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

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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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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

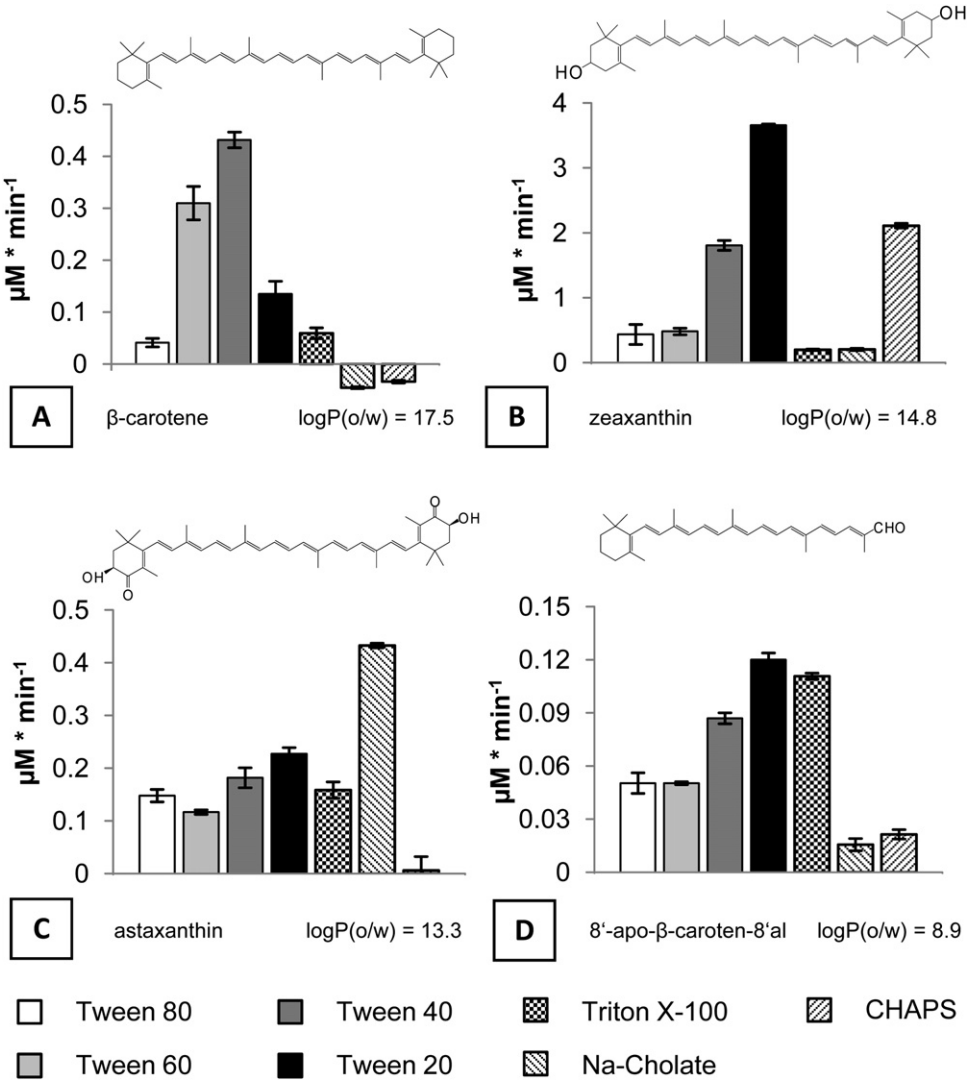

Fig. 1. Relative comparison of reaction velocities for the conversion of different carotenoids and octanol/water partitioning coefficients log *P* (o/w) of substrates tested. β-Carotene (A), zeaxanthin (B), astaxanthin (C) and 8′-apo-β-caroten-8′-al (D) using native AtCCD1. Substrate concentration: 4.8 μM. Surfactant concentration: 16 mM.

