PORPHYRIN BIOSYNTHESIS—IMMOBILIZED ENZYMES AND LIGANDS X. A NOVEL APPROACH TO THE STUDY OF THE RELATIONSHIP BETWEEN THE QUATERNARY STRUCTURE OF AMINOLAEVULINATE DEHYDRATASE AND ITS ACTIVITY

Alcira M. Del C. Batlle, Ana María Stella, Ana María Ferramola, Yolanda Sopena, Eva A. Wider de Xifra and Horacio A. Sancovich

Centro de Investigaciones sobre Porfirinas y Porfirias. Departamento de Quimica Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Núñez, 1428 Buenos Aires, Argentina

(Received 26 November 1977)

Abstract—1. Evidence for dissociation, renaturation, re-association and re-hybridization of bovine liver aminolaevulinate dehydratase attached to Sepharose 4B is reported.
2. When insolubilized enzyme was treated with 3 and 6 M urea, non covalently bound subunits were dissociated and detected in the eluate; these subunits can be re-associated into a soluble functioning enzyme with a specific activity close to that of the original pure soluble dehydratase preparation.
3. After being washed with a renaturing buffer mixture, the matrix-bound subunits recovered a level of enzymatic activity equal to 50 and 20% of that of the immobilized native aminolaevulinate dehydratase.
4. The reversibility of the dissociation process was investigated. Bound subunits dehydratase can associate with nascent soluble bovine liver aminolaevulinate dehydratase subunits in situ. The product of such treatment, bound-re-associated enzyme, has the same activity as that of the original bound-dehydratase. The matrix-bound-dissociated bovine liver enzyme was also re-hybridized with soluble dehydratase subunits from E. gracilis.
5. The apparent $K_m$ and optimum pH of the immobilized subunits were the same as those of the bound-octameric enzyme.
6. A scheme is proposed, explaining the sequence of reactions leading from the bound-octameric dehydratase to the possible different derivatives, formed during the dissociation and re-association experiments.

INTRODUCTION

The importance of quaternary interactions is indicated by the fact that most soluble globular proteins exist as oligomers (Klotz et al., 1970). The reversibility of denaturation and dissociation of these proteins has largely been investigated and can be used to advantage for studying the mechanisms by which a polypeptide chain folds into a specific conformation: also the assumption is often implicitly made that the assembly of subunits, giving rise to the quaternary protein structure, involves binding domains which are unique for every set of subunits. Immobilization of enzymes has proved useful for elucidating the problem of subunit assembly, inter-subunit interactions and structure-function relationships in oligomeric proteins.

A novel approach to the study of matrix-bound subunits was first introduced by Chan (1970), who demonstrated the existence of catalytically active aldolase subunits bound to Sepharose. This valuable procedure was then applied to the investigation of several other multi-subunit enzymes (Chan & Mawer, 1972; Chan, 1973; Nagradowa et al., 1974; Feldman et al., 1976; Brueh et al., 1976; Carvajal et al., 1977). In studying the relationship between the quaternary structure of an oligomeric enzyme and its activity, an interesting point is to know whether the individual subunits are active or whether association is necessary for enzymic activity. For this purpose, the intact enzyme is attached to a matrix via a single or at least two subunits, and then, those not covalently bound are removed; so that the properties of the isolated immobilized subunits can be studied under conditions where re-association is prevented. Further, this method may also be used to investigate re-association and hybridization of the bound subunits with soluble subunits.

This approach appeared particularly attractive for studying aminolaevulinate dehydratase (ALA.D) (5-aminolaevulate-hydrolase, EC 4.2.1.24), the enzyme catalysing the condensation of two molecules of aminolaevulinate (ALA) into the monopyrrol porphobilogen (PBG); which has been extensively investigated in our laboratory (Stella & Batlle, 1977 and refs therein) and many others.

Bovine liver ALA.D was used for this study, because we can easily obtain relatively large amounts of highly purified enzyme (Batlle et al., 1967, 1970; Stella & Batlle, 1977) and we already know the properties of ALA.D attached to Sepharose (Stella et al., 1977).
It has been shown that the enzyme has a molecular weight of about 280,000 and is composed of eight similar subunits. Substantial work has been done on ALA-D and an excellent review on its structure, function and mechanism of action has been recently published by Sheehan (1976).

