Journal of Molecular Catalysis B: Enzymatic 77 (2012) 67–73

Contents lists available at SciVerse ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Micelle based delivery of carotenoid substrates for enzymatic conversion in aqueous media

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ARTICLE INFO

Article history:
Received 29 September 2011
Received in revised form
16 December 2011
Accepted 6 January 2012
Available online 16 January 2012

Keywords:
Micelle
Substrate delivery
Carotenoid
Dioxygenase
Nonionic surfactant

ABSTRACT

Substrate delivery vesicles such as micelles or liposomes can permit the conversion of strongly hydrophobic substrates in aqueous media if the enzyme used is not adaptable to organic media. Micelle based delivery of water-insoluble hydrophobic substrates was investigated with regard to qualitative and kinetic effects of vesicle properties on the enzymatic reaction. The oxidative 9′,10′ cleavage of structurally related carotenoids by *Arabidopsis thaliana* carotenoid cleavage dioxygenase 1 (AtCCD1) was applied as model reaction. Unlike commonly assumed, non-ionic surfactants differed significantly in their ability to deliver the strongly hydrophobic carotenoid substrates to the enzyme. Even small structural differences in the carotenoid substrates led to different non-ionic surfactants being required for efficient micellar delivery to the enzyme. The reaction velocities using the same substrate but different non-ionic surfactants varied by up to the 19-fold of the lowest value.

The kinetic activation of micelle based enzymatic carotenoid cleavage by organic cosolvents was investigated in order to complement published data on this recently discovered effect [1]. The length and saturation of the surfactant aliphatic side chain determined the concentration of water-soluble organic cosolvent at which maximum kinetic activation was achieved. The required cosolvent concentrations were between 6 and 15% (v/v) and led to up to 3.8-fold increased reaction velocities. For specific combinations of enzymes and non-ionic surfactants, kinetic lag phases were observed. Factors of influence on occurrence and duration were investigated and a hypothesis for the underlying mechanism was formulated.

The results complement recent studies on the properties of liposomes and micelles as substrate delivery vesicles [1–3]. The systematic study of delivery systems for hydrophobic substrates will in the long run permit their rational design for enzymatic conversions requiring to be conducted in aqueous media.

1. Introduction

Economically attractive biocatalytic conversions of hydrophobic substrates are often prohibited by the enzyme not being sufficiently stable [4,5] or active [6] in the organic media required to dissolve the substrate. Typically, this problem is approached by screening for alternative enzymes or efforts to increase enzyme stability in organic phases by genetic methods such as rational protein design or directed evolution [7]. Despite being successful in many cases, these strategies should be complemented by strategies permitting the delivery of hydrophobic substrates in the native aqueous reaction environment in order to permit the use of enzymes inadaptable to organic reaction media. The application of micelles or liposomes as substrate delivery vesicles requires systematic studies of factors influencing their effectiveness as substrate shuttle and correlating these with substrate molecular structure.

Although not the only vesicle types applicable, micelles and liposomes seem particularly well suited since they imitate the natural, membrane associated reaction environment of many enzymes converting hydrophobic substrates. Quantitative investigations on the properties of vesicle based substrate delivery systems in biocatalysis are rare. According to the literature, the formulation of reaction systems is in most cases conducted by trial and error rather than by rational design. Yet, it has been shown that the properties of a substrate delivery system have significant effect on the kinetics of an enzymatic reaction [8]. A first set of investigations on the use of liposomes [3] and micelles [1,2] was published recently and provides a starting point for a more thorough and predictive understanding of vesicle based delivery of hydrophobic substrates.

Carotenoids represent a particularly difficult to handle example of hydrophobic substrate molecules. Having octanol/water partitioning coefficients log *P* (o/w) between 17.5 (β-carotene) and 8.9 (8′-apo-β-caroten-8′-al) they are basically insoluble in water [9]

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doi:10.1016/j.molcatb.2012.01.010

and tend to form microaggregates upon contact with water which can no longer be enzymatically converted [10]. The large variety of structurally related carotenoids available permits investigating correlations between different substrate molecular structures and required adaptations of the substrate delivery system.

1.1. Surfactant suitability for substrate delivery

Although substrate delivery by surfactant micelles was used already at an early stage of the investigations on enzymes converting carotenoids or other hydrophobic substrates [11], systematic studies on general suitability and kinetic effects of different surfactant types were not available until very recently. Especially the choice of the specific surfactant type (*e.g.* Triton X-100) within a surfactant class (*e.g.* non-ionic surfactants) is usually not justified in the literature. It was assumed that within one surfactant class, the individual surfactants would not differ significantly in their suitability to deliver hydrophobic substrates to enzymes. However, two recent publications indicate that non-ionic surfactants are not equally suitable for the delivery of carotenoid substrates in biocatalysis [12,13]. These results call for an investigation of the influence of surfactant molecular structure on the micelles' ability to efficiently serve as delivery vesicles for a specific substrate. Structurally closely related surfactants such as those of the Tween series are physicochemically well characterized [14–16] and are well suited for such an investigation.

