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Purification and properties of 5-aminolaevulinate dehydratase from human erythrocytes

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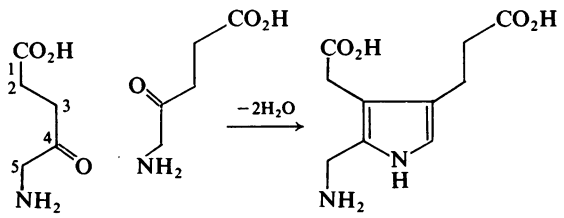
1. A new procedure for the isolation of homogeneous human 5-aminolaevulinate dehydratase (porphobilinogen synthase, EC 4.2.1.24) is described in which the enzyme is purified 35000-fold and in 65–74% yield. 2. The specific activity of the purified enzyme, 24 units/mg, is the highest yet reported. 3. An efficient stage for the removal of haemoglobin is incorporated in the method, which has general application to the purification of other erythrocyte enzymes. 4. The erythrocyte dehydratase (M_r 285000) is made up of eight apparently identical subunits of M_r 35000. 5. The enzyme is sensitive to oxygen, and its activity is maintained by the presence of thiols such as dithioerythritol. 6. Zn^{2+} is obligatory for enzyme activity, the apoenzyme being essentially inactive ($\approx$12% of control) when assayed in buffers devoid of Zn^{2+}. Addition of Zn^{2+} to the apoenzyme restores activity as long as the sensitive thiol groups are fully reduced; optimal stimulation occurs between 100 and 300 μM-Zn^{2+}. 7. The human enzyme is inhibited by Pb^{2+} in a non-competitive fashion [K_{iI} (dissociation constant for E·S·Pb^{2+} complex) = 25.3 $\pm$ 3.0 μM; K_{iS} (dissociation constant for E·Pb^{2+} complex) = 9.0 $\pm$ 2.0 μM]. 8. Modification of thiol groups, inactivation by oxidation, alkylation or reaction with thiophilic reagents demonstrates the importance of sensitive thiol groups for full enzymic activity.

5-Aminolaevulinate dehydratase (porphobilinogen synthase; EC 4.2.1.24) catalyses the conversion of 5-aminolaevulinate into porphobilinogen, the monopyrrolic precursor of haems, chlorophylls and corrins (Battersby & McDonald, 1975; Akhtar & Jordan, 1978).



The enzyme was first isolated and purified from bovine liver by Gibson *et al.* (1955). Since then the enzyme has been shown to be ubiquitous in nature and has been characterized from a wide variety of sources (Coleman, 1966; Nandi *et al.*, 1968; Shemin, 1972; Anderson & Desnick, 1979; Liedgens *et al.*, 1980).

Mammalian 5-aminolaevulinate dehydratases fall within the M_r range of 260000–285000 and appear to be composed of eight identical subunits of M_r 35000 arranged into a cubic octameric structure with dihedral (D_4) symmetry (Wu *et al.*, 1974; Despaux *et al.*, 1979). In contrast with the bacterial dehydratases, which have alkaline pH optima, the mammalian enzymes have pH optima from 6.3 to 7.1 and require Zn^{2+} for maximal catalytic activity (Shemin, 1972; Cheh & Neilands, 1976).

One common feature of all dehydratases is their remarkable sensitivity to oxygen. This sensitivity is associated with the presence of highly reactive cysteine residues, which are required for both activity and stability (Tsukamuto *et al.*, 1979; Barnard *et al.*, 1977; Seehra *et al.*, 1981). These groups may be maintained in their reduced state by the presence of a high concentration (10–20 mM) of an activating thiol (Shemin, 1976) such as 2-mercaptoethanol, dithiothreitol or dithioerythritol. Other amino acids have been shown to play an importance role in the activity of bovine 5-

Abbreviation used: SDS, sodium dodecyl sulphate.

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aminolaevulinate dehydratase, the most significant being histidine (Tsukamoto *et al.*, 1975) and lysine (Nandi, 1978).

Our current studies on the structure of the human enzyme necessitated the isolation of larger quantities of protein than could be provided by existing methods in the literature (Anderson & Desnick, 1979). Accordingly, a new method was developed for the isolation of 100mg quantities of the human enzyme. The present paper outlines this procedure, which incorporates a highly efficient step for the removal of haemoglobin during the initial stages of purification.

Experimental

Materials

5-Aminolaevulinate hydrochloride and dithioerythritol were obtained from Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K. DEAE-Bio-Gel A and Bio-Gel A 0.5m were purchased from Bio-Rad Laboratories, Watford, Herts., U.K. $(NH_4)_2SO_4$ (especially low in heavy metals), 4-dimethylaminobenzaldehyde (AnalaR grade) and all other basic laboratory reagents were obtained from BDH, Poole, Dorset, U.K.

Methods

Purification of 5-aminolaevulinate dehydratase from human erythrocytes is described in detail in the Results section.

Activation of human 5-aminolaevulinate dehydratase. 5-Aminolaevulinate dehydratase was always subjected to an 'activation' procedure immediately before use involving incubation with an excess of reducing agent and exogenous Zn^{2+} ions. The stored enzyme was dissolved in 0.1M-potassium phosphate buffer, pH6.8, containing 10mM-dithioerythritol and 100μM-$ZnCl_2$ and incubated at 37°C for 10min. The enzyme thus activated was desalted under an N_2 atmosphere on a column of Sephadex G-50 equilibrated at 0°C in 0.1M-potassium phosphate buffer and stored at 0°C under N_2 until required.

