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PIG LIVER AMINOLEVULINATE DEHYDRATASE II. STUDIES ON DIFFERENT CONDITIONS INFLUENCING ITS MOLECULAR WEIGHT DETERMINATION

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Abstract—1. By chromatography through Sephadex G-200 of increasing amounts of partially purified pig liver ALA-D, different profiles were obtained, showing that species of molecular weights ranging from 140,000 to 560,000 might exist in equilibrium, but their relative ratio was dependent on the total amount of protein sampled. In all cases, however, the main peak (60–70%) corresponded to the 280,000 MW oligomer, that is the octamer.

2. It was found that elution profiles were also dependent on column dimensions and on the purity of the enzyme preparation.

3. K^+ ions affected both catalytic activity and aggregation of the enzyme.

4. Results here reported add further support to the proposal of the existence of a minimal functional dimer.

INTRODUCTION

Aminolevulinate dehydratase (ALA-D) (EC 4.2.1.24) has been isolated and studied from very many sources, and different molecular weights (MW) have been reported in the literature, Thus, the MW of the bovine liver dehydratase calculated by sedimentation equilibrium has been found to be 282,000 (Wu *et al.*, 1974) and reported to be 140,000 by gel filtration (Batlle *et al.*, 1967); MWs of 250,000 280,000; 283,000 and 270,000 were reported for the enzyme isolated from *Rh. spheroides*, soybean callus, human erythrocytes and mouse liver (Van Heyningen & Shemin, 1971; Tigier *et al.*, 1970; Coleman, 1966; Calissano *et al.*, 1966). This discrepancy might be due not to the source of ALA-D, but to the experimental conditions under which measurements have been made, that could influence the state of aggregation of the protein.

It is now accepted however, that ALA-D independently of the source, has a MW of about 280,000 and appears to be formed by 8 similar subunits of MW 35,000.

Studies on the dissociation, renaturation, reassociation and rehybridization of bovine liver ALA-D attached to Sepharose (Gurne *et al.*, 1977; Batlle *et al.*, 1978) are in agreement with the proposed octameric structure for ALA-D.

In view of the varying MWs reported for enzyme preparations from the same and different sources and our large experience in this area, we have decided to redetermine the MW of the dehydratase under several experimental conditions using the enzyme isolated and purified from pig and bovine liver. We will only report here, data obtained with the pig liver ALA-D.

MATERIALS AND METHODS

All chemicals were purchased from Sigma Chemical Co., unless otherwise stated. Sephadex and Sepharose 4B gels were from Pharmacia Fine Chemicals, Uppsala.

Source material of enzyme

Fresh pig liver was obtained from a local slaughter house, immediately frozen in a solid CO_2 and transported to the laboratory within half an hour. ALA-D was purified and assayed according to Polo, Stafforini, Stella *et al.* (1980). The experiments were carried out as indicated with two different preparations of ALA-D:

(a) partially purified enzyme, obtained after fractionation with a 30–55% ammonium sulphate (15-fold):
(b) purified enzyme, obtained after chromatography through Sephadex G-200 (200-fold).

Specific activities are expressed as the amount of enzyme catalysing the formation of 1 μmol of PGB/min, per mg of protein.

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

Gel columns were prepared following the usual technique described by Batlle *et al.* (1965) and Batlle (1968). The molecular weights were estimated according to Batlle (1967) and Locascio *et al.* (1967). The percentage of each protein peak with ALA-D activity was calculated by the method of integrating surfaces.

