Int. J. Biochem. Vol. 12, pp 745 to 749

0020-711X/80/1101-0745$02.00/0

STUDIES ON ERYTHROCYTE AMINOLAEVULINATE DEHYDRATASE I. ITS PURIFICATION AND POSSIBLE THERAPEUTIC APPLICATIONS

NORMA BUSTOS, ANA MARIA STELLA, EVA A. WIDER DE XIFRA and ALCIRA M. DEL C. BATLLE

Centro de Investigaciones Sobre Porfirinas y Porfirias (CIPYP), Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires y Consejo Nacional de Investigaciones Cientificas y Técnicas (CONICET), Ciudad Universitaria, Pabellón II, 4 Piso Nuñez 1428, Buenos Aires, Argentina

Abstract—1. A method for purifying human erythrocytes ALA-D, using a mixture of *n*-butanol and chloroform, which denature hemoglobin, followed by ammonium sulphate fractionation and affinity chromatography yielding a 1600-fold purified enzyme, is described.

2. By oxidation of Sephadex G-25 with $NaIO_4$, a polyaldehyde, is obtained which can be covalently bound to the ALA-D; however the immobilized enzyme is inactive, because essential ϵ-amino groups at the active site were involved in the coupling. Similar experiments with another enzyme, Rhodanese, resulted in an active insolubilized preparation.

3. By suspending the carrier-enzyme in buffer, slow solubilization with simultaneous release of protein occurs, indicating that this approach might find important therapeutical applications in the treatment of enzyme deficiencies.

INTRODUCTION

Aminolaevulinate dehydratase (ALA-D) (EC 4.2.1.24) occurs very widely in nature. After Bonsignore (1966) there has been an increasing interest in the determination of erythrocyte ALA-D activity as a possible diagnostic criterion for acute and chronic lead poisoning, and as a very useful monitor for the health control of people exposed to lead and some other environmental factors, for it is well known that ALA-D, present in red blood cells, is very sensitive to inhibition by heavy metals.

However, decreased ALA-D activity was also observed in different liver (Rubino *et al.*, 1960; Ivanov, 1968) and kidney diseases (Ivanov, 1968), some tumors (Heilmeyer, 1963; Hano & Akashi, 1964; Rasetti *et al.*, 1966), alcoholism (Moore *et al.*, 1971), some anemia (Rubino *et al.*, 1960; Heilmeyer, 1963) and more recently in polychlorinated biphenyls poisoning (Maines, 1976) and in unusual cases of acute porphyria (Doss *et al.*, 1979).

We consider that in any instance where ALA-D activity is reduced due either to inactivation or to an inherited enzyme deficiency, a therapeutic approach could be the exogenous supply of the malfunctioning ALA-D, which of course should, after administration, reach and act into its target site. However direct enzyme-replacement therapy, is associated with a number of difficulties, such as hypersensitivity reactions, production of antibodies, and rapid inactivation and removal of the foreign protein. These problems might be overcome by developing a biodegradable and non-toxic carrier which would eventually transport the enzyme to the target tissue and once there, facilitate its uptake by disease cells and allow to perform its task.

Many of the requirements for a good carrier are fulfilled by liposomes (Gregoriadis & Buckland, 1973) and immobilized enzymes (Chang, 1976).

We have therefore undertaken studies to investigate the possible therapeutic applications of a suitable immobilized preparation of erythrocyte ALA-D.

In the hope of finding a means for the controlled and safe release of ALA-D in the diseased tissues, as a prelude, we had to develop a simpler method for the purification of ALA-D from the homologous source human erythrocytes. The enzyme has to be highly purified to remove endotoxins and other contaminants.

The second step was to find the carrier which better satisfied our particular requirements.

A major difficulty in purifying enzymes from erythocytes is their separation from hemoglobin (Hb). A method for purifying ALA-D using a mixture of *n*-butanol and chloroform, which denature Hb, followed by ammonium sulphate fractionation and affinity chromatography, yielding a 1600-fold purified enzyme, will be here described.