Assay for enzymic activity. The incubation mixtures contained potassium phosphate buffer, pH6.8, (100μmol), dithioerythritol (10μmol), activated 5-aminolaevulinate dehydratase (max. 0.015 unit) and 5-aminolaevulinate (5μmol), neutralized with 0.1M-NaOH before use, in a final volume of 1ml. The incubations were carried out at 37°C for 10min and were terminated by the addition of a solution (1ml) of trichloroacetic acid (10%, w/v) containing $HgCl_2$ (0.1M). The solution was centrifuged and the supernatant was added to an equal volume of freshly prepared modified Ehrlich's reagent (4-dimethylaminobenzaldehyde), (1g) in acetic acid (40ml) and $HClO_4$ (60–62%, w/v; 10ml). The coloured complex formed with porphobilinogen was measured spectrophotometrically ($\lambda_{max.}$ 555nm; ε_{555} 60200 litre·mol^{-1}·cm^{-1}) (Mauzerall & Granick, 1956).

One unit of enzyme activity produces 1μmol of porphobilinogen/h at 37°C under the above conditions. Specific activity was calculated after the determination of protein by the methods of Lowry *et al.* (1951) or Bradford (1976), with bovine serum albumen as standard, or by measuring A_{280} (ε_{280} 28570 litre·mol^{-1}·cm^{-1}).

The effect of inhibitors was determined by incorporating them into the assay mixture. Inactivating agents were diluted out or removed by rapid gel filtration by the method of Seehra & Jordan (1981) before assay for enzyme activity.

When the enzyme activity was assayed in the absence of thiol, the incubations were carried out as described above except that all buffers were saturated with N_2 before use. Incubations were carried out in stoppered tubes for 2min under an atmosphere of N_2 with up to 0.05 unit of enzyme.

Detection of 5-aminolaevulinate dehydratase activity in gels after polyacrylamide-gel electrophoresis was accomplished by incubating the gel for 30min at 37°C in 5ml of 0.1M-potassium phosphate buffer, pH6.8, containing 5mM-5-aminolaevulinate and 20mM-2-mercaptoethanol. The porphobilinogen produced during the incubation was detected by the addition of 2.5ml of 0.1M-$HgCl_2$ containing 10% trichloroacetic acid, followed by 2.5ml of modified Ehrlich's reagent (Mauzerall & Granick, 1956).

Polyacrylamide-gel electrophoresis. Polyacrylamide gels (5%) were prepared from 3ml of solution A (acrylamide, 300g/litre; methylenebisacrylamide, 8.2g/litre), 2.25ml of solution B (Tris, 363g/litre; *NNN'N'*-tetramethylethylenediamine, 4.6ml/litre; adjusted to pH8.9 with 1M-HCl) and 12.75ml of water containing ammonium persulphate (15mg). The gels were cast in tubes of internal diameter 6.3mm and of length 12cm. Bromophenol Blue was used as a tracker dye, and electrophoresis of the protein samples (5–100μg) was carried out for 4–5h (5mA/gel). The reservoir buffer contained Tris (2.89g/litre) adjusted to pH8.3 with 1M-HCl. Polyacrylamide-gel electrophoresis in the presence of SDS (0.1% w/v) was carried out by the method of Weber & Osborn (1969). Both types of gels were stained with Coomassie Brilliant Blue as described in this method.

Isoelectric focusing. Isoelectric focusing of the purified dehydratase enzyme was performed on LKB Ampholine polyacrylamide-gel plates (pH3.5–9.5) by using a LKB Multiphor apparatus, according to the manufacturer's instructions. Protein samples (15μl) were applied on sample application papers (5mm × 10mm) and electro-

focused for 1.5h. The power was supplied with an LKB 2103 power pack set at 15W (limited to 1500V and 50mA). After focusing, the gel was fixed and stained according to the manufacturer's instructions. After destaining for 24h in ethanol/acetic acid/water (25:8:67, by vol.), the plate was placed in preserving solution (40ml of glycerol in 400ml of the destaining solution) and dried.

Determination of the molecular weight of human erythrocyte 5-aminolaevulinate dehydratase. (*a*) Gel filtration on Bio-Gel A 0.5 m. The M_r of the native human enzyme was determined by applying 7mg of enzyme (200nmol; 1ml) to a Bio-Gel A 0.5m gel-filtration column and developing at a flow rate of 0.75ml/min in 60mM-potassium phosphate buffer, pH6.8, containing 20mM-2-mercaptoethanol and 0.5M-KCl. The elution volume (V_e) of the enzyme was compared with those of proteins of known M_r.

(*b*) SDS-polyacrylamide-gel electrophoresis. The subunit M_r of the human dehydratase enzyme was determined by polyacrylamide-gel electrophoresis (15%, w/v, acrylamide gels) under denaturing conditions (6M-urea and 1%, w/v, SDS) by the method of Weber & Osborn (1969). The relative mobility (R_F) of the enzyme was compared with the mobilities of standard proteins of known M_r.

The M_r standards used were: urease (479000), catalase (252000), muscle aldolase (158,000), lactate dehydrogenase (140000), bovine serum albumin (68000), opsin (40000), trypsin (23300) and cytochrome *c* (11700).

