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Purification and Characterization of Animal Porphobilinogen Synthases

I. Bovine Liver Porphobilinogen Synthase

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Summary: Porphobilinogen synthase was purified from ox liver by ammonium sulfate fractionation, heat denaturation and column chromatography (purification: 400-fold; specific activity 4.72 nkat). The molecular weight of the native enzyme obtained by thin-layer gel filtration is about 280000. Using 8M urea in the presence of dithiothreitol as reducing agent, the molecule breaks down into 8 subunits of molecular weight 36000 (dodecylsulfate gel electrophoresis); the preparation of aminoethylated subunit is described.
According to the above-mentioned molecular weight and to the quantitative amino acid analysis after total hydrolysis, the following composition of the enzyme subunit was calculated Asx_{23-25} Thr_7 Ser_{23-24} Glx_{29-31} Pro_{22-23} Gly_{22-24} Ala_{36-37} Val_{23-26} Met_7 Ile_9 Leu_{34-35} Tyr_{10} Phe_{11-12} Lys_{11-12} Cys_{6-7} His_{6-8} Arg_{22} Trp_{1-2}. The subunits, having two free sulfhydryl groups, therefore consist of a chain of about 306 amino acids. The Dansyl-Edman procedure did not enable identification of any free *N*-terminal amino acid. The acyl group blocking the *N*-terminus is an *acetyl* group. It was identified, after hydrazinolysis of the enzyme, by means of chromatographic comparison with 1-formyl-2-dansylhydrazine and 1-acetyl-2-dansylhydrazine, whose syntheses and UV spectra are described.

Reindarstellung und Charakterisierung von tierischen Porphobilinogen-Synthasen, I: Das Enzym aus Rinderleber

Zusammenfassung: Phorphobilinogen-Synthase wurde aus Rinderleber durch Ammoniumsulfat-Fraktionierung, Hitzedenaturierung und Säulenchromatographie gereinigt (Anreicherung 400fach; spezif. Aktivität 4.72 nkat). Das durch Dünnschicht-Gelfiltration ermittelte Molekulargewicht des nativen Enzyms beträgt etwa 280000. Durch 8M Harnstoff in Gegenwart von Dithioerythrit als Reduktionsmittel zerfällt das Enzym in 8 Untereinheiten vom Molekulargewicht 36000 (Dodecylsulfat-Gelelektrophorese); die Gewinnung von aminoäthylierter Untereinheit wird beschrieben.
Unter Zugrundelegung des genannten Molekulargewichtes und der quantitativen Aminosäure-Analyse nach Totalhydrolyse wurde für die En-

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Enzyme: Aminolevulinate synthase, succinyl-CoA:glycine *C*-succinyltransferase (decarboxylating), (EC 2.3.1.37); Porphobilinogen synthase, 5-aminolevulinate hydro-lyase (adding 5-aminolevulinate and cyclizing), (EC 4.2.1.24).

Abbreviation: mcm = mean common multiple.

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zym-Untereinheit die folgende Zusammensetzung berechnet: Asx_{23-25} Thr_7 Ser_{23-24} Glx_{29-31} Pro_{22-23} Gly_{22-24} Ala_{36-37} Val_{23-26} Met_7 Ile_9 Leu_{34-35} Tyr_{10} Phe_{11-12} Lys_{11-12} Cys_{6-7} His_{6-8} Arg_{22} Trp_{1-2}. Die Untereinheit, welche 2 freie Sulfhydrylgruppen enthält, besteht somit aus einer Kette von etwa 306 Aminosäuren. Der Dansyl-Edman-Abbau ließ keine freie *N*-terminale Aminosäure erkennen; der *N*-Terminus wird durch eine *Acetyl*-Gruppe blockiert. Ihre Identifizierung nach Hydrazinolyse der Porphobilinogen-Synthase erfolgte durch Vergleich mit 1-Formyl-2-dansylhydrazin und 1-Acetyl-2-dansylhydrazin, deren Synthese und UV-Spektren beschrieben werden.

Key words: Porphobilinogen synthase, *N*-acyl terminus, hydrazinolysis, 1-acyl-2-dansylhydrazines.

5-Aminolevulinic acid is the precursor of tetrapyrrole biosynthesis. The end-products are the porphyrin, chlorophyll and the corrinoid systems.

The condensation of two molecules of 5-aminolevulinic acid to the aromatic ring system porphobilinogen is catalysed by the enzyme porphobilinogen synthase[1-3]. Depending on its origin, the enzyme may or may not require various metal ions for its activation[3-7]. It is present in the cytoplasm of all aerobic cells and is, as a sulfhydryl enzyme, particularly sensitive to various heavy metals, these forming sulfides of low solubility. Cadmium, copper, mercury and silver, and especially lead, show pronounced inhibitory effects on the enzyme activity[5,7-12]. The observation that zinc ions can compensate for the inhibitory effect of heavy metal ions on mammalian tissue porphobilinogen synthase indicates a re-activation of the zinc-containing enzyme[13] by displacement of the foreign metal. The re-activation of the enzyme by $Zn^{2\oplus}$ following ethanol inhibition[5] is, however, based on another mechanism[14]. It could be shown for various micro-organisms and mammalian cells[4] that 5-aminolevulinate synthase and porphobilinogen synthase are the rate-limiting enzymes for corrinoid and porphyrin biosynthesis. On the one hand, an increase in the endogenous heme level lowers the porphyrin synthesis from lactate by repressing the aminolevulinate synthase, just as an increase in the 5-aminolevulinate level results in an increase in the rate of porphyrin synthesis. On the other hand, the lowering of the rate of porphyrin synthesis through higher heme levels in the presence of exogenous 5-aminolevulinate or the constant rate of prophyrin synthesis from porphobilinogen indicates a repression of the porphobilinogen synthase. With respect to the allosteric control of the porphobilinogen synthase[15,16] by *feedback inhibition*, coproporphyrinogen is substantially more effective than hemin or protoporphyrin IX[17].

