

Enzymatic conversion of apolar compounds in organic media using an NADH-regenerating system and dihydrogen as reductant

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A combined enzyme system, consisting of hydrogenase, lipoamide dehydrogenase and 20 β -hydroxysteroid dehydrogenase has been enclosed in reversed micelles. This system catalyzes the stereo- and site-specific enzymatic reduction of apolar, poorly water-soluble ketosteroids to their corresponding 20 β -hydroxyform using an in situ NADH-regenerating enzyme system and H₂ as ultimate reductant.

Enzymatic steroid conversion

Reversed micelle

NADH regeneration

1. INTRODUCTION

Enzymes possess several properties that make them suitable as catalysts in synthetic organic chemistry. They catalyze, under mild conditions, site- and/or stereo-specific reactions that are otherwise difficult or impossible to perform and furthermore, produce pure compounds. Despite these obvious merits, the application of enzymes to organic synthesis is seriously limited by two factors.

Firstly, enzymes preferentially function in aqueous environments, while in case of apolar compounds chemical reactions are carried out in non-polar solvents that are usually harmful for enzymes. Several approaches to overcome this problem have been reported. Enzymatic reactions have been carried out at the interface of a two-phase system, one of the phases being water, the other a water-immiscible solvent. Furthermore, mixtures of water and water-miscible solvents have been studied [1-3]. To protect enzymes against inactivation by the organic solvent in these systems, they have been immobilized in gels or on solid supports [4-7]. A relatively new approach is entrapment of enzymes in reversed micelles [8-12].

Secondly, certain classes of enzymic reactions of general interest involve cofactors which are expensive and are consumed during catalysis. Hence, the cofactor has to be regenerated from inexpensive reagents to make an enzymic process economically feasible. To date, several cofactor regenerating systems have been described, but only for aqueous media [13-18].

This paper describes an NADH-regenerating system that functions in a reversed micellar medium in combination with an enzyme that uses NADH for the conversion of an apolar steroid.

2. MATERIALS AND METHODS

2.1. Chemicals

Cetyltrimethylammonium bromide (CTAB) was from Serva; steroids, methyl viologen (MV²⁺) and NAD⁺ from Sigma and all organic solvents from Merck.

2.2. Enzymes

Hydrogenase (H₂ase, EC 1.12.2.1.) from *Desulfovibrio vulgaris* strain Hildenborough NCIB 8303 was purified as in [19] Pigheart lipoamide dehydrogenase (lipDH, EC 1.6.4.3.) and 20 β -hydroxysteroid dehydrogenase (HSDH, EC

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1.1.1.53) from *Streptomyces hydrogenans* were from Sigma.

2.3. Preparation of reversed micelles

An aqueous solution (70 μ l) was injected into 1.5 ml of a Vortex-stirred 0.2 M solution of CTAB in hexanol/octane 1:4 (v/v) containing 1.0 mM steroid unless stated otherwise. Stirring was continued until the solution became clear. The aqueous solution contained lipDH (2.5 μ M), HSDH (3.0 μ M), methylviologen (2.5 mM) and NAD^+ (1.0 mM) in 50 mM HEPES pH 7.6. After 6 cycles of 30 s evacuation, 15 s flushing with scrubbed Argon and 5 min of bubbling with O_2 -free H_2 , 10 μ l H_2 ase (final concentration of 0.6 μ M in the waterpool) was added to initiate the reaction. Incubation temperature was 25°C.

2.4. Determination of steroids

Since CTAB interferes with the detection procedure, it was precipitated by adding 100 μ l acetonitrile to a 25 μ l sample. The steroids remained in solution and were analyzed by HPLC (Varian) equipped with a Phase Sep Spherisorb 10 μ MODS column, eluted with acetonitrile and water. For progesterone and its 20 β -hydroxy-form the ratio was 70:30, and for prednisone 25:75. The detection wavelength was 240 nm.

3. RESULTS AND DISCUSSION

A multi-enzyme system, consisting of H_2 ase, lipDH and HSDH was enclosed in reversed micelles (fig. 1). In such a reversed micellar

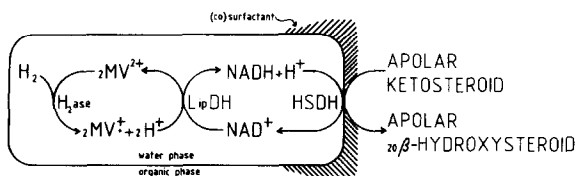


Fig. 1. Scheme for the H_2 -driven regeneration of NADH and the subsequent reduction of an apolar steroid in a reversed micellar medium. For clarity all the water soluble components of the system are drawn in one micelle. A more realistic view is that all components are distributed among all micelles that are in rapid exchange with each other [20,22]. For explanation of the abbreviations, see text.

medium the enzymes are confined to tiny waterpools that are stabilized by surfactants in an organic, water-immiscible solvent. As has been shown by several authors [8–12], reversed micelles provide a unique microenvironment for enzymes, which protects them against denaturation by the surrounding organic solvent, and enables the enzymes to convert apolar compounds present in the bulk organic phase. These systems have an enormous interfacial area (for our system $\sim 100 \text{ m}^2/\text{ml}$) compared to ordinary two-phase systems.

