Biosynthesis of Porphyrinogens in Etiolated *Euglena gracilis* Z. II. Identification of a Regulatory Pteridine

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Porphyrinogens, *Euglena gracilis* Z., Porphyrin Biosynthesis, Pteridines, Porphobilinogenase

A low molecular weight compound which stimulates the synthesis of porphyrinogens from PBG was isolated and purified from etiolated *Euglena gracilis* Z. Absorption and fluorescence excitation and emission spectra, as well as the molecular weight, revealed its structure as an unconjugated pteridine.

FTIR bands and ^{1}H NMR signals of the purified compound were identical with those of an authentic sample of 6-biopterin. The structural formula of this compound is given in Fig. 1.

Introduction

In 1981, Juknat *et al.* [1] isolated an endogenous factor, which stimulates the synthesis of porphyrinogens from PBG in homogenate and supernatant fractions of *Euglena gracilis* Z.

In a previous paper [2] we reported the isolation and purification of an endogenous factor from etiolated *Euglena gracilis* Z., which showed pteridine – like features and stimulated the formation of porphyrinogens in the tetrapyrrol biosynthesis pathway [1]. Up to now several examples of pteridines acting as cofactors have been reported in literature. In rat liver a tetrahydrobiopterin (BH4) was identified in 1963 by Kaufman as the phenylalanine-hydroxylation cofactor [3], demonstrating for the first time the metabolic role of an unconjugated pteridine. Subsequently, BH4 was found to be the cofactor for the alkylglycerol monooxygenase in rat liver [4, 5] and also to play a central role in catecholamine and indolamine neurotransmitter synthesis [6–11].

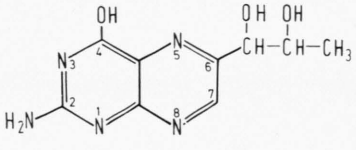

Fig. 1. Structural formula of 6-biopterin.

The importance of unconjugated pteridines in microorganisms has already been demonstrated [12, 13], showing that biopterin is a nutritional requirement for the protozoon *Crithidia fasciculata.* In this organism the synthesis of the pteridine ring [14] is blocked, which leads to a dependence of growth on dietary pteridines [15]. *Crithidia* has the ability to cleave the bond between C-9 and N-10 of folic acid, releasing 2-amino-4-hydroxy-6-hydroxymethylbiopterin which is then transferred to biopterin.

A different pathway for pteridine biosynthesis is described for *Tetrahymena pyriformis* which synthesizes its unconjugated pteridines from guanosine [16]. Böhme *et al.* [17] could isolate three yellow fluorescing compounds from *Euglena gracilis,* which were identified as 2-dimethylamino-6-trihydroxypropyl-4-oxo-3,4-dihydropteridine (Euglenapterin) and its phosphate derivatives.

Results reported here suggest that the newly isolated compound stimulating the formation of porphyrinogens from PBG is an unconjugated pteridine [2].

Materials and Methods

Organism and special chemicals

Growth and harvesting of *Euglena gracilis* Z. strain, preparation of supernatant fraction, measurement of enzymatic activity as well as all other methods not specified here, were described earlier [1, 2].

Abbreviations: (BH4), tetrahydrobiopterin; FTIR, Fourier-transformation-infrared spectroscopy; PBG, porphobilinogen.

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6-Biopterin (2-amino-4-hydroxy-6-[1,2-dihydroxypropyl]pteridine) and pterine (2-amino-4-hydroxypteridine) were obtained from Sigma Chemical Co.

Spectrophotometric and fluorimetric assays

UV-VIS absorption spectroscopy was carried out with a Kontron spectrophotometer Uvikon 820.

Fluorescence excitation and emission spectra were recorded with a Shimadzu spectrofluorometer RF 540. The excitation and emission wave-lengths were 360 and 450 nm, respectively, with a spectral bandwidth of 10 nm.