spectrophotometrically. All samples were measured in triplicate at a volume of 100 μl per well. Unless mentioned otherwise, a single assay was composed of 70 μl 20 mM Tris pH 8.5, 10 μl carotenoid loaded micelles suspended in 20 mM Tris pH 8.5 and 20 μl of AtCCD1 containing cell extract in 20 mM Tris pH 8.5. Experiments concerning the effect of pH on the duration of lag phases were performed at a total ion concentration of 150 mM which was set using sodium chloride after titration of the buffer to the desired pH. Buffers used were: pH 10.5 and 9.5 ethanol amine, pH 9, 8.5, 8 and 7.5 Tris, pH 6.5 Bis–Tris, pH 5.5 and 4.5 acetate. Negative controls were conducted with extract of cells transformed with the empty expression vector and cultivated identical to cells expressing AtCCD1. Unless mentioned otherwise, all measurements were conducted at a temperature of 30 °C using an Infinite 200 microtiter plate spectrophotometer equipped with a dispensing unit (Tecan GmbH, Groedig, Austria). All components except for the enzyme solution were pipetted robotically into the wells and tempered before the enzyme was added and the plate was shaken automatically at maximum intensity for 10 s before beginning the measurement. The reaction was measured photometrically by detecting the decrease in carotenoid absorbance during the carotenoid cleavage reaction. Measurements with different substrates were conducted at the optimum wavelength for the respective substrate: 8′-apo-β-caroten-8′-al and astaxanthin 510 nm, β-carotene as well as zeaxanthin 490 nm. The wavelengths chosen were not at the absorption maxima of the respective substances but bathochromic of it in order to avoid interference by the absorption spectra of the dialdehydes formed during the reaction [23].

3. Results and discussion

3.1. Suitability of surfactants for the delivery of carotenoid substrates in aqueous media

A series of surfactants was investigated for their ability to deliver carotenoid substrates of different hydrophobicity to the dioxygenase AtCCD1. The surfactants investigated were a series of structurally related non-ionic surfactants (Tween 20, 40, 60, 80 and Triton X-100) as well as a common representative of the class of anionic (sodium cholate) and zwitterionic (CHAPS) surfactants. Supplementary Table 1 lists the physicochemical data of the surfactants investigated.

The enzymatic conversions were conducted with identical substrate concentrations using the carotenoids β-carotene, zeaxanthin, astaxanthin and the apocarotenoid 8′-apo-β-caroten-8′-al.

As is shown in Fig. 1, the substrates differ in structure and hydrophobicity which is represented by the octanol/water partition coefficient log *P* (o/w). The substrates used cover the entire range of hydrophobicities encountered among carotenoid substrates.

All experiments were performed in microtiter plates at a substrate concentration of 4.8 μM and a surfactant concentration of 16 mM which is well above the CMC of all surfactants tested. Non modified AtCCD1 was used in order to exclude potential influence by fused polypeptide tags. In order to avoid temperature and mixing influences, pipetting, temperature control and mixing were performed automatically. The maximum reaction velocities obtained for the different reaction system configurations are shown in Fig. 1. Reaction velocities using different surfactants should only be compared among the results obtained with the same substrate. A comparison of the velocities obtained with different substrates is not advisable. In order to avoid effects caused by separate processing of expression cell batches for each substrate tested, a common enzyme solution was used which underwent a slight loss of specific activity over the series of experiments conducted. All measurements concerning a specific substrate were conducted simultaneously.