Measurement of the activity of porphobilinogen synthase is important both for tests on the influence of chemicals such as urea or guanidine derivatives[18], triazole herbicides[19], 1,3,5-triazines[20], polychlorinated biphenyls[21], hormones[22] or antibiotics[23], and for various hematologic problems, in which measurement of the activity of the individual heme enzymes, apart from determination of the porphyrin metabolites, is also of great importance. Owing to the possibility of carrying out these analyses with microliter samples of whole blood[24], the detection of defects within the heme pathway (porphyrias) has also become possible, such as a quick differentiation between heavy metal poisoning effects on erythroid cells during their maturation in the bone marrow (protoporphyrin ↑, porphobilinogen synthase ↓) and an iron-deficiency anaemia (protoporphyrin ↑, porphobilinogen synthase ↑). Rare hereditary diseases (erythropoietic protoporphyria, congenital erythropoietic porphyria) can be detected analogously.

Further possible applications are the determination of metabolite and enzyme activity patterns in biologically modified cells (eg. in hepatomas or after virus infection)[25-27].

Results and Discussion

Ox liver porphobilinogen synthase was obtained by a large-scale preparation (see "Methods and Materials" for experimental details) with a specific activity of 4.72 nkat, which falls within the range of literature values[28,29]. The reductive cleavage of the gel-electrophoretically pure native enzyme into monomers using dithiothreitol in the

presence of 8M urea and the aminoethylation of the subunits with ethylene imine (azirane)[30] resulted in a product which, after dodecylsulfate electrophoresis, showed some weak subsidiary bands. These could either be by-products formed during the reductive alkylation or else peptide fragments which were formed by partial autolysis of the porphobilinogen synthase chains while still in the tissue, during the time-lapse between extirpation and extraction of the organ, and which after the reductive unfolding of the enzyme had been set free. The separation of these subsidiary bands was achieved through re-chromatography on Sephadex G-100.

The determination of molecular weights by dodecylsulfate electrophoresis using a 7.5% polyacrylamide gel[31-33] and reference proteins with polypeptide chains of well-known molecular weights resulted, both for the native and also for the aminoethylated subunit, in a molecular weight of 36000. Contrary to other investigations[6] these subunits could not be further cleaved. Since the native enzyme from ox liver consists of 8 subunits with a di-hedral symmetry (D_4)[34,35], the enzyme from our preparation would have a molecular weight of 288000. This value is in good agreement with the molecular weight derived by gel filtration, as well as with results from other laboratories[6,35,36].

Porphobilinogen synthase from mouse liver[37], however, was different. Denaturation with 6M guanidine hydrochloride in the presence of reducing agents resulted in subunits of molecular weight 39500. With a molecular weight for the native mouse-liver enzyme of 250000[37], this enzyme would have to consist of only 6 polypeptide chains.

The results of the quantitative amino acid analyses of the native ox liver enzyme and its aminoethylated subunit after 20 or 200-h hydrolysis and after performic acid oxidation are given in Table 1. The approximate total of amino acids per enzyme subunit as well as the number of individual amino acid residues per chain were calculated according to the *factor* and to the *mcm method* (see "Methods and Materials") using the nanomole values obtained from the amino acid analyses and the known molecular weight of the enzyme. The numbers of the amino acid residues per subunit calculated according to the *mcm method* agree well with those determined by the *factor method*. This indicates the assumed molecular weight of 288000 - 289000 rather than a smaller molecular weight.

In a comparison with the amino acid composition of the subunit of hexameric mouse liver porphobilinogen synthase[37], which with about 274 amino acids is substantially shorter than the ox liver enzyme with about 306 amino acids, agreement can be found not only for the aromatic amino acids Phe, Tyr and His, but also for some neutral, acidic and basic amino acids (Gly, Met, Ile, Leu, Glu and Lys). Different experimental findings[35,38] substantiate the assumption that besides cysteine, the two amino acids histidine and lysine are functional amino acids of considerable importance for the enzymic activity of porphobilinogen synthase. For one thing, after histidine blocking with diethyl pyrocarbonate or photo-oxidation, the enzyme activity is lowered according to pseudo-first order kinetics[38]; secondly, the enzymic condensation of 5-aminolevulinate to porphobilinogen necessitates an enzyme-substrate binding, by which the carbonyl group of the 5-aminolevulinate forms a Schiff's base with the ϵ-amino group of a lysine residue[3]. By reduction of the Schiff's base with sodium borohydride[35] it could be shown that only 4 of the 8 subunits bind the substrate (5-aminolevulinate), and that the enzyme exhibits either half-site reactivity or negative cooperativity.

