Biosynthesis of Porphyrinogens in Etiolated *Euglena gracilis* Z. I. Isolation and Purification of an Endogenous Factor Stimulating the Formation of Porphyrinogens

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A low molecular weight, heat-stable factor has been purified from *Euglena gracilis* supernatant fraction by employing gel filtration, cation and anion exchange and paper chromatography.

This endogenous compound stimulates porphobilinogenase (PBG-ase) (EC 4.3.1.8) activity, an enzyme of the porphyrin biosynthetic pathway.

10^{-7} M folic acid and 10^{-4} M 6-biopterin produced a significant activation, equivalent to 2–4 units of the purified factor.

Elution patterns from the columns and fluorescence and UV absorption peaks suggest that this compound is a pteridine. This conclusion is further supported by the fact that both, folic acid and 6-biopterin can replace the action of the isolated factor on PBG-ase. The mechanism of stimulation is discussed.

Introduction

Uroporphyrinogen III (Urogen III) is the biological precursor of some of the most important pigments of living organisms, such as heme, chlorophylls, cytochromes and corrins like vitamin B_{12}. The biosynthesis of the tetrapyrrols requires the cooperation of two enzymes: uroporphyrinogen I synthase (URO-S) or hydroxymethylbilane synthase (PBG-deaminase, EC 4.3.1.8) and uroporphyrinogen III synthase (Co-synthetase, EC 4.2.1.75). This dual enzymatic system is known as porphobilinogenase (PBG-ase). Sancovich *et al.* [1] have detected a factor by ultrafiltration in partially purified cow liver preparations, that stimulates urogen synthesis from porphobilinogen (PBG). A pteroylpolyglutamate factor, which activates URO-S, has also been isolated from rat hepatic cytosol [2].

A C_1-transfer mechanism may be involved in the intramolecular rearrangement, whereby urogen III-cosynthase produces urogen III from hydroxymethylbilane [3, 4]. This enzyme is considered to be a folate-binding protein which supplies the reduced pteroylpolyglutamate necessary for the proposed mechanism [4]. Moreover, Kohashi *et al.* [5] reported that this reduced pteroylpolyglutamate associated to rat hepatic urogen III cosynthase may function as a coenzyme in this reaction. A methylene tetrahydrofolate was also suggested to be involved in porphyrin biosynthesis [6]. More recently Hart and Battersby [7] purified the cosynthetase from *Euglena gracilis* reporting that cofactors and folate derivatives were not established as components of the enzyme.

These observations together with our report about the existence of a low molecular weight, heat-stable factor in *Euglena gracilis* which is able to modify enzymatic synthesis of porphyrinogens and whose activation properties can be replaced by folic acid [8] suggested to isolate and purify this endogenous factor, in order to elucidate its structure.

Materials and Methods

Chemicals

Bacto-peptone, Bacto yeast extract and Bacto-agar employed in the growth media were purchased from Difco Laboratories (Detroit, Michigan, U.S.A).

PBG was obtained from Sigma Chemical Co. and estimated by the method of Moore and Labbe [9]. Solutions of PBG were prepared in 0.05 M sodium

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phosphate buffer, pH 7.4, which was also used throughout this work. Sephadex gels were supplied by Pharmacia Fine Chemicals (Freiburg, W. Germany), Dowex 2-x8 from Bio-Rad and Servacel phosphocellulose from Serva (Heidelberg, W. Germany).

Cyanocobalamin, 6-biopterin (2-amino-4-hydroxy-6-[1,2-dihydroxypropyl]pteridine) and *o*-phthalaldehyde were obtained from Sigma Chemical Co. and folic acid from Merck.

All other reagents commercially obtained were of analytical or the purest available grade.

Euglena growth and harvesting

Euglena gracilis Z. was grown in the dark at 26–28 °C in a medium containing 0.5% peptone, 0.2% yeast extract, 1% (v/v) ethanol and cyanocobalamin (5 μg/100 ml). Cells were harvested after 7 days of growth, washed twice with 50 mM sodium phosphate buffer, pH 7.4, and resuspended in the same buffer (1 g wet wt./2 ml buffer) [10].

