mM	*0.20±0.01*
6.7 mM	*0.49±0.04*
14.4 mM	*0.63±0.03*
Other electrondonors	
Triethylamine	*0.08±0.03*
Tributylamine	*0.20±0.01*
Trioctylmine	*0.27±0.01*
Concentration of the electron donor	
5 mM	*0.03±0.00*
25 mM	*0.15±0.01*
50 mm	*0.20±0.01*
75 mM	*0.23±0.02*

In all experiments P_{16} was used as the sensitizer. The standard reversed micellar solution was 0.2 M CTAB in a 1:4 (vol/vol) mixture of pentanol to octane, containing a waterphase (w_o=10) of 50 mM phosphate buffer, pH=7.0. The standard photosystem was 4 mM methylviologen, 50 mM tributylamine and 50 μM P_{16} All concentration are denoted with respect to the total volume of the reaction medium. For further details: See section Materials & Methods

The effect of the tail length of the porphyrin and the composition of the reversed micellar solution on Φ_{cs} was investigated. In all reversed micelles some methylviologen reduction could be recorded (Table 3.1.3). No reaction occurred if one of the components of the photosystem or light was omitted. The rate of charge separation is enhanced by an increased amount of pentanol in the reversed micellar solution. The length of the alkyl chain of the porphyrin does not affect the yield of photoinduced electron transfer from the donor to methylviologen.
The same experiments were carried out in reversed micelles using a different alcohol as cosurfactant. Table 3.1.3 shows that an increased polarity of the alcohol (from octanol to butanol) results into an increase in efficiency of the photosystem. In this case too, the difference in using P_1 or P_{16} is not significant.

Table 3.1.3 Quantum efficiency of the photosystem in various reversed micellar media.

reversed micelles		P_1	P_{16}
	concentration alcohol (M)		
10% pentanol	*0.92*		*0.07±0.01*
20% pentanol	*1.85*	*0.24±0.02*	*0.20±0.01*
30% pentanol	*2.77*		*0.39±0.06*
40% pentanol	*3.70*		*0.82±0.02*
50% pentanol	*4.62*		*1.10±0.02*
20% butanol	*2.19*	*0.33±0.00*	*0.38±0.05*
20% pentanol	*1.85*	*0.24±0.02*	*0.20±0.01*
20% hexanol	*1.59*		*0.15±0.04*
20% heptanol	*1.41*		*0.14±0.01*
20% octanol	*1.27*	*0.10±0.01*	*0.11±0.01*

3.1.5. DISCUSSION

3.1.5.1. Localization of porphyrin in the interphase of the reversed micelles

The fluoro-aldehydes (3-fluoro-4-methoxybenzaldehyde and 3-fluoro-4-hexadecyloxybenzaldehyde) were used to relate the chemical shift of a methyl and hexadecyl tail to the polarity of its environment.
These experiments showed that a decreased polarity of the solvent results in a shift of the fluorine resonance to higher frequency. The alkyl chain attached to the porphyrin also induces a shift to higher frequency.
The direction of these fluorine shifts can be explained by the formation of a hydrophobic pocket at the fluorine atom by the alkyl moieties of the alcohol or the porphyrin tail.
An alternative explanation, in analogy with the theory of Taft and coworkers [23], is the stronger electron donating ability of the longer alkyl groups in comparison with short chain groups. In this approach changes in the environment affect the electron distribution of the molecule, which determines the electron density at the fluorine atom and thus the shielding of the magnetic field at the fluorine nucleus.
Hydrogen bonding causes electron density rearrangement, resulting in a shift to lower frequency.
The fluorine resonance in porphyrins is shifted to a lower frequency as compared to the aldehydes. The similar dependence of theshift on the polarity of the medium for both the aldehydes and the porphyrins shows that the fluorine atom of both in the relatively small aldehydes and that of the porphyrins probes an environment that changes with the polarity of the solvent. These results demonstrate that the fluorine atom of the porphyrin can be used to probe the porphyrin environment *via* its NMR resonance frequency.

The environment of the porphyrin in a reversed micellar solution differs from that in a mixture of pentanol and octane representing the continuous phase of the reversed micellar solution (Table 3.1.1). Therefore, we may conclude that the porphyrin is not located in the continuous phase.
Obviously the insolubility of the porphyrin in water precludes a location in the aqueous phase. Solubilized porphyrins in aqueous CTAB micelles have similar fluorine shifts as compared to reversed micelles indicating that

the porphyrin is located in the interphase of the reversed micelle. In addition, the similar values for the fluorine shifts for $^{F}P_1$ and $^{F}P_{16}$ as compared to homogeneous solutions, show that the alkyl tail *per se* is not required to position the porphyrin in the interphase.

From the experimental results more information on the location in the interface can be extracted. The position of the resonance in standard reversed micelles (0.2 M CTAB, 20 volume percent of pentanol, 80 percent of octane), as compared to that in homogeneous solution showed that the environment of the porphyrin-tail is even more apolar than that of octanol and decanol. This indicates that the fluorine atom located at the tail of the porphyrin does not experience the polar surfactant head groups in the interphase but the apolar alkyl chains. This means that it is sandwiched between the arranged detergent and cosurfactant alkyl chains. In aqueous micelles the charged porphyrin head group is expected to be directed to the aqueous phase and the tail to stick into the hydrocarbon core of the aqueous micelle [12]. The fact that the effective solvent polarity at the tail of the porphyrin in aqueous detergent solutions is found to be very close to that observed in reversed micelles, supports the conclusion that the porphyrin is located in the hydrophobic part of the interphase.

The environment at the tail of P_{16} is not changed by replacement of butanol by octanol as cosurfactant (Fig. 3.1.2B). It is known that the amount of alcohol in the interphase and thus the polarity is similar for those reversed micelles, but the amount in the continuous phase differs considerably [21]. The fact that the fluorine atom of P_{16} probes the same polarity in butanol reversed micelles and in octanol reversed micelles shows that it does not probe the continuous phase, but the interphase. Upon increasing the concentration of the pentanol in the system the amount of pentanol in the interphase as well as in the continuous phase increases [21], so the polarity of both phases increases. The fluorine atom of the $^{F}P_{16}$ experiences an increased polarity in reversed micelles with a high pentanol concentration. Most probably the increased alcohol to detergent ratio in the interphase is probed.
An increase of the cosurfactant alkyl chain results in an increased polarity of the porphyrin environment. As stated earlier the molar concentration of butanol and octanol in the interphase is identical. This means that, unlike $^{F}P_{16}$, which always experiences an apolar environment, the fluorine atom of $^{F}P_1$ can distinguish butanol and octanol molecules in the interphase. So it has to be concluded that on the average $^{F}P_1$ is close to the alcohol groups in the interphase, whereas $^{F}P_{16}$ is closest to the alkyl chains in the interphase of the reversed micelle. Moreover, the observation that $^{F}P_1$, more than $^{F}P_{16}$, experiences both the hydroxyl group and the alkyl chain of the alcohol molecule indicates that the former moves rather freely in the interphase.

Summarizing the data ^{19}F-NMR measurements show that (i) both porphyrins are located in the interphase. (ii) Upon addition of pentanol to the reversed micellar medium the polarity of the environment of both porphyrins increases. (iii) Probing the polarity changes of the interphase is more pronounced for the porphyrin with a shorter tail.

3.1.5.2. The photosystem
Photoreduction of methylviologen using trialkylamines as donor and P_1 or P_{16} as sensitizer, was only observed in reversed micelles, and not in homogeneous solution. Obviously, the presence of a microstructure is

necessary for methylviologen reduction. Reversed micelles can prevent a fast charge recombination in two ways. (i) By creating an efficient orientation of the reactants (stabilization the charge transfer state (P^+A^-) or inducing fast rereduction of the porphyrin by a donor D) and (ii) by effectively removing one of the reactants by intermicellar exchange [8]. As seen in Table 3.1.2, the polarity of the trialkylamines affects the reaction rate. Triethylamine was less effective as an electron donor than tributylamine or trioctylamine. This difference is not caused by a changed electrochemical potential of the different donors, because they were all approximately 1.05 V *vs.* NHE. Therefore the effect must be provoked by an altered environment of these donors. The high solubility of triethylamine in the waterpool of the reversed micellar solution can prevent an efficient electron transport [8,14].
The lenght of the tail of the porphyrins is assumed only to affect the location and the dynamics of the porphyrin in the reversed micelle and not to affect the redoxpotential. This was verified by measuring the oxidation potential of the zinc modified P_1 and P_{16}. For both porphyrins the values were within equal experimental error (0.86 and 0.88 V for P_1 and P_{16}, respectively) validating the assumption.
The yields of photoinduced charge separation using P_1 and P_{16} were similar although both the location and the dynamics of these porphyrins in the interphase were shown to be different. This result indicates that in reversed micelles an efficient photoinduced electron transport from a water insoluble donor to a water soluble acceptor using a surface active porphyrin does not depend on the location or the mobility of the porphyrins in the interphase.

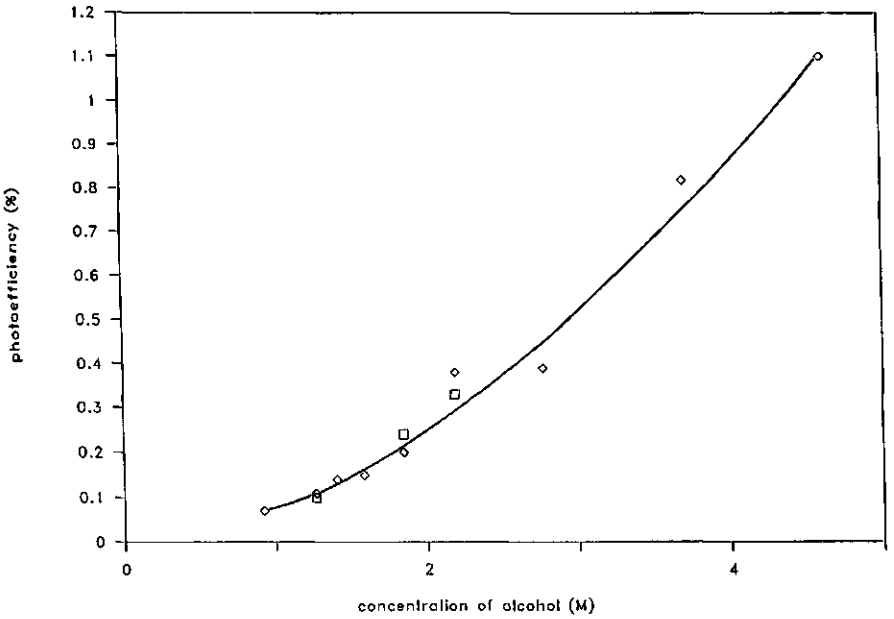


Fig. 3.1.3 Dependence of the rate of the photoinduced methylviologen reduction on the concentration of alcohol in the medium. Symbols: □ P_1 porphyrine; ◇ P_{16} porphyrine.

In preparing the reversed micellar solution the alcohol concentration is expressed with respect to its volume in the organic phase. For example, the molar concentration of 20 volume percent butanol in the reversed micellar solution is 2.2 M and the molar concentration of 20 volume percent octanol is 1.2 M. The yields in the different reversed micelles can be compared taking into account only the concentration of alcohol. Fig. 3.1.3 indicates a relationship between the concentration of alcohols in the reversed micellar solution and Φ_{cs}. This increased alcohol concentration can affect the yield in two ways: (i) The flexibility of the reversed micelles is increased [24]. As stated earlier a higher exchange rate of the reversed micelles can effectively remove photo-products and thus retards the charge recombination reaction ($k_{\alpha}[P^{+}][MV^{+\cdot}]$) (ii) The location of the reactants is altered [25]. An increased polarity of the interphase will induce partitioning of tributylamine into this interphase, resulting in an increased effective electron donor concentration in the vicinity of the porphyrin.
Our findings demonstrate that the mobility and the location of a porphyrin in the interphase is not the main factor determining the photoinduced charge separation, but rather the composition of the reversed micelles.

3.1.6. REFERENCES

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4.1. DESCRIPTION OF ENZYME KINETICS IN REVERSED MICELLES: 1. THEORY

4.1.1. SUMMARY

In the literature the measurements of kinetic data of enzymes in reversed micelles have been interpreted in two ways. In the first case all enzyme parameters are expressed with respect to the volume of the whole reversed micellar solution. In the second approach the enzymatic conversion is related only to the fraction of the volume consisting of aqueous solution (pseudophase model). In this paper equations are derived describing the rate of an enzymatic reaction for three different kinds of enzymes: Enzymes obeying Michaelis Menten kinetics, enzymes following a Ping Pong Bi Bi mechanism and enzymes which convert substrates according to an ordered mechanism. In deriving these equations, a distinction is made between intermicellar exchange reactions of substrate(s) and product(s) and the enzymatic reaction which takes place in the core of a reversed micelle. In the description all intrinsic rate constants of the enzyme were assumed to be independent of its environment. The rate equations show that the intermicellar exchange reaction, which supplies the enzyme of substrate and removes the product and the efficiency of this exchange, can affect the rate of an enzymatic reaction under conditions such as commonly met in the experimental setup.
Whereas kinetic parameters as derived from double reciprocal plots often seem to be affected by enclosure in reversed micelles, these apparent deviations from kinetics in aqueous media can be explained by the model presented here as arising from exchange phenomena. Neither v_{max} nor the Michaelis constant are affected by the incorporation of the enzyme in reversed micelles. The deviations of kinetic parameters from the aqueous values are shown to depend strongly on the concentration of reversed micelles, the intermicellar exchange rate and the volume fraction of water, a dependence in agreement with findings reported in the literature.

4.1.2. INTRODUCTION

Reversed micelles are tiny water droplets, dispersed by means of a surfactant, alone or in combination with a cosurfactant, in an organic solvent. By this layer of surface-active compounds the enzymes in the water droplets are protected from the adverse effects of the organic solvent. It has been well established now that enzymes can be incorporated in reversed micelles in an active form [1,2]. Till now, no evidence has been obtained of enzymes being located in the interface, although evidence for the existence of interactions between the surfactant head groups and charged groups of the protein has been presented [3,4]. Effects of the enclosure in a reversed micelle on enzyme kinetics have been studied [5,6], but the interpretation of the results has caused problems. It was remarked [7-9] that, in case of a water-soluble substrate, both its concentration and its Michaelis constant, can be expressed in two ways. First with respect to the total volume of the micellar solution and second with respect to the volume fraction of water present in such a solution. An argument for the first possibility is that, as the reaction is taking place under non-saturating conditions, substrate supply to the enzyme is limiting. Because substrate supply depends on the total volume of the reaction medium, this same volume should be considered [10].

Arguments for the second vision were that the reaction is taking place in the aqueous volume only, so the aqueous concentration is the one relevant to the enzyme [5,7]. Furthermore, it was argued that, as the exchange rate is much faster than the maximal turnover rate, a pseudo phase approach is allowed [1].
Experimental evidence was not conclusive. It was found for most enzymes that the Michaelis constant approached the aqueous value when expressed in overall concentration [5,8] but this was not the case for *Thermoanaerobium brockii* Alcoholdehydrogenase [11] and Liver Alcoholdehydrogenase [12].
In favour of the second approach was the observation that the Michaelis constant, expressed with respect to the overall volume, did depend on the volume fraction of water (w_o) present [5,8] and on the micelle concentration [5,8,9]. Neither approach was able to explain deviations from normal kinetic behaviour.
Here we present a model to describe enzyme reactions in reversed micelles that combines features of both approaches. This model is not only elaborated for a one substrate reaction, but also for two particular cases of two substrate enzyme reactions, *i.e.* a Ping Pong Bi Bi mechanism and an ordered mechanism. Furthermore a short remark is made on the effects of partitioning of the substrate between the aqueous phase and the organic phase on enzyme kinetics. In two subsequent papers the validity of this approach will be tested on two enzymes following either mechanism [13,14].

4.1.3. THEORY

The mechanism of the enzymatic conversion in reversed micelles can be described by separating the reaction into two steps:
In the first step diffusion of micelles containing substrate to enzyme-filled micelles takes place, followed by exchange. All reactants are concentrated inside the core of the reversed micelle. In the second step, which is confined to the water pool of the reversed micelle, the actual enzymatic conversion - substrate-enzyme interaction and catalytic reaction - is described.
To explain the two different steps and predict theoretically the rate of the enzyme activity, the intramicellar substrate concentration, the efficiency of the intermicellar exchange of reversed micelles and the rate constants of the overall system will be defined and calculated.
Using these values the effects on the rate equation of a Michaelis Menten enzyme can be deduced. However, since most enzymes do not react according to the simple one substrate Michaelis Menten pattern, also the kinetics of enzymes that catalyse the conversion of two substrates following two different mechanisms (Ping Pong Bi Bi and Ordered Bi Bi) will be described and discussed. Finally some remarks will be made on substrate-partitioning.

4.1.3.1. Definition of the concentration of substrate in reversed micelles

The definition of the substrate concentration in a reversed micellar solution is a controversial problem. To understand the presence and the effect of an effective, local substrate concentration, first the simple general definition of a concentration has to be reviewed.

In general the concentration of a substrate in any solution is given by

$$[S] = \frac{\text{number of molecules (mol)}}{\text{volume of the solution (l)}} \qquad (1.1)$$

In reversed micelles two different volumes can be distinguished. First the overall volume, *i.e.* the volume of all components together, and second the volume of the aqueous phase only. The volume of the latter is only a fraction of the total volume. When a substrate is located solely in the aqueous phase, two obvious ways of expressing its concentration can be proposed.
First, when the volume referred to in a reversed micellar solution is the total volume, while the total amount of the substrate present is related to the total volume, the concentration is the overall substrate concentration ($[S_{ov}]$). If only the aqueous phase is taken into account while the total amount of substrate is expressed only with respect to the volume of that aqueous phase, the substrate concentration is equal to $[S_{wph}]$. $[S_{ov}]$ and $[S_{wph}]$ are related by

$$[S_{wph}] = \frac{[S_{ov}]}{\Phi} \qquad (1.2)$$

in which Φ is the volume fraction of water (l_{H2O}) with respect to the total volume of the reversed micellar solution (l_{total}).
A third way of looking at the concentration of a substrate in reversed micelles also needs to be considered.
When the amount of substrate present in a given reversed micelle is related to the volume of the same reversed micelle, an effective substrate concentration is defined, taking a microscopic view at the reversed micelles. To derive this expression and to extend the expression to the overall volume, first the volume of the interior of a reversed micelle has to be defined.
Assuming that the total volume of all reversed micelles is equal to the total volume of aqueous phase in the system, *i.e.* all water pools together make up the complete interior of the reversed micelles and reversed micelles contain all water, and furthermore that all reversed micelles are assumed to have the same volume, this volume is given by

$$\Phi/[M] \text{ (l/mol)} \qquad (1.3)$$

in which [M] is the concentration of reversed micelles (mol/l). It must be emphasized that the dimensions of Eq. 1.3 are rather peculiar: they represent the interior micelle volume in liters per mole of reversed micelles but this interior is composed of water. To avoid confusion, a distinction will be made between l_{H2O} and the expression l_{in} (from interior). When the micellar core is regarded, l_{in} will be used and l_{H2O} is applied when the water phase in the reversed micellar solution is considered.
For a practical use of the microheterogeneous substrate concentration, this concentration has to be expressed as an macroscopic concentration but defined with respect to the tiny volume of one reversed micelle. So in this definition of the concentration, the number of substrate molecules per single reversed micelle is related to the average volume of the reversed micelles and to the volume of the reversed micellar solution. This microscopic, intramicellar concentration $[S_{in}]$ is given as

$$\frac{\text{moles of substrate in one mole of reversed micelles (mol/mol)}}{\text{volume of one mole reversed micelles } (l_{total}/\text{mol})}$$

If we denote the number of substrate molecules per reversed micelle as n, the intramicellar concentration is

$$[S_{in}] = n \frac{[M]}{\Phi} \qquad (mol/l_{in}) \qquad (1.4)$$

in which n = 0, 1, 2 .. ∞
It is clear that due to the small volume of the reversed micelle the concentration in a reversed micelle increases discontinuously with increasing amount of substrate in the solution. Only discrete values for the concentration can be obtained.

The average intramicellar concentration ($[\bar{S}_{in}]$), however, does not change stepwise. The value is obtained by averaging the value of n. So

$$[\bar{S}_{in}] = \bar{n} * \frac{[M]}{\Phi} \qquad (mol/l_{in}) \qquad (1.5)$$

As can be seen the value of the average intramicellar concentration is easily related to the concentration in the aqueous phase since the average number of substrate molecules in a reversed micelle is

$$\bar{n} = \frac{[S_{ov}]}{[M]} \qquad (mol/mol) \qquad (1.6)$$

and

$$[S_{wph}] = \frac{[S_{ov}]}{\Phi} \qquad (mol/l_{H2O}) \qquad (1.7)$$

Combining equations 1.5 and 1.6, the expression for the average intramicellar substrate concentration becomes

$$[\bar{S}_{in}] = \frac{[S_{ov}]}{[M]} \frac{[M]}{\Phi} = \frac{[S_{ov}]}{\Phi} = [S_{wph}] \qquad (mol/l_{H2O}, mol/l_{in}) \qquad (1.8)$$

As could be expected, the concentration of the substrate related to the volume of the water phase is equal to the concentration of the substrate related to the total volume of all water pools. However in one step of the description of the enzyme reaction in reversed micelles, we take into account only those reversed micelles actually containing one or more substrate molecules, that is: n is not 0. In these reversed micelles the intramicellar concentration of substrate cannot easily be related to the water phase concentration. This concentration will be denoted as $[S'_{in}]$. To determine the value of the intramicellar substrate concentration in substrate filled reversed micelles, only those reversed micelles that actually contain one or more substrate molecules have to be considered. The intramicellar concentration of these reversed micelles and the relative frequency of occurrence of these micelles has to be calculated to obtain a value for the intramicellar concentration. Finally this number is averaged for all substrate-filled reversed micelles.

