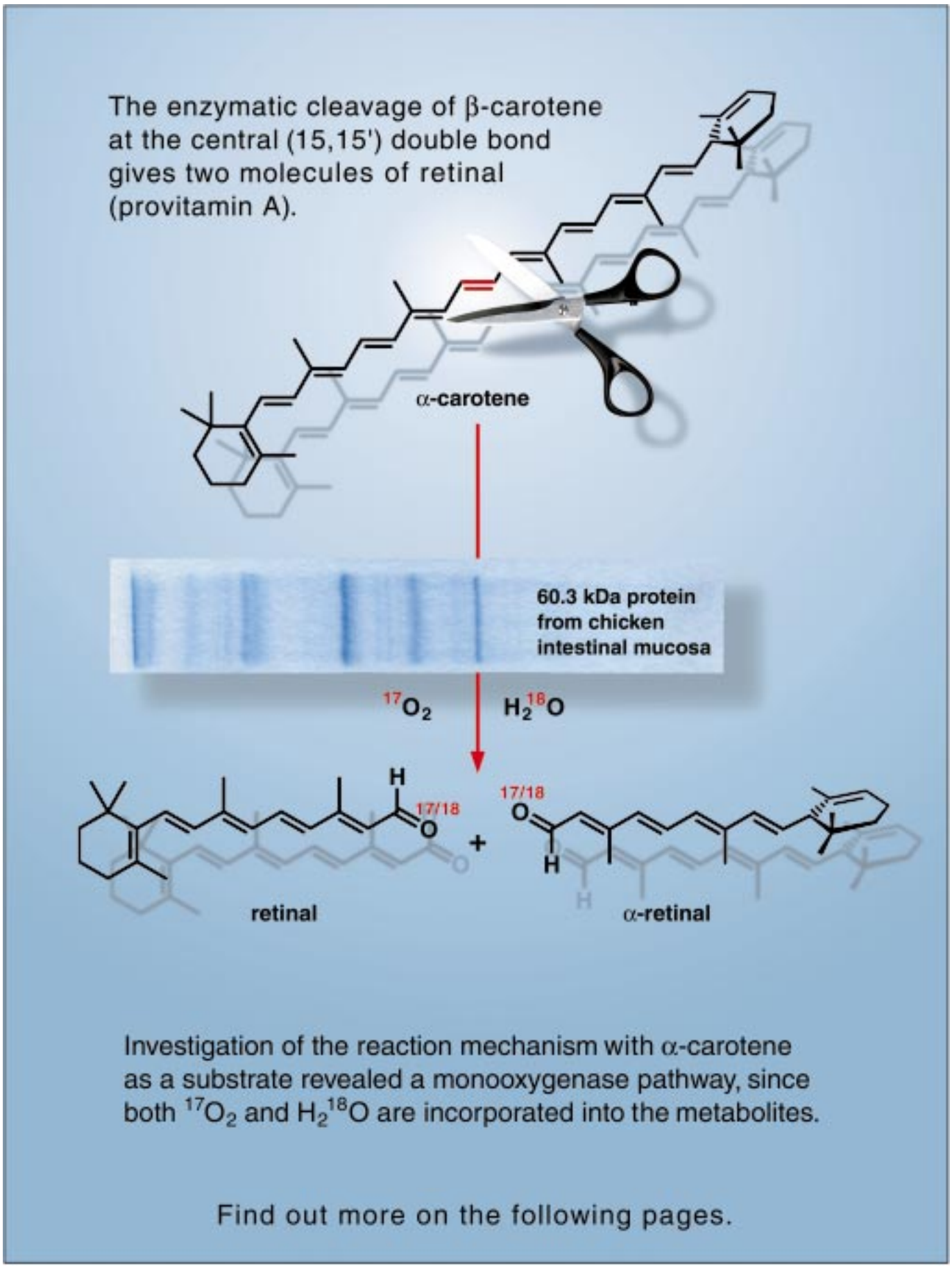

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The Reaction Mechanism of the Enzyme-Catalyzed Central Cleavage of β-Carotene to Retinal**

