BEHAVIOUR OF ENZYMES IN REVERSE MICELLES IN NON-AQUEOUS SOLVENTS

S S KATIYAR, ANIL KUMAR and AJAY KUMAR

Department of Chemistry, Indian Institute of Technology, Kanpur 208 016, India

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The enzymatic activity of pig-heart malate dehydrogenase solubilized in cationic reverse micelles of cetyltrimethylammonium bromide in CHCl₃-isooctane (1 : 1 v/v) and water has been investigated. The magnitude of enzymatic activity depends on the water content and the pH of the reverse micellar system. Under optimum conditions (i.e. pH 10.3 and W₀ 25.55) it corresponds to around 80% of the activity found in water. The dependence of malate dehydrogenase activity on pH in reverse micelles is different than that in bulk water, with the optimum pH shifted to about 3 units higher in reverse micelles. Maximum enzyme activity is not found at the highest water content but instead at medium water content.

Key Words: Reverse Micelles; Enzyme Catalysis, Malate Dehydrogenase; Micellar Enzymology

INTRODUCTION

In vivo many enzymes are either bound to biological membranes or located inside them. In other words most of the enzymatic reactions occur very close to the interface or at the interface. On the other hand in vitro studies of enzymes are usually carried out in aqueous (buffer) solutions.¹ ² It is also well known that properties of water at the interface are different from that of bulk water.³ Some workers have raised questions if the characteristics and properties of enzyme obtained in aqueous studies are truly reflective of their behaviour in the environment of the cell.⁴⁻¹⁰ ¹⁵ They have suggested that reverse micellar media because of its ability to solubilize enzymes/proteins may present an environment which is quite similar to cellular environment. Further, recently the use of enzymes in the syntheses of fine organic compounds, drug preparations and other technological processes had opened a new biotechnological area.⁵⁻⁷ But majority of synthetic reactions are carried out only in non-aqueous solvents with their substrates and products having good solubility in non-aqueous media. The major drawback of the enzymes is that they lose their catalytic activity, specificity and get denatured in non-aqueous solvents.⁸ As a consequence it became necessary to generate a media which can mimic biological membranes as well as provide a microenvironment to keep enzymes functional in non-aqueous solvents. This has been accomplished by entrapping enzymes in the hydrated core of reversed micelle formed by the surfactants in non-aqueous solvents.⁹⁻¹⁰ The core of reverse micelle probably provides a micro-environment, where enzymes function in a manner similar to that in vivo. In such reverse micellar media few enzymes¹⁰⁻¹⁷ have been investigated successfully where they retain good catalytic activity. Most of these studies have been carried out with enzymes that are simple and less complex.
In this paper, we report the behaviour of pig-heart malate dehydrogenase (MHD), which is composed of 2 subunits of 35000, dalton each. Its activity is completely dependent on the proper subunit-subunit interaction. It catalyzes the following reaction.

\[
\text{Oxaloacetate} + \text{NADH} + \text{H}^+ \rightleftharpoons \text{Malate} + \text{NAD}^+
\]

This enzyme system has been entrapped in the reverse micelle of cetyltrimethylammonium bromide (CTAB), a cationic surfactant, in isooctan-chloroform (1 : 1; v/v). Biotechnological implication of present studies and that of other enzymes in micellar media have been discussed.

**Materials and Methods**

Pig-heart malate dehydrogenase (MDH) and oxaloacetic acid were procured from Boehringer Mannheim and NADH from Sigma Chemical Co., USA. Cetyltrimethyl ammonium bromide (CTAB) extrapure grade reagent was obtained from SISCO Research Labs India. It was purified as previously described and dried over \( P_2O_5 \) in an evacuated desiccator. Isooctane puriss was obtained from Fluka and chloroform from BDH. Buffer components used were from Sigma Chemical Co., USA.