RESULTS AND DISCUSSION

Influence of the amount of protein in MW determinations by gel filtration

It has been clearly demonstrated by Shemin *et al.* (Heyningen & Shemin, 1971; Nandi & Shemin, 1978a,b) that the enzyme of *Rh. spheroides* readily

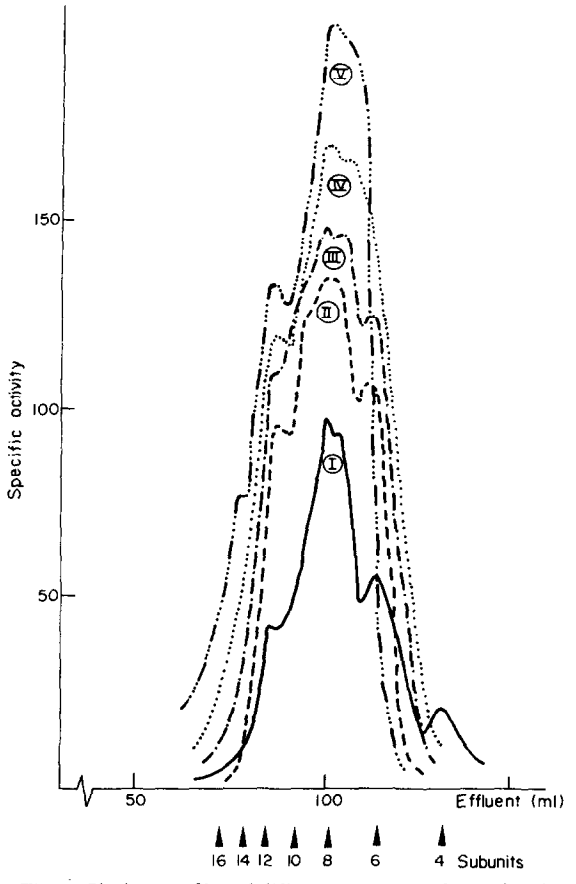

Fig. 1. Elution profiles of different amounts of protein of a partially purified preparation of pig liver ALA-D on a Sephadex C-200 column (2.4 cm × 54 cm; V:97 ml; flow rate:18 ml/hour; temperature: 4–8°C). Protein was applied to the column and eluted with 0.05 M Na^+ phosphate buffer pH 6.8 until adsorption due to protein fell to zero. I. 1.45 mg protein (——); II. 70 mg (- - - -); III. 105 mg (—·—). IV. 150 mg (. . . .); V. 340 mg (—· · ·—).

forms aggregates under certain conditions; however, depending on the method, different MW profiles are obtained. These authors have also observed that *Rh. spheroides* ALA-D dissociates either in dilution or in 1 M urea to protein species of MW 120,000. Gel filtration studies with bovine liver ALA-D from this laboratory (Batlle *et al.*, 1967) also provided indications that this enzyme, as well as others, can dissociate to subunits. We then reexamined that particular set of experiments, where dissociation of ALA-D had been observed and found that several factors could have been responsible for the association-dissociation equilibrium between protomers and oligomers.

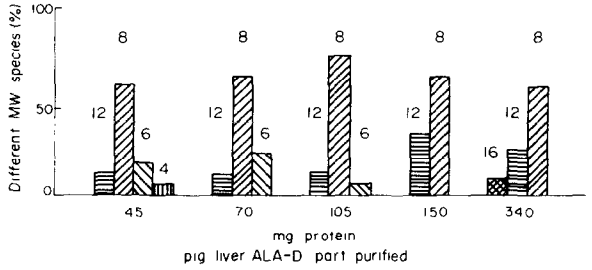

Fig. 2. Effect of the amount of protein in the relative ratio of the different MW species in equilibrium. Results here represented were obtained from the profiles illustrated in Fig. 1; with 4, 6, 8, 12 and 16, we indicate the number of subunits corresponding to each MW species.