Michele G. Leuenberger, Caroline Engeloch-Jarret, and Wolf-D. Woggon*

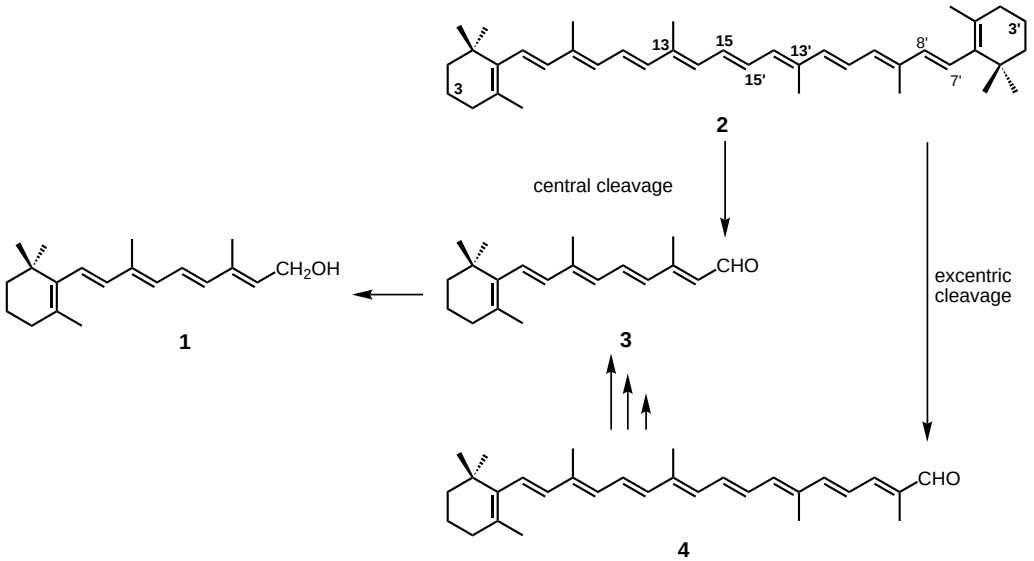
Dedicated to Professor Synnøve Liaaen-Jensen

Although it has been known since 1930 that vitamin A or retinol (**1**) derives in vivo from β-carotene (**2**),[1] the enzymatic origin of β-carotene cleavage was only shown in 1965 when Olson and Hayaishi reported the identification of in vitro activity of an enzyme from rat liver and rat intestine. This enzyme catalyzes the central cleavage of **2** to retinal (β-retinal, **3**; Scheme 1).[2] Later an alternative, probably less significant, pathway was discovered involving excentric cleavage of **2** to yield apo-carotenals such as **4**, which are subsequently degraded to **3**.[3]

During the last 35 years many groups have tried unsuccessfully to purify the enzyme catalyzing the central cleavage of β-carotene **2**,[4] and quite a number of mechanistic investigations have been published which involved crude enzyme preparations or in vivo experiments.[2, 5, 6] Solid information, however, could only be obtained for two aspects of the problem: The enzyme requires molecular oxygen, and the central cleavage proceeds stoichiometrically to yield approximately two moles of retinal from one mole of β-carotene.[7] Most of the other experiments regarding the incorporation of oxygen from water and concerning the metal involved in catalysis can be valued as inadequate.[8] Nevertheless the enzyme that catalyzes the central cleavage of **2** was termed β-carotene 15,15′-dioxygenase (EC1.13.11.21) and often the enzyme was believed to be an iron dioxygenase.[2, 9]

Recently we[10a] and others[10b] have been able for the first time to identify the protein which catalyzes the central cleavage. We have developed a purification protocol for the enzyme from chicken intestinal mucosa and it has become possible to overexpress the functional 60.3 kDa protein in BHK (baby hamster kidney) cells.[10a,c] We have also investigated the substrate specificity of the enzyme with the aim of identifying a nonsymmetrical carotenoid that could be utilized for investigation of the mechanism.[11] This aspect was mainly overlooked in earlier work, we believe. However, only the use of a nonsymmetrical carotenoid as a substrate to yield different aldehydes can provide exact information on the incorporation of oxygen from water and/or air into cleavage products and, hence, distinguish a monooxygenase from a dioxygenase mechanism.