On the basis of investigations on porphobilinogen synthase from ox liver and soy bean callus concerning the reactivity[36,39] of the sulfhydryl groups, these can be divided into three groups $SH_I > SH_{II} > SH_{III}$. SH_I and SH_{II} groups should lie at the protein surface, whereby SH_I groups should be primarily responsible for the enzyme's main activity. SH_{III} groups are demasked only after unfolding by means of 8M urea.

The total number of sulfhydryl groups was determined from the total amino acid analysis after 20-h hydrolysis by quantitative determination of the *S*-aminoethylcysteine from the aminoethylated enzyme subunit (see Table 1), as well as from the cysteic acid after oxidation of the native enzyme with performic acid. According to this, ox liver porphobilinogen synthase contains 56 cysteine residues per molecule or 7 cysteine residues per subunit.

Table 1. Quantitative amino acid analysis of the native enzyme after performic acid oxidation and of the aminoethylated enzyme subunit after 20-h and 200-h hydrolysis, respectively.

In the columns 2, 3, 6 and 8 the amino acid values found are listed in nmol or in mol-%, respectively. In columns 4, 5, 7 and 9 the amino acid residues per subunit as calculated according to the "factor method", *F*, or the "mean common multiple method", mcm, are given. The mcm value (mcm ≃ 6.3) is determined from the nanomolar amounts of the following amino acid pairs: Asp-Gly, Pro-Gly, Val-Gly, Ala-Leu, Met-Ile, Tyr-Cys($C_2H_4NH_2$), Tyr-His, Lys-Cys($C_2H_4NH_2$), Lys-Arg and Lys-His. See text for definitions of the factor, and of the mcm value.

Amino acid	20-h hydrolysis						200-h hydrolysis	
			Residues per subunit		Performic acid oxidation			Residues per subunit
						Residues per subunit		
	[nmol]	[mol-%]	*F*	mcm	[nmol]	*F*	[nmol]	*F*
Asp (Asx)	148.3	7.62	23.4	23.5	67.8	25.1	68.8	24.1
Thr	43.4	2.23	6.8	6.9	18.3	6.9	18.2	6.4
Ser	151.0	7.76	23.7	24.0	61.8	23.3		
Glu (Glx)	183.8	9.44	28.9	29.2	82.2	30.9	86.8	30.2
Pro	144.0	7.40	22.7	22.9	57.8	21.7		
Gly	137.6	7.07	21.6	21.8	62.9	23.6	64.8	22.7
Ala	232.3	11.94	36.6	36.9	95.3	35.8	105.9	37.0
Val	149.3	7.67	23.4	23.7	66.4	25.0	74.1	25.9
Met	44.8	2.30	7.0	7.1				
Met sulfone					19.5	7.3		
Ile	59.0	3.03	9.3	9.4	24.5	9.2	27.2	9.5
Leu	214.2	11.0	33.7	34.0	92.1	34.6	98.0	34.2
Tyr	64.1	3.29	10.1	10.2				
Phe	72.4	3.72	11.4	11.5	31.9	12.0	34.2	12.0
Lys	73.5	3.78	11.6	11.7	31.7	11.9		
	50.0[a]		13.6[a]					
Cysteic acid					20.2	7.6		
Cys($C_2H_4NH_2$)	40.0	2.06	6.3	6.3				
	27.0[a]		7.4[a]					
His	41.5	2.13	6.6	6.6	22.6	8.5		
	29.6[a]		8.1[a]					
Arg	139.0	7.14	21.9	22.1	58.0	21.8		
Trp	8.0	0.41	1.3	1.3				
	5.5[a]		1.5[a]					

[a] values from a tryptophan analysis of the aminoethylated enzyme subunit.

The free sulfhydryl groups were determined with the very sensitive 4,4′-bis(dimethylamino)-diphenylmethanol[40], as about 10 μmol-SH is required by the standard technique. In the presence of 6M guanidine hydrochloride (sequanal grade) at a pH of 5.1, the sulfhydryl groups react with the carbonium-immonium cation, this being resonance stabilized in solution, and form *S*-4,4′-bis(dimethylamino)diphenylmethyl derivatives with a concomitant quantitative loss of the 614-nm absorbance. On the basis of the decrease in absorbance we obtained for the porphobilinogen synthase 16 free sulfhydryl groups per mol, or 2 free sulfhydryl groups for each monomeric peptide chain.