Oxidation of the human enzyme. The human dehydratase enzyme was oxidized as previously described (Gibbs *et al.*, 1985).

Results and discussion

Purification of 5-aminolaevulinate dehydratase from human erythrocytes

Human erythrocyte 5-aminolaevulinate dehydratase has been partially purified on a small scale (Despaux *et al.*, 1979; Bustos *et al.*, 1980) and purified to homogeneity in larger amounts (30mg) by Anderson & Desnick (1979). Our requirement for quantities of homogeneous enzyme in excess of 100mg for protein chemistry and sequencing studies prompted us to develop a more rapid large-scale procedure. One of the major hurdles in dealing with large quantities of erythrocytes is in the removal of haemoglobin, which accounts for 99% of the protein. For this method an efficient batch system using DEAE-Bio-Gel was developed that can be applied to the removal of haemoglobin from any erythrocyte protein which has an acidic isoelectric point. Because of the acute sensitivity of the 5-aminolaevulinate dehydratase to oxygen, all procedures were necessarily carried out in buffers containing 20mM-2-mercaptoethanol or 10mM-dithioerythritol at 4°C unless otherwise stated.

Preparation of crude extract

Whole blood (2.4 litres) was centrifuged at 7000*g* for 5min in a Sorvall RC-3B centrifuge (4 × 1-litre rotor). The plasma was removed by aspiration and the cells were washed by gentle stirring with an equal volume of 0.9% NaCl containing 1mM-2-mercaptoethanol. The washing process was repeated twice.

The packed red cells (1.2 litres) were lysed by adding an equal volume of deionized water containing 40mM-2-mercaptoethanol. The mixture was then subjected to ultrasonication in 800ml portions for 30s at low power, followed by 30s at high power by using an MSE Soniprep 150 fitted with a 20mm-diameter probe. Cell debris was removed by centrifugation at 18000*g* for 45min in an MSE 21 centrifuge (6 × 500ml rotor), and 1M-potassium phosphate buffer, pH7.5, was added to the supernatant to give a final phosphate concentration of 10mM.

Removal of haemoglobin

The extract was carefully stirred into DEAE-Bio-Gel A (600ml packed wet volume) that had been equilibrated previously in 10mM-potassium phosphate buffer, pH7.5. Stirring was continued gently for 90min by using a Gallenkamp overhead stirrer with a Z-shaped glass rod (at 25rev./min), after which time all the 5-aminolaevulinate dehydratase had bound to the resin. The suspension was then centrifuged at 7000*g* for 1min in a Sorvall RC-3B centrifuge (4 × 1-litre rotor) and the supernatant was discarded. The DEAE-Bio-Gel 'cake' was washed four times in 3.5 litres of 10mM-potassium phosphate buffer, pH7.5, followed by centrifugation in each case. Weakly bound proteins were removed from the DEAE-Bio-Gel by stirring the gel for 10min in 3.5 litres of 10mM-potassium phosphate buffer, pH7.5, containing 50mM-KCl. The suspension was centrifuged at 7000*g* for 1min in a Sorvall RC-3B centrifuge (4 × 1-litre rotor) and the supernatant was discarded. This process was repeated three times. The enzyme was then eluted with four 500ml portions of 10mM-potassium phosphate buffer, pH7.5, containing 350mM-KCl or until all the enzyme activity had been recovered. The active enzyme fractions were pooled, treated with $(NH_4)_2SO_4$ (to 60% saturation; 390g/litre) and the precipitate was collected by centrifugation at 18000*g* for 25min in an MSE 21 centrifuge (6 × 500ml rotor). The pellet was resuspended in 0.1M-potassium phosphate buffer, pH6.8, containing 20mM-2-mercapto-

ethanol to give a protein concentration of 30mg/ml (as determined by A_{280}). This stage removes almost all the haemoglobin, the presence of which greatly hinders the purification of most erythrocyte enzymes.

Heat treatment

The enzyme solution was heated to 60°C in a 250ml conical flask by using a water bath controlled at 75°C. The temperature (60°C) was maintained for 3min, after which the flask was cooled rapidly in an ice/salt/water mixture until the temperature was below 10°C. Denatured protein was removed by centrifugation at 50000*g* for 10min in an MSE 21 centrifuge (8 × 50ml rotor).

$(NH_4)_2SO_4$ fractionation

The supernatant (170ml) was treated with $(NH_4)_2SO_4$ (30% satn.; 176g/litre) and the precipitate was collected by centrifugation at 50000*g* for 10min. The pellet, which contained the enzyme, was resuspended in 35ml of 30mM-potassium phosphate buffer, pH7.9, and the enzyme was dialysed overnight against 20 litres of the same buffer.

Chromatography on DEAE-Bio-Gel A

The dialysed enzyme was applied to a DEAE-Bio-Gel column (5.5cm × 8cm high) that had been equilibrated previously with 30mM-potassium phosphate buffer, pH7.9, and the column was washed to remove all unbound protein. A linear gradient (600ml total; 0–0.4M-KCl in 30mM-potassium phosphate buffer, pH7.9) was applied to the column and the active fractions were pooled, precipitated with $(NH_4)_2SO_4$ (60% satn.; 390g/litre) and the precipitate collected by centrifugation at 50000*g* for 10min in an MSE 21 centrifuge (8 × 50ml rotor). The pellet was resuspended in 20ml of 50mM-potassium phosphate buffer, pH6.8, and was dialysed against 20 litres of the same buffer overnight.