Attempts to immobilize ALA-D on a carrier which will be able to slow solubilization with simultaneous release of the active enzyme bound to carrier fragments, will also be reported.

MATERIALS AND METHODS

Most of the chemicals were obtained from Sigma Chemical Co. unless otherwise indicated.

Sephadex and Sepharose gels were from Pharmacia Fine Chemicals, Uppsala.

The biospecific absorbent, HOOC-ALA-succinyl-aminoethyl-sepharose (HOOC-ALA-S-AE-Sepharose) was prepared according to Stella & Batlle (1977).

Source material of ALA-D

Fresh blood was obtained from the blood bank of Ramos Mejía Hospital, Buenos Aires.

ALA-D was assayed as described by Batlle *et al.* (1967). Specific activity is expressed as the amount of enzyme catalysing the formation of 1 μmol of PBG/min per mg of protein.

Purification of rhodanese

Rhodanese was prepared from *Rh. spheroides* and measured by the procedure described by Wider de Xifra *et al.* (1976).

Protein was measured by the Lowry *et al.*'s method (1951).

Hemoglobin content was determined as described by Batlle & Grinstein (1964).

Gel columns were prepared according to Batlle *et al.* (1965) and run as described by Stella & Batlle (1977, 1978).

Method for removing Hb

It was carried out following the procedure of Scott (1976) modified as described in Results.

Oxidation of a polysaccharide matrix

Oxidation of Sephadex G-25 was carried out by suspending the gel in acetate buffer (pH: 4.0) (10 ml/ml gel) and reacting with 50 mM $NaIO_4$ (10 ml/ml gel) for 30 min at room temperature with shaking, as shown below.

$$\text{Sephadex—OH} \xrightarrow{NaIO_4} \text{Sephadex—CHO}$$

The gel was then washed with water and etilenglicol 0.1 M (50 ml/ml gel).

The degree of oxidation, that is the aldehyde groups content was determined by the method of Corcoran & Page (1947).

Immobilization of enzyme

It was performed by adding the oxidized Sephadex to different quantities of the enzyme suspended in 0.05 M phosphate buffer (pH: 8.0) (1:5, v/v) at 4°C for 30 min with shaking.

Then, after 20 and 40 min, $NaBH_4$ (0.25 mg/ml gel) was added. 20 min after the last addition, the reaction was terminated by destroying the excess $NaBH_4$ with 0.1 M acetic acid.

Nonbound enzyme was removed by washing with 100 ml of water, 100 ml of 2 M KCl and 100 ml of water. The amount of protein bound to the carrier was determined by the difference between the amount of protein added to the oxidized Sephadex and the amount present in the washings.

The enzyme is coupled to the solid support through the formation of a Schiff base between the ϵ-amino groups of lisine residues and the aldehyde groups of the oxidized Sephadex as shown below.

$$\text{Enzyme—NH}_2 + \text{OCH—Sephadex} \longrightarrow \text{Enzyme—N=CH—Sephadex}$$

$$\text{Enzyme—N=CH—Sephadex} \xrightarrow{NaBH_4} \text{Enzyme—NH—CH}_2\text{—Sephadex}$$

To determine the activity of the bound enzyme, the same standard incubation conditions described for the soluble enzyme were used, but certain modifications of the assay technique were required as described by Stella *et al.* (1977).

RESULTS AND DISCUSSION

Purification of ALA-D (Table 1)

Step 1. Preliminary experiments were carried out to determine the optimal conditions for denaturing Hg, such as the composition of the *n*-butanol–chloroform mixture, the volume to be added to 1 volume of red cells, temperature, time, dialysing buffer and period of dialysis. According to results obtained the procedure finally adopted was the following: red cells were obtained after centrifuging, washed twice with NaCl isotonic solution and adjusted to pH 8 with concentrated NH_4OH. Afterwards, a cold (−20°C) mixture of *n*-butanol and chloroform (1:0.4, v/v), was added to the packed cells (at the ratio of 1:0.2, red cells:mixture) with vigorous mechanical stirring, at 4–6°C, within 15 min. The resulting mixture was left to stand for 60 min in a water bath at 20°C and then centrifuged at 24,000 *g* for 20 min. The supernatant was dialysed for 18–20 hr against 0.01 M NaH_2PO_4, Na_2HPO_4 buffer pH 6.8 (1:200, v/v).