Determination of the ultimate *N*-terminal amino acid revealed that in analogy with the mouse liver enzyme,[37] the *N*-terminus of ox liver porphobilinogen synthase is blocked. In order to determine the nature of the blocking (*N*-acetyl-, *N*-formyl-, *N*-pyroglutamyl) groups we subjected the aminoethylated subunit to hydrazinolysis

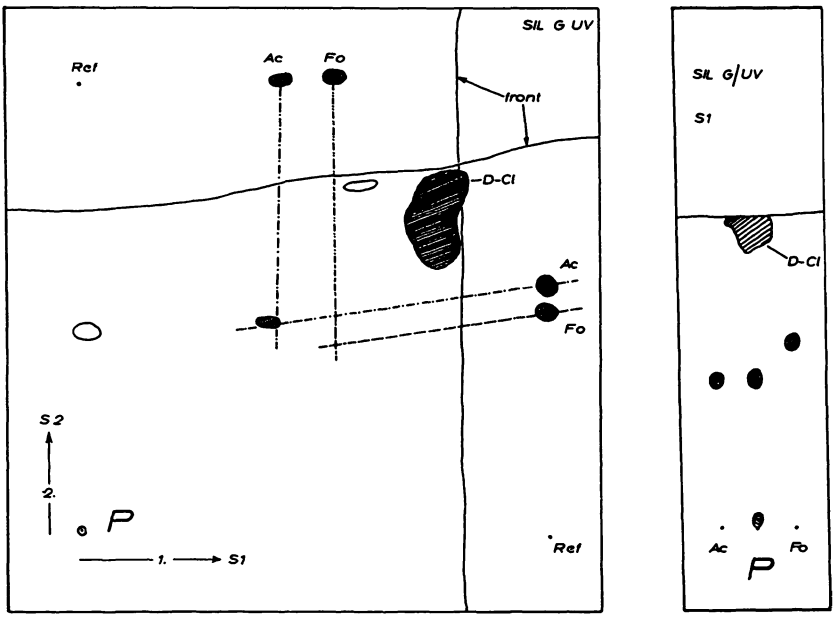

Fig. 1. Two- and one-dimensional thin-layer chromatography (from left to right) of the dansylated acyl hydrazide (P) derived from porphobilinogen synthase after hydrazinolysis. Layer: silica gel (SIL G/UV; 20 x 20 cm and 5 x 20 cm, respectively). Solvent systems were S 1 (1st dimension) and S 2 (2nd dimension as described in Methods and Materials.

Abbreviations:

Ref = start of the references; Fo = 1-formyl-2-dansylhydrazine;
Ac = 1-acetyl-2-dansylhydrazine; D-Cl = dansyl chloride.

[41-43], labelled the acylhydrazide obtained with dansyl chloride[44] at pH 3 and were able to prove, by comparison with synthetic 1-acetyl-2-dansylhydrazine and 1-formyl-2-dansylhydrazine, using one- and two-dimensional thin-layer chromatography on silica gel layers, as well as on Nano-Plates, that the *N*-terminus is *acetylated* (see Figs. 1 and 2). Controls were carried out on *N*-formylated and *N*-acetylated amino acids and on trypsin, which has no acyl group[45].

We obtained dansylated hydrazides of formic acid and of acetic acid by hydrazinolysis of their alkyl esters and subsequent dansylation at pH 3. When excess hydrazine is completely removed they are stable, contrary to the statements of other authors[41,42].

N-Terminal acylation or acetylation seems to be characteristic for animal porphobilinogen synthase; this could be demonstrated by us for this enzyme from the livers of other animals*.

Methods and Materials

All solvents and chemicals were of analytical grade; acetone for preparation of the acetone powder was of practical grade; urea, guanidine hydrochloride (both of sequanal grade), anhydrous hydrazine and 4,4'-bis(dimethylamino)diphenylmethanol were from Pierce (Pierce Eurochemie B.V., NL-Rotterdam); reference proteins (cytochrome *c*; bovine serum albumin; yeast alcohol dehydrogenase; rabbit muscle pyruvate kinase; bovine liver glutamate dehydrogenase; trypsin; carboxypeptidase A; ovalbumin) were from Boehringer Mann-

* Kreutzer, M., Schmidt, M., Stadler, E. & Zeitler, H.-J. (1977), in preparation.

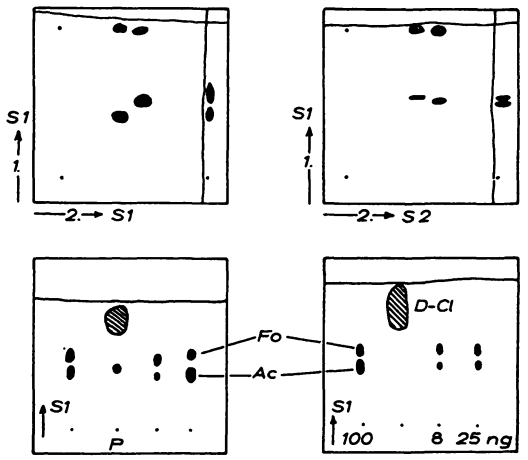

Fig. 2. Micro-thin-layer chromatography of the dansylated acyl hydrazide (P) derived from hydrazinolyzed bovine porphobilinogen synthase.

The upper pictures show the two-dimensional thin-layer chromatography of the dansylated reference hydrazides in the same solvent (S 1) and in two different solvents (S 1; S 2), respectively (from left to right).

The lower pictures show the one-dimensional cochromatography of the dansylated acyl hydrazide (P) with the references and the thin-layer chromatography of the reference substances at different concentrations in the ng range (from left to right).

Abbreviations as in Fig. 1.

Plates: 5 x 5 cm Nano-Plates SIL-20 for thin-layer chromatography.

heim GmbH, D-8132 Tutzing/Obb.; ethylene imine (azirane) and dansyl chloride were from Fluka, D-7910 Neu-Ulm; water was glass-bidistilled in vacuo (Schott Bidistillator, Art. 24 733). The pH of buffers was determined with a Knick pH meter, Type 350.