Chromatography on hydroxyapatite

The dialysed enzyme was applied to a column of hydroxyapatite (5.5cm × 8cm high) that had been equilibrated previously with 50mM-potassium phosphate buffer, pH6.8, and the column was washed with 1 litre of the same buffer to remove unbound protein. A linear gradient (500ml total; 50mM–300mM-potassium phosphate buffer, pH6.8) was applied to the column and 10ml fractions were collected. The active fractions were pooled and the enzyme was collected by precipitation with $(NH_4)_2SO_4$ (60% satn.; 390g/litre) and centrifuged as described above. The pellet was resuspended in 3ml of 60mM-potassium phosphate buffer, pH6.8, containing 0.5M-KCl.

Chromatography on Bio-Gel A 0.5m

The enzyme from the previous stage was applied to a Bio-Gel A 0.5m gel-filtration column (2.8cm × 1m) and the column was developed in the same buffer. Fractions with enzymic activity were pooled, precipitated with $(NH_4)_2SO_4$ (60% satn.; 390g/litre), and the precipitate was collected by centrifugation as described above.

The pellet was resuspended in 0.1M-potassium phosphate buffer, pH6.8, containing 10mM-dithioerythritol and 0.1mM-Zn^{2+} and was incubated at 37°C for 15min to activate the enzyme fully. The enzyme was stored as an $(NH_4)_2SO_4$ precipitate under N_2 in a sealed screw-top centrifuge tube at 4°C.

By using this new procedure, 5-aminolaevulinate dehydratase was purified 35000-fold from 2.4 litres of human erythrocytes to yield 27.5mg of enzyme protein with a recovery of 65%. The final specific activity was 24.0 units/mg when assayed in the presence of 0.1mM-Zn^{2+} (18.8 units/mg in the

Table 1. *Purification of 5-aminolaevulinate dehydratase from human erythrocytes*

Stage	Volume (ml)	Total activity (units*)	Total protein (mg)	Specific activity (units/mg)	Yield (%)	Purification factor
Whole blood	2360	N.D.†	1.463×10^3	–	–	–
Lysed + sonicated erythrocytes	2290	578	996×10^3	58×10^{-5}	–	1.5
Batch DEAE-Bio-Gel (pH7.5)	2240	793	2.74×10^3	0.29	100	530
60°C Heat treatment	175	666	795	0.84	84	1550
$(NH_4)_2SO_4$ precipitate	36	633	382	1.66	80	3100
DEAE-Bio-Gel (pH7.9)	97	605	151	4.01	76	7400
Hydroxyapatite (pH6.8)	84	574	34.8	16.5	72	30300
Bio-Gel A 0.5m (−zinc)	14.7	517	27.5	18.8	65	34600
Gel filtration (+0.1mM-zinc)		660		24.0		

* 1 unit = 1 μmol of porphobilinogen produced/h at 37°C.
† N.D., not determined.

absence of added Zn^{2+}). A typical purification is shown in Table 1.

Quantities of purified enzyme in excess of 100 mg could be isolated by a direct scale-up of this procedure. One such purification yielded 115 mg of enzyme from 7.2 litres of time-expired blood with an overall yield of 74%. This is equivalent to a 600-fold improvement in yield of enzyme protein over other published methods.

Purity of the enzyme

The purified dehydratase gave, on polyacrylamide-gel electrophoresis, a single broad diffuse protein band that was associated with the catalytic activity (Fig. 1). The diffuse band could be attributable either to a range of partially oxidized species that arose during the electrophoresis or to the presence of glycosidic residues forming part of the protein structure. Since the carboxymethylated enzyme gave a sharper band on polyacrylamide gels, the presence of several enzyme species with different oxidized states seems the most likely explanation for the broadness of the native dehydratase band. The purified enzyme did not contain contamination from 5-aminolaevulinate synthetase or porphobilinogen deaminase as judged by 1 h incubations with 100 μg quantities of protein and the appropriate substrates for these latter enzymes.

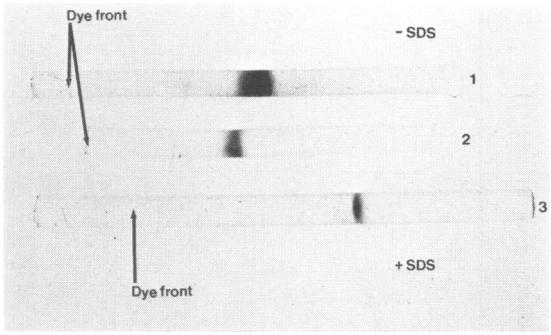

Fig. 1. *Purity of human erythrocyte 5-aminolaevulinate dehydratase*

Polyacrylamide-gel electrophoresis of the human enzyme was performed in the presence or absence of SDS (Weber & Osborn, 1969). Enzymic activity was detected as described in the text and was shown to be located in a single protein band. The gels were stained by the method of Weber & Osborn (1969). Gels 1 and 2 were 5%-polyacrylamide gels of the native (40 μg) and carboxymethylated (25 μg) enzymes respectively, Gel 3 contained denatured protein (20 μg) that had been electrophoresed on a polyacrylamide gel (15%) in the presence of SDS (0.1%, w/v).