It has been found that treatment with the organic solvent mixture removed 96–97% of Hb and yielded a fraction of ALA-D 24-fold purified.

Step 2. Ammonium sulphate precipitation. The dialysed supernatant was then fractionated with $(NH_4)_2SO_4$; the fraction precipitating at 30–55% saturation was collected by centrifugation, dissolved in the minimum value of 0.05 M phosphate buffer pH 6.8 and dialysed for 2 hr against 500 ml of the same buffer. The 30–55% fraction contained practically no Hb and was purified nearly 100-fold.

Step 3. Around 80 mg of protein of the dialysed 30–55% fraction (in 4 ml) were applied to a HOOC-ALA-S-AE-Sephadex column previously equilibrated with 0.05 M phosphate buffer pH 6.8. The column was run at 4°C with a flow rate of 0.4 ml/min, and developed with buffer alone and containing 0.05 M and 0.1 M KCl as indicated.

Fractions of 2 ml were collected. A typical elution profile is shown in Fig. 1. Erythrocyte ALA-D was purified about 18-fold in this step and a total of 1.600-fold following a rather simple procedure.

We can add that, the chromatographic behaviour of erythrocyte ALA-D on affinity columns was very similar to that of *E. gracilis* and bovine liver ALA-D (Stella & Batlle, 1977, 1978) and also to the pig liver enzyme (Polo *et al.*, 1980).

Table 1. Purification of ALA-D from human erythrocytes

Fraction	Protein mg/ml	Hb %	Specific activity	Purification (−fold)
Red cells	120	100	0.0014	1
Step 1. Supernatant after solvent treatment	28.807	3.7	0.0341	24.3
Step 2. 30–55% $(NH_4)_2SO_4$ fraction	20.961	0.25	0.1338	95.6
Step 3. Affinity chromatography	0.155	0	2.2485	1605

Experimental conditions are described in the text.

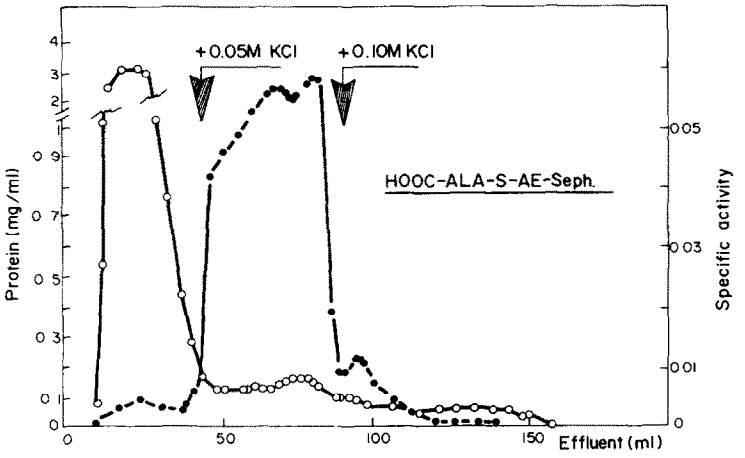

Fig. 1. Affinity chromatography of pig liver ALA-D on HOOC-ALA-S-AE-Sepharose. Column (1.4 × 1.5 cm) equilibrated with 0.05 M phosphate buffer pH 6.8. Protein (15 mg, in 2 ml) was applied to the column and this eluted with 50 ml of phosphate buffer pH 6.8; then with 50 ml of the same buffer containing 0.05 M KCl (first arrow) and then with 50 ml of buffer containing 0.1 M KCl (second arrow). Protein (○); specific activity (●).