For every substrate tested, a different spectrum of surfactants was found to form micelles permitting optimal substrate delivery and conversion by the enzyme. Among the results obtained with non-ionic surfactants, the results for Triton X-100 are particularly interesting. This surfactant in combination with the readily cleaved model substrate 8′-apo-β-caroten-8′-al was applied widely in investigations on the properties of the CCD enzyme family. The results show that only highly oxyfunctionalized xanthophylls like astaxanthin and apocarotenoids can be delivered efficiently to AtCCD1 using Triton X-100 micelles. In contrast, the less oxyfunctionalized zeaxanthin and the fully non-polar β-carotene are converted only very poorly out of Triton X-100 micelles. This might be one of the reasons why the biocatalytic conversion of the economically very attractive substrate β-carotene has been described repeatedly in *in vivo* test systems, but could not be accomplished with industrially relevant yield *in vitro* using isolated enzymes. Although *in vitro* cleavage of β-carotene has been described before, cleavage activity could only be achieved in the low picomole per mg range [24], as non-quantitative trace measurements [25] or in non-regiospecific reactions [26]. In most investigations published, Triton X-100 micelles were used for substrate delivery. It is likely that the results obtained for AtCCD1 are valid for all enzymes of the CCD enzyme family but vary for different enzyme classes. The only data set published for a carotenoid converting enzyme deals with a 15,15′-monooxygenase [12] and describes Tween 80 instead of Tween 40 as the most suitable surfactant for β-carotene conversion. The widely used standard surfactant Triton X-100 is also described as a poor surfactant for β-carotene delivery. Generally, *in vitro* studies on substrate preferences of CCD enzymes conducted with micelle based substrate delivery [21,24,25,27,28] should be critically reviewed because as shown here, the specific surfactant type used can significantly influence the substrate preference of CCD enzymes.

3.2. Influence of surfactant structure on the cosolvent based acceleration of carotenoid conversion

Enzymatic conversions of highly hydrophobic substances delivered by surfactant micelles can sometimes be accelerated by the presence of water-soluble cosolvents in the reaction media. Among carotenoid cleavage oxygenases, this effect was first observed for AtCCD7/AtCCD8 [24] and VvCCD1 [29]. Schilling and co-workers [1,23] investigated the phenomenon in more detail and found a linear correlation between the log *P* (o/w) value of the cosolvent and the concentration necessary for maximum acceleration of the reaction. In technical applications, this property could be used to increase the effective specific activity of enzymes in bioconversions of hydrophobic substrates. According to the current state of knowledge, the acceleration is caused by four independent physicochemical effects on micelle structure [23]: an increase of the critical micelle concentration (CMC) due to a lowered polarity difference between reaction media and surfactant [30–32], an increased diffusion coefficient of the substrate, a lowered aggregation number of the surfactant and lowered dynamic viscosity of the media [31]. According to the investigations of Schilling [23], a conformational change of the enzyme or the increase in substrate solubility in the media play no significant role in the acceleration effect.

The investigations mentioned did not take into account the role of the surfactant molecular structure on the accelerating effect of cosolvents in micelle based biocatalysis. Most investigations were performed with one specific non-ionic surfactant, Triton X-100. In order to gain further insight into the underlying mechanism, the role of the surfactant molecular structure on the occurrence and on the degree of acceleration of the bioconversion were investigated. In order to allow for the identification of trends in influence, the Tween surfactant series was used among others. Its members differ in the length and saturation of the hydrophobic chain while the hydrophilic headgroups of the surfactants are identical (Fig. 2). Non modified AtCCD1 was used in the investigation in order to exclude potential influence by a fused polypeptide tag. Two types of substrates were applied in the investigation: the asymmetric apocarotenoid C30 substrate 8′-apo-β-caroten-8′-al and the symmetric C40 substrate zeaxanthin. Experiments were performed at a substrate concentration of 48 μM and a molar substrate to surfactant ratio of 1:338. Ethanol was used as cosolvent because of its low log *P* allowing for a more precise pipetting due to the higher volumes transferred into the microtiter plate wells. For both substrate types a similar correlation between the surfactant structure and the cosolvent concentration necessary for maximum activation was observed: Fig. 2 shows that the cosolvent concentration for maximum activation increased with the length of the hydrophobic chain of the surfactant. A non saturated carbon–carbon bond in the hydrophobic chain dramatically reduced the ability of the formed micelles to deliver the substrate 8′-apo-β-caroten-8′-al to AtCCD1. For the conversion of zeaxanthin, the same behavior of the enzyme/micelle system as in Fig. 2 was observed.