Source of enzyme

Cells were disrupted by ultrasonic treatment in a MSE ultrasonic power unit for 30 sec at 8 μ amplitude. The resulting homogenate was centrifuged at 24,000 × *g* for 30 min. The pellet was washed twice with a total volume of buffer equal to that of the supernatant and the final sediment was discarded. The pooled supernatant was employed as enzyme source [10].

All steps were carried out at 4 °C.

Determination of enzyme activity

The standard incubation system contained (unless otherwise stated): 2 ml enzyme preparation, 50 mM sodium phosphate buffer, pH 7.4, and PBG (300 μg/ml) in a final volume of 3 ml. All solutions were degassed and flushed with N_2 before starting the incubation. Incubations were carried out with mechanical shaking in the dark at 37 °C for 4 h in a N_2 stream to prevent oxidation reactions.

Blanks were always run with PBG omitting the enzyme. Non-enzymically formed porphyrins were subtracted in each case.

The incubation was stopped with trichloroacetic acid (TCA, final concentration 5%). The porphyrinogens were oxidized by exposing them to white light (20 Wm^{-2}) for 20 min, the precipitated protein was filtered off and the total porphyrins were determined in acidic solutions [11] using a Kontron Uvikon 820 spectrophotometer.

Protein determinations

Protein was determined by the method of Lowry *et al.* [12], by measurement of the absorbance at 280 nm or by the *o*-phthalaldehyde procedure [13, 14] using crystalline bovine serum albumin as standard.

Preparation of columns

Sephadex G-25 coarse and fine columns (1.6 cm ∅; height 45 cm) were employed. Dowex 2-x8, chloride form (200–400 mesh, capacity 3,22 meq/g dry weight) and phosphocellulose ion exchange columns (capacity: 0.85 meq/g dry weight) were prepared and regenerated as described by Rembold [15]. The size of these columns was 1.5 × 17.5 cm and 2 × 3.5 cm, respectively.

Paper chromatography

Ascending paper chromatography was carried out on Whatman No. 1 filter paper. The chromatograms were developed with 1-butanol/glacial acetic acid/water (20/3/7, v/v/v). The resultant blue-fluorescing band was cut out, eluted with water and the fluorescent material was concentrated *in vacuo*.

Monitoring

Column fractions and eluates from paper chromatography were monitored spectrophotometrically by determining absorbance at 260 nm in a Kontron spectrophotometer Uvikon 820 and fluorometrically with an uncorrected Shimadzu spectrofluorometer RF 540. Excitation wave-length was 360 nm, emission measured at 450 nm. The bandwidths were both 10 nm. The eluates were also monitored for biological activity by testing aliquots for their ability to increase the activity of PBG-ase preparations [8].

Results and Discussion

Purification of the stimulating endogenous factor from etiolated Euglena gracilis Z.

PBG-ase activity seems to be regulated by a low molecular weight, heat-stable factor, which has pteridine-like properties. This endogenous com-

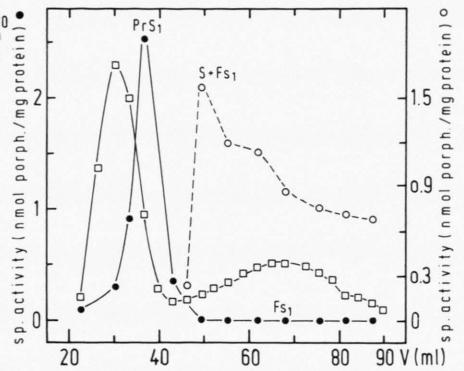

Fig. 1. Elution diagram of *E. gracilis* supernatant from Sephadex G-25 (coarse) column with distilled water. □, Absorbance of eluate at 280 nm; ●, specific activity of the eluated supernatant. Peak fraction was designated PrS_1, fractions from 45 to 90 ml, containing the factor, were called F_{s1}; ○, 1 ml unseparated supernatant + 1 ml F_{s1}-fraction showing a 7-fold increase in activity compared to S alone.