Some properties of the purified enzyme

By gel filtration through Sephadex G-200 using 0.068 M Na phosphate buffer containing 0.1 M K, the molecular weight of the dominant peak was 280,000. The optimum pH was 6.8–7.0 and the K_m 0.9×10^{-4} M. As expected at 10^{-4} M Pb^{2+}, inhibited 75%. Between 1 and 10^{-3} mM, Fe^{2+} did not significantly modify activity and from 0.01 to 0.05 mM Zn^{2+} activated 100% and overcome Pb inhibition. Thiol reagents such as PCMB, NEMI and iodoacetamide at 1 mM inactivated 70–80%, while preincubation with 0.0025 M CySH or GSH activated the enzyme.

Immobilization of the enzyme on a carrier slowly solubilized

Selection of the carrier. It is known that injected liposomes can transport enzymes into the lysosomes of the liver and spleen cells, therefore they can be excellent carriers for the treatment of enzyme deficiencies. Simultaneously extensive research have resulted from studies related to the possible therapeutic uses of immobilized enzymes. Taking into account the target site where ALA-D should act and our large experience working with insolubilized systems, we decided to prepare an enzyme-carrier complex which should be able to experiment slow solubilization with simultaneous release of the active enzyme bound to support fragments.

Periodate oxidation of polysaccharides such as Sephadex, produces polyaldehydes containing varying number of aldehydes groups; these derivates can be slowly solubilized in water solution (Ham, 1967).

Therefore, by oxidating Sephadex, we can prepare a carrier having a determined rate of solubilization and containing suitable active groups for enzyme coupling.

Preliminary experiments were designed to find the best conditions for oxidating Sephadex G-25, using different concentrations of $NaIO_4$ and various time intervals. Results are shown in Fig. 2, from them we selected 50 mM $NaIO_4$ and 1 hr as the standard oxidation conditions.

Immobilization of the enzyme

We must remember here that the ε-amino group of a lysine residue at the active site of ALA-D forms a Schiff base intermediate with the carbonyl group of its substrate, ALA. We know that in the bonding of the carrier to the enzyme, ε-amino groups are implicated; however previous studies (Stella *et al.*, 1977) have shown that the amino groups involved in the formation of the enzyme-substrate complex are rather well protected. Therefore, experiments were carried out to define the optimal conditions for coupling.

Either using a fixed amount of protein and different times, or increasing amounts of enzyme and a fixed length of time, and two temperatures, the resulting immobilized ALA-D had no activity. Attempts made to protect the ε-amino groups at the active site by treating the enzyme with ALA, prior to or during its insolubilization, were also unsuccesful, as the resulting immobilized enzyme was still inactive. Therefore

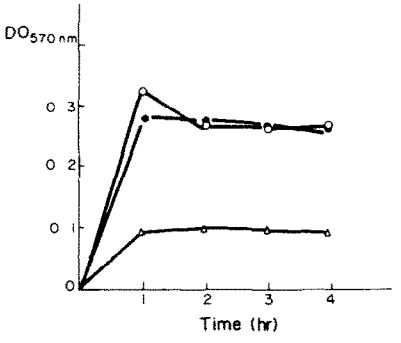

Fig. 2. Effect of $NaIO_4$ concentration and time on the oxidation of Sephadex G-25. Reaction conditions are described in Material and Methods.

Table 2. Binding of Rhodanese and ALA-D to polyaldehyde-Sephadex G-25

Fraction	Rhodanese Units	Rhodanese Total protein (mg)	ALA-D Units	ALA-D Total protein (mg)
Control	36.7	17	18.83	58.8
Gel-enzyme	1.8	4.1	0	4.5
Supernatant	19.0	12.5	1.17	47.4
Washings	0.6	1.4	0.23	5.8

Experimental conditions are described in the text.
As a control we are referring to the activity and amount of protein of the soluble purified enzyme used in the coupling reaction.

it is highly probable that the ε-amino groups in the enzyme are the primary sites of reaction with the carrier.