If we assume a Poisson distribution of substrate molecules over the total number of reversed micelles [15,16], the probability of observing n molecules in a certain reversed micelle is given by

$$\pi(n) = \frac{\exp\left(-\frac{[S_{ov}]}{[M]}\right) * \left\{\frac{[S_{ov}]}{[M]}\right\}^{n}}{n!} \qquad (1.9)$$

Using this function for $\pi(n)$, the weighed average substrate occupation ($\bar{n}'$) in a filled reversed micelle is given by

$$\bar{n}' = \frac{\sum_{1}^{\infty} n\,\pi(n)}{\sum_{1}^{\infty} \pi(n)} \qquad (1.10)$$

Note that summation is carried out from one to infinite, omitting n=0. As can be appreciated, the average intramicellar substrate concentration in filled reversed micelles becomes

$$[\bar{S}'_{im}] = \frac{\sum_{1}^{\infty} n\,\pi(n)}{\sum_{1}^{\infty} \pi(n)} * \frac{[M]}{\Phi} \qquad (1.11)$$

Summation from n = 1 to n = ∞ results in an expression for the substrate concentration in a reversed micellar solution, that is not only defined with respect to the overall concentration and the waterphase concentration, since $[S_{ov}]$ is present in equation (1.9), but also depends on the concentration of reversed micelles in the solution.

Analyzing equation 1.11, two extremes for $[\bar{S}'_{im}]$ can be deduced.
When the overall concentration of substrate is considerably higher than the concentration reversed micelles, *i.e.* $[S_{ov}] \gg [M]$, the probability of observing any empty reversed micelles becomes very small, so

$$\sum_{1}^{\infty} n\,\pi(n) \approx \sum_{0}^{\infty} n\,\pi(n) \qquad (1.12)$$

In this case the average intramicellar substrate concentration of filled reversed micelles (Eq 1.11) becomes equal to the average intramicellar concentration of all reversed micelles (Eq. 1.7), that is

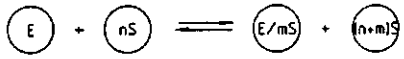
$$[\bar{S}'_{im}] = \frac{[S_{ov}]}{\Phi} = [S_{wph}] \qquad (1.13)$$

In the second case, when $[S_{ov}] \ll [M]$, most reversed micelles are empty and the probability that more than one substrate molecule is present in a reversed micelle is very small. In most micelles therefore the substrate concentration is zero. In the substrate-filled reversed micelles the concentration is (n=1)

$$[\bar{S}'_{im}] = \frac{[M]}{\Phi} \qquad (1.14)$$

Now an apparent contradiction is observed: the intramicellar substrate concentration in the filled reversed micelles becomes independent of the substrate concentration in the solution! It will be shown that this phenomenon strongly affects the enzyme activity.

A.



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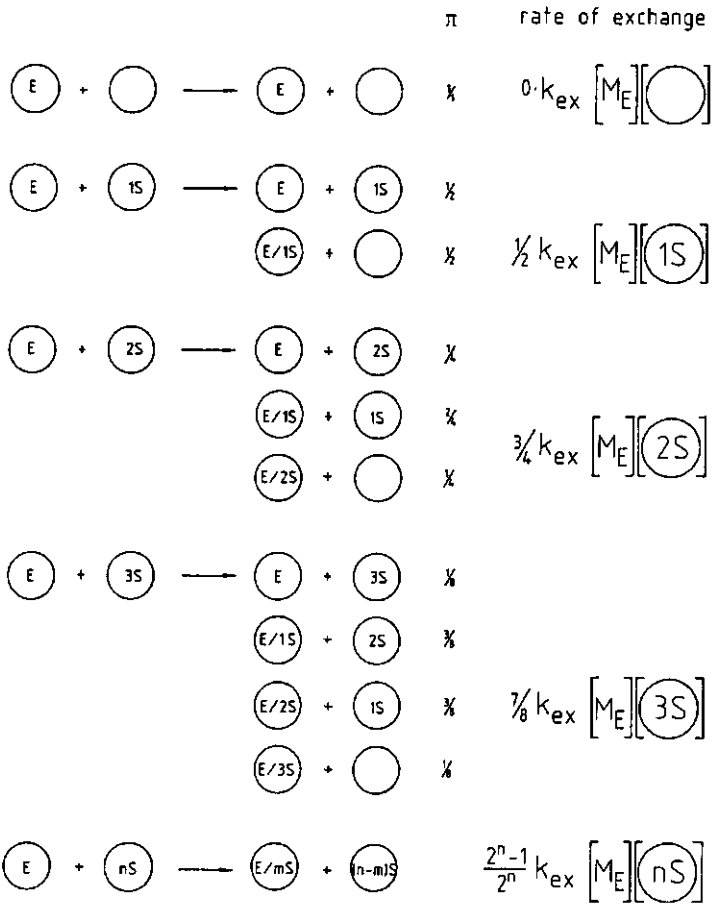

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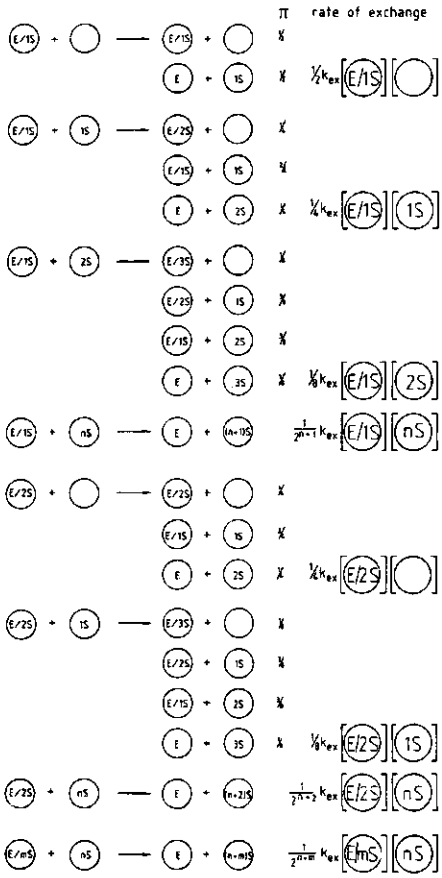


Fig. 4.1.1. Schematic representation of the various intermicellar exchange reactions and the probability of their occurence.
A) Reaction of the intermicellar exchange which is responsible for supply of the enzyme with substrate and the reversal reaction wich depletes the enzyme.
B) Scheme of the various exchange reactions which together determine the overall rate of substrate supply into the enzyme-filled reversed micelle. The probability (π) of the event is depicted for each exchange reaction.
C) Scheme of all reactions which remove substrate from a micelle containing an enzyme molecule and one or more substrate molecules.

4.1.3.2. The rate and the efficiency of intermicellar exchange
The description of the intermicellar exchange of substrates and products into and out of the enzyme-filled reversed micelle is straightforward. Clearly the result of any exchange reaction depends on the content of the colliding micelles. If, for instance a reversed micelle containing five substrate molecules collides to exchange with a micelle containing no substrate molecules at all, the probability that an empty reversed micelle

is formed is very small and most probable a micelle containing two and a micelle containing three substrates are formed. If the exchange process is determined by chance, that is, if during the exchange reaction there is a 50% probability of a solute being transferred into the new micelle and a 50% probability of it remaining in the original micelle, the probability can be calculated using Pascals triangle.
It must be emphasized that the exchange rate used throughout this work is the rate of all intermicellar exchange reactions. In the literature the experimental setup is such that commonly only the rate of formation of micelles containing both desired compounds is considered. Therefore, the maximal rate of intermicellar exchange that can be reached is upto a factor two lower than the actual number of collisions leading to intermicellar exchange, depending on the relative occupancy of the reveised micelles [15, 16].
For our specific problem the efficiency of the intermicellar exchange providing an enzyme with a substrate is most important. As suggested above this efficiency is higher when the occupancy level of the colliding reversed micelle is higher. If the enzyme-filled reversed micelle exchanges with a micelle containing only one substrate molecule the probability that after the exchange both the enzyme and the substrate are located in the same micelle is 1/2 (Fig. 4.1.1). Exchange with a micelle containing two substrate molecules results in a 3/4 probability that the enzyme-filled micelle contains at least one substrate molecules, collision of an enzyme-filled reversed micelle (containing no substrate at all) and a micelle containing three substrate molecules would yield in 7/8 of all cases a micelle containing both an enzyme and substrate. For n substrate molecules the probability is $2^{n-1}/2^n$. These probabilities are depicted in Fig. 4.1.1.

For a solution containing micelles with various occupation numbers the average probability depends on the appearance of a filled reverse micelle. This average probability determines the efficiency ϵ of the intermicellar exchange of a solute S into the enzyme filled micelle. In formula:

$$\epsilon^{S}_{i} = \sum_{0}^{\infty} \pi(n) \frac{2^n-1}{2^n} \qquad (2.1)$$

In Eq. 2.1 the subscript i denotes transport of the solute molecule into an enzyme filled reversed micelle.
The efficiency is less, if the micelles with a low occupation number are abundant, and reaches one if all micelles contain a high number n of solute molecules.

The rate of transport of solute molecules out of the enzyme filled reversed micelle
To obtain an expression for transport of solutes out of the enzyme-containing reversed micelle is more complicated because, the number of possible exchange patterns increases. As depicted in Fig. 4.1.1c, a distinction has to be made between reactions starting from a micelle containing an enzyme and either one, two, three or more substrate molecules. Starting with a micelle with one enzyme and m substrate molecules and another micelle with n substrate molecules the probability is $1/2^{n+m}$ to obtain in one exchange reaction a micelle containing only an enzyme molecule and a micelle containing n+m substrate molecules.
For the description of the enzyme reaction in a reversed micelle, and for the average efficiency of this event, the average number of $\bar{m}'$, which is

the average occupation number in a substrate filled micelle will be used. Assuming an equal distribution of substrate molecules over non-enzyme containing and enzyme filled reversed micelles, this number $\bar{m}'$ can be calculated by Eq. 1.10. The average efficiency for the transport of all substrates out of the reversed micelle becomes:

$$\epsilon^{s}_{o} = \sum_{0}^{n} \pi(n) \frac{1}{2^{n+\bar{m}'}} \qquad (2.2)$$

in which the subscript o denotes transport out of the enzyme filled reversed micelle. Using equation 2.1 and 2.2 the average rate of intermicellar transport of substrate into the enzyme filled reversed micelle can be calculated by

$$k_{ex}\epsilon^{s}_{i}[M_{E}][M] \qquad (2.3)$$

in which $[M_E]$ is the concentration enzyme filled and $[M]$ is the total concentration reversed micelles and k_{ex} is the exchange rate between reversed micelles (see below). For the transport of product out of the enzyme-filled micelle, a similar equation can be derived.
Two interesting limits are present. First if the concentration of the substrate is much higher than the concentration of reversed micelles, then ϵ^{s}_{i} becomes:

$$\epsilon^{s}_{i} = \frac{2^{n}-1}{2^{n}} \approx \frac{2^{n}}{2^{n}} = 1 \qquad (2.4)$$

and since $2^{n+\bar{m}'} \gg 1$ the expression for ϵ^{s}_{o} is:

$$\epsilon^{s}_{o} = \frac{1}{2^{n+\bar{m}'}} \approx 0 \qquad (2.5)$$

If the substrate concentration is low compared to the concentration reversed micelles, the probability of observing more than one substrate in a micelle is neglible and the efficiency is given by Eq. 2.6.

$$\epsilon^{s}_{i} = \left(\pi(0) * \frac{2^{0}-1}{2^{0}} + \pi(1) * \frac{2^{1}-1}{2} \right) = \pi(1) * 0.5 \qquad (2.6)$$

In this case the probability of observing one substrate molecule in a reversed micelle is (Eq. 1.9):

$$\pi(1) = \frac{[S_{ov}]}{[M]} \qquad (2.7)$$

and therefore the exchange rate is given by

$$k_{ex}[M_{E}][S_{ov}] \qquad (2.8)$$

The probability of transport of S out of the micelle, in this case is given by

$$\epsilon^{S}_{o} = \left(\pi(0) * \frac{1}{2^{0+1}} + \pi(1) * \frac{1}{2^{1+1}} \right) \qquad (2.9)$$

and since $\pi(0) \gg \pi(1)$. Eq. 2.9 becomes

$$\epsilon^{S}_{o} = 0.5 \qquad (2.10)$$

Initially the same limitations also holds for the product, so

$$\epsilon^{P}_{i} = \frac{[P_{ov}]}{[M]} * 0.5 = \frac{0}{[M]} 0.5 = 0 \qquad (2.11)$$

and

$$\epsilon^{P}_{o} = P(0) \frac{1}{2^{0+1}} = 0.5 \qquad (2.12)$$

4.1.3.3. Kinetics of an enzyme obeying Michaelis-Menten type reaction in reversed micelles

In aqueous solution the initial rate of the enzyme reaction can be derived using steady state conditions. In the case of steady state no overall change in the concentration of free enzyme and in the concentration of enzyme substrate complex occurs. The following equations (Fig. 4.1.2A) can be derived [17]:

$$\frac{d[ES]}{dt} = 0 = k_1[E][S] - (k_2 + k_{-1})[ES] \qquad (3.1)$$

$$[E_o] = [E] + [ES] \qquad (3.2)$$

$$v = k_2[ES] \qquad (3.3)$$

$$v_{max} = k_2[E_o] \qquad (3.4)$$

Substitution of Eqs. 3.1, 3.2 and 3.4 into Eq. 3.3 and rearranging gives

$$v = \frac{v_{max}}{[S] + K_m} \qquad (3.5)$$

in which

$$K_m = \frac{k_{-1} + k_2}{k_1} \qquad (3.6)$$

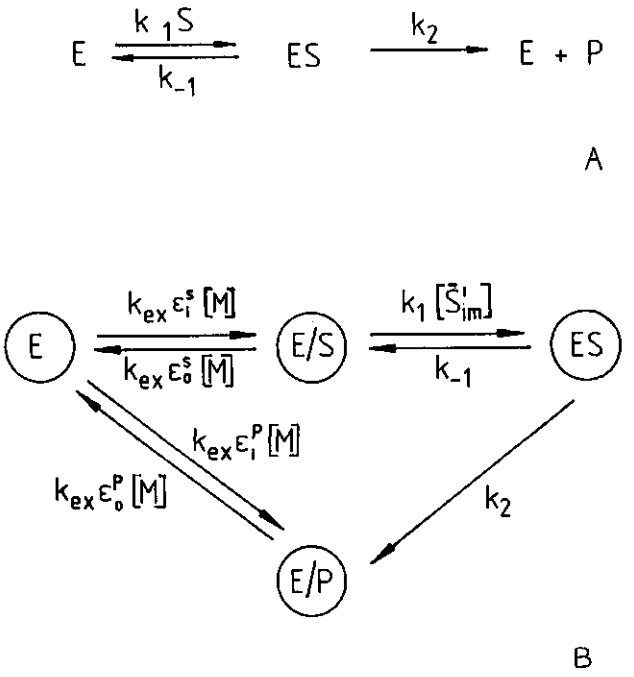


Fig. 4.1.2. Scheme of a reaction obeying classical Michaelis-Menten kinetics in aqueous solution (A) and in reversed micelles (B). E/S denotes the presence of E and S in one reversed micelles without a complex being formed. [M] is the total concentration of reversed micelle in the medium, $[\epsilon_i^s]$ and $[\epsilon_o^s]$ are the rate of which substrate is transported in and out of the enzyme filled reversed micelle, respectively. The average concentration of substrate in all reversed micelles containing substrate is given by $[S'_{im}]$. The rate constant governing the intermicellar exchange reaction is k_{ex} ($M^{-1}s^{-1}$). Further explanation: See text.

The enzyme reaction described above can be compared with the same reaction in a smaller volume containing the same amount (moles) of substrate and enzyme. If the original volume is decreased by a factor f, both the concentrations enzyme and substrate are increased by the same factor. The reaction rate, measured in the same, small volume is also increased. In formula

$$v' = k_2[ES'] = \frac{k_2[E_o'][S']}{[S'] + K_m} \qquad (3.7)$$

which is equal to

$$v' = k_2 f [ES] = \frac{f^2 k_2[E_o][S]}{f [S] + K_m} \qquad (3.8)$$

But if the reaction were to take place in this small volume but was measured in a larger volume, e.g. a reversed micellar medium, the rate would be

$$v'' = \frac{1}{f} v' = \frac{k_2 [E_o] f [S]}{f [S] + K_m} \qquad (3.9)$$

The rate actually measured is:

$$v'' = \frac{k_2 [E_o][S']}{[S'] + K_m} \qquad (3.10)$$

implying that by concentrating both substrate and enzyme into a smaller volume apparently only the substrate concentration affects the rate of the reaction, but, since the rate is actually related to the total volume, implicitly a correction for the enzyme concentration is made.
Applying this consideration, it is possible to relate the reaction of the enzyme in reversed micelles to the well known rate equations. As was already mentioned earlier, in order to do this, a distinction has to be made between reactions occurring between reversed micelles and reactions occurring in the interior of the micelle. The reaction scheme as depicted in Fig. 4.1.2B describes the reaction for the conversion of substrate by a Michaelis-Menten enzyme. First formation of a micelle containing enzyme and substrate has to take place. Then the enzymatic conversion is carried out as if it were in an ordinary aqueous environment. Since equations like Eq. 3.1 to 3.10 grow complex very rapidly, the method of King and Altman [18] is most useful to derive the rate equation.

The kinetic terms of the different species of E are

$$[E] \quad : \quad k_1 k_2 k_{ex} \epsilon^P_o [M][\bar{S}'_{im}] + k_2 k^2_{ex} \epsilon^S_o \epsilon^P_o [M]^2 + k_{-1} k^2_{ex} \epsilon^S_o \epsilon^P_o [M]^2 \qquad (3.11)$$

$$[E/S] \quad : \quad k_2 k^2_{ex} \epsilon^S_i \epsilon^P_o [M]^2 + k_{-1} k^2_{ex} \epsilon^S_i \epsilon^P_o [M]^2 \qquad (3.12)$$

$$[ES] \quad : \quad k_1 k^2_{ex} \epsilon^S_i \epsilon^P_o [M]^2 [\bar{S}'_{im}] \qquad (3.13)$$

$$[E/P] \quad : \quad k_1 k_2 k_{ex} \epsilon^S_i [M][\bar{S}'_{im}] + k_1 k_2 k_{ex} \epsilon^P_i [M][\bar{S}'_{im}] + k_2 k^2_{ex} \epsilon^S_o \epsilon^P_i [M]^2 + k_{-1} k^2_{ex} \epsilon^S_o \epsilon^P_i [M]^2 \qquad (3.14)$$

In these equations [M] is the total micelle concentration, ϵ^S_i, ϵ^S_o, ϵ^P_i and ϵ^P_o are the factors describing the efficiency of the intermicellar exchange of the substrate and product into and out of the reversed micelles. [E/S] denotes reversed micelles that contain both enzyme and m substrate molecules in free form.
Summation of all enzyme species to relate the rate to the overall concentration of enzyme yields:

$$[E_o] = [E] + [E/S] + [ES] + [E/P] \qquad (3.15)$$

The initial rate of the reaction ($\epsilon^P_i = 0$) is given by

$$v = \frac{k_1 k_2 k^2_{ox} \epsilon^S_i \epsilon^P_o [M]^2 [\bar{S}'_{in}] [E_o]}{\text{constant} + \text{coef S} * [\bar{S}'_{in}]} \qquad (3.16)$$

in which

$$\text{constant} = k_2 k^2_{ox} \epsilon^S_i \epsilon^P_o [M]^2 (k_2 + k_{-1}) \left(1 + \frac{\epsilon^S_o}{\epsilon^S_i}\right) \qquad (3.17)$$

$$\text{coef S} = k_1 k_2 k^2_{ox} \epsilon^S_i \epsilon^P_o [M]^2 \left(1 + \frac{k_2}{k_{ox} \epsilon^S_i [M]} + \frac{k_2}{k_{ox} \epsilon^P_o [M]}\right) \qquad (3.18)$$

Rearranging and substitution of

$$K_m = \frac{k_2 + k_{-1}}{k_1} \qquad (3.19)$$

gives for the initial activity ($\epsilon^P_i = 0$)

$$v = \frac{k_2 [E_o]}{1 + \frac{\left(\frac{\epsilon^S_o}{\epsilon^S_i} + 1\right) K_m}{[\bar{S}'_{in}]} + \frac{k_2}{k_{ox}[M]} \left(\frac{1}{\epsilon^S_i} + \frac{1}{\epsilon^P_o}\right)} \qquad (3.20)$$

As can be deduced from eq 3.20, the K_m that is observed, depends on the relative occupation of reversed micelles with substrate. An extra term representing the exchange of reversed micelles containing substrate and a term containing all reversed micelles is incorporated. This term is present because the reversed micelle containing the enzyme has to be supplied of substrate and to be relieved of product. It can be seen easily that only under conditions of a very high exchange rate in the reversed micellar solution in comparison with the turnover rate of the enzyme and a relatively high concentration of substrate filled micelles, the maximum velocity of the enzyme reaction observed in reversed micelles is comparable to that in aqueous solution. However, the apparent K_m always depends on the conditions of the reaction.
More detailed information about the effect on the Michaelis Menten constant in reversed micelles is obtained analyzing two limits of the reaction: The case of a very low and the case of a very high substrate concentration (both with respect to the concentration reversed micelles).