Column chromatography was carried out at 5 °C in a LKB Combi-ColdRac in combination with a LKB Uvicord III, an LKB Fraction Collector and an LKB Gradient Mixer Ultrograd.

The gels for column chromatography were from Deutsche Pharmacia GmbH, D-7800 Freiburg (Sephadex) and from LKB Instrument GmbH, D-8032 Lochham bei München (Ultrogel). Thin-layer chromatography plates (Polygram plastic sheets; Nano-Plates) were purchased from Macherey & Nagel, D-5160 Düren. Thin-layer gel-filtration was carried out with the Multi-Separation-Chamber DCZ-5 (DBGM according to Dr. Zeitler), H. Hölzl, D-8250 Dorfen.

Polyacrylamide electrophoresis was done with a Buchler Polyanalyst, U.S.A.-Searle Inc.

Melting points (Leitz Mikroskopheiztisch, type 350) have not been corrected.

Infrared und ultraviolet spectra were obtained by a recording infrared (ultraviolet) spectrophotometer Philips-Unicam SP. 200 G (SP. 800 B).

Enzyme purification

Coarsely-cut pieces of fresh liver (ca. 4 kg) were minced together with the 5-fold volume cold acetone in a mixer and the mixture then filtered on a Büchner suction filter. The residue was stirred twice on the filter with the 4-fold volume of acetone and after removal of the acetone was air-dried. At a temperature of 4 °C, the acetone dry powder (ca. 1200 g) was extracted with the 10-fold volume of 0.025M potassium phosphate buffer, pH 6.8, containing 0.01M 2-mercaptoethanol (buffer I) for 8 h (mechanical stirrer), and the supernatant obtained by 20 min centrifuging at 33000 x *g* (crude extract; spec. act. see Table 1) was fractionated by addition of solid ammonium sulfate. The material precipitating at 35% ammonium sulfate saturation was discarded. The protein fraction (fraction 1) obtained between 35 and 50% ammonium sulfate saturation was centrifuged down, dissolved in buffer I (1 m*l* buffer I per 1 g acetone powder extract) and heated for 2 min to 72 - 75 °C. The coagulum was centrifuged off and the supernatant brought to 70% saturation with solid ammonium sulfate. The protein fraction centrifuged off (fraction 2) was dissolved in buffer I and then dialysed against buffer I; the dialysate, concentrated in an Amicon ultrafiltration cell, was fractionated by column chromatography on *DEAE-Sephadex A-50* (column dimension 25 x 600 mm; flow rate 20 ml/h; 10 m*l* fractions). A non-linear 0.4M KCl gradient* was superimposed on the elution buffer (buffer I). The fraction with porphobilinogen synthase activity (fraction 3) was re-chromatographed on *CM-Sephadex C-50*, (column dimension 25 x 600 mm, flow rate 20 ml/h; 10-m*l* fractions) using a linear ionic strength and pH gradient (0.01M potassium phosphate buffer, pH 6.2, as equilibrium buffer; 0.01M potassium phosphate buffer, pH 7.2, containing 0.2M KCl, as elution buffer). The column chromatography of the fraction containing the porphobilinogen synthase (fraction 4) on *Ultrogel AcA 22* (column dimension 25 x 1000 mm; flow rate 12 ml/h; 10-m*l* fractions) with buffer I resulted finally in a preparation which was uniform in polyacrylamide disc electrophoresis (fraction 5). With a purification factor of 405 (see Table 2), the yield was 25% of the initial activity.

* Kreutzer, M., Schmidt, M., Stadler, E. & Zeitler, H.-J., in preparation.

Table 2. Specific activity of porphobilinogen synthase at different steps of purification.

Step	Spec. act.		Purif. factor
	[mU/mg]	[nkat/mg]	
Crude extract	0.7	0.012	1
35 - 50% Ammonium sulfate fraction	2.0	0.033	3
Heat-treated fraction	17.7	0.29	25
DEAE-Sephadex fraction	117	1.94	166
CM-Sephadex fraction	242	4.03	352
Ultrogel AcA 22 fraction	283	4.72	405

Determination of the specific enzyme activity

The specific activity of the porphobilinogen synthase was obtained from the determination of the porphobilinogen[46] formed from 5-aminolevulinate and the protein quantity[47,48]. 1 U/mg protein = 16.67 nkat/mg protein = 1 μmol porphobilinogen/ (min x mg protein).

Reductive aminoethylation

50 mg of native enzyme was reduced for 5 h at room temperature with 10 ml of a solution containing 0.1M dithiothreitol, 0.01M EDTA (disodium salt), 8M urea and 2M Tris/HCl buffer, pH 8.6. In a nitrogen atmosphere three portions each of 0.2 ml ethylene imine (azirane) were added under stirring, at intervals of 10 min. After 30 min reaction time, the pH was brought to 5 with glacial acetic acid, the reaction mixture dialysed against water and against 0.1N acetic acid, the dialysate lyophilised and purified by gel-filtration over Sephadex G-100 using 1N acetic acid as eluant (column dimension 25 x 1000 mm; flow rate 12 ml/h; 3 ml fractions).

