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Liposome based solubilisation of carotenoid substrates for enzymatic conversion in aqueous media

Christoph Nacke, Jens Schrader*

Biochemical Engineering, Karl Winnacker Institut, DECHEMA e.V., Theodor-Heuss-Allee 25, 60486 Frankfurt, Germany

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ABSTRACT

Enzymatic conversions of strongly hydrophobic substrates can be conducted in non-denaturing aqueous reaction media if appropriate substrate delivery systems such as micelles or liposomes are applied. We investigated liposome based substrate delivery with regard to qualitative and kinetic effects of vesicle properties on the enzymatic reaction. The cleavage of highly hydrophobic carotenoid and xanthophyll substrates by *Arabidopsis thaliana* carotenoid cleavage dioxygenase 1 (AtCCD1) was used as model reaction for the investigation. Conversions of the partly polar xanthophylls and the fully non-polar carotenes showed different responses to variations in the phospholipid composition of the delivery vesicles. Furthermore, the cellular reaction environment of AtCCD1 was imitated by incorporation of the galactolipid monogalactosyl-diacylglycerol (MGDG) into the liposome membranes. This led to an increase of the specific AtCCD1 activity towards the fully non-polar β-carotene by approximately 70% at MGDG shares between 8 and 20 mol% while the specific activity towards the more hydrophilic zeaxanthin decreased with increasing MGDG content. Liposome based systems proved to be less suitable for the delivery of amphiphilic and non-symmetric substrate molecules than micelles due to substrate orientation within the liposome membrane. The results represent a starting point for a systematic design of liposome based delivery of hydrophobic substrates in enzymatic conversions performed in aqueous media.

1. Introduction

A large number of economically attractive biocatalytic conversions of hydrophobic substrates cannot be conducted at large scale because the biocatalyst to be used is either not stable [1,2] or not sufficiently active [3] in the organic solvents required for dissolving a hydrophobic substrate. Scientific efforts to overcome such problems mainly focus on the selection of alternative enzymes or on increasing enzyme stability in organic solvents by rational protein design or directed evolution [4]. Although these strategies have led to success in many cases, they need to be complemented by strategies aiming at adapted substrate delivery. The data presented here show that technical improvement of the substrate delivery has the potential to permit biocatalytic conversion of hydrophobic substrates even in aqueous reaction systems and thereby complement or substitute the aforementioned enzyme engineering approaches.

The term liposome describes a wide variety of structures and membrane configurations [5,6]. Despite of being an established tool in medical and biochemical research as well as cosmetic applications, liposomes have only very rarely been quantitatively investigated as tools for technical biocatalysis. However this vesicle type has a number of characteristics rendering it advantageous for delivering hydrophobic substrates to biocatalysts in aqueous media. Liposomes, for example, provide a membrane-like environment required by many enzymes capable of converting highly hydrophobic substrates *in vivo*. Furthermore, the physicochemical membrane properties of liposomes can be modified, permitting an imitation of the natural reaction environment of the enzyme used. It is also known that the liposome lipid composition can activate or inactivate associated enzymes [7,8] and is even used *in vivo* to actively control the level of enzyme activity [9]. Depending on their structural features, hydrophobic substrates such as carotenoids are incorporated into liposome membranes in very different orientations [10] which also have significant effect on their availability for biocatalytic conversion. Besides lipid composition and substrate structure, lipid derivates also play an important role in the regulation of membrane-associated enzymes by providing surface structures essential to substrate recruitment as was shown recently by Latowski and coworkers [12,13]. To date, the formulation of liposome based reaction systems is often conducted by trial and error rather than by rational design. As shown by Zhou and Roberts [14], the kinetics of enzymatic reactions performed with

* Corresponding author. Tel.: +49 69 7564422; fax: +49 69 7564388.
E-mail address: schrader@dechema.de (J. Schrader).

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hydrophobic substrates are highly dependent on the properties of the substrate delivery system.

In the present work we investigated liposome based substrate delivery with regard to the influence of the vesicle properties on the qualitative and kinetic characteristics of an enzymatic model reaction, the oxidative 9′,10′ cleavage of carotenes and xanthophylls by *Arabidopsis thaliana* carotenoid cleavage dioxygenase 1 (AtCCD1) [15]. Upon enzymatic cleavage, volatile C13-norisoprenoids such as β-ionone are produced which are valuable flavour and fragrance compounds [16]. Carotenoids represent a particularly difficult to handle example of hydrophobic substrate molecules. With 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 [17] and furthermore tend to form microaggregates upon contact with water which are no longer available to enzymatic conversion [18].