FTIR spectra

Fourier transformation infrared-spectroscopy (FTIR) was performed with a Nicolet 5 DX FTIR spectrometer. The sample was dissolved in water, evaporated under N_2 and redissolved in distillation-purified methanol, the most volatile organic solvent for solubilizing the sample (nearly insoluble in ethanol, acetone, ether). An aliquot of samples was applied to NaCl plates as a small spot. In a stream of N_2 the solvent was evaporated and samples were then thoroughly dried *in vacuo*. The factor-loaded plate was scanned 2000 times. The background, measured by scanning 100 times an unloaded plate, was subtracted.

^1H NMR spectrum

Euglena biopterin, dissolved in water, was transferred to an NMR tube and evaporated under N_2. To remove traces of water, the sample was again thoroughly dried *in vacuo*. Spectra were recorded at room temperature in 1 N NaOD using a Bruker WH 400 NMR spectrometer. As internal standard the HDO-peak at 4.8 ppm was chosen.

Results

Purification of Euglena biopterin

An endogenous pteridine was isolated and purified from etiolated *Euglena gracilis* Z. as described previously [2]. Gel filtration [16, 18], ion exchange [19–21] and paper chromatography [3, 16, 19] were employed in order to isolate and purify this pteridine, which seems to regulate porphyrinogen biosynthesis [1].

Paper chromatography showed a single blue-fluorescent spot ($R_f = 0.32$) when the chromatogram was developed in 1-butanol : glacial acetic acid : water (20:3:7; v:v:v). Rechromatography of the factor under the same conditions showed a R_f value identical to that of the cochromatographed authentic 6-biopterin. After separation of the endogenous pteridine in the above mentioned solvent system it was eluted from the paper, using as little water as possible and concentrated *in vacuo*.

Spectrophotometric properties

Spectral properties of the authentic 6-biopterin and the purified endogenous pteridine in the UV- and visible region at various pH-values are shown in Table I.

Table I. Absorption maxima of 6-biopterin and the endogenous pteridine in various solvents.

Compound	Solvent	λ_{max} [nm]				
6-Biopterin	Water		238	270	(345)	(362)
	0.1 N HCl		245		320	
	0.1 N NaOH		255			367
Purified	Water	205		270		(365)
Pteridine	0.1 N HCl	210	245		320	
	0.1 N NaOH		255			
PrS	0.05 M	218	255	280		(365)
S	Na-Phosphate	224	251	280		(365)
F_s	Buffer pH 7.4		260			

Spectra were recorded as described in Materials and Methods.
( ) shoulder.
S, crude supernatant fraction; PrS, protein fraction after filtration on Sephadex G-25 (20–40 ml eluate); F_s, factor containing fraction after filtration on Sephadex G-25 (45–90 ml), see ref. [2].

Absorption maxima of the enzyme fraction S of the supernatant of a cell homogenate before gel filtration and after filtration on Sephadex G-25 (fine), designated PrS and F_s [1, 2] are also given in Table I. Spectra were recorded in 0.05 M phosphate buffer, pH 7.4. For a detailed characterization of the different fractions see ref. [2]. As expected, the shoulder at 365 nm, characteristic for the factor, is not present in the PrS fraction.

Fluorescence properties

The blue fluorescence of the emission peak of the endogenous factor appeared in water at 450 nm when excited with light of 360 nm (Fig. 2), while the excitation spectrum (Fig. 3) showed two peaks at 275 and 350 nm. Fluorescence and excitation spectra of the endogenous factor were identical to those of 6-biopterin as demonstrated in Fig. 2 and 3.

Determination of the molecular weight of Euglena biopterin

The molecular weight of the factor was estimated by gel filtration on Sephadex LH 20. The column (1.5 × 17 cm) was equilibrated with water and calibrated with ATP, folic acid, glutathion and ascorbic acid (Fig. 4). The results show that the elution volume for the purified *Euglena* biopterin corresponds to a molecular weight of 230 Da ± 10, being in agreement with the authentic 6-biopterin (237.2 Da) and slightly lower than the value of the isolated F_sL_1 obtained after the first purification step [2].

