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Reconstitution of Apomyoglobin with Bile Pigments

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Summary. Apomyoglobin was reconstituted with bile pigments of the verdinoid, 2,3-dihydroverdinoid, pterobilinoid, and violinoid type. Absorption and circular dichroism data as well as formation constants of the complexes were measured. From these results it was concluded that chromophore binding and induced chirality of these pigments are mainly governed by a lipophilic region opposite to the propionic side chain(s) and the asymmetric position of the hydrogen bonding acceptors of the propionic acid side chain(s) at the entrance of the protein pocket.

Keywords. Apomyoglobin; Bilindiones; 2,3-Dihydrobilindiones; Protein–Bile Pigment Complexes; Formation Constants; Absorption Spectra; Circular Dichroism.

Rekonstitution von Apomyoglobin mit Gallenpigmenten

Zusammenfassung. Apomyoglobin wurde mit Gallenfarbstoffen des verdinoiden, 2,3-dihydroverdinoiden, pterobilinoiden und violinoiden Typs rekonstituiert. Absorptions- und Circulardichroismus-Daten, ebenso wie die Bildungskonstanten der dabei gebildeten Komplexe wurden gemessen. Aus diesen Ergebnissen konnte abgeleitet werden, daß die Bindung des Chromophors und seine induzierte Chiralität hauptsächlich durch eine lipophile Region, die der Seite mit Propionsäurekette(n) gegenüber liegt sowie durch die asymmetrische Position der Wasserstoffbrückenakzeptoren für die Propionsäureketten an der Öffnung der Proteintasche bestimmt werden.

Introduction

Apomyoglobin reconstitutes not only with its natural prosthetic group heme [1] or other cyclic tetrapyrrolic compounds [1, 2], but also with linear oligopyrroles like the bile pigments bilirubin [3] or biliverdin [4]. The latter, rather artificial chromoproteins, may be used as model systems for the final steps of heme metabolism or may also be investigated with respect to chromophore – protein interactions (e.g. [5]), which is of interest with respect to the structural aspects of phytochrome or the phycocyanins.

Different formation constants of such protein pigment complexes [1–4] indicate different stabilization of these systems which, in pinciple, may be due to Coulombic or other nonbonded interactions. Varying the substitution pattern and type of pigment and introducing them into the heme pocket of apomyoglobin should lead

* Dedicated to Prof. Dr. A. Eschenmoser on occasion of his 65th birthday

to model systems from which a better understanding of the relative importance of such stabilizing forces might result. Therefore it seemed worthwile to investigate systems consisting of apomyoglobin and appropriate bile pigments, thus providing a means to probe the heme pocket.

Experimental

Melting points were taken on a Kofler hot stage microscope (Reichert, Vienna). NMR-, UV-VIS-, M-, and IR-spectra were recorded on the Bruker WM 360-, Hitachi U-3210-, Finnigan MAT 115-, and Zeiss IMR-spectrometers; CD data were obtained by means of an Auto Dichrograph Mark V (Jobin-Yvon).

Apomyoglobin was prepared from sperm whale myoglobin (Sigma) by acid cleavage [6] and purified by gel chromatography on Sephadex-G 15.

Biliverdin (**1**) was prepared by oxidation of bilirubin (Sigma) as described by McDonagh [7].

(Z,Z,Z)-3,17-Diethyl-1,19,23,24-tetrahydro-2,7,13,18-tetramethyl-1,19-dioxo-21H-bilin-8,12-dipropanoic Acid [**2**; $C_{33}H_{38}NO_6$]