2. Experimental

2.1. Reagents and materials

Highly purified phosphatidylcholine and phosphatidylethanolamine were provided by Lipoid GmbH, Friedrichshafen, Germany. Galactolipids were purchased from Nutfield Nurseries, South Nutfield, UK. Carotenoids were purchased from Fluka Chemicals, Buchs, Switzerland. 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, expression and preparation of cell extracts

AtCCD1 was amplified as described by Schwartz et al. [15] 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. *E. coli* BL21 DE3 cells (Novagen, UK) were heat-shock transformed with pGEX-4T-1-AtCCD1 and *AtCCD1* expression was verified by SDS-PAGE. Protein expression was conducted as described by Schilling et al. [19], but at 20 °C in a shake-incubator. After sonication, 1.85 mM Triton X-100 was added for optimum protein solubilisation. After mixing and incubation on ice for 2 min, cell debris was removed by centrifugation at 5600 × g and 4 °C for 25 min. Protein concentrations were determined by bicinchoninic acid assay with an eight-point calibration using BSA as a reference substance.

2.3. Preparation and analysis of carotenoid loaded liposome solutions

Carotenoids and lipids were dissolved in stabilized tetrahydrofurane (THF). A carotenoid containing lipid film was formed by a complete removal of the solvent via a stream of nitrogen and subsequent evaporation at 230 mbar and 50 °C for 60 min. The elevated temperature was necessary because undershooting the lipid phase transition temperature during evaporation led to precipitation of carotenoid microcrystals. The film was detached from the glass vessel wall and converted into multilamellar vesicles (MLV) by intense mixing with buffer. Prior to mixing, the lipid film and the buffer were heated to 50 °C in order to avoid carotenoid precipitation during the following steps. The MLV were converted into small unilamellar vesicles (SUV) by sonication for 120 s at 15% amplitude in a 30 °C water bath using a Branson Sonifier II 250 Digital equipped with a 5 mm microtip [21]. Due to the reduction in particle diameter, the previously opaque MLV suspension became a clear ULV suspension. The particle size distribution was determined by dynamic light scattering (DLS) using a Malvern Nano ZS device in 170° backscatter mode. Samples were measured at 25 °C in polystyrol cuvettes and diluted with lab water for which a viscosity of 0.8872 cP was calculated. The refraction indices used were 1.33 for water and 1.37 for phospholipids. Measurements were conducted according to the respective procedures described in the device handbook.

2.4. Carotenoid cleavage activity assay

AtCCD1 activity assays were conducted in lid covered polystyrol microtiter plates and carotenoid cleavage was measured spectrophotometrically. All samples were measured in triplicate at a volume of 100 μl per well. A single assay was composed of 70 μl 50 mM Tris pH 8.5, 10 μl carotenoid loaded liposomes suspended in 50 mM Tris pH 8.5 and 20 μl of AtCCD1 containing cell extract in 50 mM Tris pH 8.5. Negative controls were conducted with extract of cells transformed with the empty expression vector and cultivated identical to cells expressing AtCCD1. Measurements concerning the effect of the phosphatidylcholine (PC) to phosphatidylethanolamine (PE) ratio were conducted at a total lipid concentration of 2 mM and a substrate concentration of 0.5 mM. Measurements concerning the imitation of the natural membrane environment by the galactolipid monogalactosyl-diacylglycerol (MGDG) were conducted at a total lipid concentration of 5 mM and a carotenoid concentration of 0.2 mM. Unless mentioned otherwise, all measurements were conducted at a temperature of 30 °C using a Tecan Infinite 200 microtiterplate spectrophotometer. 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 concerning the effect of lipid composition were performed at λ = 490 nm for both substrates while measurements concerning kinetic parameters with different substrates were conducted at the optimum wavelength for the respective substrate: 8′-apo-β-caroten-8′-al 510 nm, β-carotene as well as zeaxanthin 490 nm and astaxanthin 510 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 [22].

3. Results and discussion

3.1. Influence of lipid composition

The most prominent phospholipids in natural membranes are phosphatidylcholine (PC) and phosphatidylethanolamine (PE). The influence of the lipid composition on the ability of small unilamellar vesicles (SUV) to deliver hydrophobic carotenoid substrates to AtCCD1 was investigated. The molecular structures of all lipids used in this investigation are shown in Fig. 1.