The first parameter to be tested was the amount of protein applied to the column. Therefore, with the same Sephadex G-200 column, several runs were performed using increasing amounts of a partially purified enzyme preparation. Results obtained are shown in Fig. 1. Different profiles were obtained indicating that species of MWs ranging from 140,000 to 560,000 might exist in equilibrium; however their relative ratio was dependant on the total amount of protein chromatographed. This is better illustrated in Fig. 2. In all cases, the main peak (60–70%) corresponds to the 280,000 MW species, that is, the octamer, which is in equilibrium with larger aggregates of MW 420,000 (12 subunits) and 560,000 (16 subunits) and other species of MW 210,000 (6 subunits) and 140,000 (4 subunits), but their relative ratios were always much lower. It can also be observed that when sampling more than 110 mg, the proportion of the 12 subunits oligomer significantly increases, and concomitantly diminishes the octomer, disappears the hexamer, and an even larger aggregate of 16 subunits is formed. All of these species are enzymically active, very likely due to their dissociation or association to the active oligomer, under the assay conditions. However, it has not yet been definitely clarified which is actually the structure necessary for maximum activity, although we assume that is the 280,000 MW species.

Influence of column dimensions and purity of the enzyme preparation

It was also evident that when we were chromatographing the same amount of protein of a partially

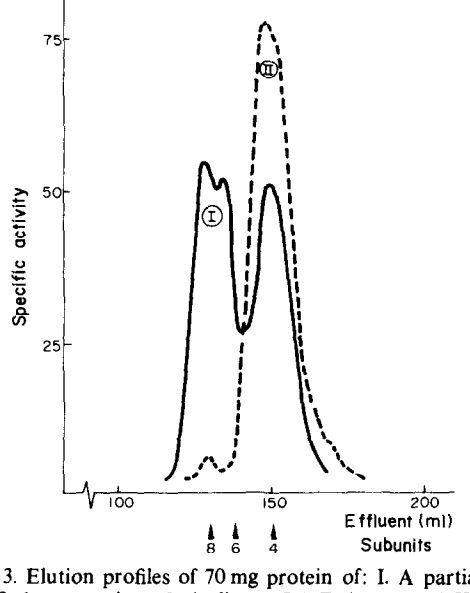

Fig. 3. Elution profiles of 70 mg protein of: I. A partially purified preparation of pig liver ALA-D (——) and II. A purified preparation (- - - -) on a Sephadex G-200 column (2.4 cm × 70 cm; V_o:111 ml; flow rate:18 ml/hour; temperature: 4–8°C). Protein was applied to the column and eluted with 0.05 M Na^+ phosphate buffer pH 6.8.