Previous work with the Rh. sphaeroides ALA-D (Nandi, 1971) has shown that this enzyme, like few others, undergoes reversible inhibition by urea, guanidine and methyl derivatives of urea, and that this inhibition is competitive in nature. However, it was simultaneously demonstrated that the inactivation of ALA-D by urea is produced by its dissociation into subunits. Therefore, we may expect that bovine liver ALA-D can be dissociated by urea into individual polypeptides, and that by removing or diluting this reagent, the subunits would then reassociate to form the active octameric oligomer.

We report here evidence for dissociation, renaturation, re-association and rehybridization of bovine liver ALA-D attached to Sepharose and we propose a scheme for describing the different matrix-bound derivatives obtained.

**MATERIALS AND METHODS**

Im mobilized bovine liver ALA-D was prepared following the procedure reported by Stella et al. (1977), except that Sepharose 4B was activated using 30-50 mg of cyanogen bromide per ml of packed gel. *Euglena gracilis* ALA-D was purified according to Stella & Batlle (1978).

Determination of enzyme activity of both soluble and different derivatives of matrix-bound protein, units of dehydratase activity and specific activity of the enzyme; as well as all other materials and methods not specified here were those already described (Stella et al., 1977; Stella & Batlle, 1978).

Soluble enzyme was pretreated for 5 min at 0 with different concentrations of urea. Then, enzyme activity was assayed under the standard conditions of incubation. When reversibility of urea inhibition was investigated, pretreated enzyme was freed of urea by passage through a Sephadex G-25 column equilibrated and eluted with 0.134 M phosphate buffer pH 6.8 containing 0.1 M KCl. It was found that gel filtration or dialysis were equally effective for removing urea; however the former technique was the one routinely used for it was quicker than the latter. When activity was measured in the presence of urea, corrections were made for its interference in the determination of PBG.

Dissociation of immobilized ALA-D was carried out at 6 by washing a column packed with 10-15 ml of gel-enzyme (1.2 cm width) with 0.134 M phosphate buffer pH 6.8/0.1 M KCl also containing 3 or 6 M urea as indicated, until no further protein was eluted. The course of elution was followed by measuring the absorbance of the eluates at 280 nm. Both activity and total amount of protein eluted were determined in the combined eluates after removal of urea by gel filtration.

Renaturation of the resultant matrix-bound dissociated enzyme was accomplished at 6 by washing the column with about 100 ml of the renaturing buffer mixture: 0.134 M phosphate buffer pH 6.8/0.1 M KCl and 25 mM GSH without urea; then activity was measured in the slurry.

Re-association or rehybridization studies of the matrix-bound subunits formed by denaturation of the bound-octameric bovine liver ALA-D with urea, were carried out by passing a discontinuous gradient (100 ml: 2.0 0.8: 0.4: 0 M urea, in 0.134 M phosphate buffer, pH 6.8/0.1 M KCl; 25 mM GSH) containing 200 units of soluble bovine liver enzyme (or 120 units of soluble *E. gracilis* ALA-D) per ml of gel, through a packed column, and finally washing with renaturing buffer mixture until no further protein and activity was eluted.

From preliminary comparative experiments performed by using a packed column and a batch procedure, it was found that both dissociation and re-association of the bound enzyme was superior when employing the former system.

The protein eluates obtained after treatment of the bound-enzyme with urea and samples of soluble ALA-D both native and urea treated, were subjected to electrophoresis in polyacrylamide-gels, following Weber & Osborn (1969), with slight modifications.

**RESULTS AND DISCUSSION**

**Inhibition by urea**

Taking into account data reported by Nandi (1971), preliminary experiments were carried out to study the *in vitro* effect of different concentrations of urea on the activity of soluble bovine liver ALA-D (Fig. 1). Inhibition rapidly increases with increasing concentrations of urea: 1 M urea brings about 80% inhibition which is practically 100%, at 2 M. It is also shown that the inhibition by 1 M and 2-3 M urea is 80% and 70%, respectively. This work seems to be in line with that of Nandi (1971), therefore supporting his hypothesis about the dual effect of urea on the activity of ALA-D.

