Activity and Stability of Catalase in Nonionic Micellar and Reverse Micellar Systems

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Catalase activity and stability in the presence of simple micelles of Brij 35 and entrapped in reverse micelles of Brij 30 have been studied. The enzyme retains full activity in aqueous micellar solution of Brij 35. Catalase exhibits "superactivity" in reverse micelles composed of 0.1 M Brij 30 in dodecane, *n*-heptane or isooctane, and significantly lowers the activity in decaline. The incorporation of catalase into Brij 30 reverse micelles enhances its stability at 50 °C. However, the stability of catalase incubated at 37 °C in micellar and reverse micellar solutions is lower than that in homogeneous aqueous solution.

Key words: Catalase, Nonionic Surfactants, Reverse Micelle

Introduction

Catalase is an ubiquitous enzyme present in aerobic organisms, which catalyzes the disproportionation of hydrogen peroxide to molecular oxygen and water. Apart from the natural substrate, H_2O_2, some alkyl hydroperoxides can act as catalase substrates as well. It has been shown that catalase can be used in bioconversion-type reactions in organic, low-water media (Borzeix *et al.*, 1995; Magner and Klibanov, 1995; Karra-Chaabouni *et al.*, 2002). Water is fundamental for the action of all enzymes and some amount of water is needed to retain enzyme activity in organic solvents. One of the methods which enables the use of the enzymes in organic solvents is the entrapment of the enzyme in reverse micelles, *i.e.* spherical water droplets dispersed by means of a surfactant in a solvent of low polarity (Savelli *et al.*, 2000). In reverse micelles the polar heads of the surfactant molecules are directed toward the interior of the aggregate while the hydrophobic tails of the surfactant are in contact with the surrounding organic solvent. The main parameter characterizing a reverse micelle is the molar ratio of water solubilized to the surfactant. It is called the hydration ratio and is denoted w_o. Reverse micelles are optically transparent, so enzymes entrapped in a space of low water content remain suitable for spectroscopic studies. Many enzymes retain activity after being encapsulated inside the water pool of reverse micelles. It has been reported, that some enzymes, incorporated into reverse micelles of the anionic surfactant sodium bis(2-ethylhexyl)sulfosuccinate (AOT), show "superactivity" (Martinek *et al.*, 1986). Catalase is among them (Haber *et al.*, 1993; Gebicka and Gebicki, 1998).

Simple micelles (hydrophobic tails of the surfactants are directed inside the micelle, while polar heads are in the contact with surrounding water) are also applied to solubilize hydrophobic substrates for enzymatic catalysis. However, the surfactant-enzyme interactions in aqueous solutions are in some cases stronger than those in reverse micelles composed of the same surfactant. For example, catalase undergoes denaturation by AOT in aqueous solution. It has been suggested, that the presence of bound water in the water pools of reverse micelles protects catalase from the denaturing effect of AOT (Gebicka and Gebicki, 1998).

In this work we investigate the catalase activity and stability in simple (aqueous) and reverse micelles composed of nonionic surfactants of the class of poly(oxyethylene) alkyl ethers: Brij 35 [forms simple micelles when its concentration exceeds 0.06 mM (Berthod *et al.*, 2001)] and Brij 30 (forms reverse micelles in a number of nonpolar solvents).

Materials and Methods

Bovine-liver catalase (twice crystallized) with RZ = 0.8 was purchased from Sigma. The enzyme concentration was determined spectrophotometri-

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cally at 405 nm, using $\varepsilon_{405} = 3.24 \cdot 10^5$ м$^{-1}$ cm^{-1} (Samejima and Yang, 1963). Water from a MilliQ Plus (Millipore) system was used throughout. Brij 30 [$C_{12}H_{25}(OCH_2CH_2)_nOH$, n ~ 4] and Brij 35 [$C_{12}H_{25}(OCH_2CH_2)_{23}OH$] from Aldrich were used as received. Brij 30 was dissolved in cyclohexane, decaline, dodecane, *n*-heptane or isooctane. All organic solvents used in these studies were obtained from Fluka. Reverse micelles were formed by injection of an appropriate amount of aqueous solutions, either of the buffer alone (10 мм phosphate buffer, pH 7.0) or of one of the reagents (enzyme or hydrogen peroxide) in the buffer, into the solution of the surfactant in the organic solvent to obtain the desired w_o. The mixture was shaken until a completely transparent solution was obtained. One should remember, however, that the introduction of a large hydrophilic enzyme molecule into the reverse micellar system most likely causes the rearrangement of micellar aggregates. Thus, after the newly established equilibrium, w_o may differ from that of the system without enzyme (Luisi, 1991). In this work we always document w_o values resulting from the amount of water and surfactant molecules added to the system. For nonionic surfactants, w_o should be corrected for the critical microemulsion concentration, cμc, to take into account the concentration of the surfactant that is not incorporated into the micelle, but is present as a monomer:

$$w_o = \frac{[H_2O]}{[\text{surf}] - [\text{surf}]_{c\mu c}}$$

The cμc was obtained by the plots of maximum water solubilized versus surfactant concentration in organic solvent (Komives *et al.*, 1994). The values read from the x-intercept were 50, 33, 40, 38 and 40 мм for Brij 30 in cyclohexane, decaline, dodecane, *n*-heptane and isooctane, respectively. We found that the highest obtainable w_o ratios for 0.1 м Brij in cyclohexane, decaline, dodecane, *n*-heptane and isooctane were 10, 4.7, 11, 4.5 and 5, respectively. All these values refer to 23 °C.

