Int. J. Biochem., Vol. 9, pp. 401 to 406

0020-711x/78/0601-0401$02.00/0

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 Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Nuñez, 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; Nagradova *et al.*, 1974; Feldman *et al.*, 1976; Bruch *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 porphobilinogen (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 Shemin (1976).

Previous work with the *Rh. spheroides* 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 also 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

Immobilized 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 enzymic 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 enzymic 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 CySH, without urea; then, activity was measured in the slurry.

Re-association or re-hybridization 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 CySH) 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% reversible, 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

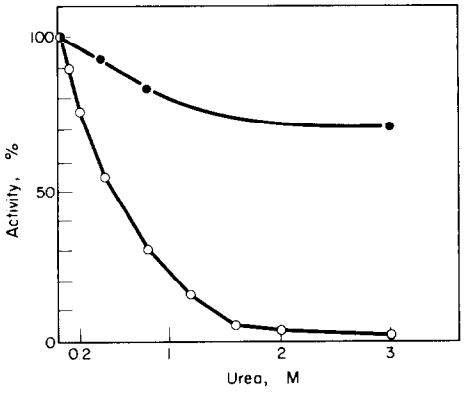

Fig. 1. Inhibition of soluble bovine liver ALA.D by Urea and its reversibility. The conditions of incubations and assays are described in the text. (○—○): activity was measured in the presence of urea. (●—●): activity was measured after removing urea by gel filtration.

Table 1. Activity of insolubilized ALA.D derivatives

Enzyme form	Total activity (units)	Percentage activity	Specific activity
Seph-ALA.D_{native}	8347	100	9.1
Seph-ALA.$D_{dis-3\ M}$	4175	50	4.0
Seph-ALA.$D_{dis-6\ M}$	1743	21	2.0
Solubilized protein*	941	—	19.2

Seph-ALA.D_{native} native ALA.D covalently attached to Sepharose.
Seph-ALA.$D_{dis-3\ M}$ Seph-ALA.D treated with 3 M Urea.
Seph-ALA.$D_{dis-6\ M}$ = 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%.

with specific activity as high as 70–75% that of the enzyme in solution, but it might be recalled that both the amount of cyanogen bromide used in the activation of the gel and that of the protein added were higher than those used in these experiments. We have already discussed the reasons why the activity of the bound enzyme is lower than that of the native soluble enzyme. These amounts were deliberately diminished, to reduce the degree of multi-point attachment of ALA.D.

When a column of immobilized bovine liver ALA.D was treated with 3 and 6 M urea, as is seen in Table 1, enzymic activity was detected in the eluate; samples of the gel removed from the column and washed with the renaturing buffer mixture, also showed activity in all cases, although the percentage of the original activity regained was dependent on the concentration of urea used.

After elution with 3 M urea the matrix-bound subunit recovered a level of enzymic activity equal to 50% of the immobilized native ALA.D, treatment with 6 M resulted in a derivative with only 20% remaining activity; while specific activities were close to 4 and 2 respectively. It is noteworthy that the specific activity of the solubilized subunits was twice that of the insolubilized ALA.D and near that of the original pure soluble preparation. The fact that after treatment with urea, 50% and close to 25% of the activity bound to the matrix remained, suggests a 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.

Re-association 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

Table 2. Re-association and hybridization experiments

Conditions	Total activity (units)	Percentage activity	Specific activity
Seph-ALA.D_{native}	3970	100	9.13
Seph-ALA.$D_{dis-3\ M}$	2089	52	4.5
Seph-ALA.$D_{dis-6\ M}$	827	20	2.0
Seph-ALA.$D_{dis-6\ M}$			
(1) Treated with buffer mixture + urea	1100	25	2.0
(2) Treated with buffer mixture + urea, in the presence of soluble subunits.			
(2a) From bovine liver ALA.D	4089	102	9.93
(2b) From *E. gracilis* ALA.D	3000	75	7.27
Seph-ALA.$D_{dis-3\ M}$			
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.$D_{dis-3\ M}$	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 without the urea gradient, showed practically no increase in the activity of the insolubilized dissociated 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 enzymic 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 aminoacid 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_ms 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

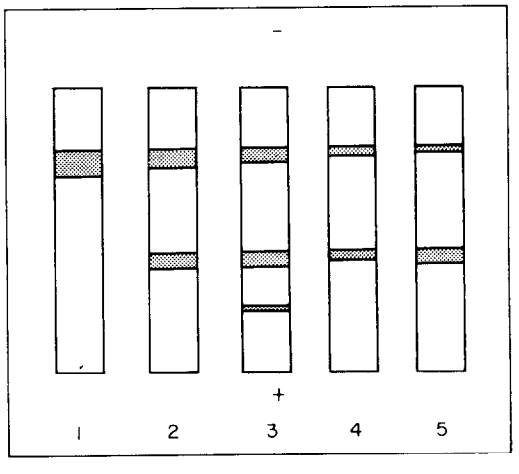

Fig. 2. Polyacrylamide gel-electrophoresis of soluble ALA.D and its subunits obtained after urea treatment as indicated. 1: Native soluble ALA.D; 2: Native soluble ALA.D pretreated with 3 M urea and run in the presence of 3 M urea; 3: as in 2, but using 6 M urea; 4: Solubilized subunits of ALA.D obtained after treatment with 3 M urea of the Sepharose-ALA.D and then freed of urea by gel filtration; 5: the same preparation as that used in 4, but run in the presence of 3 M urea.