Molecular weight determination

a) *Native enzyme.* 10 g Sephadex G-200 superfine was swelled in 1000 ml 0.2M sodium phosphate buffer, pH 6.6, for 72 h at room temperature with stirring (magnetic stirrer). The gel suspension was strained through a filter until the suspension no longer ran off a tilted glass plate. Using a spreading apparatus (Desaga, D-6900 Heidelberg) gel layers of 0.5 mm thickness were spread onto glass plates 20 x 20 cm, an approximately 1 cm-broad margin being removed from the long sides of each gel-plate with a glass sheet, in order to avoid edge effects. The gel suspension obtained from the above quantity was enough for about 6 plates.

The coated gel plates, on whose undersides the starting positions were marked with a felt pen, were laid on the horizontally-positioned bearing surface of a multi-separation chamber[49] developed by us and were covered at the top and bottom ends with strips of filter paper soaked in buffer (e.g. Macherey & Nagel, 2214 ff; Whatman 3 MM). The strips of paper, which reach to the bottom of the eluant trough, should cover about 1.5 - 2 cm of the gel layer. After laying on a cover plate (20 x 20 cm glass plate, provided on its long sides with slit silicon rubber tubings or Camag Sandwich Cover Plate with permanently-fixed glass spacers) the gel layer was equilibrated with 0.2M sodium phosphate buffer, pH 6.6, for 12 h, the "sandwich" being tilted at an angle of about 25°. The proteins were dissolved in 0.2M sodium phosphate buffer, pH 6.6 (concentration: 0.5 - 1 mg/ml buffer); to every protein sample the same quantity of cytochrome *c* was added as indicator protein. The reference proteins and the sample solution (5 - 10 μg in 1 μl) were spotted onto the gel layer in a horizontal position; the gel filtration was carried out at a tilting angle of about 20 - 25° over a period of about 4 - 7 h. After the gel filtration the cover plate and the paper strips were removed, and, with the glass plate horizontally positioned, a sheet of filter paper 20 x 20 cm was laid over the gel layer in order to obtain a paper replica. After 3 - 5 min the replica, with the gel side uppermost, was dried for 15 min at 80 - 90 °C in a drying oven, treated for 5 min with a 12.5% aqueous solution of trichloroacetic acid, stained in a 0.25% aqueous solution of Coomassie Brilliant Blue G-250 for about 30 min and subsequently rinsed in three successive rinsing baths (7 - 10% aqueous acetic acid, about 30 min each).

The time needed for staining and de-staining can be considerably shortened by using hot solutions. The method of protein staining with Coomassie Brilliant Blue on a paper replica is considerably more sensitive than that of the protein reaction with an alkaline solution of diazotized sulfanilic acid.

The distance moved by the proteins was divided by the distance moved by the cytochrome *c*, and the $R_{cyt\ c}$ values so obtained were plotted against the logarithm of the molecular weights of the standards. The molecular weight of the unknown protein sample was obtained directly from its $R_{cyt\ c}$ value and the calibration line (see Fig. 3).

b) *Porphobilinogen synthase subunit*

(1) *Acrylamide solution.* 22.2 g acrylamide, 0.6 g *N,N'*-methylene-bis(acrylamide), distilled water ad 100 ml.

(2) *Gel buffer.* 0.2M sodium phosphate buffer, pH 7.0, 0.2% of sodium dodecylsulfate.

(3) *7.5% acrylamide gel.* 15.8 ml acrylamide solution (1), 22.5 ml gel buffer (2), 5.5 ml distilled water, 0.07 ml tetramethylethylenediamine, 1.5 ml of an aqueous 2% ammonium peroxydisulfate solution.

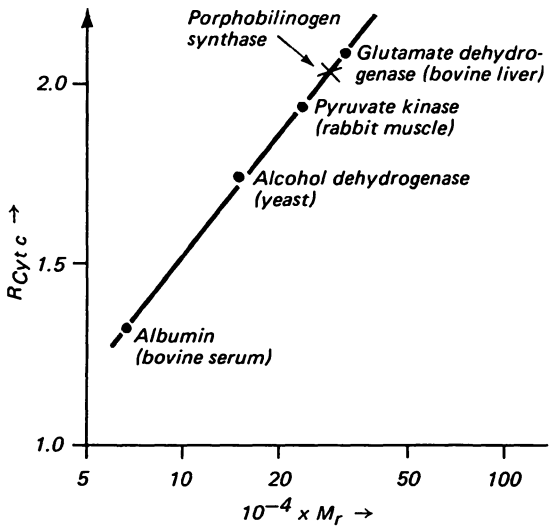

Fig. 3. Thin-layer gel filtration of native bovine porphobilinogen synthase and proteins with well characterized molecular weights (cytochrome *c* 12500; bovine serum albumin 67000; yeast alcohol dehydrogenase 150000; rabbit muscle pyruvate kinase 237000; bovine liver glutamate dehydrogenase 320000) on Sephadex G-200 superfine.

(4) *Electrophoresis buffer.* 1 part gel buffer (2) and 1 part distilled water.
(5) *Sample buffer.* 0.01M sodium phosphate buffer, pH 7.0, containing 1% sodium dodecylsulfate.
(6) *Reference proteins.* cytochrome *c* (M_r 12500), trypsin (M_r 23300), carboxypeptidase A (M_r 34400), ovalbumin (M_r 45000), bovine serum albumin (M_r 67000).