When the substrate concentration is low, i.e. $[S_{ov}] \ll [M]$, then ϵ^S_i is equal to $0.5*[S_{ov}]/[M]$, $\epsilon^S_o = \epsilon^P_o = 0.5$ and $[\bar{S}'_{in}] = [M]/\Phi$. For this limit it follows:

$$v = \frac{k_2 [E_o]}{1 + \frac{\left(\Phi K_m + \frac{2 k_2}{k_{ox}}\right)}{[S_{ov}]} + \frac{\Phi K_m}{[M]} + \frac{2 k_2}{k_{ox}[M]}} \qquad (3.21)$$

In words: when the concentration of the substrate is low, the apparent K_m is not only modified by a term Φ, but also by the ratio between the turnover of the enzyme and the exchange rate of the reversed micellar medium. Implications of this term will be discussed later.

In the second case when the concentration of substrate is much higher than the concentration of reversed micelles, all reversed micelles contain one or more substrate molecules, therefore $\epsilon^s_i \gg \epsilon^s_o$, $\epsilon^p_o = 0.5$ and $[\overline{S}'_{in}] = [S_{ov}]/\Phi$.

Now

$$v = \frac{k_2[E_o]}{1 + \frac{\Phi K_m}{[S_{ov}]} + \frac{3 k_2}{k_{ex}[M]}} \qquad (3.22)$$

The observed K_m will be equal to the K_m observed in a "ordinary" aqueous solution multiplied with a factor Φ. Again the observed v_{max} is modified by a factor depending on the ratio of the turnover rate of the enzyme and the intermicellar exchange.
Rearranging Eq. 3.22 results in

$$v = \frac{\frac{k_{ex}[M]k_2[E_o]}{k_{ex}[M] + 3k_2}}{1 + \frac{\Phi K_m k_{ex}[M]}{k_{ex}[M] + 3k_2} * \frac{1}{[S_{ov}]}} = \frac{v_{max}^{app}}{1 + \frac{K_m^{app}}{[S_{ov}]}} \qquad (3.23)$$

Therefore we can calculate for this extreme:

$$\frac{v_{max}^{app}}{K_m^{app}} = \frac{v_{max}}{\Phi K_m} \qquad (3.24)$$

From Eq. 3.24 it can be envisaged that the ratio v_{max}^{app} over K_m^{app} can be calculated from the known parameters Φ, K_m and v_{max}. It has to be stressed, however, that this relation is only valid when $[S_{ov}] \gg [M]$. It must be emphasized that for many enzymes the K_m value for the substrate varies between 0.1 - 10 mM. The range of the concentration of reversed micelles is in the same order of magnitude [16,19]. Under experimental conditions therefore the limits given above (Eq. 3.21 to 3.24) are reached rarely.
In a similar way it can be derived for $[S_{ov}] \ll [M]$ that

$$\frac{v_{max}^{app}}{K_m^{app}} = \frac{v_{max}}{\Phi K_m + \text{constant}} \qquad (3.25)$$

in which the constant contains both k_2 and k_{ex}. In this case no straightforward relation can be derived to relate the enzyme activity in reversed micelles directly to the reaction in "ordinary" aqueous solution.

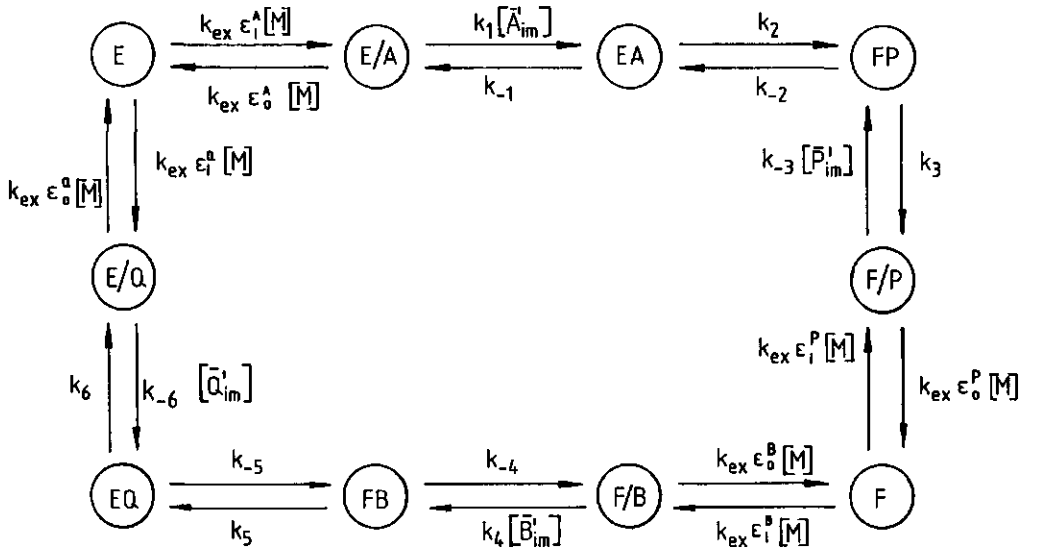


Fig. 4.1.3. Schematic representation of a Ping Pong Bi Bi reaction in reversed micelles. A and B are the substrates, P and Q the two products. E and F are the two forms the enzyme oscillates between during one reaction cycle. Other symbols are the same as or defined in analogy with Figs. 4.1.1 and 4.1.2.

4.1.3.4. Kinetics of a reaction in reversed micelles of an enzyme following a Ping Pong Bi Bi mechanism.

Using the same principles as were used to derive the rate equation for a Michaelis-Menten enzyme in reversed micelles, the reaction rate of more complex conversions can be calculated. The reaction scheme for a Ping Pong Bi Bi reaction occurring in reversed micelles is depicted in Fig. 4.1.3. In analogy with the approach in aqueous solution the rate equation for the initial activity can be derived using the King-Altman method. In this case ϵ^P_i and $\epsilon^Q_i = 0$. As will be discussed later terms containing $[\bar{P}'_{im}]$ and $[\bar{Q}'_{im}]$ do not affect the enzyme reaction. The concentrations of the various enzyme species are given by:

$$\begin{aligned}E \;:\; & k^3_{ex}\,\epsilon^B_i\epsilon^P_o\epsilon^Q_o\,[M]^3\,k_1k_2k_3k_4k_5k_6\,[\bar{A}'_{im}][\bar{B}'_{im}] + \\ & + k^4_{ex}\,\epsilon^B_i\epsilon^P_o\epsilon^Q_o\epsilon^A_o\,[M]^4\,k_4k_5k_6(k_2k_3 + k_3k_{-1})\,[\bar{B}'_{im}] + \\ & + k^4_{ex}\,\epsilon^B_i\epsilon^P_o\epsilon^Q_o\epsilon^A_o\,[M]^4\,k_{-1}k_{-2}k_4k_5k_6\,[\bar{B}'_{im}] + \\ & + k^3_{ex}\,\epsilon^B_i\epsilon^Q_o\epsilon^A_o\,[M]^3\,k_{-1}k_{-2}k_{-3}k_4k_5k_6\,[\bar{B}'_{im}][\bar{P}'_{im}] \qquad (4.1)\end{aligned}$$

$$\begin{aligned}E/A \;:\; & k^4_{ex}\,\epsilon^A_i\epsilon^B_i\epsilon^P_o\epsilon^Q_o\,[M]^4\,k_4k_5k_6(k_2k_3 + k_3k_{-1} + k_{-1}k_{-2})\,[\bar{B}'_{im}] + \\ & + k^3_{ex}\,\epsilon^B_i\epsilon^P_o\epsilon^Q_o\,[M]^3\,k_{-1}k_{-2}k_{-3}k_4k_5k_6\,[\bar{B}'_{im}][\bar{P}'_{im}] \qquad (4.2)\end{aligned}$$

$$\begin{aligned}EA \;:\; & k^4_{ex}\,\epsilon^A_i\epsilon^B_i\epsilon^P_o\epsilon^Q_o\,[M]^4\,k_1k_4k_5k_6\,(k_3 + k_{-2})\,[\bar{A}'_{im}][\bar{B}'_{im}] + \\ & + k^3_{ex}\,\epsilon^A_i\epsilon^B_i\epsilon^Q_o\,[M]^3\,k_1k_{-2}k_{-3}k_4k_5k_6\,[\bar{A}'_{im}][\bar{B}'_{im}][\bar{P}'_{im}] \qquad (4.3)\end{aligned}$$

$$FP : k^4_{ox}\,\epsilon^A_1\epsilon^B_1\epsilon^P_o\epsilon^Q_o\,[M]^4\,k_1k_2k_4k_5k_6\,[\bar{A}'_{in}][\bar{B}'_{in}] + + k^3_{ox}\,\epsilon^A_1\epsilon^B_1\epsilon^Q_o\,[M]^3\,k_1k_2k_{-3}k_4k_5k_6\,[\bar{A}'_{in}][\bar{B}'_{in}][\bar{P}'_{in}] \quad (4.4)$$

$$F/P : k^3_{ox}\,\epsilon^A_1\epsilon^B_1\epsilon^Q_o\,[M]^3\,k_1k_2k_3k_4k_5k_6\,[\bar{A}'_{in}][\bar{B}'_{in}] \quad (4.5)$$

$$F : k^3_{ox}\,\epsilon^A_1\epsilon^P_o\epsilon^Q_o\,[M]^3\,k_1k_2k_3k_4k_5k_6\,[\bar{A}'_{in}][\bar{B}'_{in}] + + k^4_{ox}\,\epsilon^A_1\epsilon^B_o\epsilon^P_o\epsilon^Q_o\,[M]^4\,k_1k_2k_3\,(k_5k_6 + k_6k_{-4})\,[\bar{A}'_{in}] + + k^4_{ox}\,\epsilon^A_1\epsilon^B_o\epsilon^P_o\epsilon^Q_o\,[M]^4\,k_1k_2k_3k_{-4}k_{-5}\,[\bar{A}'_{in}] + k^3_{ox}\,\epsilon^A_1\epsilon^B_o\epsilon^P_o\epsilon^Q_o\,[M]^3\,k_1k_2k_3k_{-4}k_{-5}k_{-6}\,[\bar{A}'_{in}][\bar{Q}'_{in}] \quad (4.6)$$

$$F/B : k^4_{ox}\,\epsilon^A_1\epsilon^B_1\epsilon^P_o\epsilon^Q_o\,[M]^4\,k_1k_2k_3\,(k_5k_6 + k_6k_{-4} + k_{-4}k_{-5})\,[\bar{A}'_{in}] + + k^3_{ox}\,\epsilon^A_1\epsilon^B_1\epsilon^P_o\,[M]^3\,k_1k_2k_3k_{-4}k_{-5}k_{-6}\,[\bar{A}'_{in}][[\bar{Q}'_{in}] \quad (4.7)$$

$$FB : k^4_{ox}\,\epsilon^A_1\epsilon^B_1\epsilon^P_o\epsilon^Q_o\,[M]^4\,k_1k_2k_3k_4\,(k_6 + k_{-5})\,[\bar{A}'_{in}][\bar{B}'_{in}] + + k^3_{ox}\,\epsilon^A_1\epsilon^B_1\epsilon^P_o\,[M]^3\,k_1k_2k_3k_4k_{-5}k_{-6}\,[\bar{A}'_{in}][\bar{B}'_{in}][\bar{Q}'_{in}] \quad (4.8)$$

$$EQ : k^4_{ox}\,\epsilon^A_o\epsilon^B_1\epsilon^P_o\epsilon^Q_o\,[M]^4\,k_1k_2k_3k_4k_5\,[\bar{A}'_{in}][\bar{B}'_{in}] + + k^3_{ox}\,\epsilon^A_1\epsilon^B_1\epsilon^P_o\,[M]^3\,k_1k_2k_3k_4k_5k_{-6}\,[\bar{A}'_{in}][\bar{B}'_{in}][\bar{Q}'_{in}] \quad (4.9)$$

$$E/Q : k^3_{ox}\,\epsilon^A_1\epsilon^B_1\epsilon^P_o\,[M]^3\,k_1k_2k_3k_4k_5k_6\,[\bar{A}'_{in}][\bar{B}'_{in}] \quad (4.10)$$

Special attention deserve the terms containing $[\bar{P}'_{in}]$ and $[\bar{Q}'_{in}]$. At the start of the reaction the values for $[\bar{P}'_{in}]$ and $[\bar{Q}'_{in}]$ are $[M]/\Phi$ (see Eq. 1.14). As can be seen in Eq. 4.1 the contribution of the term containing $[\bar{P}'_{in}]$ is small if $k_{-3}[\bar{P}'_{in}] \ll k_{ox}\epsilon^P_o[M]$, that is, if $k_{-3} \ll \Phi k_{ox}$. From Eq 4.6 it can easily be deduced that also the $[\bar{Q}'_{in}]$-term is neglible if $k_{-6}[\bar{Q}'_{in}] \ll k_{ox}\epsilon^Q_o[M]$. Since typical values for k_{-3} and k_{-6} are 10^3 ($M^{-1}s^{-1}$) [30] and k_{ox} is in the order of 10^6 $M^{-1}s^{-1}$, negligence of the $[\bar{P}'_{in}]$ and $[\bar{Q}'_{in}]$ terms is legitimate.

The initial activity becomes:

$$v = \frac{k^4_{ox}\,\epsilon^A_1\epsilon^B_1\epsilon^P_o\epsilon^Q_o\,[M]^4\,k_1k_2k_3k_4k_5k_6\,[\bar{A}'_{in}][\bar{B}'_{in}][E_o]}{(\text{coef A})'[\bar{A}'_{in}] + (\text{coef B})'[\bar{B}'_{in}] + (\text{coef AB})'[\bar{A}'_{in}][\bar{B}'_{in}]} \quad (4.11)$$

in which

$$(\text{coef A})' = k^4_{ox}\,\epsilon^A_1\epsilon^B_o\epsilon^P_o\epsilon^Q_o\,[M]^4 + k^4_{ox}\,\epsilon^A_1\epsilon^B_1\epsilon^P_o\epsilon^Q_o\,[M]^4\ )\ \text{coef A} \quad (4.12)$$

$$(\text{coef B})' = k^4_{ox}\,\epsilon^A_o\epsilon^B_1\epsilon^P_o\epsilon^Q_o\,[M]^4 + k^4_{ox}\,\epsilon^A_1\epsilon^B_1\epsilon^P_o\epsilon^Q_o\,[M]^4\ )\ \text{coef B} \quad (4.13)$$

$$(\text{coef AB})' = k^4_{ox}\,\epsilon^A_1\epsilon^B_1\epsilon^P_o\epsilon^Q_o\,[M]^4\ \text{coef AB} + + k^3_{ox}\,k_1k_2k_3k_4k_5k_6\,\epsilon^A_1\epsilon^B_1\epsilon^P_o\epsilon^Q_o\,[M]^3\,(1/\epsilon^A_1 + 1/\epsilon^B_1 + 1/\epsilon^P_o + 1/\epsilon^Q_o) \quad (4.14)$$

with

$$\text{coef A} = k_1k_2k_3(k_5k_6 + k_{-4}k_6 + k_{-4}k_{-5}) \quad (4.15)$$

$$\text{coef B} = k_4k_5k_6(k_{-1}k_{-2} + k_{-1}k_3 + k_2k_3) \quad (4.16)$$

$$\text{coef AB} = k_1k_4(k_3k_5k_6 + k_{-2}k_5k_6 + k_2k_5k_6 + k_2k_3k_6 + + k_2k_3k_{-5} + k_2k_3k_5) \quad (4.17)$$

which are the coefficients for the reaction in aqueous solution [17,21].

Substitution of:

$$K_A = \frac{\text{coef B}}{\text{coef AB}} \qquad (4.18)$$

$$K_B = \frac{\text{coef A}}{\text{coef AB}} \qquad (4.19)$$

$$V = \frac{k_1k_2k_3k_4k_5k_6\ [E_o]}{\text{coef AB}} \qquad (4.20)$$

and rearranging gives (4.21):

$$v = \frac{V}{1 + \frac{(\frac{\epsilon^A_o}{\epsilon^A_i} + 1)K_A}{[\bar{A}'_{in}]} + \frac{(\frac{\epsilon^B_o}{\epsilon^B_i} + 1)K_B}{[\bar{B}'_{in}]} + \frac{V}{[E_o]k_{ex}[M]} * (\frac{1}{\epsilon^A_i} + \frac{1}{\epsilon^B_i} + \frac{1}{\epsilon^P_o} + \frac{1}{\epsilon^Q_o})}$$

The similarity between Eq. 3.20 and 4.21 has to be emphasized. In the latter the turnover rate of the enzyme is not expressed as one k-value (k_2) but as $V/[E_o]$, but again the ratio of the turnover rate and the intermicellar exchange rate determines the enzymatic conversion. Considering the concentrations of the substrates and the concentration of reversed micelles, again two limits can be distinguished:

a) both substrate concentrations are much higher than the concentration of reversed micelles.
b) only a small fraction of all reversed micelles contains any substrate.

a) if $[A_{ov}]$ and $[B_{ov}]$>>$[M]$ than $\epsilon^A_i = \epsilon^B_i = 1$, $\epsilon^P_o = \epsilon^Q_o = 0.5$ and $[\bar{A}'_{in}] = [A_{ov}]/\Phi$ and $[\bar{B}'_{in}] = [B_{ov}]/\Phi$.