Liposomes with defined PC and PE content were generated using highly purified phospholipids and the reaction velocity of carotenoid conversion by AtCCD1 from these vesicles was measured. The site and orientation of carotenoid substrates incorporated into phospholipid bilayers can differ significantly depending on the structure of the substrate molecule. The molecular structures of all carotenoid substrates used in this investigation are shown in Fig. 2.

Therefore, two different substrate types were investigated: the fully non-polar β-carotene which intercalates in a surface-parallel

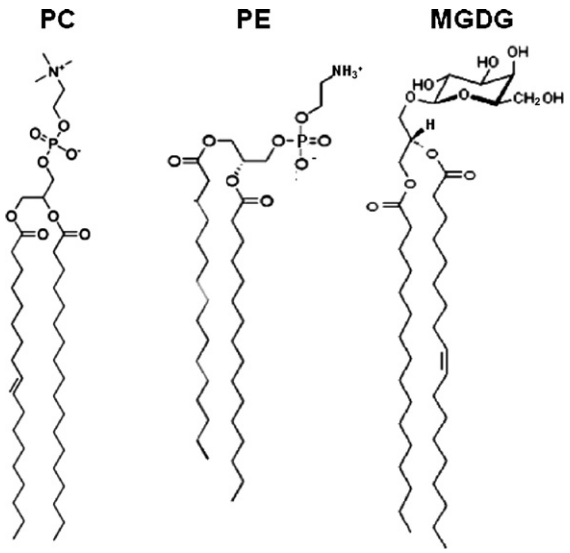

Fig. 1. Molecular structures of the lipids used in this investigation: phosphatidylcholine (PC), phosphatidylethanolamine (PE) and monogalactosyl-diacylglycerol (MGDG).

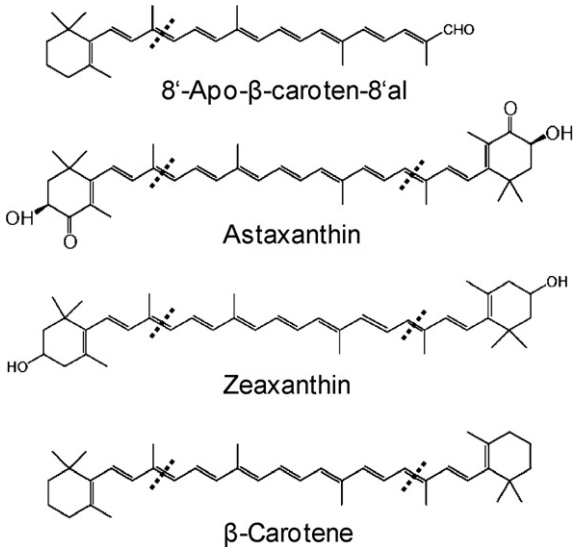

Fig. 2. Molecular structures of all carotenoid substrates used in this investigation. Enzymatic cleavage sites of the *Arabidopsis thaliana* carotenoid cleavage dioxygenase AtCCD1 are indicated by dotted lines.

manner into the hydrophobic core of phospholipid bilayers [10] (Fig. 3A) and the xanthophyll zeaxanthin, a symmetrically oxy-functionalized carotenoid, which intercalates into phospholipid membranes perpendicular to the membrane surface [20] (Fig. 3B).

For both substrate types, the lipid composition of the delivery vesicles influenced the reaction velocity obtained. For β-carotene, the optimum PC to PE ratio was determined as 40:60 mol% whereas the optimum for zeaxanthin conversion was 50:50 mol% (Fig. 3C). At PC shares higher than the optimum, different characteristics were observed for the two substrate types investigated: the conversion of zeaxanthin was still possible with high reaction velocities while the conversion of β-carotene became slower and came to a stop at a liposome composition of 100 mol% PC. Zeaxanthin conversion rates were reduced 15–20% by an increasing PC share, whereas β-carotene conversion decreased linearly to zero when the PC share in the liposome composition was increased (Fig. 3C). The data show the importance of a suitable liposome composition for the delivery of highly hydrophobic substrates such as β-carotene. The use of commercially available, non-defined phospholipid mixtures commonly referred to as lecithin can have significant negative effect on the efficiency of the pursued biocatalytic reaction. This is illustrated by a comparison with the results of Schilling [22] on the biocatalytic conversion of liposome-delivered β-carotene by AtCCD1 using lecithin for liposome formation: only trace amounts of the product β-ionone were detected via GC-MS.