0.1 mg each of the reference proteins, of the native and of the aminoethylated subunit were dissolved in 0.3 m*l* buffer (5) and after addition of 10 μl 2-mercaptoethanol, heated for some minutes in a boiling water bath. After cooling, 1 drop of glycerol and 10 μl of a 0.05% aqueous solution of bromophenolblue was added to the reaction mixture and 50 μl of this solution was used for the dodecylsulfate electrophoresis. After 4 h electrophoresis (21 °C; 8 mA per gel tube), the gel was stained with a 0.25% aqueous solution of Coomassie Brilliant Blue G-250, and then excess stain removed with 7% aqueous acetic acid. The molecular weight was determined by means of the calibration curve (ordinate: electrophoretic distance moved in mm; abscissa: logarithm of the molecular weights).

For the polyacrylamide gel electrophoresis we observed that the sodium dodecylsulfate used must be chemically pure (purity > 99%). Dodecylsulfate of lower purity (e.g. from Merck-Schuchardt, Art. No. 822 050) contains by-products with which homogeneous proteins (e.g. reference proteins) are cleaved into chains of different length during the electrophoresis.

Amino acid analysis

1) The peptides were hydrolysed for 20 and 200 h, respectively, with 6N HCl at 110 °C and the amino acids separated with a Beckman Amino Acid Analyzer, model 121, in combination with a Beckman Chromocomp. For the determination of tryptophan, 6% thioglycolic acid was added to the hydrolysis mixture. Cysteine and methionine were additionally determined after oxidation with performic acid[50] as cysteic acid and as methionine sulfone, respectively.

2) *Factor-method.* The mean number, $\bar{A}_S$, of amino acids per subunit was obtained through division of the subunit molecular weight (M_r = 289000/8 = 36125) by a mean molecular weight of the amino acids ($\simeq$ 118). A subunit chain length of about 306 amino acids results. The factor, *F*, is calculated from the quotient of the number of amino acids per peptide chain, $\bar{A}_S$, and the sum of the molar amounts of all amino acids ($F = \bar{A}_S / \Sigma$ mol) known from the amino acid analysis.

3) *mcm-method.* Since the amino acids in a peptide exhibit a whole number relation to one another, a *mean common multiple (mcm)* can be obtained from the molar values. Similarly, the number of amino acid residues in a peptide can be obtained from the quotients of the individual values and the mcm-value.

Determination of the sulfhydryl groups

4,4'-Bis(dimethylamino)diphenylmethanol stock solution: 5.019 mg of the reagent per 10.00 m*l* acetone.
Acetate buffer: 0.04M sodium acetate buffer, pH 5.1, containing 6M guanidine hydrochloride.

The carbonium-immonium cation of the reagent under the conditions used has an absorption maximum at 614 nm and an apparent[40] molar absorption coefficient of 100000 ($M^{-1} \times cm^{-1}$).

1.742 mg native enzyme free of 2-mercaptoethanol was dissolved in 1 m*l* acetate buffer. To five 2-m*l* measuring flasks, each with 10 μl of the reagent stock solution, 20, 30, 40, 50 or 60 μl of the enzyme solution was added, the measuring flasks were made up to 2.0 m*l* with acetate buffer, and after 30 min the absorbance was read at 614 nm.

Synthesis of the N-acyl hydrazides and their dansyl derivatives

a) *Formohydrazide* (formylhydrazine; CH_4N_2O; M_r = 60.05).

A mixture of 30 ml (ca. 0.6 mol) of 100% hydrazinium hydroxide and of 50 ml of absolute ethanol was placed in a 250 ml round-bottom flask fitted with a reflux condenser. The solution was heated in a waterbath to 70 - 80 °C, slowly mixed with 24 ml (ca. 0.4 mol) methyl formate, and stirred for 2 h at this temperature. After cooling to room temperature, excess hydrazinium hydroxide was distilled off in vacuo using a rotation evaporator at a temperature of about 50 °C (waterbath temperature). The process was then repeated after addition of 50 ml methyl formate to the liquid residue. The pink residue solidified to large crystals immediately after cooling. The yield was 21 g (87.5%). 13.1 g of formohydrazide, having been dried overnight in a vacuum desiccator over concentrated sulfuric acid, was recrystallized twice from a boiling mixture (10 - 12 fold-volume) of absolute ethanol/chloroform/petroleum ether (2:10:3) and the crystals air-dried (yield 8.66 g). The first, pink-coloured mother liquor was discarded. From the second, colourless mother liquor a further quantity of hydrazide (yield 2.01 g) was obtained by addition of the same volume of petroleum ether (b.p. 60 - 80 °C). The total yield was 10.67 g (81.4%).

Formic acid hydrazide can be further recrystallized with a 95% yield from the 4-fold volume of chloroform/petroleum ether (3:1); m.p. 55 - 57 °C. Crystallization is best induced by stirring while the hot solution cools and then seeding at a temperature below 50 °C. The crystals obtained were long, colourless needles or pearly-shining leaflets.

b) *Acetohydrazide* (acetylhydrazine; $C_2H_6N_2O$; M_r = 74.08).

