Efficient NAD$^+$-recycling System for ADH-catalysed Oxidation in Organic Media†

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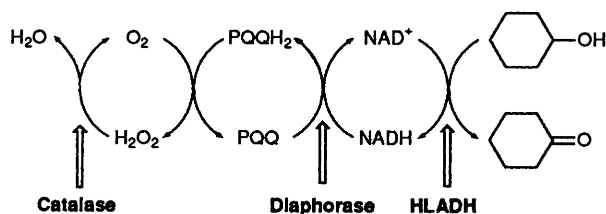
An efficient NAD$^+$-recycling system with O_2 as an oxidant, diaphorase and a heterocyclic *o*-quinone as electron-transfer catalysts and catalase as an H_2O_2-scavenger was developed for ADH-catalysed oxidation in organic media.

Enzymatic catalysis in organic solvents has attracted much recent attention as a new and promising biotechnology.[1] In particular, heterogeneous systems, where enzymes are suspended in organic solvents, have several potential advantages over enzymatic reactions in aqueous media, so that those systems have been successfully applied to development of new methodologies in organic synthesis.[1] However, most of the enzymes so far investigated in detail are hydrolytic enzymes, and the use of oxidoreductases is restricted because of difficulties in constructing an efficient electron transfer system under such heterogeneous conditions.

Among oxidoreductases, NAD(P)(H)-requiring dehydrogenases are one of the most useful and widely investigated,[2] therefore the catalytic activities of these enzymes have also been studied in organic solvent systems.[3–7] Most effort has been devoted to the clarification of the effects of added water[4] and organic solvents[3,5] and to the development of preparative methods for biocatalysts,[3,6,7] however little attention has been focused on the method of coenzyme regeneration in such systems. So far the only method employed is that using a second substrate for NAD(P)(H)-recycling.[8] Here we report an efficient NAD$^+$-regeneration system of O_2–diaphorase–heterocyclic *o*-quinone–catalase that can be adapted for the ADH-catalysed oxidation of alcohols in organic media (Scheme 1).[9]



Scheme 1

A biocatalyst, prepared by lyophilization of a phosphate buffer solution (pH 8.6) containing HLADH, NAD$^+$, diaphorase, catalase and PQQ (a newly found quinone coenzyme[10]), was suspended in a solution of cyclohexanol in octane containing a small amount of water at 30 °C under an O_2 atmosphere. The time course of the formation of cyclohexanone is shown in Fig. 1. The oxidation reaction proceeded almost quantitatively within several hours and followed first-order kinetics until 95% conversion, indicating that there is no severe damage to the catalysts (k_{obsd} = 0.59 h^{-1}). The turnover numbers of HLADH, NAD$^+$ and PQQ are 2×10^3, 1×10^2 and 1×10^2, respectively. HLADH, NAD$^+$ and PQQ were essential for the reaction, and omission of diaphorase, a flavoenzyme that catalyses electron-transfer of NAD(H), considerably depressed the reactivity (k_{obsd} = 0.11 h^{-1} without diaphorase). In the absence of catalase, the biocatalyst was completely inactivated at the early stage of the reaction (within several minutes). Catalase removes hydrogen peroxide, that is formed during the catalytic cycles, from the system to protect the enzymes and/or the coenzymes from oxidative denaturation (Scheme 1).

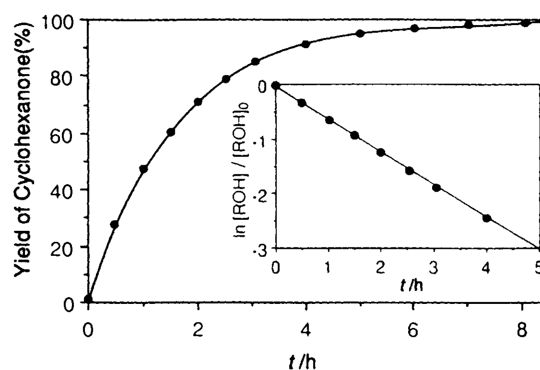


Fig. 1 Time course of the oxidation of cyclohexanol (1.0×10^{-5} mol) in the presence of HLADH (5.0×10^{-9} mol), NAD$^+$ (1.0×10^{-7} mol), PQQ (1.0×10^{-7} mol), diaphorase (40 units) and catalase (1500 units) in octane (5.0 cm^3) containing 0.6 vol% of water at 30 °C under O_2. First-order plot for the reaction (inset).