On the basis of these results, it was feasible to apply Chan's method (1970) for dissociating the Sepharose-ALA-D octamer into subunits, and characterize some of the properties of both the bound and solubilized subunits, by using urea as the dissociating agent.

**Treatment of Sepharose-ALA-D with urea**

The study of Sepharose-ALA-D subunits, implies the binding of intact octamer enzyme to the gel, followed by treatment with dissociating agents, generally protein denaturants, to remove those subunits not covalently attached and finally, renaturation of both products.

The specific activity of Sepharose-ALA-D was approximately 30%, of that of the soluble pure enzyme used in its preparation. In a previous paper (Stella & Batlle, 1977) we obtained matrix-bound ALA-D...
Table 1. Activity of insolubilized ALA.D derivatives

| Enzyme form               | Total activity (units) | Percentage activity | Specific activity |
|---------------------------|------------------------|---------------------|-------------------|
| Seph-ALA.D<sub>native</sub> | 8347                   | 100                 | 9.1               |
| Seph-ALA.D<sub>Urea, 3 M</sub> | 4175                   | 50                  | 4.0               |
| Seph-ALA.D<sub>Urea, 6 M</sub> | 1743                   | 21                  | 2.0               |
| Solubilized protein*      | 941                    | —                   | 19.2              |

Seph-ALA.D<sub>native</sub> native ALA.D covalently attached to Sepharose.
Seph-ALA.D<sub>Urea, 3 M</sub> = Seph-ALA.D treated with 3 M Urea.
Seph-ALA.D<sub>Urea, 6 M</sub> = Seph-ALA.D treated with 6 M Urea.
* Dissociated subunits, obtained after treatment of Seph-ALA.D with 3 M Urea, as described in Methods.

For the preparation of Seph-ALA.D 33,944 units of pure soluble enzyme were used; coupling yield, calculated on the basis of the amount of enzymic units added was 30%.

The change of the bound enzyme from an octamer into a tetrameric and dimeric state. We do not actually know at present the number of sites of attachment of the ALA.D to the gel; however these findings indicate that no more than two subunits are involved in the binding to the support, although we cannot exclude either, that under variable experimental conditions, the coupling could occur through a single or even through more than two subunits. Moreover, different concentrations of urea appears to specifically affect the inter-dimeric contact in the octamer.

Reassociation experiments

The reversibility of the dissociation process was investigated, to see if the matrix-bound derivatives would pick up subunits added in solution. The Sepharose-ALA.D dissociated, packed in a column was eluted with a decreasing discontinuous gradient of 2–0 M urea, in the presence of soluble ALA.D as described above. After extensively washing with the renaturing buffer mixture until the eluate was free of ALA.D activity; the gel was assayed to detect whether bound subunits had re-associated or re-hybridized with subunits in solution to re-form the matrix-bound octamer. The data of Table 2 show that the above treatment increased the activity of the bound subunits derivatives up to a value equal to that of the bound native ALA.D. These results indicate that the immobilized subunits are able to re-associate with the

| Conditions | Total activity (units) | Percentage activity | Specific activity |
|------------|------------------------|---------------------|-------------------|
| Seph-ALA.D<sub>native</sub> | 3970                   | 100                 | 9.13              |
| Seph-ALA.D<sub>Urea, 3 M</sub> | 2089                   | 52                  | 4.5               |
| Seph-ALA.D<sub>Urea, 6 M</sub> | 827                    | 20                  | 2.0               |

(1) Treated with buffer mixture + urea
(2) Treated with buffer mixture + urea, in the presence of soluble subunits.
(2a) From bovine liver ALA.D
(2b) From E. gruiclis ALA.D
Seph-ALA.D<sub>Urea, 6 M</sub> Treated with soluble bovine liver ALA.D subunits 4010 101 9.30

Experimental conditions were as described in Materials and Methods.
Table 3. Characteristics of soluble ALA.D and its insolubilized derivatives

| Enzyme form       | Apparent $K_m$ ($\times 10^{-4}$ M) | Optimum pH |
|-------------------|-------------------------------------|------------|
| Soluble ALA.D     | 1.7                                 | 6.8        |
| Seph-ALA.D$_{native}$ | 0.5                                 | 6.8 7.0    |
| Seph-ALA.D$_{diss.3M}$ | 0.5                                 | 6.8 7.0    |

Experimental conditions were as described by Stella & Batlle (1977a) or in Materials and Methods.

native subunits generated in situ, to yield a product resembling the original bound-enzyme. These effects were specific, since control experiments in which columns were washed, either with the urea gradient in the absence of soluble bovine liver ALA.D or with soluble native ALA.D in the same buffer mixture but in the absence of soluble bovine liver ALA.D, showed practically no increase in the activity of the insolubilized dissociated enzyme. It was also found that the bound species picked up an amount of protein and enzyme activity almost equal to that initially lost by the insolubilized enzyme.