50 mg (0.129 mmol) (*Z*)-9-*tert*-butoxycarbonyl-3-ethyl-2,7-dimethyl-dipyrrin-1-one-8-propanoic acid [8] were mixed with 1 ml trifluoroacetic acid under argon and heated until dissolved completely. 40.7 mg (0.129 mmol) of (*Z*)-3-ethyl-9-formyl-2,7-dimethyl-dipyrrin-1-one-8-propanoic acid was added and the mixture was stirred for 30 min at room temperature. The reaction mixture was added to 20 ml dichloromethane and extracted with 0.1 *N* NaOH. Acidification with 0.1 *N* HCl was followed by extraction with dichloromethane, drying with sodium sulfate, and evaporation. The residue was chromatographed on tlc plates (silica; acetone : dichloromethane : methanol = 50 : 50 : 10). Yield 70%; m.p. 295–298 °C, identical with a sample prepared from the corresponding dimethylester [9]. ^{1}H-NMR (CD_3OD, δ, 360 MHz): 6.95 (s, =CH-10), 5.95 (s, =CH-5,15), 2.85 (m, $-CH_2-COOMe$), 2.32 (m, $-CH_2-CH_2-COOMe$), 2.45 (q, J = 7 Hz, $-CH_2-CH_3$), 2.02 (s, $-CH_3$-2,18), 1.67 (s, $-CH_3$-7,15), 1.10 (t, J = 7 Hz, $-CH_2-CH_3$) ppm. IR (KBr): ν = 1 720, 1 635 cm^{-1}. UV-VIS (H_2O): λ = 360 (24 500), 660 (8 300) nm (ε).

(Z,Z,Z)-1,19,23,24-Tetrahydro-3,7,8,12,13,17-hexamethyl-1,19-dioxo-21H-bilin-2,18-dipropanoic Acid (**3**) [10]

It was kindly provided by Prof. Dr. A. Gossauer (Fribourg).

(Z,Z,Z)-17-Ethyl-1,2,3,19,23,24-hexahydro-3,3,7,13,18-pentamethyl-1,19-dioxo-21H-bilin-8,12-dipropanoic Acid (**4**; $C_{32}H_{38}N_4O_6$)

(E)-tert-Butyl-3-(2-methoxycarbonylethyl)-4-methyl-5-(2-nitrovinyl)-pyrrole-2-carboxylate ($C_{16}H_{22}N_2O_6$)

Preparation from *tert*-butyl-3-(2-methoxycarbonylethyl)-4-methyl-5-formyl-pyrrole-2-carboxylate analogous to [11]. Yield 75%; m.p. 129–131 °C. ^{1}H-NMR ($CDCl_3$, δ, 360 MHz): 9.38 (s, NH), 7.95 (d, J = 12 Hz, =CH−), 7.42 (d, J = 12 Hz, =CH−), 3.69 (s, $-COOCH_3$), 3.01 (t, J = 7.5 Hz, $-CH_2-COOMe$), 2.54 (t, J = 7.5 Hz, $-CH_2-CH_2-COOMe$), 2.20 (s, $-CH_3$), 1.59 (s, *t*-butyl) ppm. IR (KBr): ν = 1 726, 1 696, 1 624, 1 514 cm^{-1}. UV-VIS (CH_3OH): λ = 393 (24 600), 266 (9 100) nm (ε). MS (70 eV, 200 °C): *m*/e (%) = 338 (M^+, 31), 282 (43), 265 (35), 222 (88), 161 (100).

tert-Butyl-3-(2-methoxycarbonylethyl)-4-methyl-5-(2-nitroethyl)-pyrrole-2-carboxylate ($C_{16}H_{24}N_2O_6$)

Preparation from preceding compound analogous to [11]. Yield 57%; m.p. 114–115 °C. ^{1}H-NMR ($CDCl_3$, δ, 360 MHz): 8.78 (s, NH), 4.53 (t, J = 6.5 Hz, $-CH_2-NO_2$), 3.67 (s, $COOCH_3$), 3.26 (t,

J = 6.5 Hz, $-CH_2-CH_2-NO_2$), 2.97 (t, J = 7.5 Hz, $-CH_2-COOMe$), 2.50 (t, J = 7.5 Hz, $-CH_2-CH_2-COOMe$), 1.98 (s, $-CH_3$), 1.56 (s, *t*-butyl) ppm. IR (KBr): ν = 1744, 1660, 1562, 1454, 1430 cm^{-1}. UV-VIS (CH_3OH): λ = 278 (16800), 244 (7200) nm (ε). MS (70 eV, 200 °C): *m*/e (%) = 340 (M^+, 46), 284 (38), 253 (48), 225 (57), 177 (100).