*Preparation of Enzyme and Substrate Reverse Micellar Solutions*

The solutions were prepared by injecting the buffered enzyme stock solution or the substrates stock solutions into the CTAB/isoctane-chloroform (1 : 1; v/v) solution with a microsyringe. The desired water content was set by an additional injection of the same buffer into the reverse micellar solution which was shaken on a vortex mixer to get a clear solution. Buffers used were 0.1M potassium phosphate and 0.1M glycine-potassium hydroxide.

*Enzyme Activity Measurement*

MDH activity was measured at 30°C using oxaloacetate and NADH as substrates on a Gilford Response UV/VIS spectrophotometer. The rate of oxidation (formation of \( \text{NAD}^+ \)) was measured by following the decrease in absorbance at the absorption maxima (340nm) of NADH. The reaction was started by adding (1–2 \( \mu l \)) of an aqueous MDH stock solution to reverse micellar solution containing substrates and the desired amount of buffer. The auto oxidation-reduction of the substrates, once injected into the reverse micellar solution, was negligible compared with the enzymatic reaction. The reaction was carried out at least for 3 min. During this time interval the reaction rate was linear.

**Results and Discussion**

The specific activity of malate dehydrogenase depends on various factors like waterpool, \( W_0 (= [\text{H}_2\text{O}]/[\text{CTAB}]) \) and pH of the stock solution from which reverse micelles were prepared. \( W_0 \) defines the size and properties of the hydrated core of the reverse micelles. Fig. 1 gives the data on the variation of initial rate of enzyme MDH as a function of \( W_0 \) at different pH values of the buffer solution injected.
into reverse micelles. It may be noted that the enzyme activity increases with the increasing value of $W_0$, the lowest activity value being at the started $W_0$ value of 3.33. For each pH the enzyme activity-waterpool ($W_0$) profile is a bell-shaped curve. The maximum on the curve determines the value of $W_0$ at which enzyme shows maximum activity and is called optimum $W_0$ ($W_{0\text{opt}}$). The behaviour seems to be a common feature for enzymes in reverse micelles as some other enzymes such as $\alpha$-chymotrypsin$^{12}$ horse liver alcohol dehydrogenase$^{13}$ and lysozyme$^{14}$ have given bell-shaped curve similar to that of Fig 1. In aqueous solution the enzyme MDH exhibits maximum activity at pH 7.5, whereas in reverse micelle enzyme shows the maximum activity at pH$_{stock}$ 10.3. The pH$_{stock}$ value which is normally reported in the micellar systems is the pH of the stock buffer solution transferred inside the inner cavity of the reverse micelle. It is not the actual pH of the buffer solution inside the water pool (which is defined as pH$_{wp}$) of reverse micelle. In general it appears that pH inside the reverse micelle cannot be determined with precision. Though for AOT/isooctane system actual pH values have been indirectly calculated using $^{31}$P-nmr measurements,$^{20}$ for the cationic micellar system CTAB/CHCl$_3$-isooctane no such study has been reported so far.
Table I summarizes the data of the % control activity of enzyme in reverse micelles at different pH of the buffer solution when waterpool is kept constant. This variation of activity with change in pH was investigated at four different waterpool ($W_0$) values i.e. 10.00, 20.00, 25.00 and 30.00. It is interesting to note that activity of the enzyme shows a maximum value at a particular pH called optimum pH ($pH_{opt}$). Above and below the pH optimum, activity of enzyme decreases rapidly.