The specific activity of the re-associated bound enzyme also approached that of the insoluble ALA.D, confirming restoration of the native octamer structure. It was also found that the bound species picked up an amount of protein and enzyme activity almost equal to that initially lost by the insolubilized octamer.

In one experiment, the bound-ALA.D dissociated was re-hybridized with soluble E. gracilis ALA.D; in this instance, restoration of 75% activity also occurred. This suggests that it might be possible to form active hybrids of the bovine liver and the E. gracilis enzymes, and it further shows, that if there are any differences in the aminocacid sequence between these two dehydratases, they seem not to significantly modify the inter-subunit contact sites, so that, domains involved in subunit interaction appear to have been preserved in the evolution of these species.

Therefore, this technique proves useful for hybridization studies, between dehydratases from different species or between native and chemically modified subunits, and complementation investigations including restoration of activity by non-covalent interaction of enzyme subunits.

Characteristics of the soluble and insolubilized derivatives of bovine liver ALA.D

To obtain some insight on the enzymic properties of the insolubilized derivatives here prepared, their optimum pH and $K_m$ values were calculated. From data listed in Table 3, as already found (Stella et al., 1977), the kinetic properties of ALA.D are altered by its binding to Sepharose; a decrease in the $K_m$ of the matrix-bound enzyme has been observed, while there is practically no difference in the optimum pH between free enzyme and its insoluble analogue. Further, the same optimum pH and $K_m$ values were calculated for the matrix-bound ALA.D subunit derivative, indicating a similarity in the catalytic properties of the different oligomeric forms of the enzyme, and raising the question of why the octameric structure is actually necessary.

Polyacrylamide gel-electrophoresis

Different samples of bovine liver ALA.D, either native or treated with urea and solubilized subunits, were subjected to polyacrylamide gel-electrophoresis (Fig. 2). Molecular weights of the resulting protein bands were calculated. It was found that in the presence of 3 M urea, a mixture of octamer and tetramer enzyme is present, while in 6 M urea, octamers, tetramers and dimers are visualized. Recombination of the solubilized subunits could also be detected by this procedure, where again the main species were octamers and tetramers.

CONCLUSIONS

The aim of this work was to investigate the role of the octameric structure of bovine liver ALA.D on the conformation and function of this enzyme. Many oligomeric enzymes are formed by identical or closely similar subunits, containing each an active site; so the significance of a quaternary structure is not very clear. However, it appears that evolution has chosen, for the sake of regulation, that the potential for cooperativity must be preserved, so that many proteins might become active in regulated catalysis as an oligomer. The results reported here, tempted us to propose a scheme that, although rather speculative, would describe the sequence of events which lead from the bound octameric ALA.D to the different subunits derivatives (Fig. 3).

The immobilized native octameric ALA.D (1) seems to be covalently linked to the gel by an average of two subunits. Treatment with less concentrated urea would remove a tetramer (2) leaving therefore the other half bound to the matrix (3); after removal of urea, the solubilized subunits can recover its octamer.
Fig. 3. Scheme representing the relationship between the different insolubilized ALA:D derivatives and the sequence leading to them from the Sepharose-bound native enzyme. See the text for explanation.