(rac)-tert-Butyl-3-(2-methoxycarbonylethyl)-4-methyl-5-(2-nitro-3,3-dimethyl-methoxycarbonylbutyl)-pyrrole-2-carboxylate [$C_{22}H_{34}N_2O_6$]

Preparation from preceding compound analogous to [11]. Yield 59%; m.p. 111–113 °C. ^{1}H-NMR ($CDCl_3$, δ, 360 MHz): 8.69 (s, NH), 4.92 (dd, J_d = 2 Hz, J_d = 12 Hz, $-CH-NO_2$), 3.73 (s, $-COOCH_3$), 3.65 (s, $-COOCH_3$), 3.26 (dd, J_d = 12 Hz, J_d = 16 Hz, $-CH_2-CH-NO_2$), 3.02 (dd, J_d = 2 Hz, J_d = 16 Hz, $-CH_2-CH_2-CH-NO_2$), 2.96 (t, J = 7.5 Hz, $-CH_2-COOMe$), 2.50 (t, J = 7.5 Hz, $-CH_2-CH_2-COOMe$), 2.43 [s, $(CH_3)_2-CH_2-COOMe$], 1.94 (s, $-CH_3$), 1.56 (s, *t*-butyl), 1.23 and 1.16 [s, $(CH_3)_2-CH_2-COOMe$] ppm. IR (KBr): ν = 1744, 1720, 1696 cm^{-1}. UV-VIS (CH_3OH): λ = 278 (16200), 244 (6200) nm (ε). MS (70eV, 200 °C): *m*/e(%) = 454 (M^+, 13), 351 (78), 278 (100), 224 (42).

(rac)-tert-Butyl-2,3,4,5-tetrahydro-3,3,7-trimethyl-8-(2-methoxycarbonylethyl)-dipyrrin-1-one-9-carboxylate ($C_{21}H_{32}N_2O_5$)

Preparation from preceding compound analogous to [11]. Yield 62%; m.p. 146–149 °C. ^{1}H-NMR ($CDCl_3$, δ, 360 MHz): 9.48 (s, NH), 6.22 (s, NH), 3.69 (s, $-COOCH_3$), 3.36 (dd, J_d = 4 Hz, J_d = 12 Hz, H–4), 2.97 (m, $-CH_2-COOMe$), 2.73 (dd, J_d = 4 Hz, J_d = 14 Hz, $-CH_2-5$), 2.58 (dd, J_d = 12 Hz, J_d = 14 Hz, $-CH_2-5$), 2.48 (m, $-CH_2-CH_2-COOMe$), 2.31 (d, J = 18 Hz, $-CH_2-2$), 2.13 (d, J = 18 Hz, $-CH_2-2$), 1.96 (s, $-CH_3$), 1.53 (s, *t*-butyl), 1.22 (s, $-CH_3-3$), 1.16 (s, $-CH_3-3$) ppm. IR (KBr): ν = 1744, 1696, 1678, 1430 cm^{-1}. UV-VIS (CH_3OH): λ = 282 (17800), 244 (6200) nm (ε). MS (70 eV, 200 °C): *m*/e (%) = 392 (M^+, 0.1), 281 (32), 261 (14), 225 (94), 207 (43).

(Z)-tert-Butyl-2,3-dihydro-3,3,7-trimethyl-8-(methoxycarbonylethyl)-dipyrrin-1-one-2-carboxylate ($C_{21}H_{30}N_2O_5$)