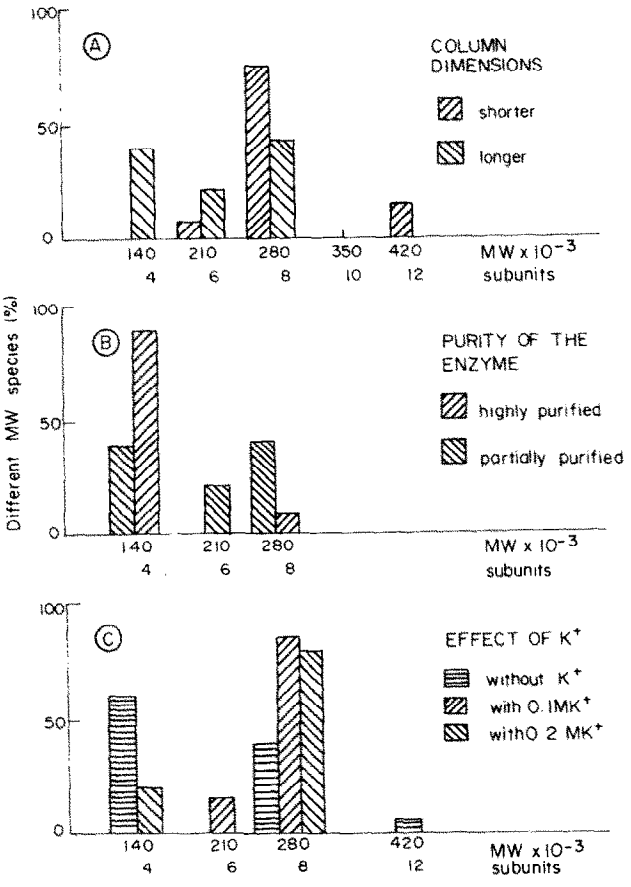

Fig. 4. A: Influence of column dimensions: two samples of 70 mg each, of partially purified pig liver ALA-D were applied on a longer Sephadex G-200 column (2.4 × 70 cm) (▨) and on a shorter column (2.4 × 54 cm) (▨). B: influence of the degree of purity: 70 mg of a partially purified ALA-D (▨) and 70 mg of a higher purified fraction (▨) were chromatographed on a larger Sephadex G-200 column. C: influence of K^+ ions: three samples of 70 mg each of a purified preparation of ALA-D were chromatographed on a shorter Sephadex G-200 (▨) eluted with 0.05 M Na^+ phosphate buffer pH 6.8; (▨) eluted with the same buffer containing 0.1 M KCl; (▨) eluted with buffer containing 0.2 M KCl.

purified preparation of ALA-D in columns differing in their dimensions, again different profiles were obtained. Furthermore, an interesting finding was that after chromatography on Sephadex G-200 of a highest purified preparation of pig liver ALA-D, a rather simple profile was found, where the 140,000 MW species was predominant (90–95%) and only 5–10% corresponded to the 280,000 MW. In Fig. 3 we can see the results of chromatographying, in a largest column, equal amounts of a partially purified (I) and a purified preparation of ALA-D (II). If we compare curve I in Fig. 3 with curve II in Fig. 1, we can clearly observe (Fig. 4, A), that the elution profile and therefore the relative ratios of the different MW aggregates is also highly dependent on the column dimensions. From Fig. 3 and Fig. 4, B, it can be added that MW determination by gel filtration, varies too with the purity of the enzyme preparation.

Influence of K^+ ions

It has been demonstrated by Nandi & Shemin (1968a) that K^+ ions are needed for activation of *Rh. spheroides*, ALA-D which easily aggregates in the presence of 0.05 M K^+ to reach an equilibrium between monomers, dimers and trimers.

In our early studies, we did not test the effect of K^+ ions on ALA-D activity nor on its MW.

Studying the action of increasing concentrations of K^+ added to the incubation mixture, it was found that above 0.1 M K^+ the enzyme activity was inhibited 10% and nearly 30% at 0.2 M K^+.

On the other hand, purified preparations of ALA-D were run on a Sephadex G-200 column, without K^+ and with elution buffer containing 0.1 M and 0.2 M K^+. Results obtained are shown in Fig. 4, C where as expected K^+ ions affected aggregation of the enzyme.

Finally it is worth noting that in the experiments here described, species corresponding to aggregates of 4, 6, 8, 12 and 16 subunits exist in equilibrium; from these, the octamer is usually the dominant species but under certain conditions the main component can be the tetramer. These results are also indicating that dissociation or association occur between dimers or tetramers and would add further support to the proposal that the minimum quaternary structure necessary for exhibiting catalytic activity can be a dimer.

formed by two kind of subunits, that although having similar composition would play a different role in Porphobilinogen synthesis (Batlle *et al.*, 1978; Batlle & Stella, 1978).

In conclusion, MW determinations by gel filtration, at least in the case of oligomeric proteins readily dissociated, such as ALA-D, seem to be dependent on the amount of protein chromatographed, on the column dimensions, on the purity of the sample and on the composition of the elution buffer.

As already suggested by Shemin (1972) our reported MW of 140,000 for the bovine liver enzyme was the result of having performed its measurement under conditions which had favored dissociation of the enzyme; in fact, only 40 mg of highly purified ALA-D were run in the absence of K^+ ions, resembling the profile II shown in Fig. 3.

Therefore, when carrying out studies similar to those here described it is necessary to clearly define the experimental conditions followed.

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