The data presented here support the hypothesis by Schilling et al. [1] that the accelerated conversion of hydrophobic substrates from non-ionic surfactant micelles occurs due to a restructuring of the micelles by the organic cosolvent. The presence of water-soluble organic cosolvents reduces the hydrophobic interactions between surfactant molecules and leads to an increase of the critical micelle concentration (CMC) [31]. Due to the increased CMC, the aggregation number N_{agg} of the micelles formed by non-ionic surfactants is reduced. As a result of the reduced aggregation number, the distance between the substrate in the hydrophobic micelle core and the micelle surface is reduced [15]. In accordance with results on liposome based substrate delivery published previously, we postulate the hypothesis that an optimum distance of the substrate from the vesicle surface exists for enzymatic conversions using AtCCD1. Surfactants with a longer hydrophobic chain form micelles of larger diameter. Their diameter therefore has to be reduced more by the addition of cosolvents until the optimum distance between substrate and vesicle surface is reached.

The presence of an unsaturated double bond in the aliphatic side chain of the surfactant rendered the respective micelles incapable of delivering substrate to the enzyme. A concise explanation for the inability of surfactants with partly unsaturated hydrophobic chains to deliver carotenoid substrates cannot be given at this point. The commercially distributed form of Tween 80 is a racemic mixture of *cis* and *trans* isomers. Possibly, the hydrophobic core

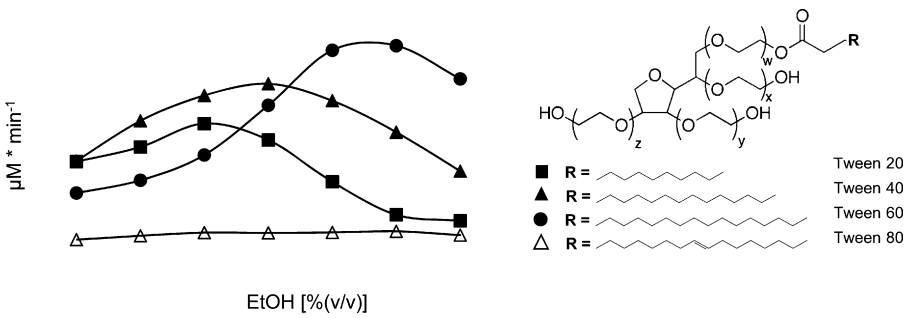

Fig. 2. Influence of surfactant molecular structure on the acceleration of carotenoid cleavage by water-soluble organic cosolvents. Substrate: 8′-apo-caroten-8′-al. Enzyme: native AtCCD1. The length of the hydrophobic side chain of Tween surfactants determines the cosolvent concentration for maximum activation of the reaction. For simplification, Tween 80 was drawn only in the *trans* form although a racemic mixture was used.

of micelles formed by this surfactant is less homogenous and less pronounced due to steric hindrance of the bent hydrophobic chains. Published data for critical micelle concentration, aggregation number and micelle diameter [33] do not indicate any other micelle properties as reasons for the observed effect. Unfortunately, measurements on the diameter and hydrophobicity of the Tween 80 micelle core in comparison to other members of the Tween group are not available yet and would be very valuable in verifying the postulated mechanism.