pound was isolated and purified by gel filtration, ion exchange- and paper chromatography, well known methods for the separation of pteridines from biological material [16, 17]. Chromatography on Sephadex G-25 (coarse) was initially employed (Fig. 1) in order to isolate the factor from the supernatant (S) of the disrupted and centrifuged *Euglena* cells. Protein content and enzymatic activity were determined in each eluate. Protein eluting between 22 and 40 ml was pooled and designated PrS_1. The specific activity of this fraction was 2.53 nmol porphyrins/mg protein, which is 12 times higher than that corresponding to the unfiltered fraction S (specific activity: 0.215 nmol porphyrins/mg protein). The high activity of this fraction indicates the absence of a low molecular weight inhibitority compound, called F_{s1}. This effect might be enhanced by the enrichment of the enzyme protein. The factor F_{s1} itself eluted between 45 and 90 ml. However, by adding 1 ml of F_{s1} to 1 ml of the S fraction, a stimulatory effect of F_{s1} could be measured. A 7-fold enhancement in activity of the S fraction was found when incubated in the presence of F_{s1}.

This apparent discrepancy between inactivation and activation of the enzyme by the same factor can be explained by two different binding sites for the low molecular compound. In the supernatant an equilibrium between the complex of inhibitorily bound factor and enzyme on the one hand and free enzyme and factor on the other hand, may exist. The once liberated factor can then immediately bind to the stimulating binding site, possibly causing a conformational change. For more details see ref. [8].

Fractions with F_{s1} activity were pooled and lyophylized (F_{sL1}). The residue was resuspended in a small volume of water (10% of original volume) and rechromatographed on a Sephadex G-25 (fine) column (Fig. 2 and 3). Fluorescence, absorbance at 260 nm, protein content and biological activity were measured as described above. Only one peak of fluorescence was obtained, which was separated from the main protein peaks and other ultraviolet absorbing material. Fluorescence and biological activity of the factor F_{s2} were in good agreement.

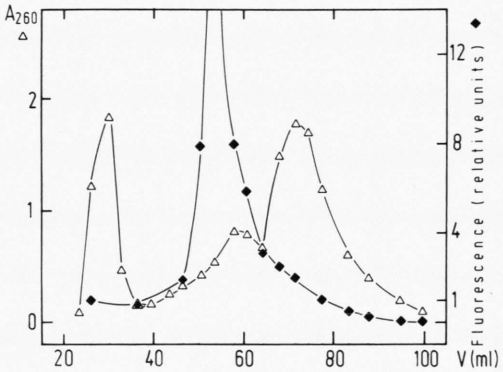

Fig. 2. Elution diagram from a Sephadex G-25 (fine) column of fractions (50–90 ml) from the Sephadex G-25 (coarse) column. △, Absorbance at 260 nm; ◆, fluorescence of fractions measured at 450 nm, excitation wavelength 360 nm.

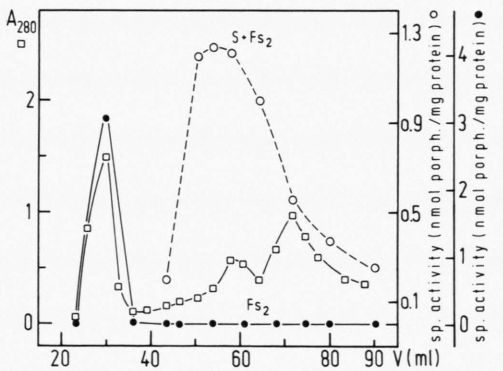

Fig. 3. Specific activity and protein elution diagram corresponding to Fig. 2. Activity was measured as described in Materials and Methods. □, Absorbance at 280 nm; ○, specific activity of supernatant after the addition of fractions of the factor from Sephadex G-25 (fine) separation; ●, specific activity of fractions from Sephadex G-25 (fine) separation.

The fractions with F_{s2} activity were combined, lyophylized and chromatographed on an anion exchange column Dowex 2-x8 (formiate form) (Fig. 4). The fluorescent material with biological activity was eluted with water between 40 and 100 ml (fractions 4 to 10) (F_{sD3}). Another small fluorescing peak without biological activity appeared at higher volumes. It has to be assumed that this compound represents a degradation product formed by the manipulations during the purification procedure or perhaps another pteridin synthesized by the organism. This contamination was not present when the concentrated fractions F_{sD3} were rechromatographed on the same Dowex 2-x8 column. The elution of the factor from the anion exchange resin with water suggests the presence of a basic or neutral compound.