Using 100% hydrazinium hydroxide in absolute ethanol, we obtained with ethyl acetate, in analogous way, colourless acetohydrazide in a 93% yield, m.p. 62 - 67 °C. 32.8 g dried acetohydrazide was recrystallized from the 7-fold volume chloroform/petroleum ether (16:7), yield 25.8 g. From the mother liquor we obtained, by addition of the same volume of petroleum ether, a further 2.4 g hydrazide. The total yield was 28.2 g (86%). The long, colourless needles were first air-dried, then stored in a vacuum desiccator for 12 h over concentrated sulfuric acid; m.p. 65 - 67 °C.

c) *1-Formyl-2-dansylhydrazine* ($C_{13}H_{15}N_3SO_3$; M_r = 293.34).

2 g dansyl chloride and 2 g powdered formohydrazide were dissolved in 25 ml of ethyl acetate and stirred for 2 h (magnetic stirrer) at room temperature. The precipitated free sulfonic acid was filtered off, washed with a few milliliters of cold ethyl acetate and discarded. The combined filtrates were extracted with 10 ml of 0.2M citrate buffer, pH 3.1. The organic layer was concentrated to 15 ml, filtered, and the filtrate evaporated to dryness in vacuo (rotation evaporator) at room temperature. The oily residue was stored in a vacuum desiccator over conc. sulfuric acid and after two days the yellow solid was powdered. The amorphous 1-formyl-2-dansylhydrazine was homogeneous in thin-layer chromatography and had a m.p. (decomp.) 52 - 54 °C.

d) *1-Acetyl-2-dansylhydrazine* ($C_{14}H_{17}N_3SO_3$; M_r = 307.37).

The formerly described procedure was also applied for the preparation of the acetyl derivative with the difference that the ethyl acetate solution was allowed to evaporate overnight after extraction with citrate buffer. The long yellow needles had a m.p. 185 - 186 °C after melting at 176 °C with a rapid recrystallization at 178 °C (phase transformation?).

Table 3. Ultraviolet spectra of the dansylated and non-dansylated hydrazides of acetic acid and formic acid.

Solvent: ca. 95% ethanol for fluorescence spectroscopy (Uvasol, Merck Art. 1002).

	λ_{max} [nm]	ϵ [$M^{-1} \times cm^{-1}$]
Acetohydrazide	210	1.596
Formohydrazide	212	1.832
1-Acetyl-2-dansyl-hydrazine	217 252 337	45.556 20.247 5.062
1-Formyl-2-dansyl-hydrazine	217 252 337	43.673 17.588 6.708

Hydrazinolysis of porphobilinogen synthase and of N-acylated amino acids

a) 1.5 mg each of lyophilized aminoethylated subunit and of trypsin were treated with 0.5 ml of 0.1N HCl in a Pierce vacuum hydrolysis tube, dried in vacuo to remove any traces of acetic acid or propionic acid, and heated with 0.5 ml of anhydrous hydrazine for 24 h at 100 °C in an oil bath. After cooling to ambient temperature and removal of excess hydrazine in vacuo, the residue was taken up in 0.5 - 1 ml of 0.2M sodium citrate buffer, pH 3.1, and mixed with a solution of 0.5 mg (forty-five-fold excess) dansyl chloride in 1 - 2 ml of absolute ethanol. The clear reaction mixture was incubated at 37 °C for 12 h and the solvents were evaporated in vacuo. The residue was dissolved in 0.5 ml of bi-distilled water and extracted three times with 1 ml of chloroform (analytical grade). The combined chloroform extracts were concentrated to about 0.3 ml by a stream of nitrogen. For one- and two-dimensional thin-layer chromatography, 1, 5 and 25 µl of the chloroform

solution were spotted on 5 x 20 cm and 20 x 20 cm plates with 0.25 mm silica gel without gypsum (precoated plastic sheets; Polygram Sil G or Sil G/UV), cochromatographed with the references, and the fluorescent spots located under ultraviolet light (254 nm; 360 nm). Thin-layer chromatography on Polygram sil N-HR was not successful.

In micro thin-layer chromatography, 1, 2 and 5 µl of the chloroform solution of the dansylated acyl hydrazides were spotted on 5 x 5 cm "Nano-Plates SIL-20 for thin-layer chromatography" (layer: 0.20 mm silica gel 60 without fluorescent indicator).

In two-dimensional thin-layer chromatography, the references were spotted on the edge of the plate before each run; before the second run the plates were activated at 100 °C for 10 min.

For developing the chromatograms, the following solvent systems were used:

(S 1) chloroform/ 1-butanol/ acetic acid (6 : 3 : 1)
(S 2) ethyl acetate/ 2-propanol/ conc. ammonia (9:7:4).

The R_F-values are rather dependent on the activity of the silica gel, the thin-layer chromatography chamber, the degree of vapor saturation and the age of the solvent mixture.

b) *N*-Acetylleucine and N^{α}-acetylhistidine were synthesized from the amino acids with acetic anhydride[51]. The hydrazinolysis of the *N*-acylated amino acids and the identification of the 1-acyl-2-dansylhydrazides were analogous to the procedure described for the protein hydrozinolysis.

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