The dependence of the delivery efficiency on the substrate structure is probably due to the different orientations of the substrate molecules within the phospholipid bilayer of the delivery vesicles. The variation of the phospholipid composition changes membrane surface polarity and structure and therefore affects the accessibility of the carotenoid substrates to AtCCD1. The surface of lipid bilayers rich in phosphatidylethanolamine is not readily hydrated and therefore hydrophobic [10,11]. The hydrophobicity of a liposome boundary layer rich in PE has two effects: firstly, non-polar substrate molecules such as β-carotene have a wider area of motional freedom within the bilayer hydrophobic core and occur closer to the liposome surface from where the substrates are recruited for enzymatic conversion. Secondly, the transfer, especially of highly hydrophobic substrate molecules, across the liposome surface to the enzyme is facilitated. In contrast to PE, PC forms a hydrated or charged membrane surface, allowing water or ions to bind to its polar headgroup [11]. The mobility of hydrophobic substrates across the membrane boundary layer is thus reduced and non-polar substrates like β-carotene remain in the hydrophobic membrane core at maximum distance from the polar surface. The enzymatic accessibility of non-polar substrates like β-carotene in liposomes

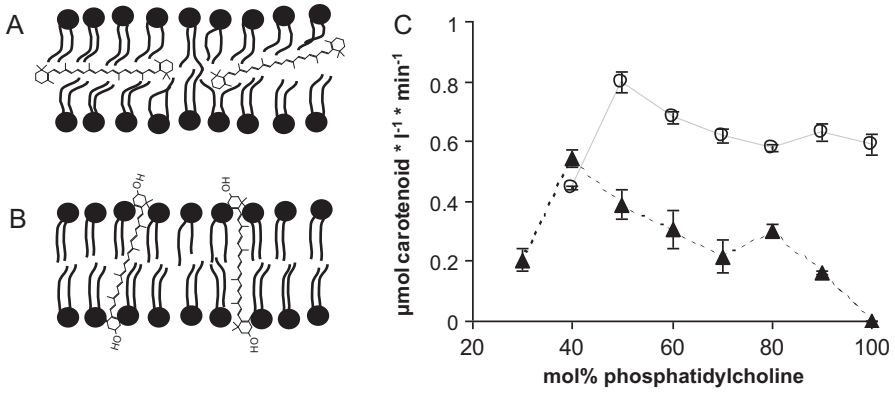

Fig. 3. Schematic depiction of the orientation of β-carotene (A) and zeaxanthin (B) in lipid membranes. (C) Reaction velocity over lipid composition for the cleavage of 0.5 mM β-carotene (▲) and 0.5 mM zeaxanthin (○) by GST-fused AtCCD1. The unilamellar liposomes consisted of phosphatidylcholine and phosphatidylethanolamine at a total lipid concentration of 2 mM in 50 mM Tris pH 8.5. Controls with non carotenoid loaded liposomes were conducted and the corresponding absorption raw data were substracted from the absorption values derived from the carotenoid cleavage experiments before calculating the data points shown.

is consequently reduced at high shares of PC. Due to their orientation perpendicular to the membrane surface, xanthophylls such as zeaxanthin are accessible to the enzyme even at a high PC share and high degree of membrane hydration. Although a low PC/PE ratio in liposomes is desirable for substrate delivery, the data in Fig. 3C shows an optimum PC share below which the reaction velocity rapidly decreases. Liposomes loaded with carotenoid substrate were not stable at PC shares below 30 mol% (β-carotene) and 40 mol% (zeaxanthin). The observed maximum most likely represents the point of minimum PC content allowing the formation of stable liposomes. The two data points obtained at PC shares below the respective optima most likely represent instable liposome populations in which the substrates undergo partial microaggregation rendering them inconvertible by the enzyme.