Substrate specificity studies revealed three nonsymmetrical carotenoids, **5**–**7**, that are readily cleaved by the enzyme (40–50% of the yield obtained for **2** under standard conditions) to furnish the corresponding aldehydes, for example, **5** →**3** and **8** (Scheme 2). α-Carotene **5** was chosen as the best candidate because it was available in isomerically pure form, and it was expected that aldehydes **3** and **8** would behave similarly in the subsequent reactions that would be required for mass spectrometry (MS) analysis of the distribution of the labeled oxygen in both cleavage products. In this context it is important to note that aldehydes such as **3** and **8** are not directly suitable for isotopic analysis of an oxygen label in the carbonyl group[12] because this label easily exchanges with the medium at the pH value of incubation (pH 7.8).[13] Thus, we decided for a combined enzyme assay with addition of horse liver alcohol dehydrogenase (HLADH) to reduce **3** and **8** in situ to the corresponding alcohols retinol (**1**) and α-retinol (**9**). Alcohols **1** and **9** are also unsuitable for tandem gas chromatography/mass spectrometry (GC-MS) analysis because both eliminate water. Thus, after quenching of the incubation and high-pressure liquid chromatography (HPLC) purification of alcohols **1** and **9** (Figure 1a), derivatization to the silyl ethers **10** and **11** was required (Figure 1b).



Scheme 1. Enzymatic cleavage of β-carotene **2**. Routes to the formation of retinol **1**.

Control experiments revealed that the rates of reduction of **3** and **8** are the same. Exchange of the carbonyl oxygen of **3** with the buffer medium was investigated under conditions similar to the incubation conditions, that is, **3** was added to a

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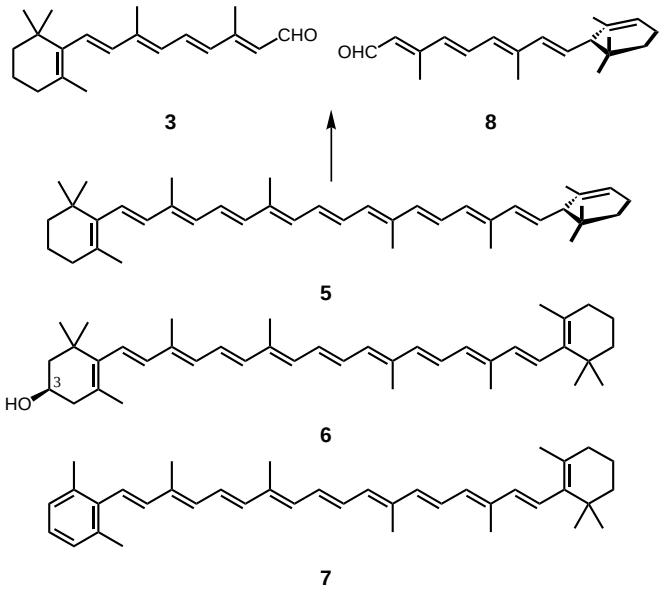

Scheme 2. Nonsymmetrical substrate analogues of the enzyme that catalyzes the central cleavage of **2**. Cleavage of α-carotene **5** to form the aldehydes **3** and **8**.

solution of HLADH in $H_2{}^{18}O$ as slowly as it would be produced by enzymatic cleavage of **5** (3.5 nmol h^{-1}). According to MS analysis of the retinyl silylether **10**, exchange of the ^{18}O label between **3** and $H_2{}^{18}O$ is $<5\,\%$.