Anyhow to compare the behaviour of ALA-D with that of another enzyme having no essential ε-amino groups, immobilization of both ALA-D and Rhodanese was performed and results obtained are shown in Table 2. First of all, we can observe that with both enzymes the yield of coupling is low, most of the protein (75–80%) is recovered in the supernatant and only 25 and 7% respectively was bound to the carrier, this means that the dependence on the amount of bound protein and on the number of aldehyde groups in the carrier, should be re-examined; an increase in the degree of oxidation could lead to rise in the amount of protein bound. However it was interesting to find out that the immobilized Rhodanese was catalytically active.

Solubilization of the enzyme-carrier

In spite that the immobilized ALA-D was inactive, the rate of enzyme release from carrier-ALA-D preparations was investigated by suspending the gel-enzyme in an equal volume of 0.05 M phosphate buffer pH 6.8. The mixture was very gently stirred at two different temperatures, 4° and 37°C. Samples (1 ml) were removed at intervals and assayed for enzymic activity in both the insoluble material and the supernatant and for protein content in the latter. Figure 3 only shows data of protein content in the supernatants because activity was nil in all cases. We can observe that protein, although inactive was release from the immobilized enzyme, and its solubilization was dependent on the temperature and the time. It was also evident that an increase in pH and in ionic strength led to a higher rate of solubilization.

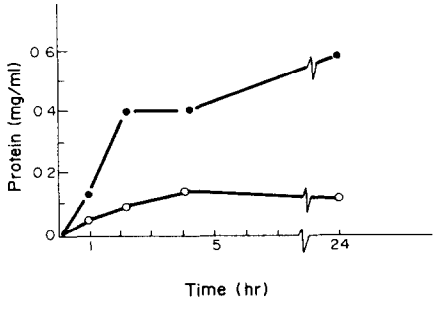

Fig. 3. Rate of ALA-D release from immobilized ALA-D in 0.05 M phosphate buffer pH 6.8. Experimental conditions are described in the text. Protein content in the supernatant obtained at different intervals at 37°C (○) and 4°C (●).

Unfortunately the binding of ALA-D to the polyaldehyde Sephadex involved the essential ε-amino groups at the active site, rendering an inactive preparation which can release the enzyme in water solutions but with its active site still blocked and consequently devoid of activity. Therefore it is necessary to re-investigate this problem, trying to bind this enzyme to the carrier through groups others than ε-amino. In conclusion, we could state that oxidized Sephadex can be used to prepare immobilized enzymes which are able to slow solubilization and simultaneous release of the enzyme into the medium. These derivatives might find a wide range of applications in the medical field.

Acknowledgements—This work was supported by grants from the CONICET. A. M. del C. Batlle; E. A. Wider de Xifra and A. M. Stella hold the Post of Scientific Researcher in the CONICET. N. Bustos thanks the CONICET for the award of a graduate fellowship. We are very grateful to Dr Elba Vazquez for her kind assistance in all the experiments carried out with the *Rh. spheroides* Rhodanese. The authors are also thankful to Miss Hilda Gasparoli for her technical assistance.

REFERENCES

BATLLE A. M. DEL C. & GRINSTEIN M. (1964) Porphyrin biosynthesis. Studies on erythrocyte preparations. *Biochem. biophys. Acta* **82**, 1–10.

BATLLE A. M. DEL C., FERRAMOLA A. M. & GRINSTEIN M. (1967) Purification and general properties of Delta Aminolaevulic Acid Dehydratase from cow liver. *Biochem. J.* **104**, 244–249.

BONSIGNORE D. (1966) Delta Aminolaevulinate dehydratase activity of erythrocytes as a diagnostic test in occupational lead poisoning. *Med. Lavoro* **57**, 647–655.

CHANG T. M. S. (1976) Methods for the therapeutic applications of immobilized enzymes. In *Methods in Enzymology*, Vol. 44 (Edited by MOSBACH K.), pp. 676–698. Academic Press, London.

