Supporting Information

"Crystal structures of substrate-free and retinoic acid-bound cyanobacterial cytochrome P450 CYP120A1" by Karin Kühnel, Na Ke, Max J. Cryle, Stephen G. Sligar, Mary A. Schuler and Ilme Schlichting

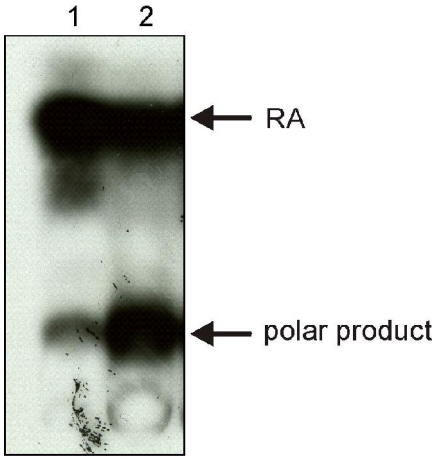
1. Metabolism of retinoic acid by CYP120A1 using Synechocystis cell lysates

Turnover assays with Synechocystis cell lysate

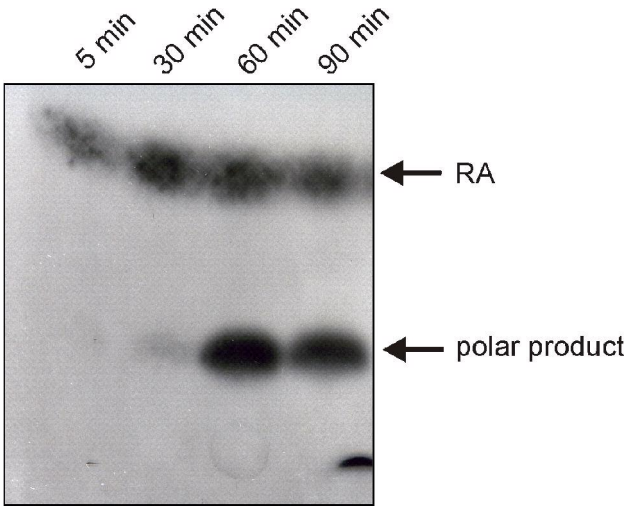
Synechocystis cells were grown in BG-11 liquid media under 50 μEinstein light at 30°C with air bubbling to an $OD_{700\ nm}$ of 0.8. Cells were collected by centrifugation at 3,000 rpm for 10 min and suspended in 1 mL 100 μM sodium phosphate buffer (pH 7.4) with protease inhibitor, pre-soaked 0.1 mm-diameter glass beads were added and cell lysates were collected.

All-*trans* [11,12-3 H (N)]-RA ([^{3}H]-RA) was purchased from DuPont-New England Nuclear (Boston, MA). To measure the catalytic activity, 10 μM CYP120A1 protein, 200 μL cell lysate, 100 μM retinoic acid, 33 nmol/L [^{3}H]-RA, 200 mM NADH or 200 mM NADPH in 0.2 mM sodium phosphate buffer (pH 7.4) in a total volume of 0.5 mL were incubated at 30°C for 2 hours in the dark. The reaction was terminated and remaining substrate and products were extracted with methanol:chloroform (1:2, v/v). The extract was dried under N_2 and redissolved in 20 μL ethanol. 10 μL of extract were spotted onto a TLC plastic sheet Silica gel 60 F254 (Merck KGaA, Damstadt, Germany). The plate was developed for 90 minutes with a mobile phase of hexane-ether-acetic acid (90:60:1.5, vol/vol/vol). The TLC plate was air-dried. Unlabeled retinoids were visualized with a 254 nm UV lamp. For visualization of ^{3}H labeled retinoic acid and its metabolites, EN3HANCE (PerkinElmer Life and Analytical Sciences, Waltham, MA) was sprayed on the TLC plate as the instruction. The plate was exposed to a Kodak film for 12 hours at –80 ^{o}C.