Since the immobilized subunit derivative still exhibits catalytic properties, it would appear that each catalytic site could function somehow independently of the other, but only within a dimer formation. On the other hand, it has already been established that only 4 of the 8 subunits of ALA:D form a Schiff base with one molecule of substrate, indicating that the enzyme exhibits the phenomenon of half-site reactivity, and according to the mechanism postulated by Nandi et al. (1968) only one of the two molecules of ALA in the reaction, forms a covalent bond with the enzyme: it is therefore possible, that we are dealing with a minimal functional dimer formed by two kinds of subunits, that, although having similar composition, play a different role in PBG synthesis. We propose that one subunit might be involved in the formation of the Schiff base with one molecule of ALA and the other in the non-covalent binding with the second molecule of substrate. Nonetheless, these are just assumptions, for the available experimental data cannot yet define which is the minimal structure necessary for activity. Experiments on this line are in progress in our laboratory and further results are eagerly awaited.

Acknowledgements—This work was supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina and the University of Buenos Aires. A. M. del C. Batlle and E. A. Wider de Xifra are members of the Career of Scientific Researcher in the CONICET. We thank Miss Hilda Garparoli for her technical assistance and Lic. Ana Lucci and Haydee Fukuda for their interest and valuable help in some of the early experiments.

REFERENCES

BATLLE A. M. DEL C., FERRAMOLA A. M. & GRINSTEIN M. (1967) Purification and general properties of delta aminolaevulic dehydratase from cow liver. Biochem. J. 104, 244-249.

BATLLE A. M. DEL C., FERRAMOLA A. M. & GRINSTEIN M. (1970) Delta aminolaevulinate dehydratase (cow liver). In Methods in Enzymology (Edited by Tabor H. & Tabor C.) Vol. 17. Part A, pp. 216-220. Academic Press.

BRUCH R., SCHNACKERZ D. & GRACY R. W. (1976) Matrix-bound phosphoglucose isomerase-formation and properties of monomers and hybrids. Eur. J. Biochem. 68, 153-158.

CARVAJAL N., MARTINEZ J. & FERNANDEZ M. (1977) Immobilized monomers of human liver arginase. Biochim. biophys. Acta 481, 177-183.

CHAN W. W.-C. (1970) Matrix-bound protein subunits. Biochim. biophys. Acta 41, 1198-1204.
CHAN W. W.-C. (1973) Active subunits of trans-aldolase bound to Sepharose. Eur. J. Biochem. 40, 533–541.

CHAN W. W.-C. & MAWER H. M. (1972) Studies on protein subunits II—preparation and properties of active subunits of aldolase bound to a matrix. Archs Biochem. Biophys. 149, 136–145.

FELDMAN K., ZEIDEL H. J. & HELMKIRCH E. J. M. (1976) Complementation of subunits from glycogen phosphorylases of frog and rabbit skeletal muscle and rabbit liver. Eur. J. Biochem. 65, 285–291.

KLOTZ L. M., LANZERMAN N. R. & DARNELL D. W. (1970) Quaternary structure of proteins. A. Rev. Biochem. 39, 25–62.

NAGARADOVA N. K., GOLOVINA T. O. & MEVKH A. T. (1974) Immobilized dimers of d-glyceraldehyde-3-phosphate dehydrogenase. FEBS Lett. 49, 242–245.

NANDI D. L. (1971) Inhibition of delta aminolevulinic acid dehydratase of Rhodopseudomonas spheroides by urea, guanidine and methyl derivatives of urea. Archs Biochem. Biophys. 142, 157–162.

NANDI D. L., BAKER-COHEN K. F. & SHEMY D. (1968) Delta aminolevulinic acid dehydratase of Rh. spheroides. I. Isolation and mechanism. J. Biol. Chem. 243, 1224–1230.

SHEMY D. (1976) 5-Aminolaevulinic acid dehydratase: structure, function and mechanism. Phil. Trans. R. Soc. B. 273, 109–115.

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

STELLA A. M. & DEL C. (1978) Porphyrin biosynthesis—immobilized enzymes and ligands VIII. Studies on the purification of delta aminolaevulinate dehydratase from Euglena gracilis. Pl. Sci. Lett. In press.

STELLA A. M., WIDER DE XIRA E. A. & DEL C. (1977) Porphyrin biosynthesis—immobilized enzymes and ligands IV. Studies on aminolaevulinate dehydratase attached to Sepharose. Molec. Cell. Biochem. 16, 97–105.

WEBER K. & OSBORN M. (1967) The reliability of molecular weight determination by dodecyl sulphate in polyacrylamide gel-electrophoresis. J. Biol. Chem. 244, 4406–4412.