100 mg (0.256 mmol) (*rac*)-*tert*-butyl-2,3,4,5-tetrahydro-3,3,7-trimethyl-8-(2-methoxycarbonylethyl)-dipyrrin-1-one-9-carboxylate were dissolved in 10 ml $CHCl_3$, 35 mg N-chlorosuccinimid were added and the solution was stirred at room temperature for 2 hours. The solution was then washed twice with 10 ml water. The $CHCl_3$-phase was dried over Na_2SO_4 and the solvent was evaporated. The product was purified by chromatography (Al_2O_3, $CHCl_3/MeOH$ = 100/2). Yield: 62%; m.p. 128–130 °C. ^{1}H-NMR ($CDCl_3$, δ, 360 MHz): 8.63 (s, NH), 7.78 (s, NH), 5.27 (s, $=CH-$), 3.69 (s, $-COOCH_3$), 2.99 (t, J = 7.5 Hz, $-CH_2-COOMe$), 2.52 (t, J = 7.5 Hz, $CH_2-CH_2-COOMe$), 2.41 (s, $-CH_2-2$); 1.95 (s, $-CH_3$), 1.57 (s, *t*-butyl), 1.36 [s, $-(CH_3)_2-3$] ppm. IR (KBr): ν = 1726, 1696, 1666, 1448 cm^{-1}. UV-VIS (CH_3OH): λ = 309 (17700), 225 (18200) nm (ε). MS (70 eV, 200 °C): *m*/e (%) = 390 (M^+, 5), 334 (32), 274 (22), 226 (100), 103 (42).

(Z)-9-tert-Butoxycarbonyl-2,3-dihydro-3,3,7-trimethyl-dipyrrin-1-one-8-propanoic Acid ($C_{20}H_{28}N_2O_5$)

80 mg (*Z*)-*tert*-butyl-2,3-dihydro-3,3,7-trimethyl-8-(methoxycarbonylethyl)-dipyrrin-1-one-2-carboxylate were dissolved in 2 ml methanol and diluted with 4 ml water. 2 g KOH were added and the solution was kept at room temperature overnight. The mixture was neutralized with SO_2, then the precipitated product was filtered off and dried in the desiccator. Yield: 92%; m.p. 184–187 °C. ^{1}H-NMR ($CDCl_3$, λ, 360 MHz): 5.43 (s, $=CH-$), 2.94 (t, J = 7.5 Hz, $-CH_2-COOH$), 2.43 (t, J = 7.5 Hz, CH_2-CH_2-COOH), 2.34 (s, $-CH_2-2$), 1.88 (s, $-CH_3$), 1.52 (s, *t*-butyl), 1.31 [s, $-(CH_3)_2-3$] ppm. IR (KBR): ν = 1732, 1684, 1448 cm^{-1}. UV-VIS(CH_3OH): λ = 310 (13200), 226 (16200) nm (ε). MS (70 eV, 200 °c): *m*/e (%) = 376 (M^+, 2), 320 (18), 276 (6), 260 (10).

(Z,Z,Z)-17-Ethyl-1,2,3,19,23,24-hexahydro-3,3,7,13,18-pentamethyl-1,19-dioxo-21H-bilin-8,12-dipropanoic Acid

The preparation is analogous to compound **2** using (*Z*)-*tert*-butoxycarbonyl-2,3-dihydro-3,3,7-trimethyldipyrrin-1-one-8-propanoic acid and (*Z*)-3-ethyl-9-formyl-2,7-dimethyl-dipyrrin-1-one-8-propanoic acid. Yield 65%; m.p. 140–142 °C. ^{1}H-NMR (CD_3OD, δ, 360 MHz): 6.84 (s, =CH−10), 5.96 (s, =CH−15), 5.39 (s, =Ch−5), 2.82 (m, $-CH_2-COOH$), 2.38 (m, $-CH_2-CH_2-COOH$), 2.82 (q, J=8 Hz, $-CH_2-CH_3$) 2.12 (s, $-CH_2-2$), 2.01 (s, $-CH_3$-7), 1.91 (s, $-CH_3$-13), 1.56 (s, $-CH_3$-18), 1.23 [s, $-(CH_3)_2-3$], 1.04 (t, J=8 Hz, $-CH_2-CH_3$) ppm. IR (KBr): ν=1 720, 1 630, 1 600, 1 230 cm^{-1}. UV-VIS (CH_3OH): λ=592 (11 900), 344 (28 800) nm (ε). No MS could be obtained.

(Z,Z,Z)-17-Ethyl-3-methoxycarbonylmethyl-1,2,3,19,23,24-hexahydro-2,2,7,8,13,18-hexamethyl-1,19-dioxo-21H-bilin-12-propanoic Acid (**5**)

It was prepared according to [12].