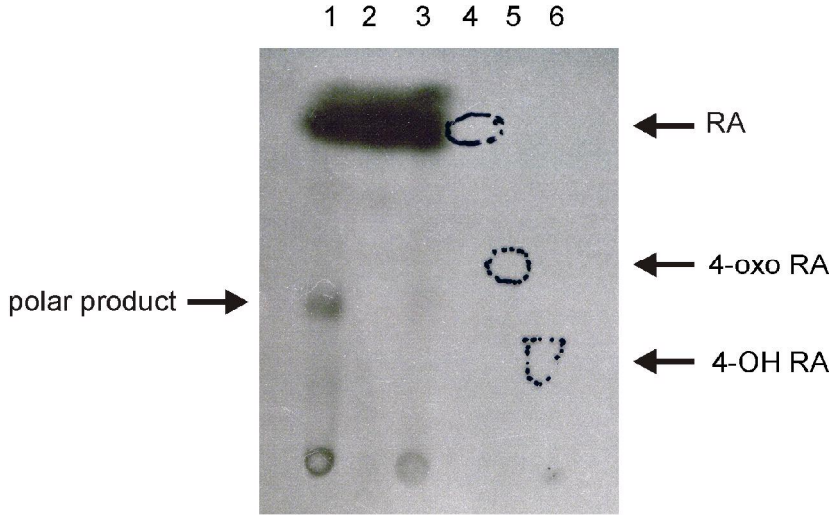
To enable sensitive detection of the CYP120 product, a mixture of [^{3}H]-retinoic acid and unlabeled retinoic acid was incubated with purified CYP120A1 and reductant with *Synechocystis* whole cell lysates as the course of redox partner. Both NADPH and NADH provide electrons to CYP120A1 with NADH-enabled CYP120A1 generating more polar product (Fig. S1). Variations in the incubation time indicate that this reaction is time-dependent (Fig. S2).



Supplemental Figure 1. Metabolism of retinoic acid with *Synechocystis* cell lysate. 10 μM retinoic acid and 33 nM [^{3}H]-retinoic acid were incubated with *Synechocystis* cell lysate, 1 μM purified CYP120A1 protein, 10 mM NADPH (lane 1) or NADH (lane 2) for 90 min, extracted and analyzed by TLC.



Supplemental Figure 2. Time-dependent product accumulation. 10 μM retinoic acid and 33 nM [^{3}H]-retinoic acid were incubated with *Synechocystis* cell lysate, 1 μM purified CYP120A1 protein, 10 mM NADH for 5 min, 30 min, 60 min and 90 min, extracted and analyzed by TLC.



Supplemental Figure 3. Metabolism of retinoic acid with *Synechoystis* cell lysate in the presence of NADH. Lane 1 is the reaction containing 10 μM retinoic acid, 33 nM [^{3}H]-retinoic acid, *Synechocystis* cell lysate, 1 μM purified CYP120A1 protein, 10 mM NADH incubated for 90 min; lane 2 is the control reaction lacking purified CYP120A1 protein; lane 3 is the [^{3}H]-RA standard; lane 4 is unlabeled RA visualized by UV; lane 5 is 4-oxo RA visualized by UV; lane 6 is 4-OH RA, which was synthesized from 4-oxo RA, visualized by UV.

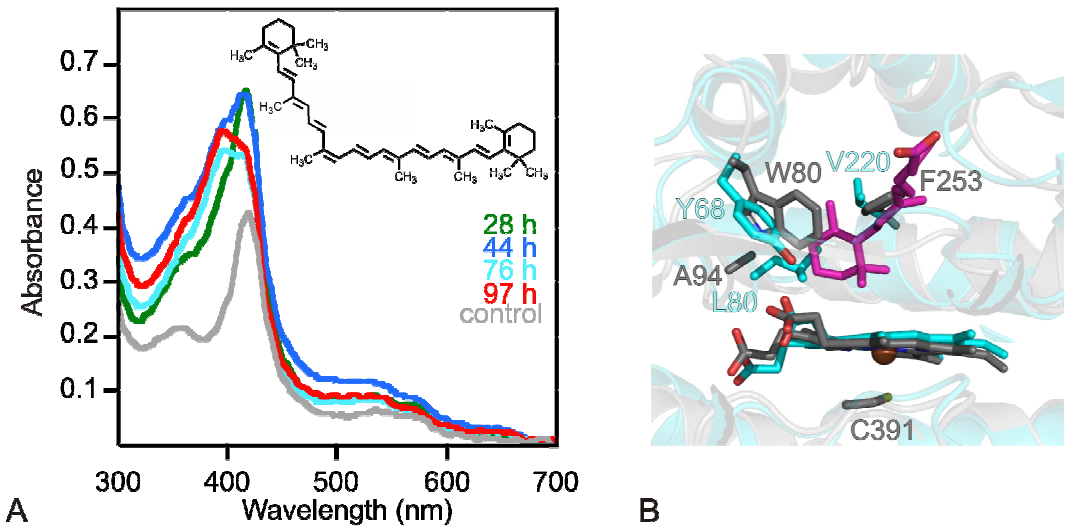
2. 13-*cis*-β-carotene binding of CYP120A1