Molecular properties of human 5-aminoaevulinate dehydratase

The M_r of the native enzyme was determined by comparing the elution volume (V_e) of the enzyme on a Bio-Gel A 0.5 m gel-filtration column with the values obtained from proteins of known M_r. A semi-logarithmic plot of the elution volumes of the standard proteins produced a straight line (Fig. 2*a*), and by extrapolating the value of the elution

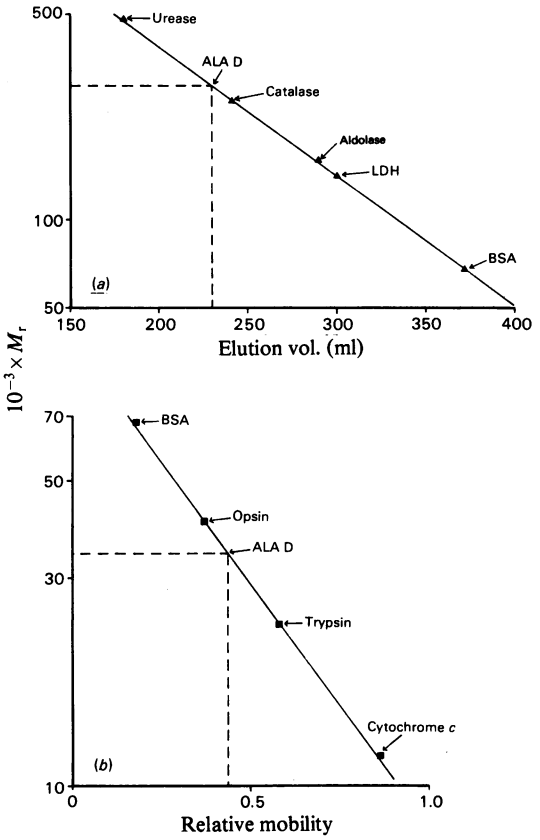

Fig. 2. *Determination of the native and subunit M_r of human 5-aminolaevulinate dehydratase*

(*a*) Gel filtration on Bio-Gel A 0.5 m. The elution volume (V_e) of the native human enzyme was determined on a Bio-Gel A 0.5 m gel-filtration column (2.8 cm × 1 m) and compared with those of various proteins of known M_r (see under 'Methods' for details). A value of 285000 was obtained. Abbreviations used: ALA D, 5-aminolaevulinate dehydratase; BSA, bovine serum albumin; LDH, lactate dehydrogenase. (*b*) SDS/polyacrylamide-gel electrophoresis. The relative mobility (R_F) of the human enzyme was determined under denaturing conditions on 15% (w/v) polyacrylamide gels (Weber & Osborn, 1969) and compared with those of standard marker proteins. A value of 35000 was obtained for the subunit M_r of human 5-aminolaevulinate dehydratase.

volume (230ml) obtained for the human enzyme to the abcissa, an M_r of 285000 ± 10000 was obtained. Similarly, the subunit M_r of the enzyme was determined by comparing its relative mobility (R_F) on 15%-polyacrylamide gels containing 0.1% (w/v) SDS (Weber & Osborn, 1969) with the mobilities of standard proteins. From these data (presented in Fig. 2*b*), a subunit M_r of 35000 was obtained. These values for the human enzyme are in close agreement with data obtained by other groups (Despaux *et al.*, 1979; Sassa & Kappas, 1983), but differ significantly from those obtained by Anderson & Desnick (1979) (native M_r 252000; subunit M_r 31000). From our data it appears that the human dehydratase, like the bovine enzyme (Wu *et al.*, 1974; Tsukamoto *et al.*, 1975), is octameric in nature and is composed of eight apparently identical subunits in a cubic octameric structure with dihedral (D_4) symmetry. This proposal has been corroborated by Despaux *et al.* (1979), who used electron microscopy to determine the quarternary structure of the semipurified human enzyme.

A single isoelectric point of 4.85 ± 0.20 was obtained for the human enzyme and was in good agreement with the value of 4.9 obtained by Anderson & Desnick (1979).

pH optimum and catalytic properties of human 5-aminolaevulinate dehydratase

The pH optimum of the enzyme was determined over the range 5.6–7.7. The enzyme was maximally active at pH6.8 in potassium phosphate buffer containing 0.1mM-Zn^{2+} and 10mM-dithioerythritol (Fig. 3). The $K_{m,app.}$ for 5-aminolaevulinate was 287 ± 31 μM at the pH optimum of 6.8. The double-reciprocal plot obtained in this case (results not shown) was linear over the substrate range 50μM–5mM and showed no evidence of more than one component. This is due to the fact that, of the two molecules of 5-aminolaevulinate which bind to the enzyme active site, the first is bound to enzyme with a very high affinity compared with the second. The double-reciprocal plot obtained thus largely reflects the dissociation constant of the second substrate molecule. This large difference between the affinities of the two substrates has been successfully exploited in single-turnover experiments reported elsewhere (Jordan & Seehra, 1980; Jordan & Gibbs, 1985). The catalytic centre activity of the enzyme was 14mol of porphobilinogen produced per min at 37°C per subunit of enzyme, assuming that all subunits are catalytically viable and based on a specific activity of 24 units/mg.