(Z,Z,Z)-3-Carboxymethyl-17-Ethyl-1,2,3,19,23,24-hexahydro-2,2,7,8,13,18-hexamethyl-1,19-dioxo-21H-bilin-12-propanoic Acid [**6**; $C_{32}H_{38}N_4O_6$)

The preparation is analogous to compound **2** using *rac*-(*Z*)-9-*tert*-butyl-3-carboxymethyl-2,3-dihydro-2,2,7,8-tetramethyl-dipyrrin-1-one-carboxylate [13] and (*Z*)-3-ethyl-9-formyl-2,7-dimethyl-dipyrrin-1-one-8-propanoic acid. Yield 58%; m.p. 250 °C dec. ^{1}H-NMR (CD_3OD, δ, 360 MHz): 6.70 (s, =CH−10, 5.95 (s, =CH−15), 5.50 (s, =CH−5), 3.61 (dd, J_d=2.4 Hz, [J_d=7 Hz, H−3), 3.06 (dd, J_d=2.4 Hz, J_d=14 Hz, $-CH_2-3^1$), 2.50 (dd, J_d=7 Hz, J_d=14 Hz, $-CH_2$-3^1), 2.78 (m, $-CH_2-COOH$), 2.41 (m, CH_2-CH_2-COOH), 2.50 (q, J=8 Hz, $-CH_2-CH_3$), 1.98 (s, $-CH_3$-8), 1.96 (s, $-CH_3$-13), 1.94 (s, $-CH_3$-7), 1.89 (s, $-CH_3$-18), 1.13 (s, $-CH_3-2$), 1.07 (s, $-CH_3-2$), 0.91 (t, J=8 Hz, $-CH_2-CH_3$) ppm. IR (KBr): ν=1 702, 1 642, 1 600, 1 395, 1 224 cm^{-1}. UV-VIS (CH_3OH): λ=596 (10 000), 331 (21 600) nm (ε). MS (70 eV, 200 °C): *m*/e (%)=530 (M^+-CO_2, 9), 474 (12), 369 (15), 351 (7), 303 (11).

The preparative scale isolation of *(Z,Z,Z)-18-Ethyl-3-ethyliden-1,2,3,19,23,24-hexahydro-2,7,13,17-tetramethyl-1,19-dioxo-21 H-bilin-8,12-dipropanoic Acid* (**7**) from *Spirulina geitleri* will be described in detail elsewhere.

(Z,Z,Z)-17-Ethyl-1,2,3,4,5,19,23,24-octahydro-3,3,7,13,18-pentamethyl-1,19-dioxo-21 H-bilin-8,12-dipropanoic Acid (**8**; $C_{32}H_{40}N_4O_6$)

rac-(Z)-9-tert-Butyloxycarbonyl-2,3,4,5-tetrahydro-3,3,7-trimethyl-dipyrrin-1-one-8-propanoic Acid ($C_{20}H_{30}N_2O_5$)

80 mg *rac-tert*-butyl-2,3,4,5-tetrahydro-3,3,7-trimehtyl-8-(2-methoxycarbonylethyl)-dipyrrin-1-one-9-carboxylate were dissolved in 2 ml methanol and diluted with 4 ml water. 2 g KOH were added and the solution was kept at room temperature overnight. The mixture was neutralized with SO_2, then the precipitated product was filtered off and dried in the desiccator. Yield: 94%; m.p. 193–196 °C. ^{1}H-NMR (CD_3OD, λ, 360 MHz): 3.44 (dd, J_d=5 Hz, J_d=11 Hz, H-4), 2.68 (dd, J_d=5 Hz, J_d=15 Hz, $-CH_2$-5), 2.54 (dd, J_d=11 Hz, J_d=15 Hz, $-CH_2$-5), 2.97 (t, J=7.5 Hz, $-CH_2-COOH$), 2.36 (t, J=7.5 Hz, $-CH_2-CH_2-COOH$), 2.15 (d, J=18 Hz, $-CH_2-2$), 2.03 (d, J=18 Hz, $-CH_2-2$), 1.87 (s, $-CH_3$), 1.46 (s, *t*-butyl), 1.05 (s, $-CH_3$-3), 1.02 (s, $-CH_3$-3) ppm. IR (KBr): ν=1 720, 1 684, 1 642, 1 460 cm^{-1}. UV-VIS (CH_3OH): λ=282 (17 800), 244 (6 200) nm (ε). MS (70 eV, 200 °C): *m*/e (%)=378 (M^+, 2), 267 (38), 211 (96), 166 (28), 112 (100).