As shown in Fig. 2, the enzyme activity greatly increased as the water content in the solvent was raised until about 2 vol% and it reached a plateau above that. This volume of optimal water content seems to be a little higher than that for hydrolytic enzymes but is comparable to that reported for other systems with coenzyme-requiring oxidoreductases.[4] Although 0.6 vol% is above the solubility of water in octane, no separate aqueous phase was observed because the excess of water was probably adsorbed onto the biocatalyst. A certain volume of water might be required to exist around the enzyme surface so that the components of the system can interact each other to achieve the efficient electron transfer.

A typical solvent-dependence of enzyme activity was observed as indicated in Fig. 3, where the organic solvents having log *P* value above 3 are good enough but no redox reaction took place in the solvents whose log *P* values are below 2. This effect of organic solvents must be the same as discussed in other enzymatic reactions in low water-content systems.[1]

In the case of the so-called, second substrate method of coenzyme regeneration, there is a certain equilibrium point

† *Abbreviations and EC numbers:* ADH, alcohol dehydrogenase; NAD(P), nicotinamide adenine dinucleotide (phosphate); diaphorase (EC. 1.6.99); catalase (EC. 1.11.1.6); HLADH, horse liver alcohol dehydrogenase (EC. 1.1.1.1); PQQ, pyrroloquinolinequinone (4,5-dihydro-4,5-dioxo-1*H*-pyrrolo[2,3-*f*]quinoline-2,7,9-tricarboxylic acid).

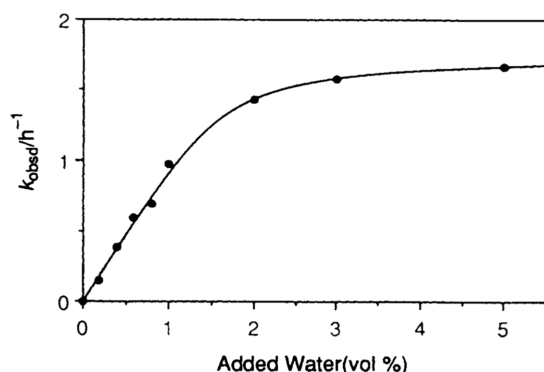


Fig. 2 Dependence of the first-order rate constant (k_{obsd}, h^{-1}) on added water contents (vol%), in octane

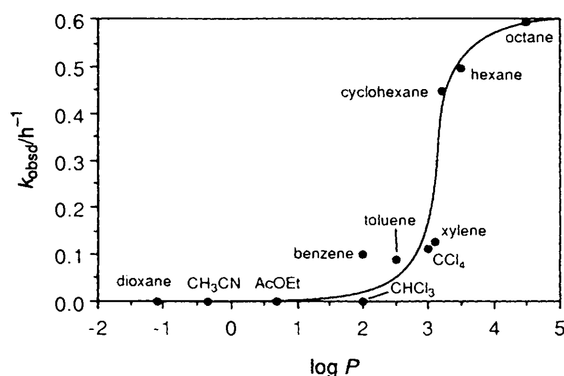


Fig. 3 Effect of organic solvents. Plot of the first-order rate constant (k_{obsd}, h^{-1}) *vs.* log *P* value of the solvents. Added water content: 0.6 vol%.

between alcohol-oxidation and carbonyl-reduction, which might cause a problem in application to organic synthesis. In the present system, on the other hand, molecular oxygen was employed as the final electron acceptor so that the equilibrium could be completely directed toward the alcohol-oxidation. It should be also emphasized that our present system is the first example of the multi-enzymatic reaction that works very well under low water conditions. Mechanistic details of the electron transfer, catalytic efficiency of other quinonoid compounds and substrate- and enzyme-specificity of our system are now under investigation.

Experimental

HLADH, NAD$^+$, diaphorase, catalase and PQQ were obtained commercially and were used without further purification. Organic solvents were dried over 4 Å molecular sieves and distilled before use. The log *P* values were taken from the literature.[11] The biocatalyst was prepared by lyophilization of 0.1 mol dm^{-3} phosphate buffer solution (pH 8.6) containing HLADH, NAD$^+$, diaphorase, catalase and PQQ.

Typically, a small portion of the biocatalyst preparation containing HLADH (5.0×10^{-9} mol), NAD$^+$ (1.0×10^{-7} mol), diaphorase (40 units), catalase (1500 units) and PQQ (1.0×10^{-7} mol) was suspended in a solution of cyclohexanol (1.0×10^{-5} mol) in octane (5 cm^3), and a certain amount of water (usually 0.6 vol%) was added. The heterogeneous mixture was stirred gently at 30 °C under O_2-atmosphere and progress of the reaction was followed by monitoring the formation of cyclohexanone and/or disappearance of cyclohexanol by GLC.

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