Now eq. 4.21 simplifies to:

$$v = \frac{V}{1 + \frac{(\frac{\epsilon^A_o}{\epsilon^A_i} + 1)K_A}{\frac{[A_{ov}]}{\Phi}} + \frac{(\frac{\epsilon^B_o}{\epsilon^B_i} + 1)K_B}{\frac{[B_{ov}]}{\Phi}} + \frac{V}{[E_o]k_{ex}[M]} * (\frac{1}{\epsilon^A_i} + \frac{1}{\epsilon^B_i} + \frac{1}{\epsilon^P_o} + \frac{1}{\epsilon^Q_o})}$$

which is equal to

$$v = \frac{V}{1 + \frac{\Phi\ K_A}{[A_{ov}]} + \frac{\Phi\ K_B}{[B_{ov}]} + \frac{6\ V}{[E_o]k_{ex}[M]}} \qquad (4.22)$$

b) If $[A_{ov}]$, $[B_{ov}] \ll [M]$ then $\epsilon^{A}_{1} = 0.5*[A_{ov}]/[M]$, $\epsilon^{B}_{1} = 0.5*[B_{ov}]/[M]$, ϵ^{A}_{0}, ϵ^{B}_{0}, ϵ^{P}_{0} and ϵ^{Q}_{0} are 0.5, and $[\bar{A}'_{1s}] = [\bar{B}'_{1s}] = [M]/\Phi$. In this case substituting and rearranging gives

$$v = \frac{V}{1 + (\Phi K_A + \frac{V}{[E_o]k_{ox}})\frac{1}{[A_{ov}]} + (\Phi K_B + \frac{V}{[E_o]k_{ox}})\frac{1}{[B_{ov}]} + \frac{4V}{[E_o]k_{ox}[M]}} \quad (4.23)$$

4.1.3.5. Ordered mechanism

In a similair way the description of the enzyme reaction in reversed micelles for an enzyme following an Ordered Bi Bi reaction can be derived. To do this we again use an exchange model (Fig. 4.1.4) in combination with the King-Altman method of discriminating between all different forms of the enzyme. In this case the original equation (using the notation of reference [17]) is given by:

$$v = \frac{V}{1 + \frac{K_A}{[A]} + \frac{K_B}{[B]} + \frac{K_{IA} K_B}{[A][B]}} \quad (5.1)$$

In which

$$K_A = \frac{\text{Coef B}}{\text{Coef AB}} \quad (5.2)$$

$$K_B = \frac{\text{Coef A}}{\text{Coef AB}} \quad (5.3)$$

$$V = \frac{k_1 k_2 k_3 k_4 [E_o]}{\text{Coef AB}} \quad (5.4)$$

$$K_{IA} = \frac{\text{Constant}}{\text{Coef AB}} \quad (5.5)$$

Using the common expressions for these coefficients [17,21]

$$\text{Constant} = k_{-1}k_4 (k_{-2} + k_3) \quad (5.6)$$

$$\text{Coef A} = k_1 (k_3 k_4 + k_4 k_{-2}) \quad (5.7)$$

$$\text{Coef B} = k_2 k_3 k_4 \quad (5.8)$$

$$\text{Coef AB} = k_1 k_2 (k_3 + k_4) \quad (5.9)$$

In reversed micelles the constant, coefficient A, coefficient B and coefficient AB are modified as follows:

$$(\text{Constant})' = k^4_{ox}\,\epsilon^A_1\epsilon^B_1\epsilon^P_0\epsilon^Q_0\,[M]^4\left(\frac{\epsilon^A_0\epsilon^B_0}{\epsilon^A_1\epsilon^B_1}+\frac{\epsilon^B_0}{\epsilon^B_1}\right)*\text{Constant} \tag{5.10}$$

$$(\text{Coef A})' = k^4_{ox}\,\epsilon^A_1\epsilon^B_1\epsilon^P_0\epsilon^Q_0\,[M]^4\left(1+\frac{\epsilon^B_0}{\epsilon^B_1}\right)*\text{Coef A} \tag{5.11}$$

$$(\text{Coef B})' = k^4_{ox}\,\epsilon^A_1\epsilon^B_1\epsilon^P_0\epsilon^Q_0\,[M]^4\left(1+\frac{\epsilon^A_0}{\epsilon^A_1}\right)\left(1+\frac{k_{-1}}{k_{ox}\,\epsilon^B_1\,[M]}\right)*\text{Coef B} \tag{5.12}$$

$$(\text{Coef AB})' = k^4_{ox}\,\epsilon^A_1\epsilon^B_1\epsilon^P_0\epsilon^Q_0\,[M]^4\left(1+\left(\frac{1}{\epsilon^A_1}+\frac{1}{\epsilon^B_1}+\frac{1}{\epsilon^P_0}+\frac{1}{\epsilon^Q_0}\right)\frac{V}{k_{ox}[E_o][M]}\right)*\text{CoefAB} \tag{5.13}$$

Substitution of the equations for K_{IA}, K_A, K_B and V and rearranging gives

$$v = \frac{V}{1+\dfrac{\alpha_1 K_A}{[\bar{A}'_{is}]}+\dfrac{\alpha_2 K_B}{[\bar{B}'_{is}]}+\dfrac{\alpha_3 K_{IA} K_B}{[\bar{A}'_{is}][\bar{B}'_{is}]}+\dfrac{V}{k_{ox}[E_o][M]}\left(\dfrac{1}{\epsilon^A_1}+\dfrac{1}{\epsilon^B_1}+\dfrac{1}{\epsilon^P_0}+\dfrac{1}{\epsilon^Q_0}\right)} \tag{5.14}$$

In which

$$\alpha_1 = \left(1+\frac{\epsilon^A_0}{\epsilon^A_1}\right)\left(1+\frac{k_{-1}}{k_{ox}\,\epsilon^B_1\,[M]}\right) \tag{5.15}$$

$$\alpha_2 = 1+\frac{\epsilon^B_0}{\epsilon^B_1} \tag{5.16}$$

$$\alpha_3 = \left(\frac{\epsilon^A_0}{\epsilon^A_1}+1\right)\frac{\epsilon^B_0}{\epsilon^B_1} \tag{5.17}$$

Also for the ordered mechanism modifications in the apparent K_A, K_B, K_{IA} terms occur. Simplification for a high concentrations of both substrates with respect to the concentration of reversed micelles, taking into account the considerations given in sections 3 and 4, results in Eq. 5.18

$$v = \frac{V}{1+\dfrac{\Phi K_A}{[A_{ov}]}\left(1+\dfrac{k_{-1}}{k_{ox}[M]}\right)+\dfrac{\Phi K_B}{[B_{ov}]}+\dfrac{6\,V}{k_{ox}[M][E_o]}} \tag{5.18}$$

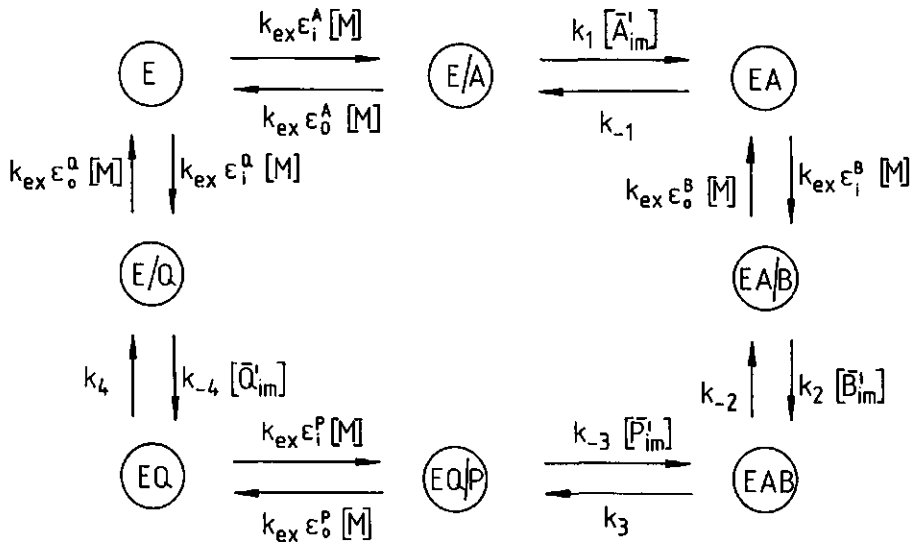


Fig. 4.1.4 Reaction scheme for an enzyme following an ordered mechanism. Symbols are explained in Figs. 4.1.1, 4.1.2 and 4.1.3.

It is very important to note that the system shows the kinetic characteristics of a Ping Pong mechanism due to cancellation of the K_{IA} term. So, although the enzyme still reacts according to an ordered mechanism, apparently another reaction sequence is followed.

For low substrate concentrations with respect to [M] Eq. 5.19 is obtained.

$$v = \frac{V}{1 + \frac{\beta_1}{[A_{ov}]} + \frac{\beta_2}{[B_{ov}]} + \frac{\beta_3}{[A_{ov}][B_{ov}]} + \beta_4} \qquad (5.19)$$

with

$$\beta_1 = \Phi K_A + \frac{2\,V}{k_{ex}[E_o]} \qquad (5.19a)$$

$$\beta_2 = \Phi K_B + \frac{\Phi^2\,K_{IA}\,K_B}{[M]} + \frac{2\,V}{k_{ex}[E_o]} + \frac{2\,\Phi\,K_A\,k_{-1}}{k_{ex}[M]} \qquad (5.19b)$$

$$\beta_3 = \Phi^2\,K_{IA}\,K_B + \frac{2\,\Phi\,K_A\,k_{-1}}{k_{ex}} \qquad (5.19c)$$

$$\beta_4 = \frac{\Phi}{[M]}(K_A + K_B) + \frac{4\ V}{k_{ex}[E_o]} \qquad (5.19d)$$

4.1.3.6. Substrate partitioning

When studying the enzymatic conversion of apolar compounds in reversed micelles, partitioning of the compound between the organic phase, the interphase and the aqueous phase has to be considered.
Enzyme kinetics are highly dependent on the amount of substrate available. When a major part of the substrate is partitioned into the organic phase, it is not immediately available for the enzyme to react with. In addition to exchange of apolar substrate molecules between waterpools also transport of the substrate from the organic phase to the water pool supplies the enzyme with fresh substrate. The rate of the latter supply, in this case, is given by the rate of the transport of the compound across the interphase, assuming that this reaction is much slower than diffusion in both aqueous phase and organic phase. The rate of transport into the reversed micelle will be denoted as k_{in}, the transport out of the micelle as k_{out}. It can be seen that the ratio of these rates determines the distribution of the solute between the phases. In formula

$$P^{\ddagger} = \frac{k_{out}}{k_{in}} \qquad (6.1)$$

In the model describing the reaction of a Michaelis Menten enzyme in reversed micellar medium, the step in which enzyme and substrate combine into one micelle is determined by the k_{in} and k_{out} and their ratio. The reaction scheme is depicted in Fig 4.1.5.

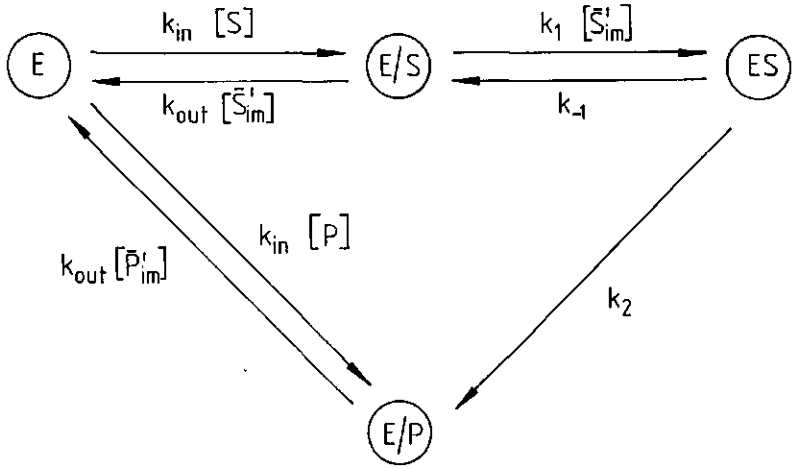


Fig. 4.1.5. Schematic representation of a Michaelis-Menten enzyme converting an apolar substrate. Note that the exchange rates have been replaced by the rate of transport of the solute into the aqueous phase and out of this phase.

Recalculating the rate equation following the same procedure as used in section 3, the velocity of the reaction is given by

$$v = \frac{k_2[E_o]}{1 + \frac{K_m\left(\frac{k_{out}[\bar{S}'_{in}]}{k_{in}[S_{ov}]} + 1\right)}{[\bar{S}'_{in}]} + \frac{k_2}{k_{in}[S_{ov}]} + \frac{k_2}{k_{out}[\bar{P}'_{in}]}} \qquad (6.2)$$

Since, due to partitioning of the substrate between the organic phase and the aqueous phase, $[\bar{S}'_{in}]$ is equal to $[M]/\Phi$, and substituting $[\bar{P}'_{in}]=[M]/\Phi$, $P^a=k_{out}/k_{in}$ and rearranging gives:

$$v = \frac{k_2[E_o]}{1 + \frac{\left(K_m P^a + \frac{k_2}{k_{in}}\right)}{[S_{ov}]} + \frac{\Phi}{[M]}\left(K_m + \frac{k_2}{k_{out}}\right)} \qquad (6.3)$$

As can be seen from Eq 6.3, the K_m that will be observed in reversed micelles now not only depends on the K_m in aqueous solutions and the overall concentration of the substrate, but also on the partitioning of the substrate and the rate of the transport of the substrate into the reversed micelle.

For a bimolecular reaction the same procedure can be followed to derive the rate equation for two apolar substrates. A more interesting case, however, is the enzymatic conversion of a polar and an apolar substrate in reversed micelles. If the first substrate (A) is polar and the second (B) is mainly present into the organic phase, for an Ping Pong Bi Bi mechanism in reversed micelles (Eq. 4.21) the rate equation becomes (Eq. 6.4):

$$v = \frac{V}{1 + \frac{\alpha_1 K_A}{[\bar{A}'_{in}]} + \frac{\alpha_2 K_B}{[\bar{B}'_{in}]} + \frac{V}{[E_o]}\left(\frac{1}{k_{ox}\epsilon^A_i[M]} + \frac{1}{k_{ox}\epsilon^P_o[M]} + \frac{1}{k_{in}[B_{ov}]} + \frac{1}{k_{out}[\bar{Q}'_{in}]}\right)}$$

$$\alpha_1 = \frac{\epsilon^A_o}{\epsilon^A_i} + 1 \qquad (6.4a)$$

$$\alpha_2 = \left(1 + P^a \frac{[\bar{B}'_{in}]}{[B_{ov}]}\right) \qquad (6.4b)$$

Simplifying for $[\bar{B}'_{in}]=[\bar{Q}'_{in}]=[M]/\Phi$ gives (6.5)

$$v = \frac{V}{1 + \frac{\alpha_1 K_A}{[\bar{A}'_{in}]} + \frac{\alpha_2}{[B_{ov}]} + \frac{V}{[E_o]}\left(\frac{1}{k_{ex}\epsilon^A_1[M]} + \frac{1}{k_{ex}\epsilon^P_o[M]} + \frac{\Phi}{k_{out}[M]}\right) + \frac{\Phi}{[M]} K_B}$$

with α_1 and α_2 as defined in Eq. 6.4a and 6.4b respectively and with

$$\alpha_3 = P^B K_B + \frac{V}{[E_o]\ k_{in}} \qquad (6.5a)$$

In the case A is a polar and B is an apolar substrate, the equations for an Ordered Bi Bi reaction in reversed micelles (Eq. 5.14-5.17) are converted into:

$$v = \frac{V}{1 + \frac{\beta_1 K_A}{[\bar{A}'_{in}]} + \frac{P^B K_B + \frac{V}{[E_o]}\frac{1}{k_{in}}}{[B_{ov}]} + \frac{\beta_1 \beta_2}{[\bar{A}'_{in}][B_{ov}]} + \frac{\Phi K_B}{[M]} + \frac{V}{[E_o]}\beta_3} \qquad (6.6)$$

In which

$$\beta_1 = \frac{\epsilon^A_o}{\epsilon^A_1} + 1 \qquad (6.6a)$$

$$\beta_2 = P^B K_B K_{IA} + \frac{k_{-1}}{k_{in}} K_A \qquad (6.6b)$$

$$\beta_3 = \frac{\beta_2}{k_{ex}\ \epsilon^P_o\ [M]} + \frac{\Phi}{k_{out}\ [M]} \qquad (6.6c)$$

4.1.4. DISCUSSION

The description of reversed micelles as being a microheterogeneous medium consisting of tiny water droplets dispersed by in organic solvent, which exchange their contents on a microsecond time scale, was used to derive a model to describe the enzymatic conversions in reversed micelles. The fact that on a long time scale (seconds) the content of reversed micelles form one continuous phase is not sufficient to understand kinetics in reversed micelles completely. Therefore the fact that on a short time scale (μs) the content of a reversed micelle is a distinct entity, separated from the rest of the solution has been used in this theory to make a distinction between the reactions that supply the enzyme with substrate and the enzymatic conversion itself.
Comparison of literature data [22,23] made us conclude that enzyme kinetics

50 μM at five fixed concentrations of 2-methylbutenoic acid ranging from 1 to 25 mM. Our data confirmed the Ping Pong Bi Bi mechanism reported by Bühler et al [16]. For NADH a K_A of 20 μM was deduced and for 2-methylbutenoic acid a K_B-value of 4.7 mM was calculated. Literature data [16] (K_A and K_B respectively 12 μM and 1.47 mM) were obtained at a higher ionic strength in a phosphate buffer at pH 6.0. No inhibition of the enzyme activity was observed in aqueous solution until the concentration of 2-methylbutenoic acid was increased up to 25 mM. At a NADH concentration over 250 μM a distict inhibition was observed.

4.2.4.2. Kinetics of enoate reductase in reversed micelles

Enoate reductase can be incorporated in various types of reversed micelles. In most reversed micellar media tested the enzyme retained its full catalytic activity (see below). However, incorporation of the enzyme in SDS (sodium dodecylsulphate) reversed micelles resulted in a complete loss of the activity. Three different reversed micellar media were used to study to which extend the kinetics of enoate reductase are modulated by incorporation of the enzyme in such microheterogeneous media. The reversed micelles used in our studies were composed of the anionogenic surfactant AOT, the cationogenic detergent CTAB and the nonionogenic Triton X-100. All reversed micelles contained an aqueous phase of 50 mM Hepes pH 7.0, I=0.05.

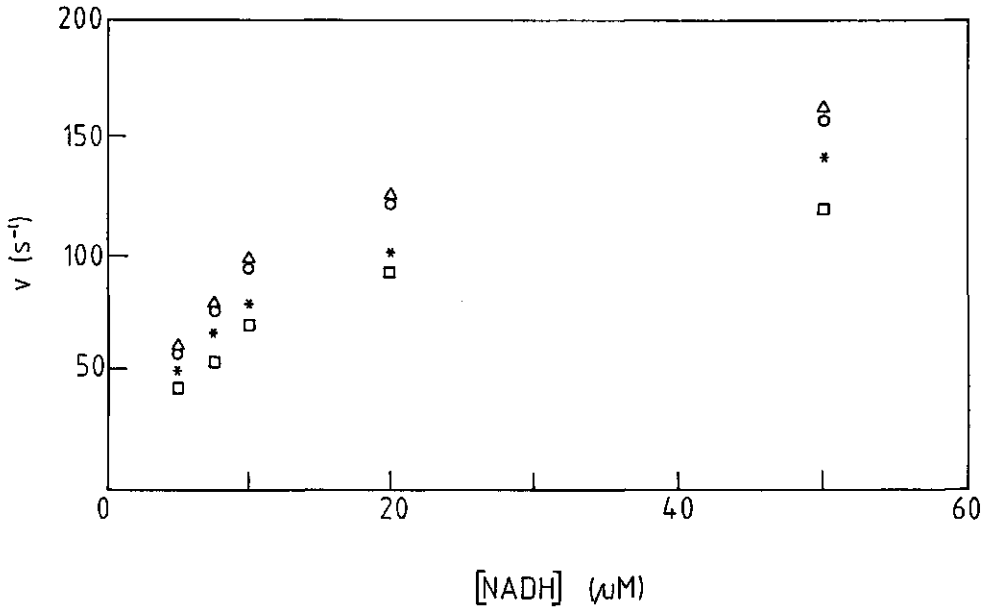


*Fig. 4.2.1. A. Turnover rates of the enoate reductase in AOT reversed micelles using concentrations of 2-methylbutenoic acid below the inhibitory level. The buffer in the reversed micelles (w_o=10) was 50 mM Hepes, pH=7.0, I=0.05. Symbols: □ 0.075 mM, * 0.15 mM, ○ 0.3 mM, Δ 0.6 mM 2-methylbutenoic acid. For further experimentals details: see Materials & Methods.*

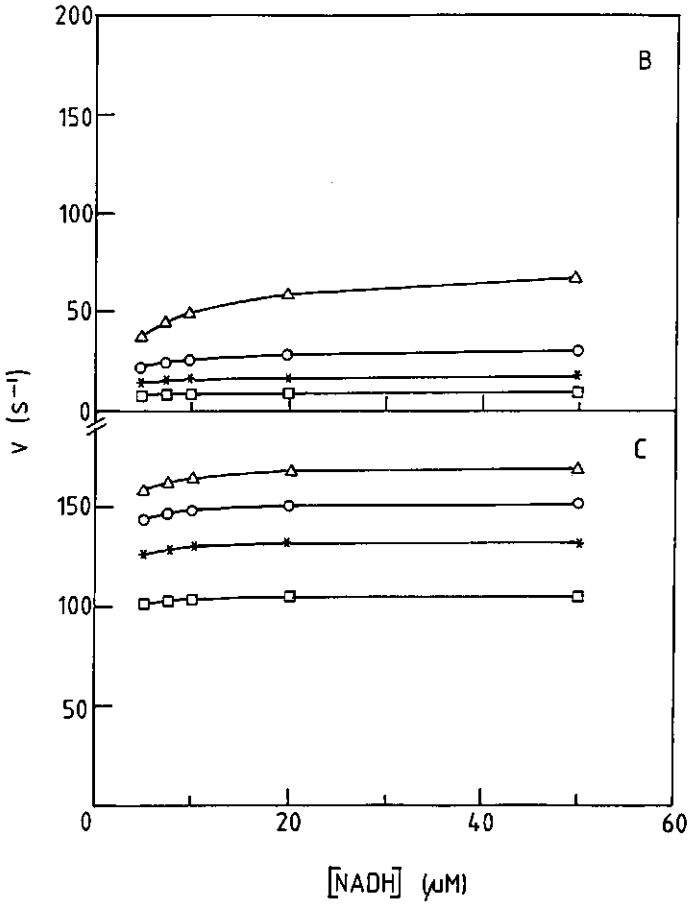


Fig. 4.2.1. B. Enzyme activities predicted assuming a homogeneous distribution of the substrate over the solution. Symbols as in A.
C. Turnover rates of enoate reductase assuming a pseudophase model. Note that all concentrations in the plot are expressed with respect to the overall volume. Symbols as in A. For further explanation: see text.