The concentrations of enzyme and substrate solubilized in reverse micelles were the overall concentrations referring to the total volume of the system. The activity of catalase was determined by the method of Beers and Sizer (1952). The measurements were done in 10 мм phosphate buffer, pH 7.0, in aqueous solution of Brij 35 in 10 мм phosphate buffer, pH 7.0, and in reverse micelles composed of Brij 30. At least three determinations of catalase activity were performed for each system. The estimated accuracy of determination was about 5%. The absorption spectra and kinetic measurements were done on a Hewlett-Packard 8452A diode-array spectrophotometer.

In the stability assays catalase (in buffer, in aqueous buffered micellar solution of Brij 35 and in reverse micelles of Brij 30 in *n*-heptane) was incubated at 37 or 50 °C. At proper time intervals a 1-ml aliquot was taken, cooled down to room temperature and the enzyme activity (measured at triplicate) was assayed. The stability was expressed through the half-life time of inactivation ($t_{1/2}$), *i.e.* the time after which the enzyme loses 50% of its initial activity. All spectrophotometric experiments were carried out at 23 °C.

Results and Discussion

We have measured the catalase activity in aqueous solutions of Brij 35 at concentrations up to 45 мм and have found that Brij 35 at concentrations below, as well as above the CMC (critical micelle concentration) does not affect the activity of catalase (data not shown). Although nonionic surfactants are in general considered "soft", in some cases they can cause enzyme inactivation. For example, Brij 35 inhibits potato acid phosphatase (Lalitha and Mulimani, 1997), glycerol 3-phosphate dehydrogenase (from rabbit muscle) (McLoughlin *et al.*, 1978) and horseradish peroxidase (Gebicka and Jurgas, 2004). On the other hand, Brij 35 in micellar concentration enhances 1.5-fold the activity of cytoplasmic glycerol 3-phosphate dehydrogenase (McLoughlin *et al.*, 1978) and 1.8-fold the activity of *Bacillus amyloliquefaciens* a-amylase (Hoshino and Tanaka, 2003). Enzyme inactivation or activation in the presence of aqueous solutions of nonionic surfactants are interpreted in terms of hydrophobic interactions between enzyme and surfactants which may induce the denaturation in some enzymes, or little activation in the others (Savelli *et al.*, 2000). The increase of the enzyme activity in the presence of surfactants can also occur as a result of surfactant-product or surfactant-substrate interactions (Savelli *et al.*, 2000). Our results indicate that catalase does not interact with Brij 35 micelles. This is in agreement with Karra-Chaabouni *et al.* (2002) who found that Brij 35 did not inactivate the alcohol oxidase/catalase system during oxidation of alcohols to aldehydes.

It was reported earlier that the activity of catalase incorporated into reverse micelles of AOT in isooctane or *n*-heptane, measured by H_2O_2 decomposition, increased with water loading and at w_o = 50 was several-fold higher than that measured in homogeneous aqueous solution (Haber *et al.*, 1993; Gebicka and Gebicki, 1998). On the other hand the catalase activity in AOT/isooctane reverse micelles at w_o = 50, measured by O_2 production, was lower by about 25% than in aqueous solution (Escamilla *et al.*, 1992). In this work we have measured the activity of catalase entrapped into reverse micelles of Brij 30 in different organic solvents. In these systems the amount of water which can be solubilized in the interior of the micelle is significantly lower than in reverse micelles formed from AOT (see Materials and Methods). The radius of the aqueous core of reverse micelles from poly(oxyethylene) alkyl ethers can be estimated from the equation: R (nm) = 0.19 w_o + 0.7 (Lipgens *et al.*, 1998). Hence, the radii of the "empty" aqueous core at w_o = 4.5 and w_o = 11 (lowest and highest hydration ratio of the investigated reverse micelles) are 1.55 and 2.79 nm, respectively. The catalase molecule is dumbbell shaped with a length of 10.5 nm and a waist of 5.5 nm. (Murthy *et al.*, 1981). Because the diameter of the catalase molecule is significantly higher than the diameter of "empty" micelles, the redistribution of all mass and the assessment of the new equilibrium have to take place after incorporation of catalase into Brij 30 reverse micelles. Large micelles containing the protein molecules together with small "empty" micelles probably exist in such a system (Luisi, 1991).