Requirement for Zn^{2+}

Human 5-aminolaevulinate dehydratase, in common with all mammalian dehydratases, requires the presence of Zn^{2+} (Tsukamoto *et al.*, 1979; Bevan *et al.*, 1980) for maximal catalytic activity. The dependence on Zn^{2+} for the catalytic activity of the human enzyme was investigated by assaying the apoenzyme (prepared by incubation with 50mM-EDTA, followed by gel filtration) in buffers devoid of Zn^{2+}. The results showed that the apoenzyme had very low activity ($\simeq$12% of control), even when assayed in the presence of 10mM-dithioerythritol. The residual activity was attributed to trace amounts of Zn^{2+}, present in the dithioerythritol, which could be detected by atomic-absorption spectroscopy. The addition of increasing quantities of Zn^{2+} to the apoenzyme revealed a concentration-dependent activation (Fig. 4), with optimal stimulation occurring in the range 100–300 μM-Zn^{2+}. This value is in good agreement with data obtained by other groups in assays using erythrocyte haemolysates (Meredith *et al.*, 1977; Davis & Avram, 1980; Trevisan *et al.*, 1980; Geisse *et al.*, 1983) or the purified enzyme (Anderson & Desnick, 1979), but is substantially different from the value of 1.5 μM for the partially purified enzyme (Despaux *et al.*, 1979). It is significant that the Zn^{2+} concentration in whole blood [135 ± 31 μM (Vallee & Gibson, 1948) and 109 ± 29 μM (Meredith & Moore, 1980)] falls within the above range, suggesting that the human enzyme is fully active *in vivo*.

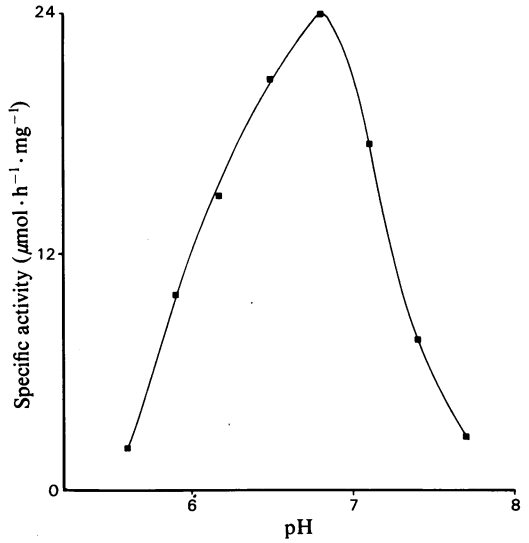

Fig. 3. *Effect of pH on the activity of the human dehydratase enzyme*

The purified human enzyme (10.5μg) was assayed at various pH values by using the appropriate 0.1M-buffer containing 10mM-dithioerythritol and 0.1mM-$ZnCl_2$ as described under 'Methods'.

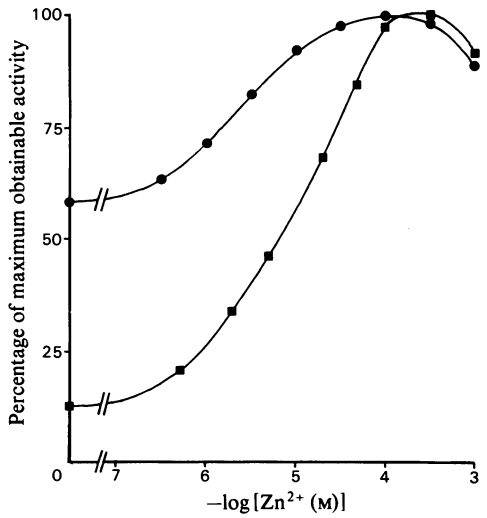

Fig. 4. *Effect of zinc-ion concentration on the activity of native and apo-(5-aminolaevulinate dehydratase)*
Native (●) and apo-(5-aminolaevulinate dehydratase) (■) from human erythrocytes was preincubated for 10min at 37°C in 100μmol of potassium phosphate buffer, pH6.8, containing 10μmol of dithioerythritol and increasing amounts of Zn^{2+} (0.5nmol–1μmol) in a final volume of 900μl. After the addition of 100μl of 5-aminolaevulinate (5μmol; neutralized with 0.1M-NaOH before use), incubations were carried out for 10min and terminated by the addition of 1ml of 10% (w/v) trichloroacetic acid containing 0.1M-$HgCl_2$. The porphobilinogen produced during the reaction was determined as described under 'Methods'. The data are presented in a graphical form as log[Zn^{2+}] versus the enzyme activity (100% represents the maximum activity of the enzyme).

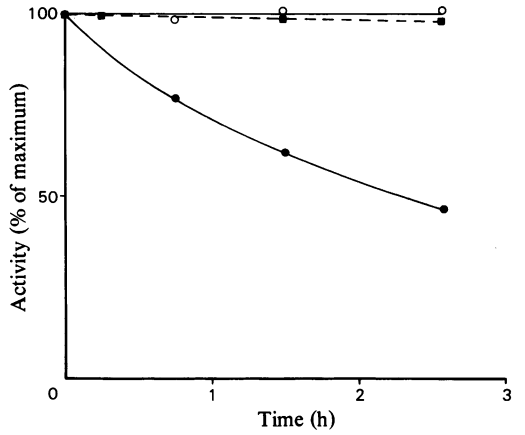

Fig. 5. *Rate of loss of activity of 5-aminolaevulinate dehydratase on oxidation by air*
After removal of exogenous thiol by Sephadex G-50 gel filtration, the activated human enzyme was exposed to air oxidation in 0.1M-potassium phosphate buffer, pH6.8, in the absence of Zn^{2+} (●), the presence of 100μM-Zn^{2+} (■) or under O_2-free N_2 (○). The solutions were incubated at 37°C in open tubes of area 1.45cm² and had a depth of 1cm. Samples were removed at timed intervals and assayed for both enzymic activity (under N_2) and protein concentration.