3.2. Imitation of natural membrane environment of AtCCD1 by incorporation of galactolipids

In vivo AtCCD1 is located on the cytoplasmic side of the chloroplast membrane [23,24]. The chloroplast membrane differs from other membranes by its high content of galactolipids, in particular monogalactosyl-diacylglycerol (MGDG, Fig. 1) [25]. The membranes of the chloroplast fraction of *A. thaliana* contain approximately 75 mol% galactolipids with 21 mol% being digalactosyl-diacylglycerol (DGDG) and 54 mol% being MGDG [26]. The natural membrane environment of AtCCD1 was imitated by the formation of liposomes consisting of phospholipid mixtures with varying shares of MGDG. The goal of the investigation was to obtain an increase in specific AtCCD1 activity through improved substrate delivery. Liposome solutions with a total phospholipid concentration of 5 mM and 0–30 mol% MGDG were generated. The remaining phospholipid share in each preparation consisted of PC and PE at a molecular ratio of 50:50 mol% as determined in Section 3.1 to be kinetically optimal (zeaxanthin) or near the optimum (β-carotene). MGDG shares higher than 30 mol% could not be tested since the stability of monolamellar vesicles formed out of such lipid mixtures was too low. Again, the liposomal substrate delivery system showed a different behaviour for the fully non-polar β-carotene and the xanthophyll zeaxanthin whose structure corresponds to a β-carotene scaffold substituted with hydroxyl groups at the ionone rings (Fig. 2). The volumetric cleavage activity of AtCCD1 towards β-carotene was increased by approximately 70% through MGDG shares between 8 and 20 mol%. In contrast to that, the cleavage activity of AtCCD1 towards zeaxanthin decreased with increasing shares of MGDG (Fig. 4). Since the same enzyme solution was used simultaneously in all assays, the data also represent the relative specific activity of AtCCD1.

A similar effect of MGDG was reported for the enzyme violaxanthin-deepoxidase which also converts a carotenoid substrate [12]. Since the two types of substrate tested incorporate into the lipid bilayer of liposomes in different orientations, the positive effect of galactolipid addition on β-carotene conversion is most likely due to a change in the site of incorporation of the substrate relative to the enzyme. In regions of reverse hexagonal structure caused by MGDG, the lipid bilayer thickness is reduced [13]. The distance of the incorporated β-carotene to the vesicle surface is therefore reduced as shown in Fig. 5.

Apparently, the distance between the membrane incorporated substrate and the vesicle surface is of influence to the efficiency of substrate conversion from liposomes. The lack of effect on the cleavage of zeaxanthin is probably due to its orientation perpendicular to the membrane surface and reaching through it. This permitted sufficient access of AtCCD1 to the substrate independent of the membrane boundary layer properties. This hypothesis would also be in accordance with the results obtained by Schilling for micelles [22]: the application of organic cosolvents caused a decrease in average micelle diameter resulting in increased efficiency of substrate delivery. A partially similar effect was also observed for the activity of phospholipase by Hoyrup and coworkers [27]. By changing the phospholipid composition, the authors were able to induce phase micro-heterogeneities in the lipid bilayers of liposomes which led to an increased enzyme activity.

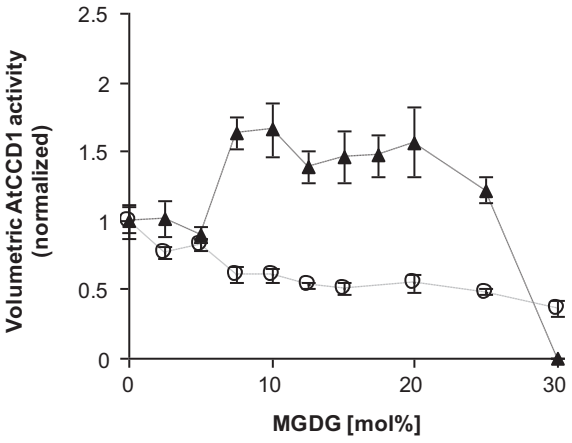

Fig. 4. Influence of the monogalactosyl-diacylglycerol (MGDG) share on the suitability of unilamellar liposomes for the delivery of β-carotene (▲) and zeaxanthin (○) to AtCCD1. The lipid share besides MGDG consisted of 50 mol% phosphatidylcholine and 50 mol% phosphatidlyethanolamine. Total lipid concentration: 5 mM. Carotenoid concentration: 0.2 mM.

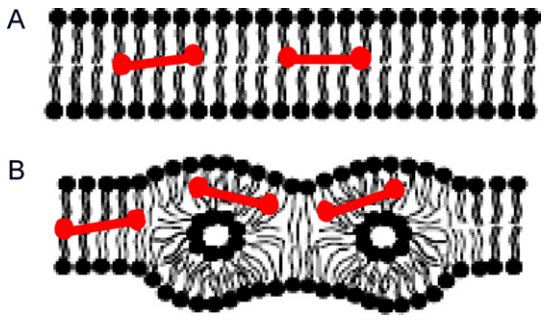

Fig. 5. Schematic depiction of phospholipid bilayer without (A) and including (B) inverted hexagonal structures formed by monogalactosyl diacylglycerol (MGDG). Potential sites of incorporation of β-carotene in the two types of lipid membranes are schematically depicted (red symbols). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Kinetic parameters for the conversion of a series of structurally related carotenoids