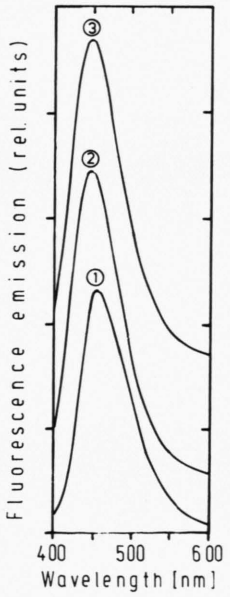

Fig. 2. Fluorescence emission spectra of the purified factor dissolved in: ① 0.1 N NaOH, ② 0.1 N HCl, ③ water. Excitation wave-length was 360 nm, bandwidth being 10 nm for both excitation and emission. Spectra were recorded with an uncorrected Shimadzu RF 540 spectrofluorometer at room temperature.

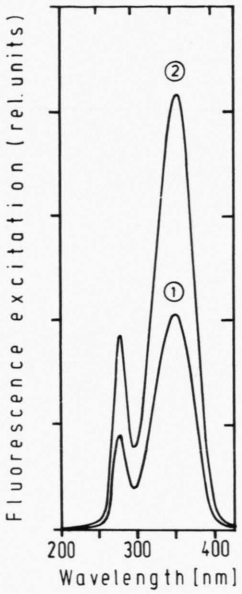

Fig. 3. Excitation spectra of the purified factor ① and of purchased 6-biopterin ② dissolved in water. Emission wave-length was set to 450 nm with a bandwidth of 10 nm. Spectra were recorded with an uncorrected Shimadzu RF 540 spectrofluorometer at room temperature.

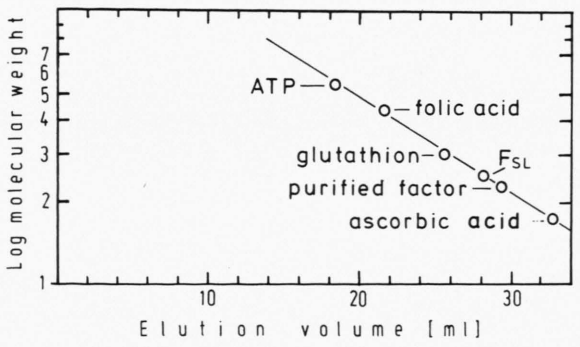

Fig. 4. Calibration curve of a Sephadex LH-20 column. Experimental details are described in the text.

FTIR spectrum of Euglena biopterin

In order to provide more evidence that the *Euglena* endogenous factor is an unconjugated pteridine, FTIR spectra of the purified compound were recorded (Fig. 5b) and compared to those of an authentic sample of 6-biopterin recorded under the same conditions (Fig. 5a). Both spectra showed characteristic maxima around 3450, 1650 and 1380 wave-numbers as well as full agreement in the finger print region, demonstrating the identity of sample and authentic standard.

^{1}H NMR spectrum of Euglena biopterin

^{1}H NMR spectrum obtained from the purified factor dissolved in NaOD, was compared with the corresponding spectrum of the authentic 6-biopterin (Table II).

The spectrum of the endogenous *Euglena* pteridine showed a singlet at 8.70 ppm, a doublet at 4.58 ppm (J = 6.7 Hz), a multiplet at 4.11 ppm (J = 6.5 Hz) and a second doublet at 1.26 ppm with a coupling constant of J = 6.2 Hz. The singlet can be attributed to the vinyl proton at position 7. The discrepancy between our data and ref. [23] are due to the fact that in our spectrum the HDO-peak at 4.8 ppm was taken as internal standard, whereas the other values were obtained with tetramethylsilane as external standard. The last doublet can be assigned to the protons of the methyl group at position 3′. The multiplet at 4.11 ppm and the doublet at 4.58 ppm can be ascribed to protons corresponding to positions 2′ and 1′, respectively.

All the signals of the isolated compound were consistent with the data from commercial 6-biopterin. For comparison, values reported in literature for 6-biopterin [22–24] are also summarized (Table II).