For the decisive incubation experiment with **5** the native enzyme was employed due to its favorable turnover, which is $\approx$2.5 times higher than the hexahistidine-tailed protein overexpressed in BHK cells. Highly enriched oxygen sources, such as 85 % $^{17}O_2$ and 95 % $H_2{}^{18}O$, were used. GC-MS analysis of the silylethers **10** and **11** with the focus on the molecular ion

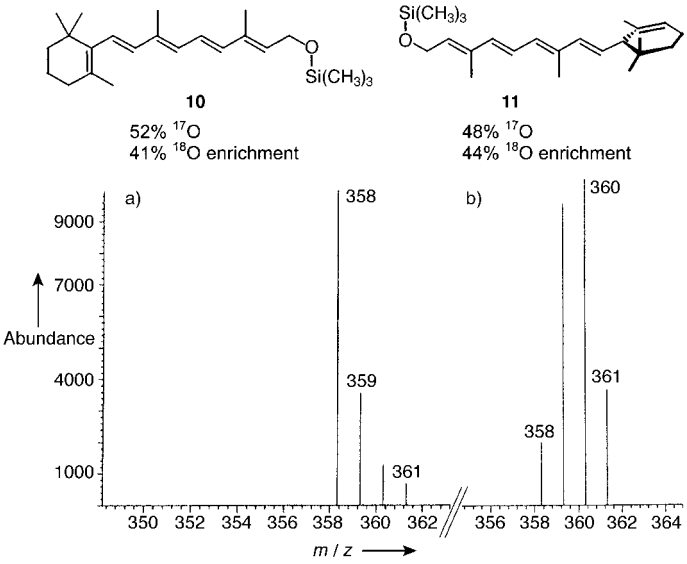

Figure 2. Mass spectra: a) silylether **10** with natural abundance of oxygen isotopes; b) $^{17}O/^{18}O$-enriched **10** from incubation of **5** in the presence of $^{17}O_2$ and $H_2{}^{18}O$.

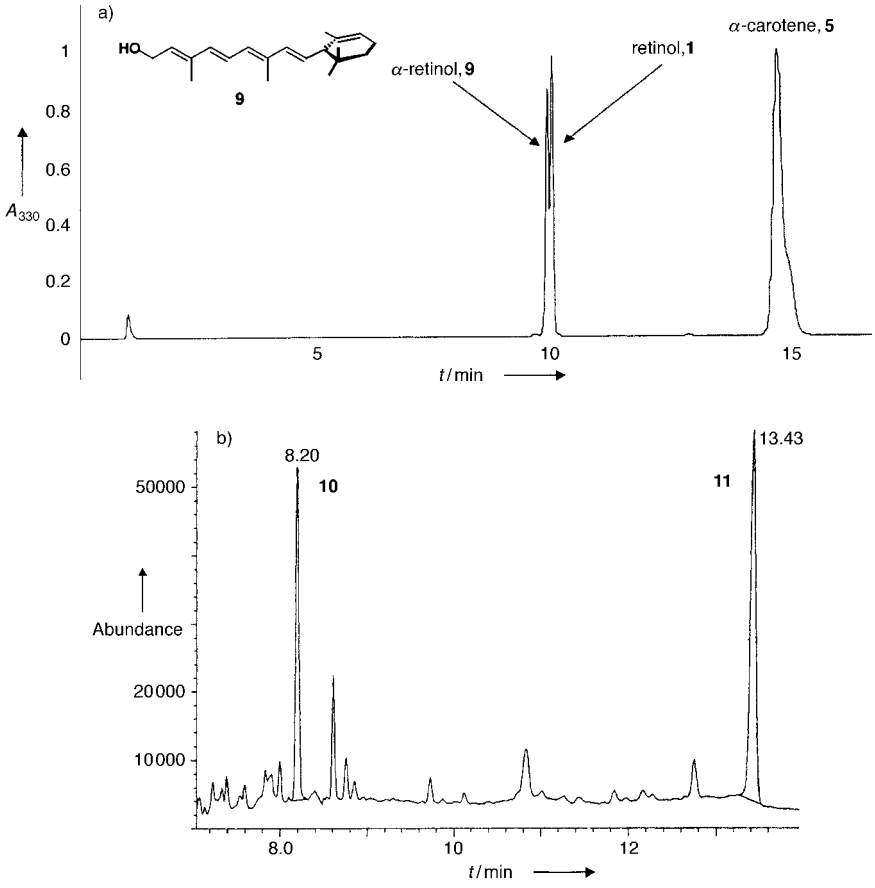

Figure 1. a) HPLC trace recorded after incubation of α-carotene **5**; b) GC trace for **10** and **11**, the silylethers of the metabolites of **5**.

area revealed, within experimental error, equal enrichment of the ^{17}O and ^{18}O label in both derivatives of metabolites **3** and **8** (Figure 2). This result proves for the first time the incorporation of one ^{17}O atom of molecular oxygen and the concomitant incorporation of one ^{18}O atom from labeled water.