In 2008, the first quantitative investigation of the structural substrate preferences of AtCCD1 was conducted by Schilling [22] using micellar substrate delivery. The investigation was conducted with a series of C40-carotenoids which differed in the substituents at the ionone rings and with one non-symmetric C27-apocarotenoid. The results indicated that in the micellar system, the V_{MAX} of AtCCD1 conversion of C40-carotenoids increases with the degree of oxyfunctionalization of the substrate's ionone rings. The conversion of β-carotene could not be shown. Cleavage of the non-symmetric C27-apocarotenoid was faster than the cleavage of all C40-carotenoids except for the highly oxyfunctionalized astaxanthin. In order to obtain further insight into the substrate preferences of AtCCD1 and the dependence of the substrate preference on the delivery system, a similar investigation was conducted using liposomal substrate delivery. A similar series of carotenoids was used and the kinetic parameters K_M, V_{MAX} and k_{CAT} of AtCCD1

Table 1
Kinetic parameters for the conversion of differently functionalized C40 carotenoids and one non-symmetric C27 apocarotenoid from 100% phosphatidylcholine liposomes with purified GST-AtCCD1. For all substrates, the same molar ratio of carotenoid to phospholipid molecules was used.

Substrate	K_M (μM)	V_{MAX} (μM min^{-1})	k_{CAT} (min^{-1})
8'-Apo-β-caroten-8'al	19.3	0.13	0.016
Astaxanthin	14.6	0.36	0.044
Zeaxanthin	94.1	1.1	0.133
β-Carotene	–	–	–

were determined. In order to permit a comparison with liposome literature data and in order to avoid bias by the phospholipid composition, liposomes consisting of 100% phosphatidylcholine (PC) were used since this phospholipid is the most common in the respective literature. The data for the liposomal system is shown in Table 1.

The order of reaction velocities for the tested substrates was different from published values for the micellar system [22]. The biggest difference was observed for the non-symmetric C27-apocarotenoid 8′-apo-β-caroten-8′-al: while this type of substrate was converted the second-fastest in the micellar system, it was converted only very poorly in the liposomal system. Due to the different sites of incorporation, the liposomal system is less well suited than the micellar system for the delivery of amphiphilic substrates such as apocarotenoids: in micelles, hydrophobic substances – amphiphilic or not – are incorporated into the hydrophobic core of the micelle, the average distance from the micelle surface being dependent on the amphiphilic character of the molecule.

In contrast to that, amphiphilic substances integrate into the lipid bilayer of liposomes with the hydrophilic moiety at the boundary layer and the hydrophobic moiety dipping into the hydrophobic interior of the bilayer. This type of incorporation can occur on the outer or inner surface of the lipid bilayer. However only the molecules on the outer surface are accessible for enzymatic conversion since no enzyme is present in the aqueous core of a liposome produced in an enzyme-free solution. Even if the membranes are a dynamic system as proposed by the fluid mosaic model of Singer and Nicolson [28] and substrate molecules can move between outer and inner membrane sides, the lowered transient substrate concentration at the outer membrane surface is most likely the reason for the low reaction velocity obtained with the weakly amphiphilic substrate 8′-apo-β-caroten-8′-al.

In contrast to non-symmetric amphiphilic molecules such as apocarotenals, the symmetric C40 carotenoids are incorporated into lipid bilayers in a symmetric manner. As described previously, they are incorporated either parallel to the membrane surface or perpendicular to it, depending on the substituents at the ionone rings. Both types of symmetric incorporation permit equal access to the substrate molecules from either surface of the membrane.

The order of reaction velocities obtained for the tested C40 carotenoids differed significantly between micelle and liposome based substrate delivery. The data on micelle based delivery obtained by Schilling [22] is shown in Supplementary Table 1. While micelle based substrate delivery led to the highest V_{MAX} for astaxanthin (4.82 μM min^{-1}) followed by zeaxanthin (0.95 μM min^{-1}) and canthaxanthin (0.95 μM min^{-1}), liposome based substrate delivery lead to a different order of reaction velocities: despite of few differences in structure and log P (o/w), the ratio of V_{MAX} for astaxanthin and zeaxanthin was inverted. The liposome based substrate delivery system led to the V_{MAX} of zeaxanthin (1.1 μM min^{-1}) being approximately three times higher than the V_{MAX} for astaxanthin (0.36 μM min^{-1}).