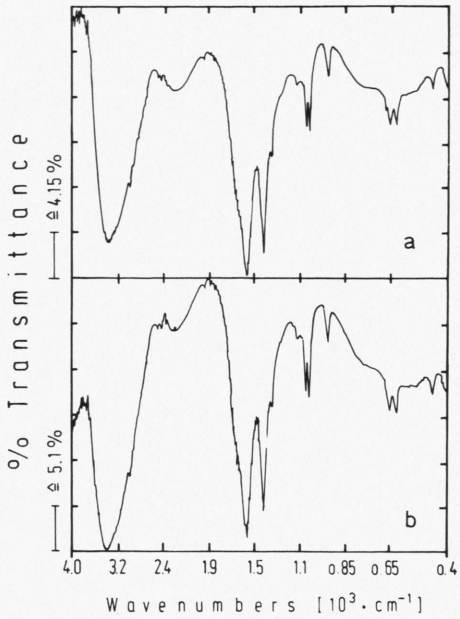

Fig. 5. FTIR spectra of purchased 6-biopterin (a) and the purified factor from *Euglena* (b). Samples were administered to NaCl-plates as a small spot and evaporated to dryness. Spectra were recorded with a Nicolet 5 DX FTIR-spectrophotometer. Transmittance in (a) ranges from about 15 to 40%, in (b) from about 20 to 50%.

Discussion

The typical absorption spectrum of the endogenous factor, which has, in alkaline solution, two maxima around 260 nm and 365 nm and the fluorescence

Table II. ^{1}H NMR of 6-biopterin. Data are given as ppm-values relative to external TMS.

Compound	7H (s, 1H)	1′-H (d, 1H)	2′-H (m, 1H)	3′-H (d, 3H)	Reference	Solvent
Authentic 6-Biopterin	8.70	4.59 (J = 6.7 Hz)	4.11 (J = 6.5 Hz)	1.26 (J = 6.2 Hz)	–	NaOD
Purified *Euglena* Biopterin	8.70	4.58 (J = 6.7 Hz)	4.11 (J = 6.5 Hz)	1.26 (J = 6.2 Hz)	–	NaOD
6-Biopterin	9.08	5.26	4.54	1.34	[22]	CF_3COOH
6-Biopterin (+)	9.22	–	–	1.77	[23]	NaOD
4-Amino-6-Biopterin (*)	8.71	4.50	–	1.12	[24]	d_6-DMSO

(+) The values were converted to the ppm-scale.
(*) 2- and 4-NH_2 signals were not given here. Spectra were recorded as described in Materials and Methods.

spectrum with an emission peak at 450 nm, as well as the molecular weight, compared to an authentic standard of 6-biopterin, demonstrate that the endogenous factor is an unconjugated pteridine.

In order to obtain a more precise determination of the molecular weight of the purified factor and, moreover, information about its structure, ^{252}Cf-plasma desorption mass spectra (PDMS) [25–28] were recorded, but it was not possible to identify a peak corresponding to the molecular ion. A negative result was obtained, when elementary analysis was considered, most probably due to the resistance of the pterine ring to combustion and to the capacity of this type of compounds to retain water in a non-stoichiometric form [29]. Authentic material showed the same properties, too.

^{1}H NMR and Fourier transformation infrared-spectroscopy (FTIR) were applied in order to determine the structure of the isolated factor. All signals present in the ^{1}H NMR spectrum of the purified pteridin were found in that of the authentic 6-biopterin, too.

An additional singlet at 8.48 ppm was found in the authentic sample. Taking into account that 6-biopterin could be co-purified with 7-biopterin, which is quite possible after chemical synthesis [24], the above described signal could be assigned to the vinyl proton at position 6. Rembold and Eder [24] found a signal at 8.47 ppm for 6-H from 4-amino-7-biopterin.

The isolated *Euglena* factor also showed an additional singlet at 8.47 ppm, which increases its intensity with time of compound storage. It was thus concluded that the existence of a degradation product might cause this signal.

Data reported in this work would suggest a 6-biopterin structure for the isolated and purified *Euglena* factor, being different from other pteridines extracted from this organism, like Euglenapterin and derivatives [17].

Results reported here are consistent with the structure of an aromatic pteridine. This is an unexpected result, since in biological reactions active forms of pteridines, so far known, were reduced and non-fluorescing pteridines.

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