(Z,Z,Z)-17-Ethyl-1,2,3,4,5,19,23,24-octahydro-3,3,7,13,18-pentamethyl-1,19-dioxo-21H-bilin-8,12-dipropanoic Acid

The preparation is analogous to compound **2** using *rac*-(*Z*)-9-*tert*-butyloxycarbonyl-2,3,4,5-tetrahydro-3,3,7-trimethyldipyrrin-1-one-8-propanoic acid and (*Z*)-3-ethyl-9-formyl-2,7-dimethyl-dipyrrin-1-one-8-propanoic acid. Yield 53%; m.p. 178–182 °C. ^{1}H-NMR (CD_3OD, δ, 360 MHz): 6.93 (s, $=CH-10$), 5.92 (s, $=CH-15$), 3.62 (m, 4-H), 2.77 (m, $-CH_2-COOH$), 2.64 (m, $-CH_2$-5), 2.43 (q, $J=8$ Hz, $-CH_2-CH_3$), 2.28 (m, CH_2-CH_2-COOH), 2.10 (d, $J=18$ Hz, CH_2-2), 2.02 (d, $J=18$ Hz, $-CH_2-2$), 1.93 (s, $-CH_3$-7), 1.88 (s, $-CH_3$-13), 1.74 (s, $-CH_3$-18), 1.05 (t, $J=8$ Hz, $-CH_2-CH_3$), 0.99 [s, $-(CH_3)_2-3$] ppm. IR (KBr): $\nu=1696$, 1642, 1612, 1395 cm^{-1}. UV-VIS (CH_3OH): $\lambda=544$ (15100), 324 (25200) mm (ε). MS (70 eV, 200 °C): *m/e* (%)= 530 (9), 316 (16), 302 (27).

Preparation of the Apomyoglobin-Pigment Complexes

For **1** and **3** the pigment was dissolved in 0.03 *N* NaOH, acidified to pH 7.5 with HCl and added to a solution of apomyoglobin. A 10% molar excess of the protein over the pigment was taken. This mixture was adjusted to pH 6.0, kept for 2 h at room temperature, filtered, and chromatographed on Sephadex G-15 with distilled water. To inhibit proteolysis about 2% of sodium azide was added. After lyophilization the complex was dissolved in water, kept at room temperature for another hour, filtered, and lyophilized again. In the case of **2** and **4–8** the pigments are water soluble and therefore their water solutions were directly added to the apomyoglobin solution.

Stoichiometric compositions were determined by the method of Job [14]. Formation constants were calculated using quantitative circular dichroism data and absorption difference spectra [15].

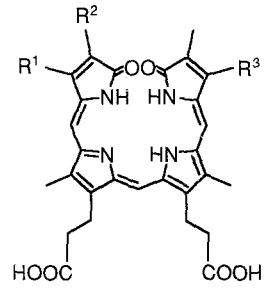
Results

Reconstitutions of apomyoglobin with the bile pigments **1–8** resulted in formation of stable apomyoglobin-pigment complexes which were purified by gel chromatography. Their absorption and circular dichroism data together with their formation constants (K, 1 mol^{-1}) are given in Tab. 1. In all cases Job plots [14] corroborated the formation of the 1 : 1 complexes. Binding of the pigments into the heme pocket is evidenced by their displacement through hemin [4].

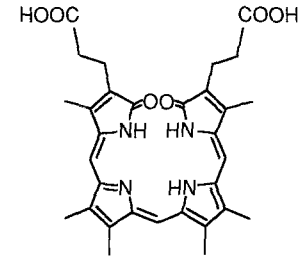
Discussion

Due to an improved preparation procedure of the apomyoglobin-bilin complexes the optical data of the system with biliverdin (**1**) as the bile pigment given in a recent communication [4] have been revised to the values given in Table 1. The absorption values are closer to those normally encountered for this chromophoric unit. Nevertheless, as pointed out earlier [4], such data have to be taken "*cum grano salis*" because of the limited stability of apomyoglobin which has a marked tendency to irreversible denaturation.