It is noteworthy that the native enzyme, after purification in the absence of added Zn^{2+}, could also be fully activated by 100–300μM-Zn^{2+}, although from a much higher basal level (60–70%), showing that 30–40% of the metal is lost during the purification process. The lower specific activity of the native enzyme in the absence of added Zn^{2+} was found to be due to a decrease in $V_{max.}$ rather than in $K_{m,app.}$

Requirement for thiols

The sensitivity of human 5-aminolaevulinate dehydratase to oxidation is similar to that found for other mammalian dehydratases (Shemin, 1972; Cheh & Neilands, 1976). When the apoenzyme was exposed to air, there was a time-dependent inactivation ($t_{\frac{1}{2}}$ = 135min), as shown in Fig. 5. The inactivation was almost completely prevented by the presence of Zn^{2+} (100μM), suggesting that, in the holoenzyme, the sensitive thiol groups are no longer readily available for reaction. When the apoenzyme was incubated in the absence of oxygen (under N_2), there was no significant loss in activity.

The oxygen-inactivated enzyme could be restored to full activity by incubation with thiols such as dithioerythritol (Fig. 6). A minimum concentration of 10mM-thiol was required for the maintenance of full catalytic activity.

Inhibition and inactivation

The effects of various inhibitors and inactivating agents of mammalian 5-aminolaevulinate dehydratase activity were determined as described under 'Methods'. The results presented in Fig. 7 clearly demonstrate a time-dependent inactivation of the human enzyme by the metal-chelating agent EDTA (30mM-final concn.), similar to that found with the bovine liver enzyme (Gibson *et al.*, 1955; Wilson *et al.*, 1972). In a parallel experiment, 94% of the labelled Zn^{2+} was displaced from the $^{65}Zn^{2+}$-labelled holoenzyme (prepared as described by Gibbs & Jordan, 1981) and was associated with an almost complete loss of activity (88%). This residual enzymic activity is almost certainly attributable to adventitious metal-ion contamination, since it is virtually impossible to decrease the concentration of Zn^{2+} below 10nM by using standard metal-extraction procedures (Vallee & Galdes, 1984).

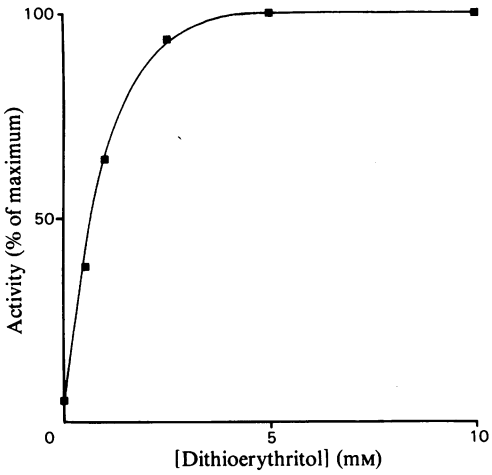

Fig. 6. *Re-activation of the oxygen-inactivated enzyme by dithioerythritol*

Oxygen-inactivated enzyme (prepared as described in Gibbs *et al.*, 1985) was incubated for 15min at 37°C in 0.1M-potassium phosphate buffer, pH6.8, containing 0.1mM $ZnCl_2$ in the presence of increasing concentrations of dithioerythritol (0.5–10mM). The enzyme activity was determined as detailed under 'Methods'.

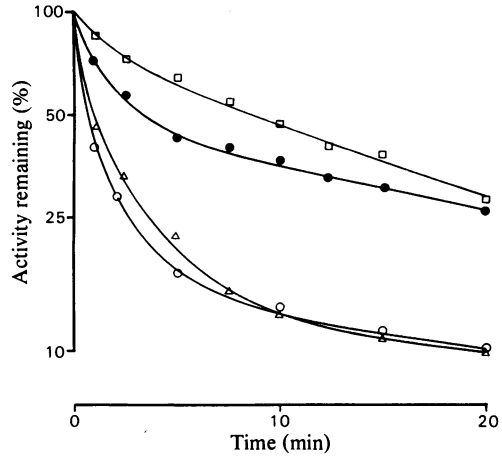

Fig. 7. *Effect of various inhibitors and inactivating agents on human 5-aminolaevulinate dehydratase activity*

For experimental details, see under 'Methods'. The data are presented as % activity (on a logarithmic scale) versus time. Additions were: ○, EDTA (30mM); △, 5,5′-dithiobis-(2-nitrobenzoic acid) (0.5mM); □, 3-chlorolaevulinic acid (10mM); ●, 5-chlorolaevulinic acid (5mM).

Inactivation of the human enzyme was observed in the presence of the thiophilic reagent 5,5′-dithiobis-(2-nitrobenzoic acid) (0.5mM final concn.) (Fig. 7), owing to the modification of catalytically important thiol groups. A more detailed investigation of the interaction of 5,5′-dithiobis-(2-nitrobenzoic acid) with the human dehydratase enzyme has been reported [see Gibbs *et al.* (1985) for further details]. Haloketones, including 3-chlorolaevulinic acid (10mM final concn.) and the active-site-directed inhibitor 5-chlorolaevulinic acid (5mM final concn.) (Seehra & Jordan, 1981), were also shown to inactivate the the human enzyme in a linear manner, with a $t_{\frac{1}{2}}$ for 7). Iodoacetic acid (10mM final concn.) inactivated the human enzyme in a linear manner, with a $t_{\frac{1}{2}}$ for inactivation of about $3\frac{1}{2}$min (results not shown).