Accordingly, and in contrast to earlier beliefs, the reaction mechanism of enzymatic central β-carotene cleavage is not in agreement with a dioxygenase-catalyzed procedure. A dioxygenase mechanism ([2+2] cycloaddition to the central C15–C15′ double bond, followed by fragmentation of the intermediate dioxetane) would require the incorporation of one complete oxygen molecule into the product aldehydes and the absence of any ^{18}O label originating from the labeled water.

Experimental evidence provided here accounts for a monooxygenase-type mechanism, as shown in Scheme 3, in which the first step is an epoxidation of the central double bond of **5**. This is followed by unselective ring opening with water and final diol cleavage to yield the aldehydes **3** and **8**.

Another small experimental detail agrees with the monooxygenase mechanism. Given the enrichment of the labels in both oxygen

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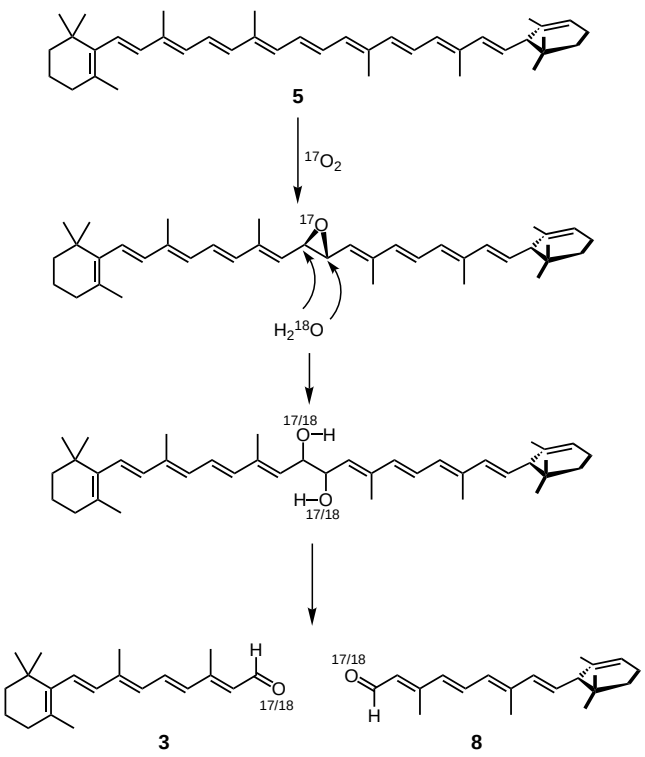

Scheme 3. The reaction mechanism of the central cleavage of α-carotene **5** catalyzed by the 60.3 kDa cytosolic monooxygenase purified from chicken's intestinal mucosa. The mechanism for β-carotene **2** is thought to be analogous.

sources, one would expect, in case of quantitative O-incorporation, the following isotopic enrichments for **10** and **11**: 10 % ^{16}O, 42.5 % ^{17}O, and 47.5 % ^{18}O. Experimentally, however, one finds 5 – 8 % higher ^{17}O enrichment than calculated along with the correspondingly lower ^{18}O enrichment (systematic deviation $\leq \pm 2$ %). This difference can be explained by assuming that ^{17}O-labeled water originating from $^{17}O_2$ cleavage in the active site "dilutes" the $H_2{}^{18}O$ oxygen source in situ.