When performing NADH-linked enzymatic reductions in reversed micelles, an NADH-regenerating system is preferred that consumes a substrate that is a strong and inexpensive reducing agent, dissolves well in organic solvents and, above all, leaves no harmful byproducts after consumption. A reductant that meets these requirements is H_2 as was shown by Whitesides and coworkers for aqueous media [16]. In our system too, H_2 is used by H_2 ase to reduce MV^{2+} to MV^+ . Two of these radicals are consumed by LipDH to regenerate NADH from the NAD^+ formed by HSDH during the enzymatic reaction. The coupling of these reactions leads to the recycling of both MV^{2+} and NAD^+ while H_2 is consumed. This is clearly seen in fig. 2, showing the net reaction for progesterone, an apolar ketosteroid.

Progesterone was chosen as a model substrate because of its low solubility in aqueous solutions ($< 50 \mu\text{M}$ [23]). In addition, only one of the two ketogroups present is reduced enzymatically to the β -hydroxyform. That the stereospecificity was preserved could be seen from HPLC-data, for the 20 α - and 20 β -isomers are well resolved, and only the β -isomer was found under our experimental conditions. This shows that the reaction is both site- and stereo-specific.

In some experiments the composition of the reversed micellar medium was varied. When

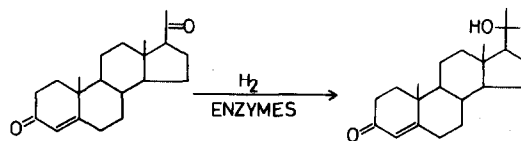


Fig. 2. Net reaction for the reduction of progesterone to 20 β -hydroxy-pregn-4-en-3-one by the multi-enzyme system shown in fig. 1.

chloroform was used instead of hexanol in a 1:1 ratio with octane, no conversion of progesterone could be detected whereas prednisone, a more polar steroid, was readily converted. The same result was obtained when octane was replaced by toluene with hexanol as cosurfactant. This indicates that, rather than changes in the properties of the enzymes, the flux of substrate from the organic phase to the enzyme is the rate-limiting factor. Apparently, the penetration of the steroid into the micelle can be influenced strongly by the composition of the system. To gain more insight into the role of the cosurfactant we varied its chainlength (C_3 - C_{10}) at a constant surfactant: cosurfactant ratio of 1:8. With progesterone as substrate, optimum conversion was obtained with hexanol, but with prednisone, butanol was more effective. This indicates that both the nature of the cosurfactant and of the steroid determine the penetration into the micelle and in this way influence the rate of conversion.

In contrast to our results, no conversion of progesterone with HSDH was found in a two-phase system consisting of water-butylacetate [3]. Apparently the composition of this interfacial system does not allow a sufficient flux of progesterone to the enzyme. Moreover the interfacial area of such two-phase systems is too small to avoid diffusion limitation.

The conclusion that the accessibility of the substrate is the rate-limiting factor is further supported by the observation that the reaction rate in the CTAB-octane-hexanol medium depends on the substrate concentration, even at progesterone concentrations far above the apparent K_m -value for water ($= 22 \mu\text{M}$ [24]) (fig. 3).

With low concentrations of progesterone the rate of reaction levels off when the conversion is almost completed ($>90\%$ conversion). At higher concentrations it proceeds steadily, indicating that the system is stable for a considerable length of time. At 5 mM the reaction slows down. This only occurs in the complete system, HSDH itself is not subject to substrate inhibition at these concentrations. The reason for this phenomenon is not understood yet. Determination of turnover numbers (mol product formed/mol enzyme or cofactor) after 28 h reaction time gave values of 50 000, 13 000 and 11 000 for H_2ase , lipDH and HSDH, respectively, whereas for NAD^+ a value

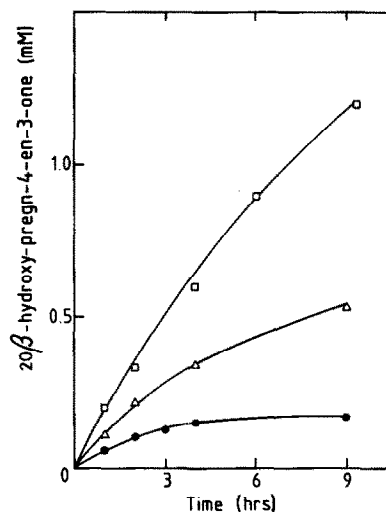


Fig. 3. Time course of 20 β -hydroxy-pregn-4-en-3-one formation at different concentrations of progesterone: (○-○) 0.20 mM; (×-×) 1.0 mM; (□-□) 5.0 mM.

of 100 was obtained. These results demonstrate that the enzymes in the redoxcycle in fig. 1 operate catalytically and that the cofactor is regenerated many times.

After the reaction had been completed the enzymes and other water-soluble components could be regenerated in fairly good yield ($\sim 80\%$) by the method in [25], and the product can be isolated by precipitation of the surfactant with acetonitrile.

Hence, a reversed micellar medium seems to have potential for the batch-like enzymic synthesis of apolar compounds.

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