3.3. Kinetic lag phases in enzymatic conversions of carotenoid substrates

Factors of influence on lag phases in biocatalytic conversions of hydrophobic substrates from micelles were investigated in order to gain insight into the underlying mechanisms. In order to determine whether the kinetic lag phases are inherent to all enzymatic carotenoid bioconversions from non-ionic surfactant micelles or specific only to the AtCCD1 enzyme, the substrate depletion curves of two carotenoid cleaving enzymes specific for the 9′,10′ double bond were compared. Besides AtCCD1, the murine β-carotene monooxygenase Bcmo2 was used [34] to cleave a Tween 40 micellar solution of astaxanthin. Pipetting and mixing were performed automatically in order to avoid influences by mixing effects. Fig. 3 shows a distinct difference in the course of the reactions. For AtCCD1, a 53 min kinetic lag phase similar to the data reported by Schilling [1] was observed. In contrast, Bcmo2 showed no lag phase when converting substrate from the same micellar solution. The highest reaction velocity of Bcmo2 was instead observed at the beginning of the reaction. The results show that the occurrence of lag

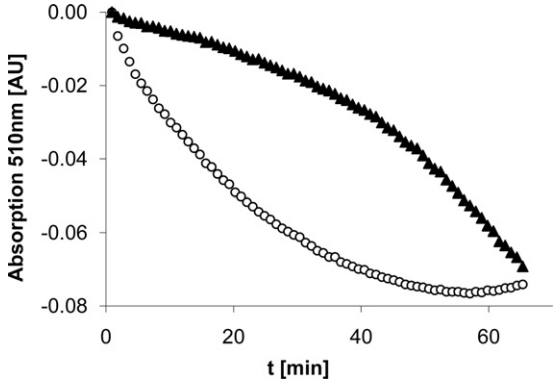

Fig. 3. Example for the lag phase phenomenon observed when using AtCCD1 with Triton- and Tween type substrate delivery micelles. Cleavage of astaxanthin from Tween 40 micelles using native AtCCD1 (▲) and native Bcmo2 (○). Carotenoid concentration: 38 µM. Tween 40 concentration: 12 mM.

phases in enzymatic conversions of carotenoids is specific to the AtCCD1/micelle interaction and not an inherent property of the micellar substrate delivery system.

The lag phase could either be due to a change in the AtCCD1/micelle interaction or due to an activation of AtCCD1 by one of the two reaction products formed. β-Ionone, one of the two reaction products of β-carotene, is described as AtCCD1 inhibitor in a recent Ph.D. thesis [23]. Therefore, only the second reaction product, a C17 dialdehyde, remains as a potential AtCCD1 activator. The effect of the C17 dialdehyde on AtCCD1 could not be investigated directly since the substance cannot be purchased and a preparative purification was not possible due to the chemical instability of the molecule. We investigated the conversions of the carotenoid substrates 8′-apo-β-caroten-8′-al and zeaxanthin which lead to two structurally different C14 and C17 dialdehydes. Since a lag phase was observed for both substrates tested, product activation of AtCCD1 by the dialdehyde products seems unlikely due to their different structural features. Instead, further investigations indicated that enzyme/micelle interaction is responsible for the observed kinetic lag phase. Solvent properties have significant influence on the physicochemical behavior of micellar solutions. Solvent hydrophobicity affects the boundary layer polarity [35] and the solvent pH influences the aggregation number and hydration [36] of non-ionic surfactant micelles. Both boundary layer polarity and aggregation number have significant influence on contact interactions between micelles and solid particles such as enzymes. The pH and solvent hydrophobicity were varied in order to investigate the factors governing enzyme/micelle interaction and kinetic lag phases during carotenoid conversion by AtCCD1. The hydrophobicity of the solvent was influenced by the addition of the water-soluble cosolvent ethanol. In accordance with a previous investigation on lag phases of CCD enzymes [1], the duration of the lag phase was expressed as percent substrate converted. A linear correlation between the duration of the lag phase and the cosolvent concentration was observed (Fig. 4A). The share of substrate converted until reaching maximum reaction velocity is 39% in cosolvent-free reaction media and decreases to 9% at a cosolvent concentration of 18% (v/v) (Fig. 4B).