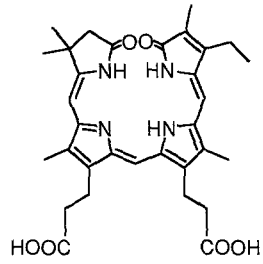
Varying the substitution pattern of **1** by making it C_{2v}-symmetrical and replacing the vinyl groups by ethyl groups leads to mesobiliverdin-XIIIα (**2**). Complexation of this bilin with apomyoglobin yields a system showing data very similar to those of the biliverdin complex. Accordingly, geometry and chirality of the chromophore is retained and the formation constant of this system is only slightly smaller than the one found in the case of apomyoglobin-biliverdin. This result points to a rather minor sensitivity of the inner heme pocket surface with respect to bilin substitution type and pattern.



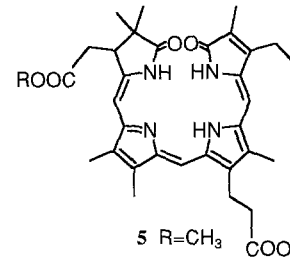
1 $R^1 = CH_3$, $R^2 = R^3$ = vinyl
2 $R^2 = CH_3$, $R^1 = R^3$ = ethyl



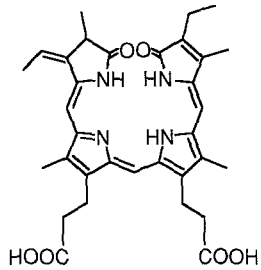
3



4



5 R=CH_3
6 R=H



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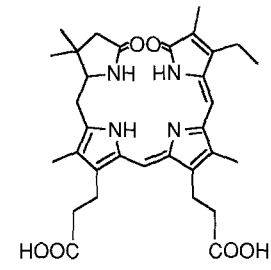

8

Reconstituting the pterobilin type pigment **3** (which formally is derived from heme through cleavage of the porphyrin ring in the γ position) into the heme pocket of apomyoglobin yields a system with absorption and circular dichroism data similar to the two bilin complexes of apomyoglobin with the α-type pigments **1** and **2** presented above. However, its formation constant is nearly one order of magnitude higher than that of the comparable protein complex of **2** and is nearly as high as the one of protoporphyrin ($5.5 \cdot 10^6$ l mol^{-1} [1, 3])! These data lead to two conclusions. First, the polarity of the lactam groups in the bilins derived from a cleavage of the porphyrin ring reduces the stability of the complex, whereas the lipophilic dipyrrin fragment (rings B and C) of **3** compares favorably with the corresponding lipophilic region of protoporphyrin which is embedded in the corresponding lipophilic region of the pocket (isoleucine-99 and leucin-104 [16]). Accordingly, the substitution pattern and type of such lipophilic residues is rather insignificant. Second, the chirality of the pigment helices as evidenced from the identical circular dichroism sign patterns of **1**, **2**, and **3** (Table 1) is not primarily determined by the chirality of the inner surface of the apomyoglobin heme pocket, but instead depends

Table 1. Absorption, circular dichroism, and formation constants of the complexes of **1–8** and apomyoglobin

Pigment	ε	nm	Δε	nm	$K(1\,mol^{-1})$
1	10 140	705	+43	700	$5 \cdot 10^5$
	38 650	385	−63	380	
2	10 000	665	+35	680	$3 \cdot 10^5$
	32 000	370	−74	370	
3	11 700	651	+65	650	$1 \cdot 10^6$
	35 900	362	−85	360	
4	12 200	606	+11	610	$1.5 \cdot 10^5$
	27 900	345	−17	350	
5	10 500	601	+19	596	$2 \cdot 10^5$
	26 100	346	−29	344	
6	1[a]	600	+1[a]	595	appr. 10^5
	2[a]	350	−1[a]	350	
7	11 770	636	+23	690	1.10^5
	33 100	371	−38	370	
8	13 900	546	−1	530	appr. 10^5
	7 500	413	+1	411	