Lead was found to be a potent non-competitive inhibitor of human erythrocyte 5-aminolaevulinate dehydratase activity, affecting both the K_m and the $V_{max.}$ of the enzyme (Fig. 8). By using computer curve-fitting analysis the two inhibition constants (K_{iI}, the dissociation constant for the enzyme–substrate–lead complex; and K_{iS}, that for the enzyme–lead complex) were found to be $25.3 \pm 3.0\,\mu M$ (K_{iI})and $9.0 \pm 2.0\,\mu M$ (K_{iS}). These data indicate that the lead interacts with the free enzyme approximately three times more readily than with the enzyme–substrate complex. Zn^{2+} was found to reverse completely the lead-induced inhibition, a finding consistent with those in the literature (Finelli *et al.*, 1975; Haeger-Aronsen *et al.*, 1976).

In summary, the present paper outlines a new procedure for the purification of 5-aminolaevulinate dehydratase from human erythrocytes that improves on the three-step purification of Despaux *et al.* (1979) and the method of Bustos *et al.* (1980) (using affinity chromatography). One of the major advantages of our method is that the haemoglobin can be removed from the enzyme solution by using a DEAE-Bio-Gel batch-elution step, a procedure that is far more rapid ($\simeq$3h) and efficient than the use of a conventional column, and which allows the processing of larger quantities of starting material.

By using this new procedure, 2.4 litres of whole blood yielded 27.5mg of homogeneous human 5-aminolaevulinate dehydratase in 65% recovery and gave the highest specific activity yet reported for this enzyme (24.0 units/mg in the presence of 0.1mM-$ZnCl_2$). In addition, the overall yield of enzyme protein (27.5mg from 2.4 litres of whole blood; 115mg from 7.2 litres of whole blood) was much higher than that obtained by Anderson & Desnick (1979) (30mg from 6 litres of packed erythrocytes; equivalent to $\simeq$13 litres of whole blood).

The purified human enzyme (M_r 285000) was shown to be essentially homogeneous on polyacrylamide-gel electrophoresis (Fig. 1) and was made up of eight apparently identical subunits of M_r 35000 (Fig. 2). The enzyme has a pH optimum

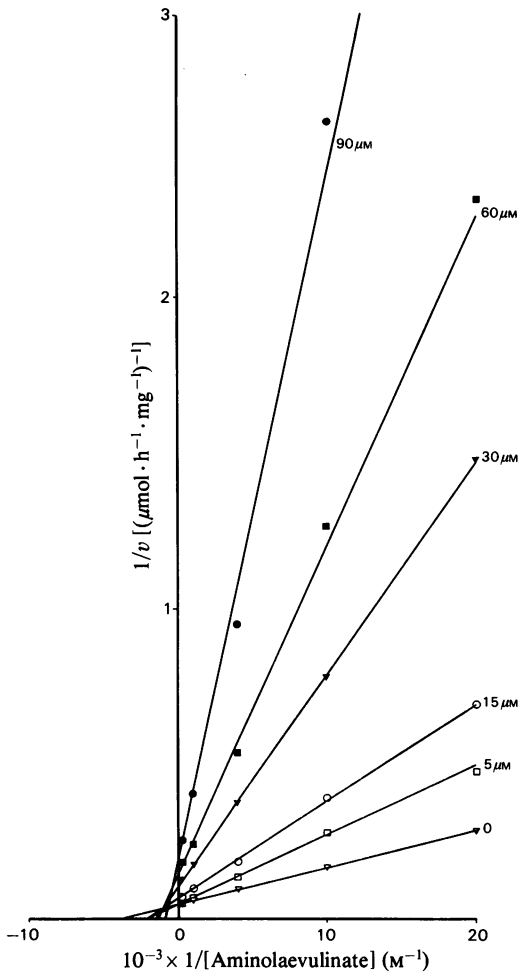

Fig. 8. *Lineweaver–Burk plot representing the inhibition of native human 5-aminolaevulinate dehydratase by lead*
The effect of lead on the activity of the native enzyme (10.5 μg) was determined as described under 'Methods'. The graph of $1/v$ versus $1/s$ is based on computer-estimated intercepts obtained by hyperbolic non-linear-regression analysis. For each plot the concentration of lead is given.

of 6.8 (Fig. 3) and has an absolute requirement for Zn^{2+} ions for maximal catalytic activity (Fig. 4). Optimal activation of the human enzyme occurred in the presence of 100–300 μM-$ZnCl_2$. Lead was found to be a potent non-competitive inhibitor of the human enzyme.

Human 5-aminolaevulinate dehydratase was found to be sensitive to air oxidation ($t_{\frac{1}{2}} = 135$ min; Fig. 5); however, full catalytic activity could be restored on return of the enzyme to reducing conditions (Fig. 6). Inactivation of the human enzyme by various alkylating and thiophilic reagents (Fig. 7) demonstrated that the sensitivity to oxidation was due to the modification of catalytically important thiol groups.

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