The presence of water-soluble organic cosolvents causes several physicochemical changes in non-ionic micellar solutions, the predominant change being an increase in the Reichardt parameter E_T which is a measure for the polarity difference at the micelle boundary layer [36]. In the system investigated the increased micelle boundary layer polarity led to a shortened kinetic lag phase of the carotenoid conversion by AtCCD1. A possible cause for the observed shortening of the lag phase is an alleviated docking of the enzyme to the vesicle surface which is necessary to recruit the substrate out of its hydrophobic core. In the system investigated, the statistical number of substrate molecules per micelle was 0.7. The velocities of

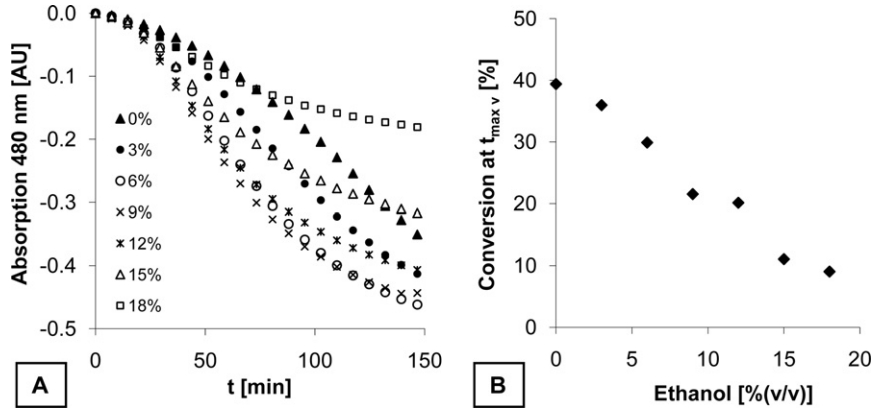

Fig. 4. (A) Courses of reaction for different concentrations of the organic cosolvent ethanol. Conversion of 48 μM zeaxanthin from 16 mM Tween 40 by native AtCCD1 in 20 mM Tris pH 9.0 at 37 °C. Substrate concentration was measured photometrically at 490 nm. (B) Influence of cosolvent concentration on the length of the lag phases until reaching $t_{MAX\,V}$.

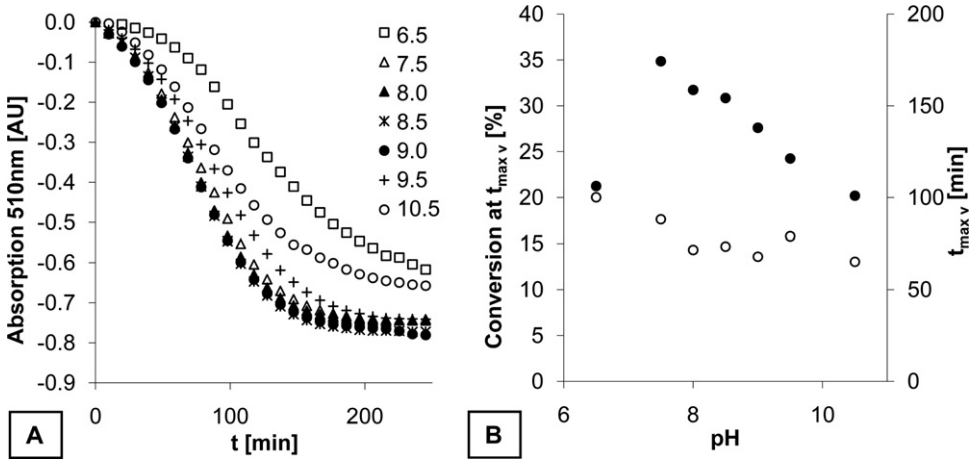

Fig. 5. (A) Courses of reaction for different pH values. Conversion of 38 μM astaxanthin from 12 mM Tween 40 micelles using native AtCCD1 at 37 °C. Buffer concentration: 75 mM, total ion concentration 150 mM. Pipetting and mixing were performed automatically to avoid falsification by mixing effects. (B) Influence of pH on length of kinetic lag phases, plotted for two frames of reference: time until reaching maximum reaction velocity (○) and substrate converted (●) when reaching maximum reaction velocity.

enzyme docking and detachment were therefore key determinants of the reaction velocity.