4.2.4.3. Determination of the kinetic parameters of enoate reductase

1. AOT-reversed micelles

2-methylbutenoic acid concentration ranging from 0.075 to 1 mM was used to study the kinetics of enoate reductase in AOT reversed micelles. The initial rates of NADH reduction were measured (Fig. 4.2.1). No inhibition of the reaction was observed up to a 2-methylbutenoic acid concentration of 0.6 mM (see below).
Calculating the Michaelis constants from these data expressing all concentrations with respect to the total volume of the medium a K_A value of

10μM and a K_s value of 0.02 mM was obtained. Comparing these values with the value in aqueous solution it is clear that the Michaelis constant for 2-methylbutenoic acid has decreased more than 200-fold. For NADH the value has changed only by a factor two.
To obtain clarity on this observation we first calculated the data which would have been observed if enzyme kinetics in reversed micelles were completely similar to kinetics in aqueous solution. If the concentrations of both NADH and 2-methylbutenoic acid are expressed with respect to the total volume of the reaction medium and the rate of the reaction is calculated with the kinetic equations for a Ping Pong Bi Bi mechanism, the plot of these simulations (Fig. 4.2.1B) differs remarkedly from the data that are experimentally determined (Fig. 4.2.1A).
Secondly, because many literature reports only consider the volume of the water phase to express the substrate concentration, we also calculated the rate of the reaction as if it were only dependent on the substrate concentration in the aqueous volume fraction. In this case the concentrations of 2-methylbutenoic acid and NADH present in the aqueous phase range from 140 to 1400 μM for NADH and from 2.1 to 17 mM for 2-methylbutenoic acid. As can be seen in Fig. 4.2.1C all reaction rates are higher now, since especially the concentration of NADH reaches a saturating level. Some dependence on the amount of 2-methylbutenoic acid, however, would still be observed, since the concentration of this substrate (only if it is expressed with respect to the volume of the waterphase) is in the range of its K_s. Comparing Fig. 4.2.1A and Fig. 4.2.1C, it is clear that this dependence is not found experimentally.

These results show that the two Michaelis constants are influenced in different ways. The obvious conclusion could be that not only apparent K_m values but all kinetic parameters in reversed micelles differ from those in aqueous solution. Neither the approach of relating the concentration of the reactants to the total volume, nor the pseudophase approach explains our data satisfactorily. Therefore these results prompted us to analyse the enzymatic reaction in reversed micelles in more detail and to propose a reaction scheme as represented in Fig. 4.1.3 [9]. In this model a distinction is made between the reactions occurring between various reversed micelles and the reactions that can, in essence, take place inside one reversed micelle. The exchange reactions between reversed micelles are essential to supply the enzyme with substrate(s). Especially at very low concentrations of solutes more intermicellar exchange reactions are necessary to convert one 2-methylbutenoic acid molecule to the reduced species. Apart from the kinetic effects imposed by the micellar environment, the enzymatic reaction in a reversed micelle is assumed to be the same as in an aqueous solution.
The rate of the reaction can according to this model be described by [9]

$$v = \frac{V}{1 + \frac{(\frac{\epsilon^A_o}{\epsilon^A_i} + 1)K_A}{[\bar{A}'_{is}]} + \frac{(\frac{\epsilon^B_o}{\epsilon^B_i} + 1)K_B}{[\bar{B}'_{is}]} + \frac{V_i}{[E_o]k_{ex}[M]}(\frac{1}{\epsilon^A_i} + \frac{1}{\epsilon^B_i} + \frac{1}{\epsilon^P_o} + \frac{1}{\epsilon^Q_o})} \quad (1)$$

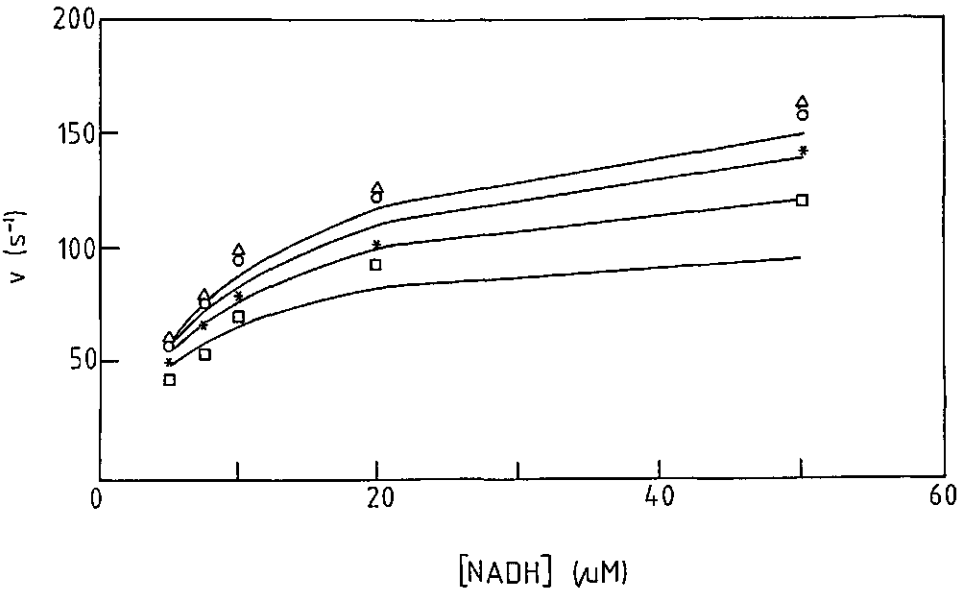
in which [M] is the concentration reversed micelles, ϵ^A_i and ϵ^B_i are the efficiencies of transport of NADH and 2-methylbutenoic acid into the enzyme

filled micelle, ϵ^{r}_{o} and ϵ^{q}_{o} are the efficiencies of product removal of NAD^{+} and 2-methylbutanoic acid out of the enzyme filled micelle. $[\bar{A}'_{in}]$ and $[\bar{B}'_{in}]$ are the previously defined [9] intramicellar concentrations of NADH and 2-methylbutenoic acid in filled reversed micelles. V is the maximum activity of the enzyme (Ms^{-1}) [18]; K_A and K_B are the Michaelis constants for NADH and 2-methylbutenoic acid (in aqueous solution) respectively. $[E_o]$ is the overall enzyme concentration in the reversed micellar medium and k_{ex} is the rate at which reversed micelles exchange their content.
It has to be stressed that all concentrations are expressed with respect to the total volume of the medium.
In deriving the rate equation it has been assumed that the intrinsic parameters of the enzyme are not altered. The deviation in the rate observed in a reversed micellar solution, as compared to the reaction in water under the same conditions, is imposed by limitations in the rate of the intermicellar exchange and the variation in effective concentrations around the enzyme due to the droplet like structure of the medium.

The initial reaction rates that were measured for enoate reductase in AOT reversed micelles were compared with those predicted by Eq. 1. In order to do this an experimentally determined [19] concentration of reversed micelles, [M], of 2.7 mM was used. The rate of intermicellar exchange of reversed micelles is reported to be in the order of 5×10^{7} ($M^{-1}s^{-1}$) [19]. Using these values and taking the values for the K_A and K_B values for NADH and 2-methylbutenoic acid determined in aqueous solution, simulations of the reaction rate in reversed micelles were carried out.

Fig. 4.2.2A shows that the patterns of the experimentally observed values are simulated rather well. The incorporation of enoate reductase in AOT reversed micelles does not alter the intrinsic kinetic parameters of the enzyme, but the kinetic pattern changes due to the microheterogeneity of the system. Although the rate of intermicellar exchange is five orders of magnitude higher than the turnover of the enzyme, this value strongly affects the rate of the reaction. A second cause for the deviating behaviour is that in the water pool of a reversed micelle very high concentrations of NADH or 2-methylbutenoic acid can be present.
Because both substrates are randomly distributed over all micelles, the intramicellar concentration, *i.e.* the concentration experienced by the enzyme, depends on the volume of the reversed micelles as well as on the concentration of reversed micelles.
For simplicity, in the simulation mentioned above, all reversed micelles are assumed to behave the same. That is, a reversed micelle containing a substrate molecule is expected to behave the same as a reversed micelle containing no solute molecule at all. Since we expected an effect of the content of the micelle on its exchange behaviour (*e.g.* fusion and mixing of the micelles) [20] the enzyme reaction was simulated assuming a slower exchange rate for NADH than for 2-methylbutenoic acid. In an AOT reversed micelles the reason for this assumption was that the size and the high charge density of NADH may slow down the effective exchange rate compared to that of the relatively small and less charged 2-methylbutenoic acid. Taking a higher exchange rate for 2-methylbutenoic acid (a factor of 2.5 increase), resulted in the simulation depicted in Fig. 4.2.2B. It has to be stressed that the deviations in exchange rates used here, are still well within the accuracy of the experimentally determined values. As can be deduced from Fig. 4.2.2A and Fig. 4.2.2B relatively small deviations in the system result in considerable variations of the experimental results.

A.



B.

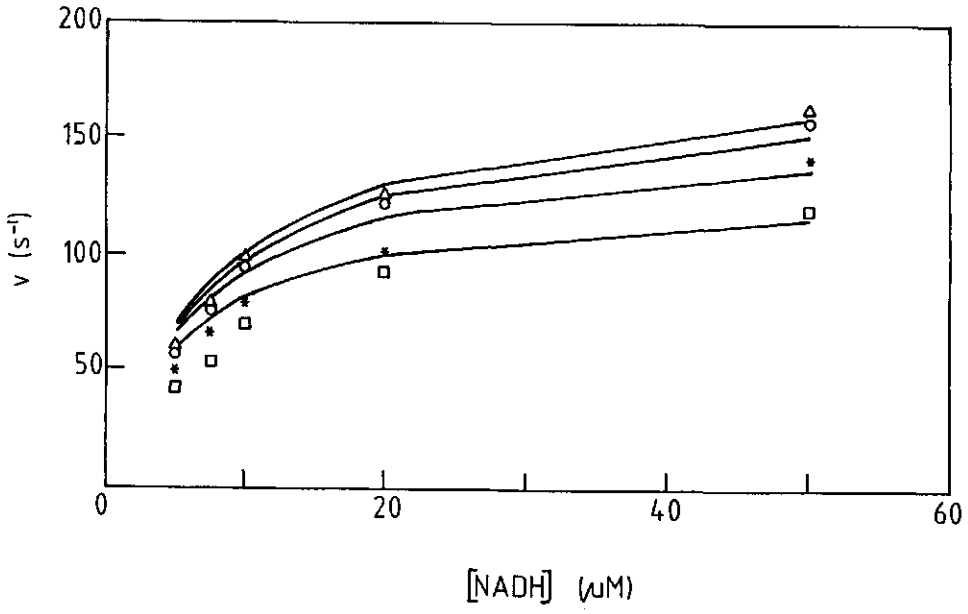


Fig. 4.2.2. A. Simulation of the enoate reductase reaction in AOT reversed micelles using Eq. 1. The symbols represent the experimental data. For K_A, K_B and $V/[E_o]$ the values observed in aqueous solution were taken. Symbols: see Fig. 4.2.1.
B. Simulation of the enoate reductase reaction using different exchange rates for differently loaded micelles. The fitting parameters are given in Table 4.2.1.

TABLE 4.2.1. Parameters used to fit the theoretical rate equation to the values experimentally determined

	[M](mM)	Φ	[NADH](μM)	[2-methylbutenoic acid (mM)	$k_{ex}(M^{-1}s^{-1})$	k^A_{ex}/k^B_{ex}
CTAB	*1.0*	*0.036*	*5.0-50*	*0.075-2.5*	*1x10⁷*	*0.05*
AOT	*2.7*	*0.036*	*5.0-50*	*0.075-0.6*	*5x10⁷*	*0.4*
TRITON X-100	*2.0*	*0.054*	*5.0-50*	*1.0-10*	*2x10⁸*	*7*

The parameters used to fit the theoretical curves to the experimental data are given in Table 4.2.1. The simulated curves are very close to the data experimentally determined (For K_A an apparent value of 9.8 μM was calculated using classical double reciprocal Lineweaver plots on the experimental data, the model predicts a value of 8 μM using the same method; for K_B these values are 0.02 and 0.03 mM respectively.). Thus the model describes the rate of enzymatic conversion of 2-methylbutenoic acid to 2-methylbutanoic acid in AOT reversed micelles rather well and the experimental rate of the reaction can be predicted without having to assume any changes of the intrinsic kinetic parameters of the enzyme itself.

2. CTAB reversed micelles

To investigate whether the interphase had any effect on the kinetics of the enzymatic reaction, also a detergent with a cationogenic headgroup was used, instead of AOT (anionogenic detergent), in which repulsion of the substrates by the surfactant headgroup could occur.
In 0.2 M CTAB reversed micelles with hexanol as a cosurfactant the enzymatic formation of 2-methylbutanoic acid was measured. The concentration of the substrate was varied from 0.075 to 2.5 mM. No substrate inhibition was observed up to this concentration (see below). The concentration of the cofactor NADH ranged from 5 to 50 μM. Again, the different rates of the reaction which were measured, did not correspond with the values that could be expected using the methods reported in the literature. The results showed either inhibition, or a 50% inactivation of the enzyme. For these reversed micelles it was investigated whether the model could explain this observation. Since the concentration of reversed micelles was within the range of 2-methylbutenoic acid concentration, that is, upon varying the concentration of the substrate all reversed micelles become filled, a marked effect of the ratio of [2-methylbutenoic acid] to [M] is expected according to our model. A good simulation was obtained using Eq.1 and values for the variable parameters k_{ex} and [M] reported in the literature [20]. No changes in the turnover rate of the enzyme, denaturation or inhibition needed to be assumed. The exchange rate of 1 x 10^7 ($M^{-1}s^{-1}$) is responsible for the apparent inhibitor observed. An even better simulation was obtained by using a twenty-fold faster exchange rate for 2-methylbutenoic acid than for NADH (Fig. 4.2.3). The fact that the agreement becomes better suggests that the exchange of a negatively charged species is hindered by association with the interface. In this case NADH (double negatively charged) will exchange slower than 2-methylbutenoic acid (one negative charge). The parameters used to simulate the experimental data are presented in Table 4.2.1.
Both the concentration of reversed micelles and the intermicellar exchange rate are well within the ranges reported in the literature [20,21].

concentration and not by the high NADH concentration when expressed in pseudophase concentration where inhibition can be expected, indicates that not simply the concentration of the solute in the aqueous phase determines enzyme kinetics. An explanation is offered by the theory reported in reference [9] which considers the intramicellar concentration. The theory predicts that a solute becomes only concentrated when the overall concentration becomes higher than the concentration of reversed micelles which is in the mM range [19]. At this level more substrate molecules are forced into one reversed micelle. The coincidence that inhibition of the enzyme activity occurs for a substrate concentration that is comparable with the concentration of reversed micelles supports the idea that also the concentration of micelles is important in the kinetics of the enzymatic conversion.

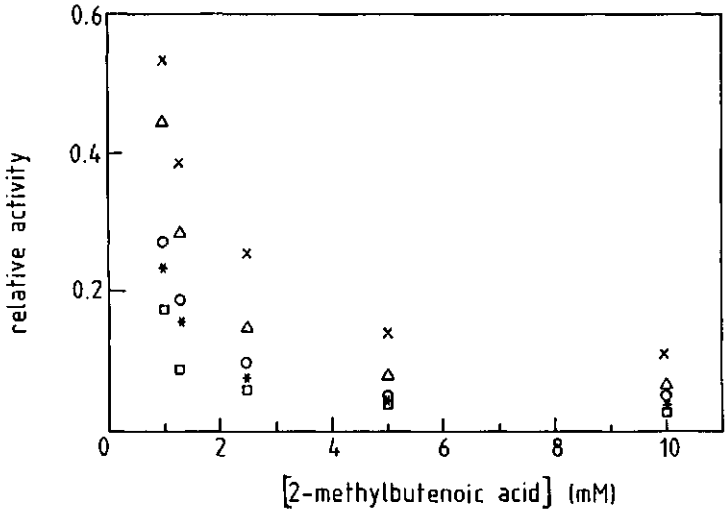


Fig. 4.2.5. Rate of the enzymatic reduction of 2-methylbutenoic acid in AOT reversed micelles. For 2-methylbutenoic acid an overall concentration range from 1 to 10 mM was chosen. The (overall) concentrations for NADH were: □ 5.0 μM, * 7.5 μM, ○ 10 μM, Δ 20 μM, × 50 μM. For further experimental details: see Materials & Methods.

This phenomena can be understood, qualitatively, using Eq. 1. The ratio between (overall) concentration of the substrate and the concentration reversed micelles determines the efficiency of the exchange and therefore, the rate of the reaction. When the ratio is between 0.2 and 1, as is the case for 2-methylbutenoic acid, the enzyme will, irrespective of the rate of the intermicellar exchange, always experience a substrate concentration which is close to the concentration of the substrate in the aqueous pseudophase. Therefore in our case with an effective concentration of 25 mM present in volume of the waterphase, when the overall concentration of substrate is *ca.* 1 mM, inhibition of the enzyme activity may occur. For NADH in AOT this value is, by far, not reached; the molar ratio between substrate and reversed micelles is maximally 0.01. In this case, the

concentration of NADH experienced by the enzyme in its microenvironment is determined by the exchange rate and the rate of the reaction.
Similar inhibitory effects have been reported by Eremin and coworkers [22]. Also in CTAB reversed micelles substrate inhibition was observed. In this case however, the inhibition was present to a minor extend and occurred at higher concentrations of 2-methylbutenoic acid *i.e.* above 5 mM overall. In this case maximally 10 % of inhibition could be observed. In Triton X-100 no inhibition was observed up to a concentration of 10 mM. Apparently the level at which the inhibitory 2-methylbutenoic acid concentration is reached in the vicinity of the enzyme varies for the different reversed micellar media.
We think that repulsion between the substrate and the interface will more easily lead to inhibitory effective intermicellar concentrations. For example interaction of the negatively charged 2-methylbutenoic acid with the cationogenic headgroup of CTAB can result in a relatively low concentration of the substrate in the core of the micelle.
Finally, also the importance of the rigidity of the interface has to be stressed: If the interface is very flexible not only the exchange of the reversed micelles is more rapid, but also the structure of the solution is less defined and the assumptions used to derive the model of Fig. 4.1.3 may not be valid anymore.

4.2.5. CONCLUDING REMARKS

From the results presented here we can conclude that all intrinsic parameters governing the rate of the enoate reductase reaction remain unchanged in reversed micelles. Application of this model also implies that enzyme kinetics depend not only on the substrate concentration in the medium but also the concentration of reversed micelles in the medium. Serious deviations from ordinary kinetics can be measured. When concentrations are used in the same order of magnitude of the concentration of reversed micelles, the observed pattern is seriously affected, leading to a wrong interpretation of the kinetic parameters of the enzyme. The effects of the different compositions (charge of the interphase) of the reversed micellar media are small compared to the effect of the rate of the intermicellar exchange.

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4.3. ENZYME KINETICS IN REVERSED MICELLES: 3. BEHAVIOUR OF 20ß-HYDROXYSTEROID DEHYDROGENASE.

4.3.1. SUMMARY

The kinetic parameters of 20ß-hydroxysteroid dehydrogenase were determined in aqueous solutions and in reversed micellar media composed with either an anionic, a cationic or a nonionic surfactant, at low and at high ionic strength. The velocity data were analysed in two ways: first by extrapolation to infinite concentrations of both substrates to determine "apparent" Michaelis constants and V values, and secondly by simulation of experimental curves using the model, presented before (Verhaert, R.M.D., Hilhorst, R., Vermuë, M., Schaafsma, T.J. and Veeger, C., submitted, Chapter 4.1). Data analysis according to the first approach reveals some differences in the kinetic parameters in reversed micelles as compared to those in aqueous solution, though the kinetic parameters of the enzyme seem not to be much affected by enclosure in reversed micelles. It is shown that the changes that do occur are not caused by a shift of the intramicellar pH or by electrostatic interactions between the enzyme and the surfactant headgroups. Interpretation of the data using the second approach assumes that the enzyme is not affected by the enclosure in reversed micelles, and that deviations with respect to the aqueous parameters are caused by exchange phenomena between distinct aqueous droplets in the organic phase and by a high effective intramicellar substrate concentration. This model is able to predict reaction rates that agree rather well with experimentally determined rates and explains why the enzyme mechanism in reversed micelles is, at all progesterone concentrations used, the same as observed at high progesterone concentrations in aqueous solution. Furthermore it clarifies the occurence of substrate inhibition in AOT reversed micelles and the observed low activity in Triton reversed micelles, as arising from the high par tition coefficient of progesterone and the slow rate of diffusion of progesterone into the reversed micelles. From these results and those reported for enoate reductase (Verhaert, R.M.D., Tyrakowska, B., Hilhorst, R., Schaafsma, T.J. and Veeger, C., (Chapter 4.2) it can be concluded that the theory presented before (Verhaert, R.M.D., Hilhorst, R., Vermuë, M., Schaafsma, T.J. and Veeger, C., (Chapter 4.1) offers a good explanation for the observed kinetic behaviour in reversed micelles, and emphasizes the importance of exchange processes between micelles.