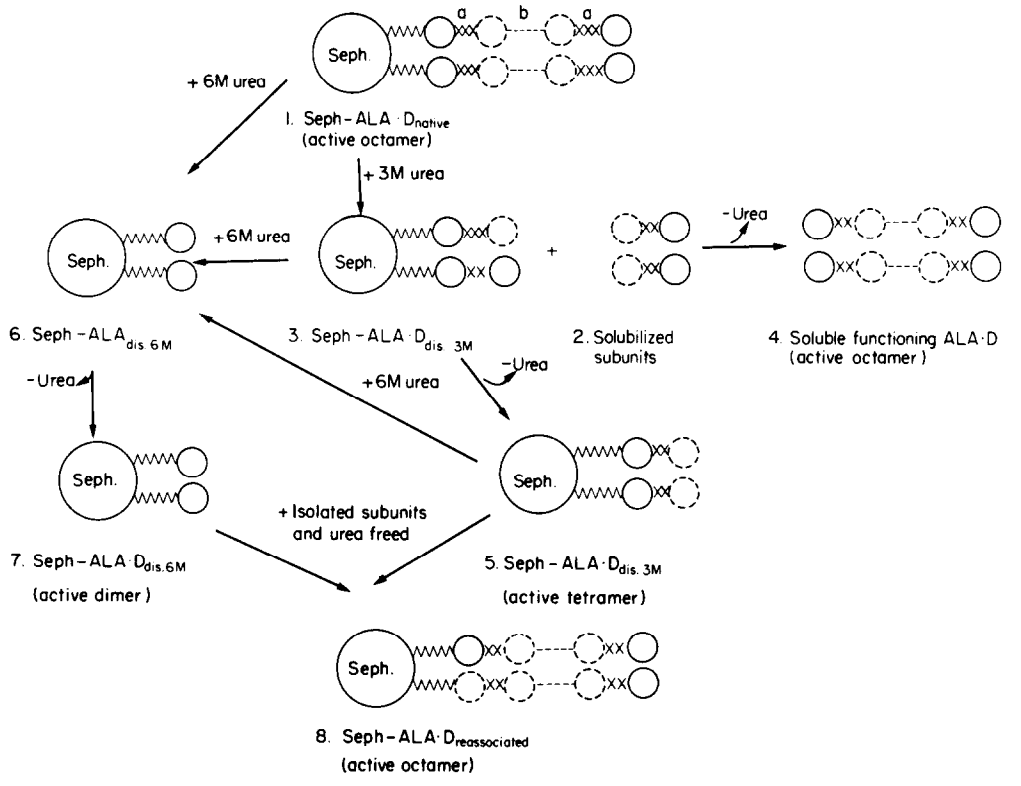

Fig. 3. Scheme representing the relationship between the different insolubilized ALA.D derivatives and the sequence leading to them from the Sepharose-bound native enzyme. See the text for explanation.

structure, re-assembling into normally soluble functioning ALA.D (4); while the bound tetramer is still enzymically active (5) it can be dissociated further by higher concentrated urea into a bound-dimeric derivative (6); suggesting that there should exist different types of binding between dimers and tetramers (shown as *a* and *b* in the insoluble derivative number 1). Finally, both of these bound-dissociated species (5 and 7), are able to pick up soluble subunits and restore the original octameric structure (8); confirming that urea has depleted the immobilized ALA.D of subunits, but that these can be replaced under convenient re-association conditions.

Since the immobilized subunit derivative still exhibit 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 Cientificas 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 Gasparoli for her technical assistance and Lic. Ana Lucci and Haydée 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., ZEISEL H. J. & HELMREICH 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 I. M., LANZERMAN N. R. & DARNELL D. W. (1970) Quaternary structure of proteins *A. Rev. Biochem.* **39,** 25–62.

NAGRADOVA 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. & SHEMIN D. (1968) Delta aminolevulinic acid dehydratase of *Rh. spheroides*. I. Isolation and mechanism. *J. biol. Chem.* **243,** 1224–1230.

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

STELLA A. M. & BATLLE 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. & BATTLE 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 XIFRA E. A. & BATLLE A. M. 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. (1969) The reliability of molecular weight determination by dodecil sulphate in polyacrylamide gel-electrophoresis. *J. biol. Chem.* **244,** 4406–4412.