The nature of the metal complex involved in O_2 cleavage and epoxidation still has to be elucidated. At present it is only certain that this first step of carotene metabolism is not a P450-catalyzed reaction because the heme-thiolate chromophore ($\lambda_{max} \sim 415$nm) is absent in the purified protein (broad absorption without fine structure between 200 – 280 nm) as well as in the overexpressed enzyme. Interestingly the monooxygenase mechanism resembles, at least in part (the epoxidation), the mechanism we previously proposed for a supramolecular enzyme model catalyzing the regioselective cleavage of **2** and **7**.[14]

Experimental Section

Enzymatic reaction conditions: α-carotene **5** ((6′*R*)-β,ε-carotene) was obtained from F. Hoffmann-La Roche (Basel) and stored at $-18\,^{\circ}C$. A stock solution of **5** in benzene (10 mM) was freshly prepared. In a glass vial the stock solution of **5** (40 μL), α-tocopherol solution (50 μL, 43 mg mL^{-1} in hexane), and tween 40 solution (200 μL, 400 μL in 10 mL acetone) were evaporated with a gentle stream of N_2 in a heated block (45 °C). Tricine buffer ($H_2{}^{18}O > 95$ %; 1 mL, 150 mM; pH 7.8, 45 °C) was added and the solution was gently mixed until almost complete solubilization. The substrate solution was added to a 25-mL flask containing tricine buffer ($H_2{}^{18}O > 95$ %; 3.5 mL, 150 mM; pH 7.8), glutathione (12 mg), sodium cholate (1 mg), and nicotinamide adenine dinucleotide, reduced form (NADH; 50 mg). The mixture was cooled to $-180\,^{\circ}C$ while connected to a high-vacuum line (3×10^{-2} mbar) and then degassed three times. Finally labeled molecular oxygen (> 85 % $^{17}O_2$; 20 mL; 2.15 bar) was condensed on the surface of the frozen solution ($-180\,^{\circ}C$). The mixture was allowed to warm up, and after reaching 25 °C the system was allowed to equilibrate over 30 min. In a separate flask the enzyme purified by hydrophobic interaction chromatography (HIC)[15] (from ≈10 g mucosa of one chicken's duodenum) was dissolved in tricine buffer ($H_2{}^{18}O > 95$ %; 500 μL, 150 mM; pH 7.8). HLADH (80 μL, 11.1 mg mL^{-1}, 2.9 U mg^{-1} protein; Fluka AG, Buchs) was added. Argon was passed through the solution for 30 min/25 °C to remove $^{16}O_2$. The enzymatic reaction was started by adding this solution to the substrate solution (final concentration of **5**: 80 μM). After incubation for 7.5 h at 37 °C in the dark, the reaction was quenched by addition of acetonitrile (4 mL). The mixture was extracted three times with chloroform (4 mL) and the collected organic phases were evaporated to dryness.

Purification by HPLC: The residue obtained as described above was separated by analytical HPLC (LiChrospher 100 RP-18 5 μm, dimensions: 125 × 4.6 mm, 25 °C, flow rate: 1 mL min^{-1}, eluents and gradients: 100 % solution of acetonitrile/1 % NH_4OAc_{aq} (1:1) →100 % solution of acetonitrile/*i*PrOH (1:1) over 10 min, the eluent remained the same for 5 min, then →100 % solution of acetonitrile/1 % NH_4OAc_{aq} (1:1) over 2 min; diode array detector at 330 nm for **1** (R_t=10.0 min) and **9** (R_t = 9.9 min), and 455 nm for **5** (R_t = 14.9 min). The mixture of retinoids was collected, evaporated, and concentrated in a 100-μL vial.

Silylation and GC-MS analysis: The mixture of the two products **1** and **9** was dissolved in hexane (5 μL). A syringe was purged several times with *N*,*O*-bis(trimethylsilyl)acetamide and then directly used to take an aliquot (1 μL) of the solution of **1** and **9**. Thus, silylation occurred in the syringe followed by immediate splitless injection (285 °C) into the GC column (cross-linked 5 % phenylmethyl silicone, dimensions: 25 m × 0.2 mm, film thickness: 0.33 μm, 30 s purge delay; temperature program: 150 °C →250 °C at 25 °C min^{-1} and then constant at 250 °C).[16] Selected ion monitoring analysis (electron ionization, 70 eV) was pursued for the [M^+] regions of the spectra for **10** (R_t = 8.2 min) and **11** (R_t = 13.4 min). The retention times of the retinoids **1** and **9** on HPLC and of the silyl derivatives **10** and **11** on GC were confirmed by injection of authentic material.