The catalase activity in reverse micelles formed of Brij 30 depends on the solvent used for solubilization of the surfactant and on the surfactant concentration (Table I). Catalase exhibits "superactivity" in the reverse micelles composed of 0.1 M Brij 30 in heptane, isooctane and dodecane, whereas significantly lower catalase activity, in comparison with that measured in bulk water, is observed in reverse micelles of Brij 30 in decaline. The efficiency of substrate supply in the reverse micellar system is limited by the exchange process between substrate-filled and enzyme-filled micelles. The exchange rates for nonionic reverse micelles are in the order of 10^7–10^8 M^{-1} s^{-1} (Gebicki, 2004) and are comparable with the k_{cat} value of catalase (Obinger *et al.*, 1997). Thus, it has been suggested that the material exchange between reverse micelles becomes the rate-determining step in the decomposition of H_2O_2 catalyzed by catalase in reverse micelles (Haber *et al.*, 1993). The activity of catalase entrapped into reverse micelles of Brij 30 decreases with an increase of the surfactant concentration (Table I). A similar behaviour was observed for catalase encapsulated in reverse micelles of AOT (Escamilla *et al.*, 1992) and can be explained in terms of the interaction of the enzyme with micellar membranes (Martinek *et al.*, 1989).

Table I. The activity of catalase incorporated into reverse micelles of Brij 30 in several solvents measured in percents of the activity determined in homogeneous aqueous solution under the same catalase and H_2O_2 concentrations. $[catalase]_{overall} = 10^{-8}$ M, $[H_2O_2]_{overall} = 10^{-2}$ M ("overall" means concentration of the reactant, referring to the total volume of the system).

Solvent	Brij 30 [M]			
	0.1	0.25	0.3	0.35
Cyclohexane, w_o = 9.0	98	55	–	–
Decaline, w_o = 4.7	19	16	–	15
Dodecane, w_o = 7.5	228	150	–	139
Heptane, w_o = 4.5	250	96	89	80
Isooctane, w_o = 3.7	200	–	–	–

We have also studied the stability of catalase, as characterized by the half-life time of inactivation ($t_{1/2}$), in aqueous micellar solution of Brij 35, as well as incorporated into reverse micelles of Brij 30 in *n*-heptane. The values of $t_{1/2}$ have been calculated, taking, as a reference, the catalase activity measured before incubation (Table II). The stability of catalase incubated at 37 °C in micellar and reverse micellar solutions of Brij 35 and Brij 30, respectively, is lower than that in homogeneous aqueous solution. On the other hand, the stability of catalase incubated at 50 °C is improved by the factor of 3.3 after incorporation into reverse micelles of Brij 30, but nevertheless it is dramatically lower than that observed at 37 °C. Escamilla *et al.* (1992) reported that catalase incorporated into reverse micelles of AOT exhibited significantly higher thermostability than in the buffer. In their experiment, samples were preincubated for 5 min at the indicated temperature and activity assays were performed at the temperature used in the preincubation. It has been suggested that in low water environment, enzymes should exhibit higher resistance to thermal denaturation than in aqueous solution where destabilization of many of the

Table II. Half-life time ($t_{1/2}$) of catalase incubated at 37 or 50 °C measured in homogeneous aqueous solution, in aqueous solution of 5 mM Brij 35 and in the presence of 0.1 M Brij 30 in *n*-heptane reverse micelles of w_o = 4.5.

System	$t_{1/2}$ [h] 37 °C	$t_{1/2}$ [h] 50 °C
Homogeneous aqueous solution	19.0 ± 1.0	0.35 ± 0.02
Aqueous solution of 5 mM Brij 35	17.5 ± 0.9	0.12 ± 0.01
Reverse micelles of 0.1 M Brij 30 in *n*-heptane	14.5 ± 0.7	1.17 ± 0.06

weak bonds may occur at higher temperatures (Tuena de Gómez-Puyou and Gómez-Puyou, 1998). On the other hand, stability studies taken in reverse micelles suggest, that the kind of surfactant plays an important role. For example, $t_{1/2}$ for *Vaccinium mirtyllus* peroxidase incorporated into CTAB/hexanol reverse micelles of w_o = 16 at 50 °C was 8-fold longer than $t_{1/2}$ at 50 °C observed in aqueous solution (20 h and 2.5 h, respectively). The same enzyme incorporated into AOT reverse micelles of w_o = 16 lost 50% of its activity after about 2 min of incubation at 50 °C, although its initial activity in this system was 4-fold higher than in aqueous solution (Setti *et al.*, 1995). Recently Costa *et al.* (2002) studied the effects of various additives on the activity of the aqueous solution of *Bacillus* species catalase under the aspect of potential application of this enzyme for degradation of residual peroxide in textile bleaching baths. They found that several additives improved the enzyme stability at 4 and 30 °C at neutral pH, but glycerol and polyethylene glycol showed the best effect (the stability at 30 °C was increased 3-fold and 7-fold, respectively).

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