The fractions from Dowex 2-x8 which contained biological activity were pooled and concentrated *in vacuo*. The residue was redissolved in about 6 to 8 ml of water and chromatographed on phosphocellulose (H^+-form) (Fig. 5). The fluorescing peak eluting in fractions 10 to 20 (between 100 and 180 ml) (F_{sC4}) was coincident with the biological activity of the corresponding eluates.

Further purification by paper chromatography in 1-butanol/glacial acetic acid/water (20/3/7, v/v/v) showed a single fluorescent zone, which could clearly be separated from a lipid-like material that remained at the origin of the chromatogram.

The degree of purification, the quantitative effectiveness of the factor and the specific activity measured at each purification step are shown in Table I.

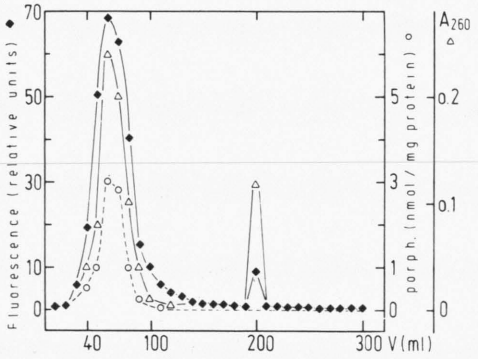

Fig. 4. Elution diagram of lyophylized F_{s2} fractions from the previous column (Fig. 2), chromatographed on Dowex 2-x8 (formate form), eluted with distilled water; fraction size was 10 ml. ◆, Fluorescence of the fractions at 450 nm, excitation wave-length 360 nm; ○, specific activity of fractions (stimulation of S); △, absorbance at 260 nm.

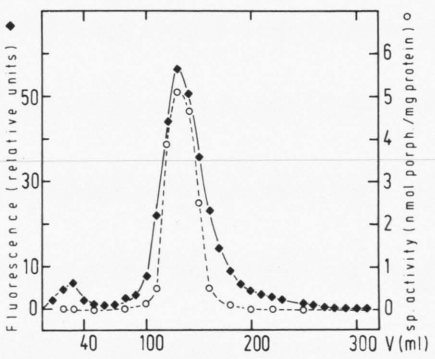

Fig. 5. Elution diagram of the fluorescent fractions from the Dowex 2-x8 column eluted from a phosphocellulose column with distilled water; fraction size was 10 ml. ◆, Fluorescence of fractions at 450 nm, excitation 360 nm; ○, specific activity of fractions.

Table I: Purification of the stimulating endogenous factor.

Steps of purification	Protein [mg/ml]	Specific activity [nmol porph/mg protein]	Factor [units]	Purification [-fold]
Supernatant (S)	8.06	0.215	–	1
Sephadex G-25 (F_{s1}) (coarse)	0.12	1.572	3.6	7.3
Sephadex G-25 (F_{s2}) (fine)	0.108	1.814	4.2	8.4
Dowex 2-x8 (F_{sD3})	0.04	3.04	7.0	14.1
P-Cellulose (F_{sC4})	0.05*	4.25	9.9	19.8
Paper chromat. (F_{sP5})	0.025*	4.74	11.0	22.0

* *o*-Phthalaldehyde reactive material.
Incubation conditions and methodology are described in Materials and Methods.

One unit of factor is defined as the amount of compound which produces a 2-fold increase in activity of the corresponding control [8]. It is shown that 11 units of a 22-fold purified factor were obtained. Proteins from the first four steps were determined by the method of Lowry *et al.* [12]. The last two fractions showed negative Lowry reaction but reacted with *o*-phthalaldehyde in the presence of 2-mercaptoethanol. This fluorimetric technique is employed in the detection of primary amines [14] and in the determination of proteins at a concentration range of 0.1 to 50 μg/ml [13]. The results suggest that the purified factor might contain a primary amino group.

Properties of the stimulating factor

As previously reported [8] the factor is heat-stable at 100 °C for 10 and 20 min, but its stimulating effect disappeared after treatment with NaOH or HCl (1–6 N). Samples of F_{sL1} and F_{sC4} could be stored at −20 °C for several months without any loss of their activating properties.

F_s is soluble in acidic and basic solutions, but nearly insoluble in organic solvents like ethanol, acetone or ether. The apparent molecular weight of F_s was determined by gel filtration on Sephadex LH-20. The column (1.5 × 17 cm) was calibrated with ATP (M_r: 551.15), folic acid (M_r: 441.41), glutathion (M_r: 307.3) and ascorbic acid (M_r: 176.13). The molecular weight of the purified factor was found to be 230 Da ± 10, being lower than that reported for the *Euglena* pterin (281 Da) [18].