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Saddle-Shaped Six-Coordinate Iron(III) Porphyrin Complexes Showing a Novel Spin Crossover between $S = 1/2$ and $S = 3/2$ Spin States**

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Spin states of iron(III) porphyrins are controlled by the number and nature of axial ligands.[1] The coordination of nitrogen bases such as imidazole (HIm) and pyridine results in the formation of low-spin ($S = 1/2$) six-coordinate complexes. In contrast, anionic ligands such as Cl^- and F^- lead to the formation of five-coordinate high-spin ($S = 5/2$) complexes. Maltempo discussed a spin-admixed $S = 3/2$, 5/2 state on the basis of quantum mechanical calculations, and suggested that the $S = 3/2$ state is an important contributor to the spin state of certain bacterial heme proteins known as cytochromes c′.[2]

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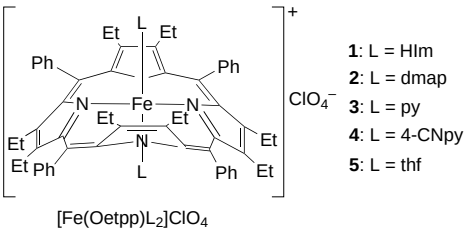
We and others recently reported that highly nonplanar (porphyrinato)iron(III) complexes with weak axial ligands show a quite pure intermediate spin state.[3, 4] The results were ascribed to the short $Fe{-}N_{por}$ bonds of the nonplanar porphyrin rings and the weak coordination ability of the axial ligands.[5] We therefore expected that the spin state of nonplanar $[Fe^{III}(oetpp)L_2]ClO_4$ (**1**–**5**) could change from



$[Fe(Oetpp)L_2]ClO_4$

the pure $S = 1/2$ to the pure $S = 3/2$ state as the axial ligand changes from strong HIm to weak THF; the order of the coordination ability is HIm > dmap > py > 4-CNpy > thf.[6] Of particular interest are the spin states of **2**–**4** because the axial ligands of these complexes are ranked between HIm and THF.

Table 1 lists the Mössbauer parameters, isomer shift (IS; relative to α-iron foil at 290 K), and quadrupole splitting (QS) measured at ambient and liquid nitrogen temperatures. The QS values for **1** and **2** at ambient temperature were within the range of low-spin complexes.[7] The IS and QS values for **4** (0.37 and 3.26 mm s^{-1}, respectively) were close to those for **5** (0.41 and 3.65 mm s^{-1}); **5** has been fully characterized as the quite pure intermediate-spin complex.[4] Thus, from the viewpoint of Mössbauer spectroscopy, **1** and **2** are the low-spin complexes, while **4** is the intermediate-spin complex at ambient temperature. Figure 1 shows the Mössbauer spectra of **3** and **4** taken at ambient temperature and 80 K. The features change as the temperature is lowered. Complex **4** exhibited a new doublet (site B) below 230 K, and the relative intensities for this site increased on decreasing the temperature. The values for sites A and B are in the range of intermediate-spin and low-spin complexes, respectively, and both spin states co-exist at low temperature. This observation implies the occurrence of a novel spin-crossover process [Eq. (1)].[8, 9]

Table 1. Mössbauer parameters and spin state (S) of **1**–**5**.

		T [K]	IS [mm s^{-1}]	QS [mm s^{-1}]	Γ_1 [mm s^{-1}]	Γ_2 [mm s^{-1}]	S
1		297	0.18	1.82	0.24	0.25	1/2
		78	0.26	1.86	0.40	0.62	1/2
2		290	0.19	2.21	0.27	0.32	1/2
		80	0.26	2.31	0.55	0.89	1/2
3		290	0.32	2.76	0.27	0.29	3/2 – 1/2
		80	0.25	2.29	0.47	0.64	1/2
4	site A	295	0.37	3.26	0.32	0.33	3/2
	site A	80	0.57	3.03	0.47	0.47	3/2
	site B	80	0.20	2.70	0.64	0.64	1/2
5		290	0.41	3.65	0.32	0.26	3/2
		80	0.50	3.50	0.77	0.49	3/2

low spin ($S = 1/2$) $\rightleftharpoons$ intermediate spin ($S = 3/2$) (1)

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