**Table I**

*Effect of pH on the activity of MDH at different waterpools ($W_0$) in CTAB/CHCl$_3$ — Isooctane (1:1; v/v) reverse micellar system*

<table>
<thead>
<tr>
<th>$W_0$</th>
<th>pH</th>
<th>% of control activity</th>
</tr>
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<tbody>
<tr>
<td>10.00</td>
<td>7.50</td>
<td>10.5</td>
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<tr>
<td></td>
<td>8.40</td>
<td>24.5</td>
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<tr>
<td></td>
<td>9.50</td>
<td>25.5</td>
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<tr>
<td></td>
<td>10.30</td>
<td>32.0</td>
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<tr>
<td></td>
<td>11.00</td>
<td>34.0</td>
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<tr>
<td></td>
<td>11.80</td>
<td>32.5</td>
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<tr>
<td>20.00</td>
<td>7.50</td>
<td>19.5</td>
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<td></td>
<td>8.40</td>
<td>36.0</td>
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<td></td>
<td>9.50</td>
<td>37.5</td>
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<td></td>
<td>11.80</td>
<td>54.0</td>
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<td>25.00</td>
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<td></td>
<td>8.40</td>
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<td>9.50</td>
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<td></td>
<td>90.30</td>
<td>70.0</td>
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Fig 2 shows the variation of $pH_{opt}$ with the change in optimum waterpool ($W_{0\ opt}$). It may be noted that by increasing pH of the buffer solution the optimum waterpool (at which enzyme activity is maximum) decreases in almost linear fashion. In other words at higher pH, maximum activity of enzyme occurs at the lower water pool. Our results show that maximum activity of MDH in reverse micelles is at waterpool 25.55 and pH 10.30. It is around 80% of the activity in aqueous buffer at its optimum conditions.

The fact that enzyme activity is very low at low waterpool range (3.33-8.88) indicates that a large fraction of enzyme molecules was probably directly
exposed to the organic solvent and were denatured. However, a small fraction of enzyme molecules which is protected somehow, might be in the centre of the inner core and could catalyse the reaction. It is also expected that at lower water content in the reverse micelle, the water droplet size may come close to the size of enzyme. In this kind of situation coenzyme binding may be affected in comparison to that at higher water content and therefore lower activity would be observed. Our results demonstrated that the water pool size extensively regulates the catalytic activity of enzyme entrapped in reverse micelles. Once the water content reaches the proper catalytic amount, the reaction rate becomes maximum. The display of maximum activity at an optimal water pool is indicative of the existence of most viable conformation of the enzyme protein. Such a dependence of activity on conformational change of α-chymotrypsin has been suggested by Barbaric and Luissi.12 Further there would be a significant effect of change in water structure on the protein conformation. Increase of reaction-rate at higher water pool may also be due to an increase in the interfacial area. With larger interfacial area, the likelihood of substrate binding with enzyme molecules near the interface will be improved. Beyond the $W_{0\text{opt}}$, the decrease in catalytic activity is probably due to the loss of interfacial tensions of enzyme in this water pool range.

The pH optimum for the MDH activity is 7.5 in aqueous solution whereas it apparently becomes 10.3 in reverse micelles. This shift of the pH optimum for maximum MDH activity to a higher value than that of aqueous solution most likely indicates that the $pK_a$ of certain amino acid residues is modified by the peculiar nature of solvent in the reverse micelles. It may also be due to a change in the behaviour of water in microreactor (or water pool) as reverse micelles provide unique micro-environment.

**CONCLUSION**

Present studies show that MDH and other dehydrogenases are able to maintain their catalytic activity in reverse micelles even at low water content. The activity
profile appears to be complex and it very much depends on various factors like waterpool, pH of the buffer solution injected, surfactant concentration, temperature etc. Our results in the case of MDH shows that MDH in reverse micelle retains activity at its optimum conditions. These optimum conditions in reverse micelles are different than that of aqueous optimum conditions. This indicates that for enzymatic study in reverse micelles, it is necessary to study the effect of various parameters, which influence the activity of enzyme, over a wide range.

Enzymes/proteins entrapped in reverse micelles are not interesting only because of their biotechnological relevance but also due to their similarity with enzymes embedded in vivo in biological membranes. The microheterogeneous medium namely a colloidal solution of water in organic solvents stabilized with amphiphilic compounds appears to be a suitable medium for the study of enzyme reactions, multienzyme systems, nucleic acids and other macromolecules.

REFERENCES