[a] Only relative values could be estimated

on the asymmetric anchoring of the propionic acid side chains as schematically shown for the α- and γ-type bilins in Fig. 1. As is envisaged by this picture the entrance of the pocket provides a nearly C_2-symmetrical environment for the docking of the propionic acid side chains of the bile pigment by means of histidine-97 and arginine-45 (corresponding shaded and labeled areas in Fig. 1) according to the X-ray structural data of myoglobin [16]. Correspondingly, the propionic acid side chains act as molecular levers to enforce the sense of chirality upon the bilin helix. If the asymmetry of the pockets inner surface would determine the chiraliy of the chromophore helix, the sign of the circular dichroism bands should invert on proceeding from the α-type to the γ-type pigments. It is quite interesting to note that the chirality (*P*) of **3** in the apomyoglobin heme pocket as derived from the sign rule for C_2 symmetric chromophores [4, 17] is the same as that of pteroblin in the naturally occurring bilin binding protein [18]. Of course, in the latter case the absolute chirality of the bilin helix is proven by the X-ray crystallographic results.

Inserting a partially saturated bilin **4** of the 2,3-dihydro type (which is characteristic of the plant biliproteins) into the heme pocket of apomyoglobin does not produce a significantly different situation compared to the fully unsaturated bilins **1** and **2**. The circular dichroism values of this complex (Table 1) are somewhat smaller than those of the bilin complexes. The formation constant is also a little lower but still in the same order of magnitude. This value indicates again the indifference of the pocket with respect to steric and functional patterns of the ligand

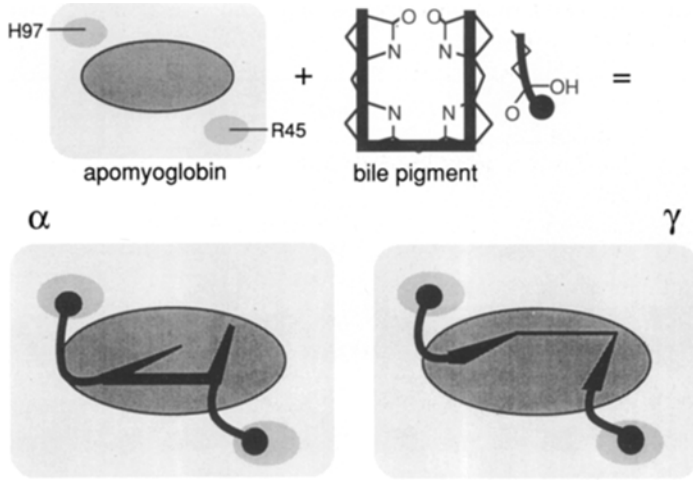

Fig. 1. Schematic drawing of the verdin (α) and pteroblin (γ) type bile pigments anchored to the heme pocket of apomyoglobin

substituents. Even altering the substitution type and pattern in a dramatic way as encountered with **5** leads to only rather small changes of the complexation data (Table 1) as compared to the bilins derived from α cleavage of a porphyrin ring. This result indicates that one propionic acid side chain is sufficient to produce the same kind of chirality as observed for the corresponding dipropionic acid derivatives (compare Fig. 1). With the dicarboxylic acid **6**, which is known to adopt an equilibrium with its lactone tautomer [13], reliable quantitative data could not be gathered. However, qualitative estimations indicate no profound changes in the complexation type and stability. The same holds for the natural phycocyanobilin (**7**) (Table 1). The violin derivative **8** yields only small dichroic signals upon reconstitution with apomyoglobin. Possibly with this violinoid pigment chiral discrimination is not as pronounced as in the cases of verdinoid systems.

These results advanced from the reconstitution of the bile pigments **1**–**7** with apomyoglobin (Table 1) indicate that chromophore binding and chirality of these pigments are mainly governed by a lipophilic region of the bilin opposite to the propionic side chain(s) and the dissymmetric positions of the hydrogen bonding acceptors of the propionic acid side chain(s) at the entrance of the protein pocket.

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