To experimentally probe 13-*cis* β-carotene binding, the absorption spectrum of CYP120A1 was measured upon addition of 13-*cis* β-carotene. In this assay, a shoulder at 395 nm appeared on the Soret band after incubation for two days in the dark at room temperature and became more pronounced after longer incubation periods (Fig. S4a). We hypothesize that the long time required until a Soret shift could be observed may be due to the extremely low solubility of carotene in water. That it is not caused by the lower affinity of the 13-*cis* isomer is ruled out by the fact that both 13-*cis* and 9-*cis* retinoic acids bind with similar affinity as all-*trans* RA.

It also remains possible that there is a higher concentration of carotene *in vivo* due to the presence of carotene carrier proteins which might facilitate binding of carotene to CYP120A1. Water-soluble carotene binding proteins have been identified in *Synechocystis* sp. PCC 6803 and *Arthrospira maxima* (*1*);(*2*). Most carotenes are present in the more stable all-*trans* conformation in the cell. Also, resonance Raman spectroscopy studies showed that carotenoids in photosystem II of *Synechocystis* sp. are almost entirely all-*trans* isomers (*3*). Therefore, the relevance of a possible hydroxylation of 13-*cis* β-carotene by CYP120A1 is unclear.

Most carotene hydroxylases are nonheme diiron enzymes, however the hydroxylation of the ε-ring during lutein (3R,3'R-β,ε-carotene-3,3'-diol) biosynthesis in *Arabidopsis thaliana* is catalyzed by CYP97C1 (*4*). Another example for a carotene modifying P450 enzyme is the thermophilic CYP175A1. Functional complementation experiments showed that CYP175A1 hydroxylates β-carotene (*5*). When a plasmid encoding the carotenoid biosynthesis genes from *Erwinia uredovora* was co-transformed into *E. coli* with the CYP175A1 gene, formation of zeaxanthin (β,β-carotene 3,3'-diol) was observed. Even so and in contrast to our observations for CYP120A1, the CYP175A protein generated no spectroscopic evidence for either carotene or retinoic binding to its catalytic site. Only addition of β-ionone resulted in a low spin to high spin Soret band shift (*6*).

So far, only the substrate-free CYP175A1 crystal structure is known (*7*). Despite the similarities of CYP120A1 and CYP175A1, which share a sequence identity of 23% and their Cα backbones superimpose with a r.m.s.d. of 2.5 Å, there are significant differences between the active sites of the two enzymes (Fig. S4b). CYP175A1 Leu80 clashes with the bound RA in the CYP120A1 structure. In CYP120A1, this position is occupied by the smaller Ala94 instead. These differences indicate that the two enzymes bind carotenoid derivatives in different manners, which would be consistent with the different expected specificities of these two enzymes (*i.e.*, hydroxylation of carbon 3 in CYP175A1 and carbon 2 (or C16, C17) in CYP120A1).



Supplemental Figure 4. (A) CYP120A1 binds 13-*cis* β-carotene. 13-*cis* β-carotene was obtained from CaroteNature (Lupsingen, Switzerland) and dissolved in tetrahydrofuran at a concentration of 18 mM. A five-fold molar excess of carotene was added to a 120 µM CYP120A1 solution in 20 mM Hepes (pH 7.5). Samples were stored in the dark at room temperature. A Soret band from 418 nm to 395 nm was observed. As a control, a CYP120A1 sample was incubated without 13-*cis* β-carotene for 96 h (grey), here the Soret peak remained at 418 nm.

(B) Superimposition of CYP120A1 (grey) and CYP175A1 (1N97, cyan) (*7*)). Leu80 from CYP175A1 clashes with RA (magenta).

References

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