Since size and general molecular structure of these two substrates do not differ significantly, the shift in substrate preference is likely due to the different substituents at the cyclic headgroups of zeaxanthin and astaxanthin. The double oxyfunctionalization at the cyclic headgroups of astaxanthin most likely impedes substrate recruitment from the liposome membrane. Since the molecule spans through the membrane perpendicularly like a "molecular rivet", one of the very hydrophilic headgroups has to be pulled through the hydrophobic lipid membrane core in order to enter the active site of AtCCD1. The cyclic headgroups of zeaxanthin carry only a single oxyfunctionalization and can be more easily recruited by the enzyme for conversion.

In Table 1, no kinetic parameters are displayed for the enzymatic cleavage of β-carotene. At the phospholipid composition of 100% PC chosen for comparability to the literature data, the reaction velocity using β-carotene is too low to permit a reliable determination of kinetic parameters as can also be seen in the data displayed in Fig. 3C. Compared to the micelle based system, the liposome based system is less suited for the delivery of non-symmetric, amphiphilic substrate molecules. Furthermore, the kinetic parameter V_{MAX} in reactions with liposome based substrate delivery seems to be closely correlated with the degree of oxyfunctionalization of the cyclic headgroups of C40 carotenoids.

When delivering hydrophobic substrates using micelles, the structural features of the substrate influence its enzymatic availability to a smaller extent due to the different nature of substrate incorporation as described above. Instead, the enzymatic substrate availability using micelles could be governed by the solubility of the substrate in the aqueous solvent which it has to be transferred across in order to enter the substrate channel of the enzyme. The octanol–water partitioning coefficients log P (o/w) of astaxanthin, zeaxanthin and 8′-apo-β-caroten-8′-al are 13.3, 14.8 and 8.9, respectively. According to the published kinetic data on micelles by Schilling [22], the dependence of V_{MAX} on the substrate solubility is as proposed for the C40 carotenoids but lower than expected for the non-symmetric 8′-apo-β-caroten-8′-al.

Although the underlying mechanisms are not fully understood – especially in the case of micelles – the two substrate delivery systems applied for carotenoids were shown to have a significant influence on the substrate preferences of the carotenoid dioxygenase AtCCD1. Since such an effect would significantly impair the *in vitro* investigation of carotenoid cleavage dioxygenase function *in vivo*, further insight into the underlying mechanisms is desirable to clarify the different, in part contradictory results and hypotheses on the function and substrate preferences of CCD1 enzymes *in vivo* [29].

4. Conclusion

The phospholipid composition of liposomes used as substrate delivery vesicles directly influenced the reaction velocity obtained for *in vitro* carotenoid cleavage with AtCCD1. The delivery of xanthophylls and fully non-polar carotene substrates were influenced differently by the phospholipid composition. The *in vitro* enzymatic conversion of the fully non-polar substrate β-carotene from liposomes could be demonstrated for the first time in this work and required a very defined liposome composition. The cellular reaction environment of AtCCD1 was imitated by incorporation of the galactolipid MGDG into the liposome membranes. While the specific activity of AtCCD1 towards β-carotene was increased by approximately 70% through MGDG shares between 8 and 20 mol%, the cleavage activity towards the xanthophyll zeaxanthin, which bears one hydroxyl group at each ionone ring, decreased with increasing shares of MGDG. Inverted hexagonal structures of reduced membrane thickness formed by MGDG were proposed as a reason for improved substrate accessibility to the biocatalyst. Furthermore, liposome based systems turned out to be less suitable than micelle based systems for the delivery of amphiphilic and non-symmetric

substrate molecules due to substrate orientation within the vesicle membrane. The results contribute to the understanding of liposomes as tools for the delivery of highly hydrophobic substrates to enzymes in aqueous media and represent a first step towards a rational reaction system design for enzymatic conversions of this type.

Acknowledgments

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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.2011.04.009.