4.3.2. INTRODUCTION

Presently, several fields of application for enzymes in reversed micelles have been suggested [1-3]. One of those is the use of reversed micellar media for the site- and stereo-specific conversion of compounds that are poorly soluble in water. Such an application is feasible because many enzymes can be incorporated in the water pool of a reversed micelle, which is stabilized by a monolayer of surface active compounds in a hydrocarbon bulk solution. Due to the presence of an aqueous domain, an interfacial domain and an organic phase, compounds that are poorly soluble in either water or in an alkane, are well soluble in a reversed micellar medium. An advantage of reversed micellar media over two phase systems for enzymatic reactions is their large interfacial area, (10-100 m^2/ml) that reduces

diffusion limitation to a minimum. For steroid conversions, an additional advantage is the effective extraction of product into the organic phase, thus preventing product inhibition [4]. It has been shown that apolar compounds like steroids and cholesterol are well converted in such media but the reaction rates at fixed substrate concentrations are strongly dependent on medium composition [5-7]. Guidelines have been given to optimize the composition of the media in such a way that a high substrate concentration in the vicinity of the enzyme is assured [7-9]. For the enzyme 20ß-hydroxysteroid dehydrogenase preliminary observations on its kinetic properties in reversed micelles have been reported [9,10], presenting evidence for an increased Michaelis constant for the apolar substrate progesterone and an influence of the type of surfactant on the maximal velocity. These measurements were carried out under semi-saturating conditions with respect to progesterone.
This study presents the apparent kinetic parameters for HSDH as obtained by extrapolation to infinite concentrations of both substrates, and analyses those data with the kinetic model presented in the accompanying paper [11].

4.3.3. MATERIALS AND METHODS

4.3.3.1. Materials

Cetyltrimethylammonium bromide (CTAB) was purchased from Serva, Heidelberg, FRG, and Triton X-100, progesterone, Mes and Hepes from Sigma, St. Louis, USA. AOT from Janssen Chimica, Beerse, Belgium, was purified according to Menger and Yamada [12]. Octane and hexanol were from Merck, Darmstadt, FRG, and were distilled before use. NADH and 20ß-hydroxysteroid dehydrogenase (*Streptomyces hydrogenans*) were supplied by Boehringer Mannheim, FRG. The enzyme was dialyzed against 5 mM phosphate buffer pH=7.6 and stored in small portions at -20°C. Water was Nanopure (Sybron-Barnstead, Boston, MA).

4.3.3.2. Determination of the composition of the organic phase and interphase

The amounts of hexanol in the interphase and in the continuous phase of CTAB and Triton reversed micelles were determined by a titration procedure according to Bowcott and Schulman [13]. The micellar solutions were prepared with 50mM Mes buffer pH 6.4 of either I=0.05 or I=0.5 [14] to give w_o=10. Determination of the partition coefficient of progesterone in organic solvent-buffer two-phase systems. Excess progesterone was added to 2 ml of octane, containing the amount of hexanol that was determined to be present in the continuous organic phase of the reversed micellar solutions. After one hour of shaking at 25°C, excess progesterone was removed by 4 min. of centrifugation in an Eppendorf microcentrifuge. 0.9 ml of the organic phase was diluted to 1 ml with organic solvent of the same composition. To this 90% saturated solution 1 ml of 50 mM Hepes buffer pH 7.6 of either I=0.05 or I=0.5 was added. The closed vials were shaken overnight at 25°C at 160 rpm. The steroid concentration in both phases was determined by HPLC (ISCO) with a Microspher C10 column, using methanol:H_2O (75:25 v/v) as eluent at a flow rate of 1 ml.min^{-1}. The detection wavelength was 240 nm. The organic phase was diluted 100 times with methanol and subsequently to 400 times by addition of three volumes of methanol:H_2O (2:1 v/v). The aqueous phase was not diluted. Progesterone concentrations were determined from a calibration curve. The reported partition coefficients are an average of two independent determinations.

4.3.3.3. Preparation of reversed micelles
Stocks of 0.2 M CTAB reversed micelles were prepared by mixing 2.2 g CTAB, 26 ml octane, 3.0 ml hexanol and 0.8 ml Hepes buffer till a clear solution was obtained. For 0.3 M Triton the composition was 5.25 ml Triton, 18 ml octane, 4.5 ml hexanol, 1.3 ml Hepes buffer. For 0.2 M AOT, it was 2.7 g AOT, 29 ml octane and 0.8 ml Hepes buffer. The solutions were used within 24 h from preparation. A stock solution of progesterone was prepared in chloroform. Appropriate amounts were pipetted into glass tubes. After evaporation of the chloroform, stock reversed micellar solutions or buffer solutions were added and the tubes were stoppered till use.

4.3.3.4. Determination of HSDH activity
The activity of 20ß-hydroxysteroid dehydrogenase was measured in aqueous buffers and in reversed micellar media at 25°C. Aqueous buffers and the water pool of the reversed micelles consisted of 50 mM Hepes pH 7.6 at an ionic strength [14] of either I=0.05 or I=0.5, unless indicated otherwise. To 1 ml of aqueous solution or 1 ml of a stock reversed micellar solution (already containing the desired progesterone concentration) 5 µl of NADH solution was added of such a concentration as to give the desired overall concentration. The final H_2O to surfactant ratio (w_o) of 10 was obtained by the addition of 5 µl enzyme solution. The reaction was followed by monitoring the change in absorption at 340 *vs.* 380 nm using an Aminco DW2A spectrophotometer, with a thermostatted (25°C) cuvette holder.

4.3.3.5. Determination of pH profile
The pH-profile of 20ß-hydroxysteroid dehydrogenase was determined under conditions approaching maximum activity, in aqueous solutions of 50 mM Mes or Hepes with an ionic strength of either I=0.05 or I=0.5. Both the NADH concentration and the progesterone concentration were 50 mM. In CTAB, Triton and AOT reversed micelles the final NADH concentrations were 87, 190 and 83 µM respectively and the progesterone concentrations were 10, 10 and 3.1 mM respectively, all with respect to the overall volume. All measurements were carried out in duplicate.

4.3.3.6. Determination of kinetic parameters
Kinetic parameters were determined by initial velocity analysis. This involved varying the NADH concentration at five fixed levels of progesterone. Concentration ranges of progesterone and NADH were varied from 0.5 to 5 times the K_m^{app} values (expressed as overall concentrations). The reaction velocity was defined as the change in absorbance between 10 and 25 s after the reaction was started. Enzyme activities were corrected for slow loss of activity during the day, as assayed under standard conditions in aqueous solution of 50 mM Hepes pH 7.6, I=0.05 using NADH and progesterone concentrations of 10 times the K_m values. In reversed micelles all measurements were carried out in duplicate. The kinetic results obtained in aqueous solution are the average of two independent sets of experiments, carried out in duplicate.

4.3.3.7. Data analysis
Experimental data points were analysed by two methods. First they were interpreted according to equation 1, that describes the reaction rate for an Ordered Bi Bi mechanism. The symbols used in this equation are defined as in [15].

$$v = \frac{V}{1 + \frac{K_A}{[A]} + \frac{K_B}{[B]} + \frac{K_{IA}\,K_B}{[A][B]}} \qquad (1)$$

K_A, K_B, K_{IA} and v_{max} values were determined from the secondary double-reciprocal plot, at infinite concentrations of the other substrate. The v_{max} was expressed as moles of substrate converted per mole of enzyme per second. The second method of analysis was according to equation 2 that is derived in the accompanying paper [11]

$$v = \frac{V}{1 + \frac{\beta_1 K_A}{[\bar{A}'_{in}]} + \frac{P^a K_B + \frac{V}{[E_o]}\frac{1}{k_{in}}}{[B_{ov}]} + \frac{\beta_1 \beta_2}{[\bar{A}'_{in}][B_{ov}]} + \frac{\Phi K_B}{[M]} + \frac{V}{[E_o]}\beta_3} \qquad (2)$$

In which

$$\beta_1 = \frac{\epsilon^A_o}{\epsilon^A_i} + 1 \qquad (2A)$$

$$\beta_2 = P^a K_B K_{IA} + \frac{k_{-1}}{k_{in}} K_A \qquad (2B)$$

$$\beta_3 = \frac{\beta_1}{k_{ex}\,\epsilon^P_o\,[M]} + \frac{\Phi}{k_{out}\,[M]} \qquad (2C)$$

Curves of v *vs.* [substrate] were calculated for the micellar systems. Values for the parameters used, are listed in Table 4.3.1. The concentrations of AOT reversed micelles were derived from reference [16], values for the concentration of reversed micelles and k_{ex} for CTAB from [17] and [18] and for Triton the micellar concentration was taken from [19]. k_{ex} and k_{in} were adjusted to give the best fits, k_{out} and k_{in} are related by the partition coefficient using the formula [11]:

$$P^a = \frac{k_{out}}{k_{in}} \qquad (3)$$

4.3.4. RESULTS

When studying the effect of encapsulation in reversed micelles on the kinetic parameters, it has to be realized that those parameters are subject to influences of pH and ionic strength. In case of reversed micelles, also the charge of the head groups might play a role [9,10,19], as has been

observed for immobilized enzymes [21-24]. We investigated the effect on the kinetic parameters of the enclosure of 20ß-hydroxysteroid dehydrogenase in reversed micelles composed of a cationic, an anionic or a nonionic surfactant. This enzyme catalyzes the reduction of the ketogroup on the 20-position of progesterone by NADH to yield 20ß-hydroxy-pregn-4-en-3-one. NADH is water-soluble and resides in the water pool. Progesterone partitions between the organic phase, interphase and aqueous phase.

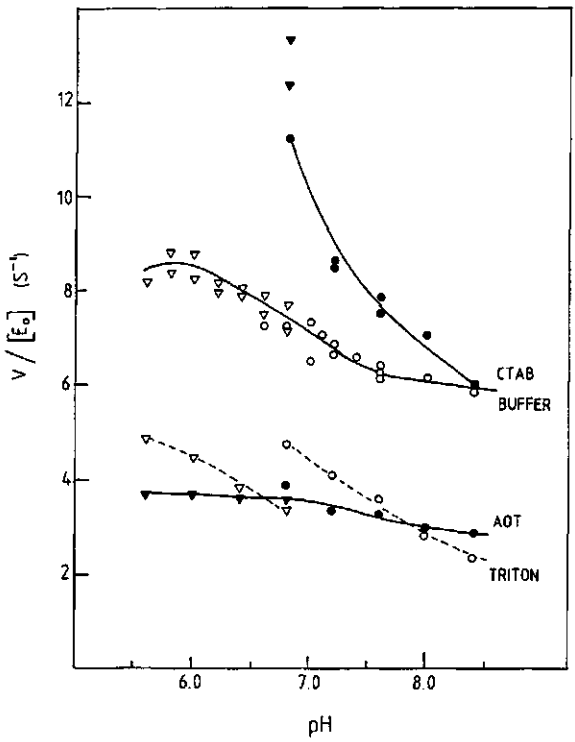


Fig. 4.3.1. pH profiles of 20ß-HSDH activity in aqueous buffer solution and reversed micelles, composed with AOT, CTAB or Triton X-100 as surfactant. Conditions were as described in Materials and Methods. ∇,▼ *Mes-buffer* ○,● *Hepes buffer.*

4.3.4.1. Activity as a function of pH

As a shift in pH may cause an apparent change in kinetic parameters, the pH profile of the reaction was determined under saturating conditions both in aqueous solution and in reversed micelles, from pH 5.6 to 8.4, with constant ionic strength (Fig. 4.3.1). In aqueous solution the activity decreased with increasing pH. No distinct optimum was found within the pH range investigated. The highest activity was found at a pH around 5.8. This result deviates from the value of 6.4 that was reported for this enzyme using cortisone as a substrate [25].

Table 4.3.1. Parameters used for the simulation of the kinetic data to Eq. 2.

				Concentration range				
	I	$[M]$ (mM)	Φ (1/1)	$[NADH]$ (μM)	$[progesterone]$ (mM)	P^a	k_{in}	k_{ex} ($M^{-1}s^{-1}$)
CTAB	*0.05*	*1.0*	*0.036*	*5-50*	*0.2-4*	*368*	*10^5*	*1 x10^6*
	0.5	*1.0*	*0.036*	*5-50*	*1.0-10*	*530*	*10^5*	*2 x10^6*
AOT	*0.05*	*2.7*	*0.036*	*16-115*	*0.4-4*	*199*	*10^6*	*1.3x10^6*
Triton	*0.05*	*2.0*	*0.054*	*15-146*	*0.075-0.75*	*671*	*7x10^3*	*10^6*
	0.5	*2.0*	*0.054*	*15-146*	*0.075-0.75*	*744*	*10^4*	*10^6*

In CTAB reversed micelles, activity was strongly pH dependent. Below pH 7 a steep increase in enzyme activity and spontaneous breakdown of NADH was observed. At a higher ionic strength with w_o=15, measurements could be extended to a lower pH. In this case a pH optimum of 6.4 was observed. In AOT and Triton reversed micelles the activity of the enzyme was about half the activity observed in aqueous solution although the substrate concentrations were saturating. As variation of pH led to minor changes in activity, a shift in internal micellar pH cannot explain this low activity. Since no clear pH optima were found, and possible pH shifts due to incorporation of buffer into the micellar water pool were not observed, we decided to perform our kinetics measurements at a fixed pH instead of at the pH-optimum for each system. For reversed micelles slight deviations from this pH caused by the micellar environment do not cause changes in enzyme activity larger than 10-15%. An additional advantage of using pH=7.6 is the fact that the measurements are not troubled by spontaneous hydrolysis of NADH.

4.3.4.2. Determination of kinetic parameters

The kinetic parameters of HSDH were determined as described in Materials and Methods. In order to exclude an effect of a change in ionic strength of the micellar water pools as compared with the aqueous solution and to shed light on a potential influence of the change of surfactant head groups on the kinetic behaviour, measurements were performed at low and high ionic strength except for AOT, where the micellar system is not stable at higher ionic strength.

In aqueous solutions, a series of straight lines converging in the third quadrant was obtained when 1/v was plotted against 1/[NADH] at low progesterone concentrations, suggesting sequential binding of NADH and progesterone to the enzyme (Fig. 4.3.2). This is in agreement with the results reported at low concentrations of the substrate deoxycortisone [26]. But at higher progesterone concentrations the point of intersection moved to the extended X-axis. These results obtained in buffer solutions at both low and high ionic strength suggest an Ordered Bi Bi (bimolecular, bireactant, Fig. 4.3.3a) mechanism at lower progesterone concentrations. But above a progesterone concentration of 7 mM, the mechanism changes into the special case of a Rapid Equilibrium Random Bi Bi mechanism [27,28] (Fig. 4.3.3b). In this case the presence of one substrate has no effect on the binding of the second substrate and all the equilibria are adjusted rapidly except the conversion of EAB to ECD (K_1=K_4 and K_2=K_3). Now K_{IA} is equal to K_A. These results differ from the Random mechanism proposed for 20ß-HSDH as observed at high cortisone concentrations [29]. Because the

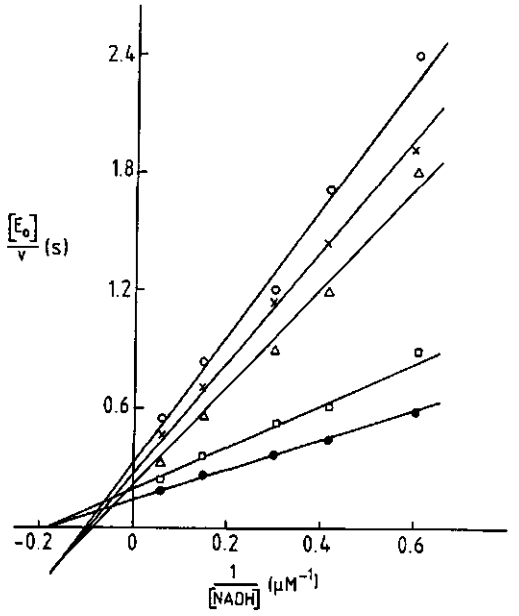


Fig. 4.3.2. Double reciprocal plot of 20ß HSDH activity in aqueous solution at I=0.5 at varying NADH concentrations and five fixed levels of progesterone. ○, *1.8 mM;* ×, *2.7 mM;* △, *3.6 mM;* □, *7.1 mM;* ●, *17.7 mM of progesterone.*

latter measurements with cortisone were carried out at pH 10.3, comparison of the reported kinetic parameters with our values is not possible. It must be emphasized that our results were obtained in two independent sets of duplicate measurements.

A linear relationship between the reciprocal initial velocity and the reciprocal concentrations of the substrate exists also in reversed micelles containing either CTAB or Triton as surfactant (Fig. 4.3.4). In AOT marked inhibition by NADH was observed but the plots were linear at lower concentrations (overall concentraton < 60 μM). For all reversed micellar systems Lineweaver Burke plots gave straight converging lines, with one point of intersection, indicating the special case of a Rapid Equilibrium Random Bi Bi mechanism when K_1=K_A, K_2=K_I and K_{IA}=K_A. The kinetic parameters K_A, K_B and v_{max} that were derived from primary and secondary double reciprocal plots are listed in Table 4.3.2.

As an alternative, we analysed our results using the model for an Ordered Bi Bi mechanism presented in a separate paper [11], which is also valid for a Rapid Equilibrium Random Bi Bi mechanism. This model is based on the assumption that the enzyme resides in the water pool, where the reaction takes place. Because the substrate molecules are enclosed in the very small volume of one reversed micelle, this can lead to a high intramicellar concentration. The basic thought behind this theory is, that V, K_A and K_B for HSDH in reversed micelles have the same values as in an aqueous solution, and that deviations from the behaviour in aqueous solutions are

caused by confinement of the enzyme to the waterpool of reversed micelles. This approach has shown to be valuable to explain the behaviour of enoate reductase in reversed micelles [19].
Using Eq. 2 and the parameters listed in Table 4.3.1, the kinetic curves for HSDH shown in Figs. 4.3.5 to 4.3.9 were obtained.

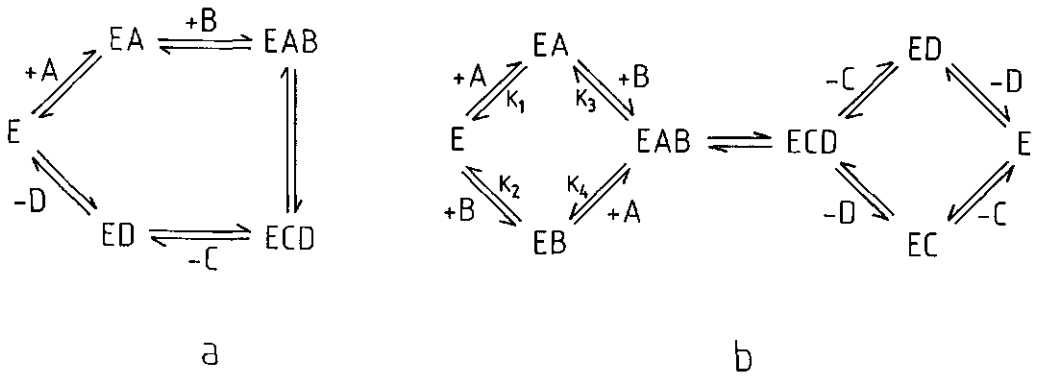


Fig. 4.3.3. Schematic representation of the reaction sequences for a: Ordered Bi Bi and b: Rapid Equilibrium Random Bi Bi Mechanism. A: NADH; B: progesterone; C: 20ß-hydroxy-pregn-4-en-3-one; D: NAD⁺.

Table 4.3.2. Kinetic parameters of 20ß-hydroxysteroid dehydrogenase in aqueous and reversed micellar media. The values were obtained by data analysis according to Eq. 1. K_s was calculated from K_s^* by taking into account the partition coefficient for progesterone.

	Ionic strength	*$V/[E_o]$ (s^{-1})*	*K_A (mM)*	*K_s (mM)*	*K_s^* (mM)*
Aqueous solution	*0.05*	*10.5*	*3.5*	*2.8*	-
	0.5	*11.1*	*5.7*	*5.0*	-
reversed micelles					
CTAB	*0.05*	*7.9*	*4.0*	*840*	*2.3*
	0.5	*13.3*	*7.9*	*1330*	*2.5*
Triton	*0.05*	*4.7*	*5.4*	*714*	*1.1*
	0.5	*5.8*	*5.2*	*1000*	*1.3*
AOT	*0.05*	*10.1*	*16.7*	*299*	*1.5*

4.3.4.3. Determination of the composition of the organic phase and interphase and of the partition coefficient for progesterone

For reactions occurring in reversed micelles, transport of reactants into the water pool is required. In case of a polar substrate transport takes place *via* collisions. In case of apolar substrates *via* transport across the interphase. In both cases the composition of the interphase is a determining factor. In AOT reversed micelles, no cosurfactant is present, the interphase is only covered with AOT molecules. In CTAB and Triton reversed micelles, hexanol is the cosurfactant used. The amount of hexanol in the interfase and continuous phase was determined following the procedure described before [8,9,13] (Table 4.3.3). These data were used to determine the partition coefficient of progesterone between aqueous buffer and an organic phase having the same composition as in the reversed micellar media. The distribution of progesterone over the water phase and organic phase is dependent on the amount of hexanol in the organic phase and on the ionic strength of the buffer (Table 4.3.3).

Table 4.3.3. The composition of the interphase and continuous phase of CTAB, Triton and AOT reversed micelles and partition coefficients of progesterone in buffer-organic solvent two-phase systems. The composition of the organic phase used when determining the partition coefficients corresponded to the composition of the continuous phase of the reversed micelles. The composition of interphase and continuous phase was determined as described in Materials and Methods.