The pH value of the solvent has significant influence on the structure and size of micelles. Both aggregation number and hydration per mass unit of surfactant are reduced by increasing pH values. In order to investigate a potential correlation between solvent pH and lag phases, astaxanthin was converted from non-ionic surfactant micelles at different pH values between 6.5 and 10.5. The reaction buffers chosen all had a pK_a value of the buffered pH ±1. The buffer concentration in all samples was 75 mM with a total ion concentration of 150 mM. The substrate depletion curves all show a sigmoidal shape with a kinetic lag phase (Fig. 5A). The lag phase duration in Fig. 5B is presented in two ways: in order to permit comparison to literature data, the lag phase length is given in percentage of converted substrate at $t_{MAX\,V}$ as was common in the data published on this topic until now. Additionally, we expressed lag phase duration also in minutes in order to give the reader a more clear picture of the kinetic effects involved. The lag phase in minutes until reaching maximum reaction velocity (v_{MAX}) changed only very slightly between pH 6.5 and 10.5 (Fig. 5B).

In contrast to that, the share of converted substrate at maximum reaction velocity varied significantly: substrate conversion was 35 mol% when v_{MAX} was reached at pH 7.5, dropping to 21 mol% at pH 10.5 (Fig. 5B). The data show that altering the pH value did not change the actual duration of the lag phase but instead changed the velocity of the enzymatic reaction. Expressing lag phase duration primarily in percent of substrate converted should therefore be done with care: this reference system can provide interesting clues as in the work of Schilling et al. [2] but can also falsify results.

The hypothesis of Schilling [1] stating that for a given surfactant/enzyme combination the share of converted substrate at v_{MAX} is constant, should be amended: the share is only constant at a given pH and a given solvent hydrophobicity. In technical applications of CCD enzymes, the different dependences of the lag phase and the specific activity of the enzyme on the pH value should be accounted for in order to achieve kinetically optimal reaction conditions.

4. Conclusions

Non-ionic surfactants differ significantly in their ability to deliver highly hydrophobic carotenoid substrates to AtCCD1. Even structurally similar carotenoids require different non-ionic surfactants for efficient micellar delivery to the enzyme AtCCD1. Published data on the *in vitro* substrate preference of CCD enzymes should be taken into account only very carefully because the surfactant applied in such investigations was shown to significantly influence the substrate preference of CCD enzymes. The conversion of carotenoids from micelles by AtCCD1 can be accelerated by the addition of water-soluble organic cosolvents. We were able to show that within a series of structurally related surfactants, the length and saturation of the surfactant's aliphatic side chain

determine the cosolvent concentration necessary for maximum acceleration. Kinetic lag phases were described for some combinations of carotenoid cleaving enzymes and non-ionic surfactants of the Tween series and both the concentration of water-soluble cosolvents and the pH have been shown to influence the occurrence and duration of such lag phases.

Although liposome based reactions were not reported to have kinetic lag phases [3], the reaction velocities which could be achieved using micelles are significantly higher. This is especially true for weakly amphiphilic hydrophobic substrates like apocarotenoids which are not incorporated symmetrically into lipid bilayers [37] of liposomes and therefore generate an accessibility problem [3]. Liposomes could, however, be especially suited for substrate delivery to enzymes which are either membrane associated or depend on their natural reaction environment at a phospholipid bilayer.

The set of results presented in this work contributes to the understanding of micelles as tools for the delivery of hydrophobic substrates to enzymes in aqueous media and represents a step toward a rational reaction system design for enzymatic conversions of carotenoids and other hydrophobic substrates in aqueous environments.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2012.01.010.

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