CORCORAN A. C. & PAGE I. H. (1947) A method for the determination of mannitol in plasma and urine. *J. biol. Chem.* **170**, 165.

DOSS M., TIEPERMAN R. V., SCHNEIDER J. & SCHMID H. (1979) Acute hepatic porphyria syndrome with porphobilinogen synthase defect. *Int. J. Biochem.* **12**, 823–826.

GREGORIADIS G. & BUCKLAND R. A. (1973) Enzyme-containing liposomes alleviate a model for storage disease. *Nature, Lond.* **244**, 170–172.

HAM D. (1967) *Methods of Carbohydrates Chemistry* (Edited by KOCHETKOV N. K.) p. 478. Mir., Moscow.

HANO K. & AKASHI A. (1964) Influences of anticancer agents on the metabolism of delta-aminolevulinic acid in normal and tumor bearing mice. *Gann.* **55**, 25–30.

HEILMEYER L. (1963) Neue Ergebnisse der Porphyrinstoffwechselforschung. *Münch. med. Wschr.* **105**, 277–287.

IVANOV E. D. (1968) Die Rolle der Delta-Aminolavulinsaure-Dehydrase (ALA-D) in der Erythrocyten bei der Erforschung der Biosynthese der Porphyrine. *Folia Haemat.* **89**, 233–240.

LOWRY O. H., ROSSEBROUGH N. J., FARR A. L. & RANDALL R. J. (1951) Protein measurement with the Folin-Phenol reagent. *J. biol. Chem.* **193**, 265–275.

MAINES M. D. (1976) Evidence for the catabolism of polychlorinated biphenyl-induced cytochrome P-448 by microsomal heme oxygenase, and the inhibition of δ-aminolevulinate dehydratase by polychlorinated biphenyls. *J. exp. Med.* **144**, 1509–1519.

MOORE M. R., BEATTIE A. D., THOMPSON G. G. & GOLDBERG A. (1971) Depression of delta-ALA-dehydratase activity by ethanol in man and rat. *Clin. Sci.* **40**, 81–91.

POLO C. F., STAFFORINI D. M., STELLA A. M., WIDER DE XIFRA E. A. & BATLLE A. M. DEL C. (1980) Pig liver aminolevulinate dehydratase I. Alternative methods for its purification and properties of the enzyme. In preparation.

RASETTI L. RUBINO G. F. & DRAGO W. (1966) Attività ferrochelatasia, ALA-deidrasica e ALA-sintetasica nei tessuti tumorali umani. *Minerva Med.* **57**, 2834–2844.

RUBINO G. F., TESO G. & RASETTI L. (1960) Erythrocyte delta-aminolaevulinic acid dehydratase in anemia. *Acta Haemat.* **24**, 300.

SCOTT C. M. (1976) Purification of red cell enzymes by treatment with *n*-butanol and chloroform. *Prep. Biochem.* **6**, 147–152.

STELLA A. M. & BATLLE A. M. DEL C. (1977) Porphyrin biosynthesis. Immobilized enzymes and ligands V. Purification of aminolevulinate dehydratase from bovine liver by affinity chromatography. *Int. J. Biochem.* **8**, 353–358.

STELLA A. M. & BATLLE A. M. DEL C. (1978) Porphyrin biosynthesis. Immobilized enzymes and ligands VIII. Studies on the purification of aminolevulinate dehydratase from *Euglena gracilis*. *Plant. Sci. Lett.* **11**, (2), 87–92.

STELLA A. M., WIDER DE XIFRA E. A. & BATLLE A. M. DEL C. (1977) Porphyrin biosynthesis. Immobilized enzymes and ligands IV. Studies on delta aminolevulinate dehydratase attached to Sepharose. *Mol. Cell. Biochem.* **16**, 97–104.

WIDER DE XIFRA E. A., SANDY J., DAVIES R. & NEUBERGER A. (1976) Control of 5-aminolevulinate synthetase activity in *Rh. spheroides*. *Phil. Trans. R. Soc. Lond.* B. **273**, 79–98.