Medium	*Ionic strength*	a_o*	*ml hexanol/ ml octane*	P^s **
CTAB	*0.05*	*2.45*	*40.7*	*368*
	0.5	*2.10*	*36.3*	*530*
Triton	*0.05*	*1.27*	*76.4*	*671*
	0.5	*1.16*	*76.7*	*744*
AOT	*0.05*	*0*	*0*	*199*
	0.5	*0*	*0*	*279*

* *a_o is the molar ratio of hexanol to surfactant.*
** *P^s is the partition coefficient of progesterone in the medium indicated.*

Because catalysis is assumed to take place in the water pool, and the K_s value is determined by the substrate concentration experienced by the enzyme, partition coefficients of the substrate have to be taken into account. K_s values were recalculated making use of the partition coefficients. The resulting K_m values are listed in Table 4.3.2.

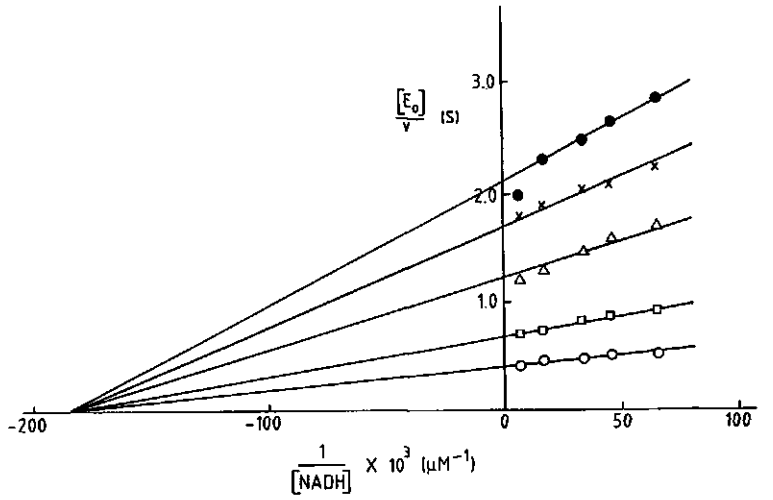


Fig. 4.3.4. Double reciprocal plot of 20ß-HSDH activity in Triton reversed micelles at I=0.05, at five fixed progesterone levels. ●, 0.075 mM; ×, 0.115 mM; Δ, 0.15 mM; □, 0.30 mM; ○, 0.75 mM of progesterone.

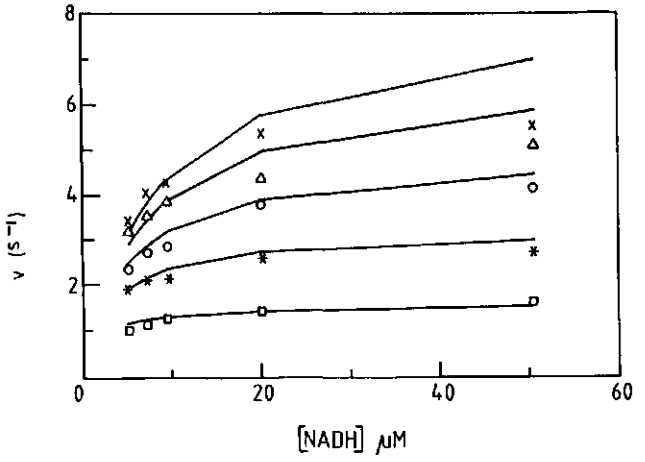


*Fig. 4.3.5. Conversion rate of progesterone to 20ß-hydroxy-pregn-4-en-3-one by HSDH in CTAB reversed micelles, I=0.05. Symbols represent experimental values, solid lines are rates calculated using Eq. 2 with parameters as listed in Table 4.3.1; □, 0.20 mM; *, 0.49 mM; ○, 0.99 mM; Δ, 1.98 mM; ×, 3.96 mM.*

4.3.5. DISCUSSION

As a first approach, the kinetic data presented in this paper were analysed as is usually done, by assuming that the reaction obeys Michaelis Menten kinetics and calculating the kinetic parameters from double reciprocal plots at infinite concentrations of both substrates. Substrate concentrations were expressed with respect to the overall volume. The values thus obtained can either reflect the kinetic parameters of the enzyme itself, or can be the result of a modification in the environment of the enzyme. For example, a change in K_m might reflect a change in the enzyme itself or might be caused by a shift in pH, ionic strength or changed local concentration in the environment the enzyme, or be a combination of several of these factors [9].
Inspection of Table 4.3.2 reveals that v_{max} obtained via traditional analysis is affected by enclosure in reversed micelles, especially in Triton reversed micelles and to a small extend in CTAB reversed micelles. K_A is also slightly affected in CTAB and Triton, but in AOT a fivefold increase in K_A is observed. An increase in ionic strenght hardly affects v_{max} in buffer and Triton reversed micelles, but has a strong effect in CTAB reversed micelles. For K_A, a similar tendency is observed in aqueous solution and CTAB reversed micelles, but K_A in Triton has hardly changed. These results indicate that the NADH-binding is not much affected by enclosure in reversed micelles.

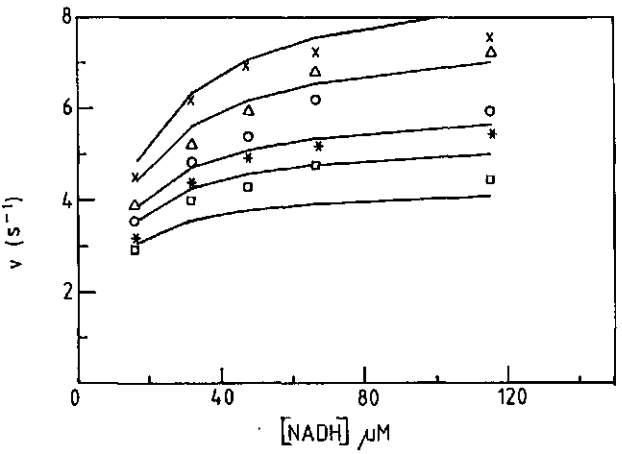


*Fig. 4.3.6. Conversion rate of progesterone to 20ß-hydroxy-pregn-4-en-3-one by HSDH in AOT reversed micelles, I=0.05. Symbols represent experimental values, solid lines are rates calculated using Eq. 2 with parameters as listed in Table 4.3.1, □, 0.40 mM; *, 0.60 mM; ○, 0.80 mM; Δ, 1.6 mM; ×, 4.0 mM progesterone.*

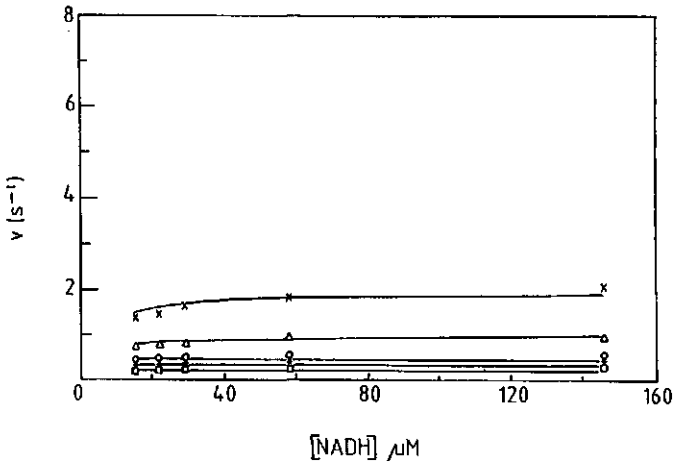


*Fig. 4.3.7. Conversion rate of progesterone to 20ß-hydroxy-pregn-4-en-3-one by HSDH in Triton reversed micelles, I=0.05. Symbols represent experimental values, solid lines are rates calculated using Eq. 2 with parameters as listed in Table 4.3.1, □, 0,075 mM; *, 0.11 mM; ○, 0.15 mM; Δ, 0.30 mM; ×, 0.75 mM progesterone.*

K_s values for progesterone in reversed micelles as expressed in overall concentrations are 100-1000 fold higher than in aqueous solutions. Such an increase in K_s values in reversed micelles as compared to an aqueous solution could lead to the conclusion that either the binding of progesterone to the enzyme is much weaker or that the substrate concentration in the microenvironment of the enzyme is lower than the overall concentration. The first possibility does not seem very likely, because the unaffected K_s reflects a state of the enzyme not very different from the aqueous situation. The second explanation is more likely, for the hydrophobic progesterone will partition over the organic phase, interphase and water pool.
Recalculation of the K_s values by taking the partition coefficient of progesterone between the water phase and organic phase into account, yielded values between 1.1 and 2.5 μM, even lower than in aqueous solution. It can be concluded that the behaviour of HSDH in reversed micelles closely resembles its behaviour in aqueous solutions when taking the overall concentrations and partition-coefficients into account.
However, this approach is not able to explain either the change in kinetic mechanism or the low v_{max} in Triton reversed micelles. Also the reason for the low activity in AOT reversed micelles (Fig. 4.3.1), under conditions where more than 75% of the maximal activity would be expected, is not understood. The presence of substrate inhibition at overall NADH concentrations above 60 μM cannot be responsible for this decrease, nor can it be attributed to changes in pH inside the reversed micelles, or to electrostatic interactions between the enzyme and surfactant head groups. As has been shown previously [8], and is confirmed here, the amount of hexanol in the interphase only influences the partitioning of the substrate

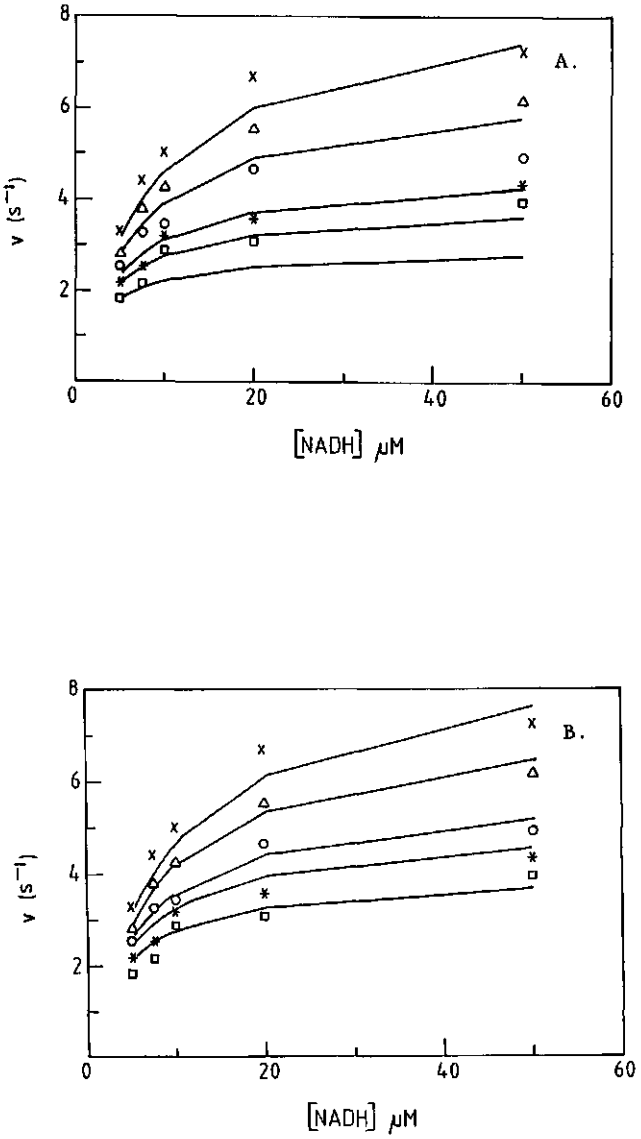
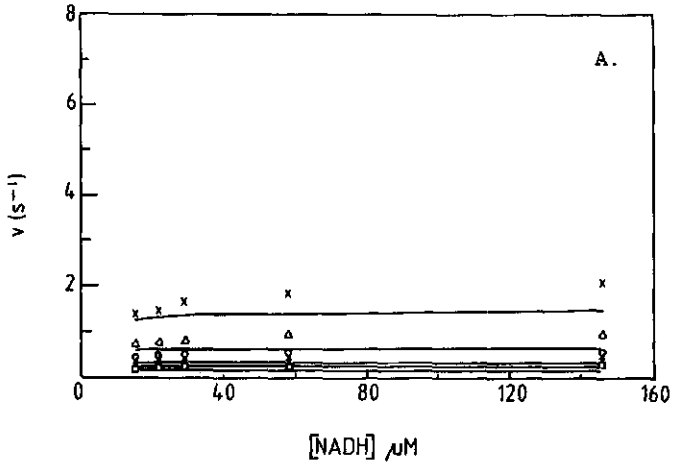


Fig. 4.3.8. *A. Conversion rate of progesterone to 20ß-hydroxy-pregn-4-en-3-one by HSDH in CTAB reversed micelles, I=0.5. Symbols represent experimental values, solid lines are rates calculated using Eq. 2 with parameters as listed in Table 4.3.1. Kinetic parameters of the enzyme are those measured in aqueous solution at I=0.5*
*B. As A, but for the calculation enzyme kinetic parameters used are those obtained at I=0.05, □, 0.98 mM; *, 1.47 mM; ○, 1.96 mM; Δ, 3.93 mM; ×, 9,82 mM progesterone.*



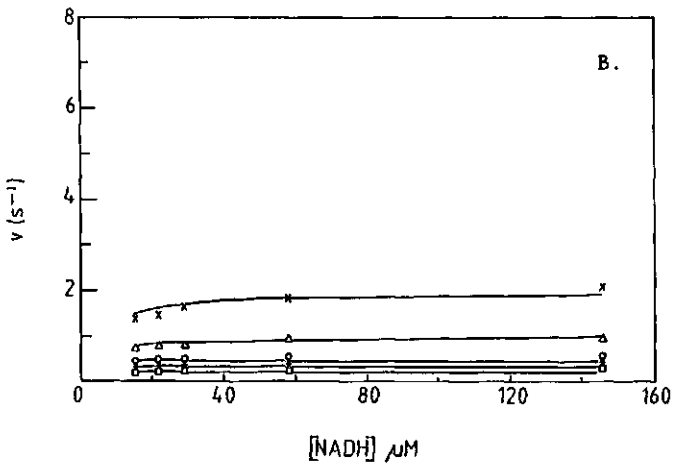


Fig. 4.3.9. *A. Conversion rate of progesterone to 20ß-hydroxy-pregn-4-en-3-one by HSDH in Triton reversed micelles, I=0.5. Symbols represent experimental values, solid lines are rates calculated using Eq. 2 with parameters as listed in Table 4.3.1. Kinetic parameters of the enzyme are those measured in aqueous solution at I=0.5*
*B. As A, but for the calculation enzyme kinetic parameters used are those obtained at I=0.05, □, 0.075 mM; *, 0.11 mM; ○, 0.15 mM; Δ, 0.30 mM; ×, 0.75 mM progesterone.*

but not the activity of hydroxysteroid dehydrogenase. In conclusion: Even though data analysis using Eq. 1 gives reasonable results, many questions concerning the reasons for the changed behaviour, remain open.
As an alternative, the reaction of HSDH in different types of reversed micelles was simulated using Eq. 2. The basis of this equation is the assumption that the reaction takes place in the waterpool of a reversed micelle, where, only if a substrate molecule is present, the local substrate concentration is high (intramicellar concentration instead of overall concentration). Assuming such an intramicellar concentration and the occurence of repulsion between NADH and the similarly charged surfactant head groups, substrate inhibition by NADH in AOT reversed micelles is not unexpected. From the concentrations of reversed micelles and the volume fraction of water given in Table 4.3.1, the intramicellar NADH-concentration in AOT reversed micelles can be calculated to be 75 mM, much higher than the values in CTAB (27 mM) and Triton (37 mM) reversed micelles. Similar results have been obtained for enoate reductase [19]. Also the observation of a Rapid Equilibrium Random Bi Bi mechanism in reversed micelles can be explained, when realising that the enzyme experiences a high progesterone concentration under conditions that only one molecule is present per reversed micelle. In the accompanying theoretical paper [11] it was observed that at high substrate concentrations the mechanism apparently changes in a Ping Pong mechanism. This phenomenon is not observed in our kinetic plots, because the condition that [S] >> [M] is not met.

Comparison of the curves, simulated by Eq. 2 using the micellar parameters of Table 4.3.1 and the kinetic parameters in aqueous solution of I=0.05 given in Table 4.3.2, with the experimental data, shows a good correlation (Figs. 4.3.5 to 4.3.7). In all cases values of k_{ex} around 10^6 $M^{-1}s^{-1}$ gave a good fit. These values for k_{ex} agree well with literature data, but are lower than the exchange rates reported previously [19]. An increase in k_{ex} enhanced the calculated rate at low NADH concentrations, giving poorer fits. The low activity in Triton reversed micelles can be ascribed to both the unfavourable partitioning of progesterone and the very low rate of entrance of this substrate in the reversed micelles. In the case of AOT, the low activity observed under semisaturating conditions is not due to unfavourable micellar parameters k_{ex} and k_{in}, but to the occurence of substrate inhibition (Fig. 4.3.6). Figs. 4.3.8a and 4.3.9a show the fits at high ionic strength, using the kinetic parameters of the enzyme measured in aqueous solution at high ionic strength. The agreement between calculated lines and experimental data is reasonable. However, better fits could be obtained (Figs. 4.3.8b and 4.3.9b) by using the values of K_A, K_B and v_{max} at I=0.05; changes in only one of these parameters did not give satisfactory results. Only redistribution of ions in the reversed micellar medium, leading to an enzyme environment that is independent of the ionic composition of the medium can explain this phenomenon. This model is capable of explaining the apparent change in enzyme mechanism that occurs upon enclosure in reversed micelles, especially the apparently lowered V values in CTAB and Triton, facts that cannot be explained using Eq. 1.

Implications of this model are that enclosure of the enzyme in reversed micelles has no effect on its kinetic parameters, and that deviations from kinetics as compared with that in aqueous solutions are caused by exchange phenomena. Furthermore the approaches used to describe enzyme kinetics either in terms of a reaction occurring in an overall volume, or as occurring in a pseudo aqueous phase are too simplified.

4.3.6. REFERENCES

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5. DISCUSSION.

The central theme in this thesis is the use of photon energy for the enzymatic production of fine chemicals. The results show that the light driven conversion of α-ß unsaturated carboxylates by enoate reductase in reversed micelles, containing a suitable photosystem, is possible (Chapter 2). The photosystem consisted of ZnMDPyTrPP (Fig. 2.1) as a photosensitizer, methylviologen as an electronacceptor and tributylamine as an irreversible donor. This photo-enzymatic system was shown to be active for over 100 hours and 100% conversion could be obtained. In Chapter 2 the system was optimized with respect to the reversed micellar composition. The separate parts of the photoinduced enzymatic system have been described in Chapter 3 (photoinduced charge separation) and Chapter 4 (enzymatic reaction). In this Chapter, some aspects of (both) parts that have not been covered in the previous Chapters will be discussed.

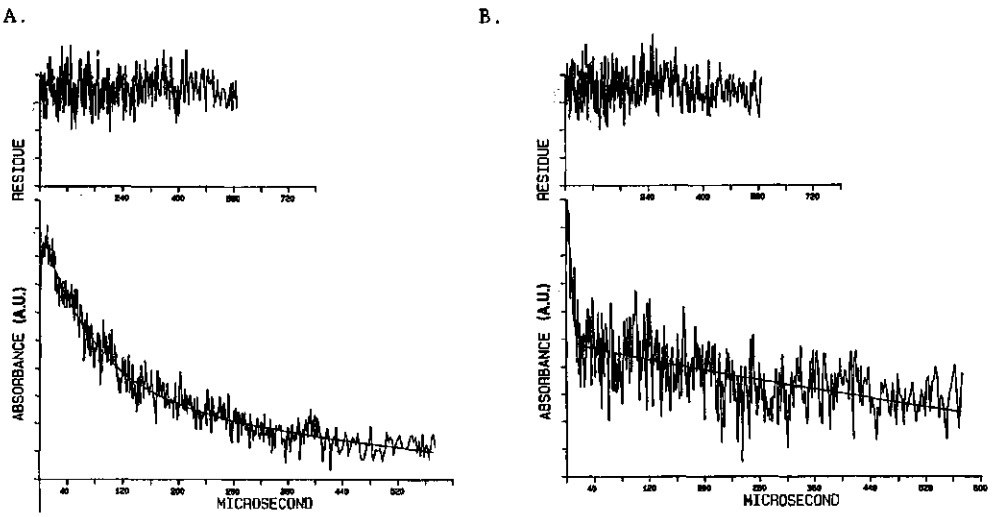

Fig. 5.1. Transient absorption of ZnMDPyTrPP in a reversed micellar solution. A. Without methylviologen; B. In the presence of 6 mM methylviologen.
The sample contained 50 µM ZnMDPyTrPP in reversed micelles composed of 0.2 M cetyltrimethylammonium bromide (CTAB) in hexanol:octane 1:9 (vol/vol), with a molar ratio of water to CTAB of 10. The waterphase consisted of 50 mM phosphate buffer pH=7.0. For excitation 12 ns, 532 nm laser pulses (Nd-Yag laser, JK Lasers System 2000) were used. Transient absorption at 598 nm and kinetic analysis was carried out as described in ref. [7].