Purified factor preparations were analyzed by ultraviolet and fluorescence spectroscopy. The absorption spectra showed characteristic maxima in aqueous, acidic and alkaline solution (Table II). Fluorescence spectra demonstrate excitation peaks at 275 nm and 350 nm with a fluorescence maximum at 450 nm.

Table II. Absorption maxima of the stimulating factor.

Fractions	Conditions	max. [nm]		
F_{sL1}	Water, pH 7.0	205	255	
	0.1 N HCl	210	255	360
	0.1 N NaOH	225	255	
F_{sD3}	Water, pH 7.0		251	(340)
F_{sP5}	Water, pH 7.0		256	(381)

( ) shoulder.
Spectra were determined as described in Materials and Methods.

The F_{s1} fluorescence spectrum can be seen in Fig. 6. The spectrum was obtained by subtracting the spectrum given by the fraction PrS_1 (without factor) from that given by the S fraction. Samples were adjusted to identical protein content. The shoulder at 510 nm disappeared during the purification procedure. Fluorescence and UV absorption peaks suggest that the factor might be a pteridine derivative. A previous report [8] showed that 10^{-7} M folic acid produced a significant activation of the homogenate fraction. It was also considered that the stimulatory effect of F_s could be replaced by folic acid. This stimulatory effect of F_s on the enzyme activity was slightly decreased by folic acid (Fig. 7a), a result also observed by Piper and van Lier [2] in rat hepatic cytosol.

In order to obtain additional evidence that the regulatory factor was a pteridine derivative, the effect of 6-biopterin and folic acid were investigated. The increase of PBG-ase activity caused by 6-biopterin was double of that produced by folic acid (Fig. 7b). The activation of enzyme activity produced by 10^{-7} M folic acid and 10^{-4} M 6-biopterin, respectively, are equivalent to that produced by 2–4 units of the

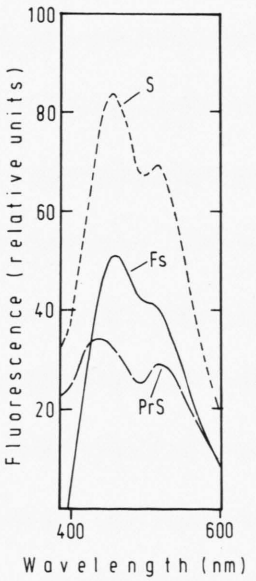

Fig. 6. Fluorescence spectrum (solid line) of F_s, obtained by subtraction of the PrS spectrum from the S spectrum as described in the text. Samples were adjusted to identical protein content. Spectra were recorded with an uncorrected spectrofluorometer Shimadzu RF 540 at room temperature in phosphate buffer.

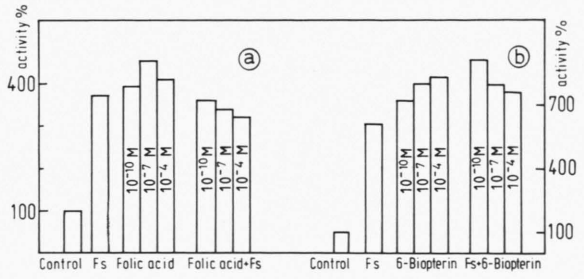

Fig. 7. Inhibiting and activating effect on F_s stimulation of PBG-ase activity by folic acid and 6-biopterin, respectively. Values are given as % of control. Control represents S-fraction without addition of F_s. Data in Fig. 7 are typical for experiments.

purified factor. It should be noted that a slight additive effect was found when F_s and 6-biopterin (10^{-10} M) were incubated together (Fig. 7b) and that a decreasing effect was found, when F_s and folic acid were incubated together. This controversal behavior could be considered in respect to the presence of two different binding sites for the stimulating factor of PBG-ase as discussed previously [8]. Although the properties of the isolated compound described in the present paper might not be sufficient for an absolute identification, we consider that they are an important proof that the factor is an unconjugated pteridine. More data will be presented in order to elucidate the structure of this regulating pteridine [19].

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