References

[1] N. Krieger, J. Tej Bhatnagar, C. Baratti, A.M. Baron, V.M. de Lima, D. Mitchell, Food Technol. Biotechnol. 42 (4) (2004) 279–286.
[2] A.M. Azevedo, D.M.F. Prazeres, J.M.S. Cabral, L.P. Fonseca, J. Mol. Catal. B: Enzymatic 15 (4–6) (2001) 147–153.
[3] G. Pencreac'h, C. Baratti, Enzyme Microb. Technol. 28 (4–5) (2001) 473–479.
[4] P.V. Iyer, L. Ananthanarayan, Process Biochem. 43 (10) (2008) 1019–1032.
[5] R.A. Deems, Anal. Biochem. 287 (2000) 1–16.
[6] E. Sackmann, T. Feder, Mol. Membr. Biol. 12 (1995) 21–28.
[7] K.H. Cheng, J.R. Lepock, S.W. Hui, P.L. Yeagle, J. Biol. Chem. 261 (1986) 5081–5087.
[8] Y.A. Isaacson, et al., J. Biol. Chem. 254 (1979) 117–126.
[9] K. Boesze-Battaglia, R. Schimmel, J. Exp. Biol. 200 (1997) 2927–2936.
[10] K. Strzalka, W.I. Gruszecki, Biochim. Biophys. Acta 1194 (1994) 138–142.
[11] D.R. Gauger, H. Binder, A. Vogel, C. Selle, W. Pohle, J. Mol. Struct. 614 (2002) 211–220.
[12] D. Latowski, H.-E. Akerlund, K. Strzalka, Biochemistry 43 (2004) 4417–4420.
[13] D. Latowski, et al., Eur. J. Biochem. 269 (2002) 4656–4665.
[14] C. Zhou, M.F. Roberts, Biochim. Biophys. Acta 1348 (1997) 273–286.
[15] S.H. Schwartz, X. Qin, J.A. Zeevaart, J. Biol. Chem. 276 (2001) 25208–25211.
[16] J. Schrader, in: R.G. Berger (Ed.), Flavour and Fragrances – Chemistry, Bioprocessing and Sustainability, Springer Berlin Heidelberg, 2007, pp. 507–574, ISBN 978-3-540-49338-9.
[17] D.A. Cooper, D.R. Webb, J.C. Peters, J. Nutr. 127 (1997) 1699–1709.
[18] S. Köhn, et al., Aggregation and Interface Behaviour of Carotenoids, Birkhäuser,Switzerland, Basel, Switzerland, 2008.
[19] M. Schilling, F. Patett, W. Schwab, J. Schrader, Appl. Microbiol. Biotechnol. 75 (2007) 829–836.
[20] C. Socaciu, R. Jessel, H.A. Diehl, Spectrochim. Acta A: Mol. Biomol. Spectrosc. 56 (2000) 2799–2809.
[21] R.C.C. New, Liposomes – a Practical Approach, 44–48, Oxford University Press, USA, 1990, ISBN 978-0199630776.
[22] Schilling, M. Gewinnung von Aromastoffen durch enzymatische Spaltung von Carotinoiden mit der rekombinanten Dioxygenase AtCCD1. Ph.D. thesis, Technical University of Munich, Department of Biomolecular Food Technology, 2008).
[23] M.E. Auldridge, et al., Plant J. 45 (2006) 982–993.
[24] M.E. Auldridge, D.R. McCarty, H.J. Klee, Curr. Opin. Plant Biol. 9 (2006) 315–321.
[25] P. Dormann, I. Balbo, C. Benning, Science 284 (1999) 2181–2184.
[26] G. Bonaventure, J.J. Salas, M.R. Pollard, J.B. Ohlrogge, Plant Cell 15 (2003) 1020–1033.
[27] P. Hoyrup, O.G. Mouritsen, K. Jorgensen, Biochim. Biophys. Acta 1515 (2001) 133–143.
[28] S.J. Singer, G.L. Nicolson, Science 175 (1972) 720–731.
[29] D.S. Floss, M.H. Walter, Plant Signal. Behav. 4 (3) (2009) 172–175.

Jens Schrader Study of Biotechnology at Technical University Braunschweig, Germany; PhD at Dortmund University; Postdoc at DECHEMA, Germany, and UC Berkeley, USA. Currently: Head of Biochemical Engineering Group and deputy head of Karl-Winnacker-Institut at DECHEMA, Germany. Teaching position at Frankfurt University, Germany. Research interests: biocatalysis; biochemical engineering; metabolic engineering; terpenoids; flavors and fragrances.

Christoph Nacke studied Biotechnology at Technical University of Braunschweig, Germany and University of Waterloo, Canada; Scholarships by Studienstiftung des Deutschen Volkes (German National Merit Foundation), DAAD (German Academic Exchange Service) and Klee Foundation for Medical Technology; Currently Ph. D. student at Technical University of Munich (Biotechnology of Natural Products, Prof. Dr. W. Schwab), working at DECHEMA Karl-Winnacker Institut, Biochemical Engineering Group.