5.1. THE PHOTOSYSTEM.

The maximally obtainable efficiency of the photosystem depends not only on the location of the reactants and the mobility of the components of the system (Chapter 3), but also on the electron transfer mechanism and on the differences between the redox potentials of the reacting species.

The mechanism of the electron transfer was investigated for the photosystem described in Chapter 2. Electron transfer from the singlet state is very unlikely because ZnMDPyTrPP fluorescence quenching by methylviologen could not be observed (Chapter 2).
Transient absorption at 598 nm was used to detect both the triplet state ZnMDPyTrPP [1] and reduced methylviologen.
In Fig. 5.1A and 5.1B the transient absorptions of ZnMDPyTrPP with and without addition of 6 mM methylviologen are represented. In the absence of methylviologen only the porphyrin triplet decay is visible ($\tau_{triplet}$=0.1 ms). Upon addition of methylviologen the porphyrin triplet lifetime is quenched ($\tau_{triplet}$=8.3 μs) by electron transfer and a methylviologen radical with a long lifetime becomes observable ($\tau_{methylviologen}$=5 ms) in agreement with literature data [2]. The rate of the triplet quenching, as determined by measuring the triplet decay rate varying the methylviologen concentration from 0 to 6 mM was 1.7×10^7 $M^{-1}s^{-1}$. This value is of the same order of magnitude as the intermicellar exchange rate: The electron transfer rate in reversed micelles composed of 0.3 M CTAB is lowered (4.4×10^6 $M^{-1}s^{-1}$). The fact that such micelles are less flexible [3,4] shows that the forward electron transfer reaction rate may be determined by the flexibility of the system, *i.e.*, the intermicellar exchange rate can influence the efficiency of the photoinduced charge separation.
This is in agreement with the results of Chapter 3, in which both the dynamics of the reversed micelles and the partitioning of tributylamine were suggested to determine the efficiency of the photosystem.
The observation that electron transfer occurs from the triplet state and that the efficiency of the photosystem increases with increasing flexibility of the reversed micelles can be exploited to optimize the photosystem in reversed micelles (*Vide infra*).

A prerequisite for electron transfer which has not been discussed in the preceding Chapters is the influence of the redox potentials of the components of the photosystem on the yield of the reaction. For any photoinduced methylviologen production to occur in the photosystems as described in Chapter 2 and 3, the oxidation potential of the porphyrin has to exceed the oxidation potential of tributylamine (1.1 V *vs.* NHE) and the oxidation potential of the porphyrin excited state from which electron transfer occurs has to be more negative than the reduction potential of methylviologen (-0.44 V *vs.* NHE).
The reduction potential of ZnP_{16} (Fig. 3.1.1) in methanol was 0.87 V *vs.* NHE (Chapter 3). Although the redox properties of a porphyrin may shift due to the environment [5], the oxidation potential of tributylamine will be too high to reduce ZnP_{16}^+. Indeed, no formation of reduced methylviologen could be observed during prolonged illumination. Although some attention has been paid to the redox properties of the porphyrins, only a few systematic studies to those properties of porphyrins with different substituents have been performed [1,6,8]. It was demonstrated that a variety of metals substituting the central hydrogen atoms can be used to modulate the electrochemical properties [1]. Chemical modification by substituents at the porphyrin core modulates both electrochemical and chemical properties. An example is the modification of the porphyrin used in Chapter 3 (P_{16}) by incorporating Pd as a central metal ion. The triplet yield is expected to rise compared to P_{16} and the ground state oxidation potential (approximately 1.3 V *vs.* NHE). At the same time this substitution does not prevent rereduction of the photo-oxidized state, as was observed for ZnP_{16} [2].
The studies presented in this thesis have demonstrated that not only the electrochemical properties, but also the chemical and spectroscopic

characteristics have to be carefully selected to obtain an efficient photosensitizer. Chapter 3.1 shows that chemical modification of porphyrins can be used to study its location in the reversed micellar interphase and to anchor the molecule in a boundary layer. The results of Chapter 3.2 indicate that a detailed study of these porphyrin properties in a variety of media can reveal valuable information about the influence of the environment on the lifetime and behaviour of the excited state.

5.2. ENZYMATIC CONVERSIONS IN REVERSED MICELLES

In reversed micelles the activity and stability of enoate reductase are at least equal to that in aqueous solution. The activity of enoate reductase is highest in reversed micelles with a short chain alcohol as cosurfactant, but the stability of the enzyme is highest in reversed micelles with a long chain alcohol. The enzyme is less stable in its reduced form (especially in apolar reversed micelles).
The peculiar dependence of the enzyme activity (measured as specific activity of enoate reductase using 10 mM 2-methylbutenoic acid and 0.2 mM NADH) on the concentration of the cosurfactant in the solution can be caused, as explained by the theory presented in Chapter 4.1, by substrate inhibition: On increasing the cosurfactant concentration the concentration of reversed micelles drops [3] and more reversed micelles become filled with NADH. This can induce an increase of the reaction rate (Chapter 4.1). Similarly, the intramicellar 2-methylbutenoic acid concentration increases further and inhibition of the reaction can occur (Chapter 4.2). This explanation implies that the specific activity measurements in Chapter 2 may be affected by substrate inhibition. The dependence of enzyme kinetics on variations of the concentrations of both substrates has to be studied in relation to the composition of the reversed micellar solution to clarify this phenomenon.

The validity of the theoretical description of enzyme kinetics (Chapter 4.1) has some other implications. In the derivation of the equations describing enzyme kinetics in reversed micelles, no assumptions were made about the dimensions and the interfacial properties of the system. The assumption made for the model was only that exchange of separate enzyme and substrate containing entities ("micro-reactors") existed. *In vivo* systems like lysosomes and parts of the endoplasmatic reticulum have in principle the same properties. The impact of microheterogeneity on enzyme kinetics as visualized in Chapter 4 shows that enzyme kinetics in cellular organelles need to be approached by similar models.

5.3. CONCLUDING REMARKS

For an efficient enzyme system the following conditions were deduced (Chapters 2 and 4).
(i) The lifetime of enoate reductase can be extended using a system with a relatively low concentration of reducing agent (NADH, reduced methylviologen) and with excess substrate. Then the enzyme reacts rapidly with substrate after it has been reduced. The overall reaction rate, however, slows down.
(ii) The stability of the enzyme is increased in rather rigid reversed micelles (Chapter 2).
(iii) The highest activity of enoate reductase can be reached using

substrate and NADH concentrations which are in the same order of magnitude as the concentration of reversed micelles (Chapter 4). It has to be stressed that this condition may conflict with (i) and that substrate inhibition has to be excluded.
(iv) The reaction rate is highest in a reversed micellar solution with a high exchange rate ($k_{ex} \gg V/[E_o]$). A drawback of such a solution is that it displays a high level of flexibility. Formation of protein detergent complexes causing inactivation may be facilitated (see (ii)). Indeed, in Triton reversed micelles, in which the activity of enoate reductase was highest and the intermicellar exchange was very fast, enzyme activity was only present for a short period of time (approximately 30 min).
(v) With respect to other substrates of the enzyme: The rate of the enzyme reaction can be increased by affecting the partitioning of the substrate (Chapter 4.1). The highest rate of the reaction can be expected if the partition coefficient is low (Chapter 4.3).
(vi) Similarly, equally charged substrate(s) and interface are beneficial for optimizing the enzyme activity in reversed micelles. Theoretical predictions of the enzyme activity in AOT reversed micelles omitting charge-charge interactions were in good agreement with experimental results, so the effects of substrate charge were small in those reversed micelles. However, in CTAB reversed micelles deviations were observed, probably caused by complexation of the substrate with the interface. This phenomenon can be studied systematically now.

The results of the study to the photosystem indicate that for chosing a photosensitizer attention has to be paid to its behaviour with respect to location (preferentially in the interphase, Chapter 2), redox potential and the occurence of intermolecular reactions (Chapter 3.2).
The yield of charge separation using this system is highest (Chapter 3) (i) using reversed micelles displaying a high degree of flexibility and (ii) using high concentrations of both electron donor and electron acceptor.
The conditions for an optimal performance of both a photoinduced charge separation reaction and an enzymatic conversion show that the selection of non-denaturating reversed micellar media with high intermicellar exchange rates is necessary for a significant improvement of photoinduced enzymatic reactions in reversed micelles.

5.4. REFERENCES

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SUMMARY

In this thesis the performance and coupling of two types of reaction, photoinduced charge separation and enzymatic conversion were studied in reversed micelles. Reversed micelles are 1 to 10 nm sized water droplets dispersed in an organic solution. The dispersant is a detergent (cationogenic, nonionogenic or anionogenic). In some solutions an additional compound (cosurfactant) is necessary to stabilize the droplets. In the studies presented here aliphatic alcohols were used for this purpose. The composition of reversed micelles can be optimized to yield both an efficient photoinduced charge separation and a high enzyme turnover number.

In Chapter 2 a photosystem was coupled to the reduction of α-ß unsaturated carboxylates by enoate reductase (EC 1.3.1.31). The photosystem was composed of a porphyrin as photosensitizer, tributylamine as electrondonor and methylviologen as electronacceptor. The photoinduced enzymatic system was active for over 100 hours. A 100% conversion could be accomplished. Systematic studies to the relation between the efficiency of the photosystem and the composition of the reversed micellar solution showed that an increase in cosurfactant concentration and cosurfactant polarity induced a higher yield of charge separation. The location of a porphyrin as a function of the composition of the reversed micellar solution was studied. The polarity of the environment of a porphyrin could very well be probed by ^{19}F-NMR. The results showed that the porphyrin was located in the interfacial region of the reversed micelle. Modification of the porphyrin with a hexadecyl tail anchored the porphyrin. Although distinct differences between the environment and mobility of the tailed and untailed porphyrin could be detected, the efficiencies of photosystems using these porphyrins were similar. Thus neither the location, nor the mobility of the porphyrin but the concentration of alcohol in the reversed micellar solution is the main factor determining the yield of the photoinduced charge separation. An increased alcohol concentration in the medium is known to increase the flexibility of the micelles and to affect the partitioning of the electron donor. Therefore, the availability of the donor and the flexibility of the reversed micellar system have to be optimized to obtain a highly efficient photosystem. The spectroscopic properties of those porphyrins are affected by the interfacial location of the molecule. An increasing resolution of the fluorescence emission spectrum and a transient fluorescence rise in detergent solutions indicate that after excitation an intramolecular rearrangement occurs.

The enzyme performance in photoinduced enzymatic reactions is determined by its stability and its activity. Increasing the chain length of the cosurfactant increased the stability of the enzyme in its oxidized state, but the stability of the reduced enzyme was lowered. The activity of enoate reductase increased with increasing cosurfactant polarity, but the relation with the concentration of cosurfactant in the reversed micellar solution was unexpected (Chapter 2). Therefore the kinetics of enoate reductase were studied in great detail using a water soluble substrate. A model was derived taking into account that both substrate supply and substrate concentration in the microheterogeneous reversed micellar solution deviate from those in aqueous solutions. Not only the, unaltered, intrinsic kinetic parameters of the enzyme, but also the reversed micellar concentration, the intramicellar substrate concentration and the rate and efficiency of the exchange of reversed micellar contents were shown to determine the enzyme

activity. This model could be used to predict the experimental enoate reductase results in reversed micelles prepared with an anionogenic, a cationogenic and a nonionogenic detergent (Chapter 4.2).
The enzyme kinetic study was extended to the case of 20ß-hydroxysteroid dehydrogenase (EC 1.1.1.53). This enzyme catalyzes the reduction of a variety of 20ß-ketosteroids. It could be demonstrated that partitioning of the substrate between the aqueous phase and the organic phase was the main factor affecting the Michaelis constant observed in reversed micelles. These results could also be interpreted with the model presented in Chapter 4.1.
The implications of both the kinetic model and the study to photoinduced charge separation are discussed in Chapter 5. It was shown that the electron transfer in forward direction emerges from the triplet state of the porphyrin and strongly depends on the flexibility of the system. For further optimization the redox potentials of the components, especially that of the porphyrin, need to be considered, too. With respect to the enzymatic conversion the model presented in Chapter 4 was used to explain the dependency of enzyme activity on the cosurfactant concentration. It is stressed that because of the general validity of the principles used to derive it, the model describing the enzymatic reactions in reversed micelles can be equally applied to other microheterogeneous systems. Finally some results of this thesis are listed as guidelines to optimize photoinduced enzymatic reactions in reversed micelles.

SAMENVATTING.

Dit proefschrift beschrijft onderzoek aan een systeem voor lichtgedreven ladingsscheiding in omgekeerde micellen en de koppeling van dit systeem aan een enzymatische omzetting. Omgekeerde micellen zijn waterdruppeltjes van 1 tot 10 nanometer doorsnede in een organisch oplosmiddel. Deze druppels blijven stabiel in oplossing door de aanwezigheid van een monolaag oppervlakte aktieve stof rond het waterdruppeltje. Soms is een extra oppervlakte aktieve stof, de zg. cosurfactant, nodig om de druppeltjes te stabiliseren. Bij het hier beschreven onderzoek zijn alcoholen met alifatische, verzadigde koolwaterstofketens gebruikt. De samenstelling van de omgekeerde micellen kan geoptimaliseerd worden om de efficientie van zowel de lichtgedreven ladingsscheiding als van de enzymatische reaktie te verhogen.
In hoofdstuk 2 is een lichtsysteem gekoppeld aan de reduktie van α-ß onverzadigde carbonzuren door enoaat reductase (EC 1.3.1.31). Het lichtgedreven systeem bestond uit een porfyrine als lichtabsorberende stof, tributylamine als electrondonor en methylviologeen als electronacceptor. Het lichtgedreven enzymsysteem was aktief gedurende meer dan 100 uur. Het substraat werd volledig omgezet.
Systematisch onderzoek naar een verband tussen de efficientie van het lichtsysteem en de samenstelling van de oplossing met omgekeerde micellen toonde aan dat een toename in de concentratie van de cosurfactant en een toename in de polariteit van de cosurfactant, resulteerde in een betere ladingsscheiding. De plaats van de gebruikte porfyrines in de omgekeerde micellen is bestudeerd voor omgekeerde micellen van verschillende samenstelling. De polariteit van de omgeving van het porfyrine bleek goed te worden weergegeven door de plaats van de ^{19}F NMR resonantie van fluor gemodificeerde porfyrines. De resultaten toonden aan dat de porfyrines zich in het grensvlak bevinden. Het porfyrine met een hexadecylstaart bleek meer verankerd in het grensvlak dan het porfyrine met een methylsubstituent op dezelfde plaats. Hoewel de porfyrines duidelijk verschillen in omgeving en beweeglijkheid, is de efficientie van de lichtgedreven reaktie voor beiden hetzelfde. Dus de opbrengst van de lichtgedreven ladingsscheiding wordt niet door de plaats of door de beweeglijkheid van het porfyrine bepaald maar door de concentratie alcohol in het micellaire medium. Het is bekend dat een hogere alcoholconcentratie in de oplossing zowel de beweeglijkheid van de micellen beinvloedt als ook de verdeling van de electrondonor over het grensvlak en de continue fase. Op grond van deze resultaten wordt geconcludeerd dat voor de optimalisering van het lichtsysteem de beschikbaarheid van de donor en de flexibiliteit van de omgekeerde micellen geoptimaliseerd moeten worden.
De fluorescentie van zowel het porfyrine met een hexadecyl staart als dat met een methyl groep bleek bepaald te worden door zijn omgeving. Een toegenomen resolutie van het fluorescentie emissiespectrum en een toename in fluorescentie in omgekeerde micellen en in andere zeepoplossingen, geven aan dat in deze moleculen een intramoleculaire verandering op kan treden waaruit ook emissie optreedt.
Het funktioneren van het enzym in de lichtgedreven reaktiecyclus hangt af van de stabiliteit en de aktiviteit. Een toename van de ketenlengte van de cosurfactant deed de stabiliteit van het enzym in de geoxideerde toestand toenemen, maar de stabiliteit van de gereduceerde toestand was minder. De activiteit van het enoaat reductase nam toe met toenemende polariteit van de cosurfactant; er werd een vreemd verband gevonden tussen de hoeveelheid alcohol die als cosurfactant gebruikt werd in de micellaire oplossing (hoofdstuk 2). Dit was de aanleiding tot een gedetaileerde bestudering van

de kinetiek van enoaat reductase in omgekeerde micellen met een wateroplosbaar substraat. Er werd een model afgeleid voor de beschrijving van de kinetiek waarbij rekening gehouden werd met het feit dat zowel de aanvoer als de concentratie van substraat in de microheterogene omgekeerde micel verschillen ten opzichte van die van een homogene waterige oplossing. De enzymaktiviteit wordt niet alleen bepaald door de intrinsieke, ongewijzigde, kinetische parameters van het enzym, maar ook door de concentratie omgekeerde micellen, de intramicellaire substraatconcentratie en de snelheid en efficientie van uitwisseling tussen de micellen. Met dit model konden de experimentele resultaten verkregen voor enoaat reductase in omgekeerde micellen, die bereid waren met hetzij een anionogene, een cationogene of een nonionogene zeep verklaard worden (Hoofdstuk 4.2). Tevens werd de kinetiek van 20ß-hydroxysteroid dehydrogenase (EC 1.1.1.53) bestudeerd. Dit enzym katalyseert de reduktie van diverse 20ß-ketosteroiden. In hoofdstuk 4.3 werd aangetoond dat de verandering in de Michaelis constante in omgekeerde micellen voornamelijk veroorzaakt wordt door de verdeling van het substraat over de waterfase, het grensvlak en de continue fase. De verkregen resultaten konden ook verklaard worden met het in hoofdstuk 4.1 gepresenteerde model.
De gevolgen van het kinetische model en van de studie naar optimalisering van de lichtgedreven ladingsscheiding worden besproken in hoofdstuk 5. Daar wordt ook aangetoond dat de voorwaartse electronoverdracht uit de triplettoestand van het porfyrine geschiedt en sterk beinvloed wordt door de flexibiliteit van het micellaire systeem. Om het lichtgedreven systeem verder te optimaliseren moet ook rekening gehouden worden met de redoxpotentialen van de componenten, met name met die van de lichtgevoelige stof. Het enzymatische gedeelte van het systeem wordt bediscussieerd in het licht van de inzichten verworven uit de kinetiekstudie. Hiermee kan de variatie van de enzymaktiviteit met het percentage cosurfactant verklaard worden. Tevens wordt aangegeven dat het kinetische model ook toepasbaar is voor andere microheterogene systemen, omdat de aannames die gemaakt zijn bij de afleiding algemeen geldig zijn. Tenslotte worden een aantal resultaten uit deze dissertatie vertaald in aanwijzingen ter optimalisering van lichtgedreven reacties in omgekeerde micellen.

CURRICULUM VITAE

De schrijver is geboren op 28 mei 1961 te Huijbergen. Na de lagere school volgde hij op de Newman Ypelaar Scholengemeenschap te Breda het gymnasium ß. In 1979 startte hij met de studie Moleculaire Wetenschappen aan de Landbouwhogeschool in Wageningen. Zijn doctoraalfase bestond uit twee hoofdvakken, Biochemie en Celbiologie, en twee bijvakken, Microbiële Genetica en Moleculaire Genetica.
Het hoofdvak Biochemie bestond uit het bestuderen van enzymatische reacties in en fysische karakterisering van omgekeerde micellen en dit voerde hij uit aan de vakgroep Biochemie (Prof.Dr. C. Veeger), onder leiding van Dr. N.C.M. Laane.
Zijn Celbiologische doctoraal projekt richtte zich op de bestudering van de recessief-erfelijke ziekte Xeroderma Pigmentosum, waarbij er een verlaagde herstelcapaciteit bestaat van door UV beschadigd DNA. Dit onderzoek voerde hij uit aan humane fibroblasten met behulp van moleculair-biologische technieken en microinjectie op de vakgroep Celbiologie, Medische Faculteit, Erasmus universiteit Rotterdam, onder leiding van Dr. J.H.J. Hoeymakers en Prof.Dr. D. Bootsma.
De bijvakken Moleculaire Genetica (onder leiding van Ir. L.H. de Graaff en Dr.Ir. J. Visser) en Microbiële Genetica (onder leiding van Dr.Ir. K. Swart) verrichtte hij op de vakgroep Erfelijkheidsleer, Landbouwhogeschool, Wageningen (Prof.Dr.Ir. J.H. van der Veen) aan respectievelijk de moleculaire karakterisering van en de inductie van gluconzuurproduktie door *Aspergillus Niger*.
Zijn onderzoeksstage heeft hij uitgevoerd aan de University of British Columbia te Vancouver. Dit onderzoek, geleid door Prof.Dr. A.P. Autor, richtte zich op de regulering en het mechanisme van de door complementfactor C5a geinduceerde $O_2^{\cdot-}$ produktie door leukocyten.
In juni 1986 sloot hij zijn studie met lof af.
Sinds 1 april 1986 werkt hij aan het onderzoek dat tot dit proefschrift heeft geleid. Dit onderzoek werd uitgevoerd in het kader van het Stichting voor de Technische Wetenschappen (STW) gesteunde projekt "Ladingsscheiding en produktie van reduktie-equivalenten in geordende systemen".