Furthermore, Schilling et al. showed in 2007 [1,2] that a restructuring of micelles by water-soluble organic cosolvents can significantly increase reaction velocities. The investigations mentioned focussed mainly on one surfactant type, Triton X-100. It remained to be shown if other non-ionic surfactants behave similarly and which structural properties of the surfactant have an influence on the magnitude of the activating effect. Again, investigation of the surfactants of the Tween series permits the identification of structure related trends.

1.2. Lag phases

The enzymatic cleavage of hydrophobic carotenoid substrates does not always display Michaelis–Menten type kinetics if the substrate is delivered using surfactant micelles. For some combinations of non-ionic surfactant and enzyme, a kinetic lag phase was reported [1]. In such cases, the maximum reaction velocity (v_{MAX}) in batch experiments is not observed at the beginning of the reaction when the substrate concentration is the highest, but at a later point in time ($t_{MAX\,V}$). It has not been investigated yet if the occurrence of such lag phases is caused by a restructuring of the micellar substrate delivery system or by a product activation of CCD enzymes. Descriptions of lag phase effects for other enzymes are sparse in the literature. Only for phospholipase A_2, lag phases have been described systematically. Lag phases of up to 83 min were reported for reactions conducted in microemulsions [17]. Other authors investigated the influence of the boundary layer structure and the solvent hydrophobicity on the duration of the lag phases in reverse micellar systems and found out, that the duration decreased as the log *P* (o/w) value of the solvent decreased [18]. Furthermore, it was shown that in a similar reverse micellar system, the concentration of the surfactant AOT linearly correlated with the length of the lag phase [19]. An investigation of the kinetic behavior of phospholipase A_2 in phospholipid vesicle systems revealed furthermore that the reaction temperature can influence reaction velocity due to gel/fluid phase transitions within the vesicle membranes [20].

In this work, micelle based delivery of strongly hydrophobic substrates is characterized qualitatively and kinetically using the oxidative 9′,10′ cleavage of structurally related carotenoids by *Arabidopsis thaliana* carotenoid cleavage dioxygenase 1 (AtCCD1) [21] as a model reaction. A detailed analysis of the influence of substrate and surfactant structure on micelle based substrate delivery is reported and novel factors of influence on kinetic lag phases in micelle based enzymatic conversions are presented.

2. Experimental

2.1. Reagents and materials

β-Carotene and 8′-apo-β-caroten-8′-al were purchased from Fluka Chemicals, Buchs, Switzerland. Zeaxanthin and astaxanthin were kindly donated by Wild Flavors GmbH & Co., Berlin, Germany. Surfactants were purchased from Sigma–Aldrich GmbH, Steinheim, Germany. All other chemicals were purchased from Roth Chemicals, Karlsruhe, Germany and were – where available – of analytical grade purity. Water was obtained from a NANOpure UV water purification system.

2.2. Transformation and expression

AtCCD1 was amplified as described by Schwartz et al. [21] and cloned under control of the *tac* promoter into pGEX-4T-1 (Amersham Biosciences) *via* the BamHI/EcoRI restriction sites yielding an *AtCCD1-GST* fusion. Orientation and correct ligation were confirmed by sequencing. *AtCCD1* was then subcloned into pET29a as described by Schilling [1], however with a different set of primers in order to receive a native, non His_6-tagged gene. The primers used were: forward: 5′-T ACA TTA ATG GCG GAG AAA CTC AGT G-3′; reverse: 5′-TA CAT TAA TGC TTA TAT AAG AGT TTG TTC C-3′. *Escherichia coli* BL21 DE3 cells (Novagen, UK) were heat-shock transformed and *AtCCD1* expression was verified by SDS-PAGE. Protein expression was conducted as described by Schilling et al. [1], but at 20 °C in a shaker incubator. *Bcmo2* of *Mus musculus* was received from PD Dr. von Lintig, University of Freiburg cloned into pBAD with the polyhistidine tag in the vector cut out [22]. The pBAD-Bcmo2 plasmid was heat shock transformed into *E. coli* BL21 DE3 cells and Bcmo2 was expressed as described by Kiefer et al. [22]. Cell extracts were prepared by sonication and subsequent centrifugation at 5600 × *g* and 4 °C for 25 min.

2.3. Preparation of carotenoid loaded micelle solutions

Carotenoids were dissolved in butylated hydroxytoluene (BHT) stabilized tetrahydrofuran and aliquots of the pre-solution were mixed with pure surfactant at 50 °C in a water bath. The solvent was removed in a rotavapor while maintaining a temperature above the surfactant cloud point in order to avoid carotenoid microcrystallization. The rotavapor was run at 50 °C water bath temperature, 230 mbar and 30 rpm rotation velocity. Buffer was heated to 35 °C before addition to the vessel with the carotenoid loaded surfactant film and vigorous mixing. Solutions were visually checked for precipitation, covered with nitrogen and stored for a maximum of two days at room temperature. The concentrations of surfactant and substrate were chosen individually for each group of experiments, depending on the substrates and surfactants used therein. Except for the data presented in Fig. 1, the highest molar substrate to surfactant ratio permitting reliable solubilization of all carotenoids used in the experiment with all surfactant types used and without risk of precipitation was used. In order to avoid confusion, the respective concentrations are given with the experimental results for each set of data presented.

2.4. Carotenoid cleavage assay

AtCCD1 activity assays were conducted in lid covered polystyrol microtiter plates and carotenoid cleavage was measured