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STUDIES ON ASPARTASE

VII. SUBUNIT ARRANGEMENT OF *ESCHERICHIA COLI* ASPARTASE

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Aspartase (L-aspartate ammonia-lyase, EC 4.3.1.1) of *Escherichia coli* is composed of four subunits of seemingly identical molecular weight (Suzuki, S., Yamaguchi, J. and Tokushige, M. (1973) Biochim. Biophys. Acta 321, 369–381). The subunit arrangement of the enzyme was studied by two distinct methods, cross-linking of subunits with a bifunctional reagent, dimethyl suberimidate, and statistical classification of negatively stained electron microscopic images. In the former method, the densitometric patterns of the cross-linked aspartase were analyzed quantitatively after separating each component by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and the results were compared with the theoretical distribution. In the latter method, a number of electron microscopic images were classified into several groups according to their characteristic appearance. The results obtained by these two methods are compatible with the possibility that the enzyme has a tetrameric structure consisting of two pairs of dimers, in which the two pairs of rod-shape subunits meet perpendicularly, being typical of D_2 symmetry.

Introduction

Aspartase (L-aspartate ammonia-lyase, EC 4.3.1.1) purified from *Escherichia coli* W cells has a molecular weight of 193 000 and is composed of four seemingly identical subunits with L-serine as the NH_2-terminal residue [1,2]. The oligomeric structure of the enzyme appears to be fairly stable and dissociation of the enzyme into subunits does not readily occur, unless treated with potent denaturants, such as guanidine-HCl and SDS. As previously reported from this laboratory [3], aspartase denatured in 4 M guanidine-HCl is renatured in vitro by simple dilution with a concomitant restoration of the activity; the enzyme renatured at low temperature (0–4°C), however, remains as inactive subunits; as the temperature is raised to 25–30°C, the activity is restored concomitant with the formation of the tetrameric structure. The inactive subunits being present at the low temperature were recently identified to be monomers [x]. In this

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Supplementary data to this article are deposited with, and can be obtained from, Elsevier/North-Holland Biomedical Press B.V., BBA Data Deposition, P.O. Box 1345, 1000BH Amsterdam, The Netherlands. Reference should be made to No. BBA/DD/188/69381/661 (1981) 261–266. The supplementary information includes equations for determining the cross-linking patterns expected for tetrameric proteins.

[x] These data were presented at the 51th Annual Congress of the Japanese Biochemical Society in Kyoto, Seikagaku 50, 648 (1978).

communication we wish to report the quaternary structure of the enzyme in more detail as estimated by the cross-linking technique and analysis of the electron microscopic images.

Experimental procedures

Monosodium-L-aspartate was a product of Kyowa Hakko Kogyo Co. (Tokyo). Guanidine-HCl and dimethyl suberimidate-2 HCl were products of Nakarai Chemical Co. (Kyoto). Rabbit muscle aldolase (EC 4.1.2.13) was obtained from Boehringer. All other chemicals were of reagent grade.

Aspartase and assay of its activity. Aspartase was extracted and purified to homogeneity from *E. coli* W cells as described previously [1]. The activity was routinely determined spectrophotometrically by measuring the formation of fumarate following the increase in absorption at 240 nm and 30°C. The standard assay mixture contained 100 μmol sodium L-aspartate (pH 8.8)/2 μmol $MgCl_2$/100 μmol Tris-HCl (pH 8.8)/and enzyme in a total volume of 1.0 ml. The molar extinction coefficient of fumarate, 2 530 $mol^{-1} \cdot cm^2$ reported by Emery [4] was used. 1 unit of enzyme was defined as the amount producing 1 μmol fumarate/min under the standard assay conditions. Specific activity was expressed as units/mg protein at 30°C.

Cross-linking with dimethyl suberimidate. Cross-linking reaction of the enzyme was performed with the diimidate freshly dissolved in 0.2 M triethanolamine-HCl buffer (pH 8.5) according to the method of Davies and Stark [5].

Electron microscopy. The enzyme molecules were negatively stained by a modification of the method described in a previous paper [3]. A JEM-7A electron microscope (JEOL, Tokyo), fitted with an anticontamination device, was used at an accelerating voltage of 80 kV. Micrographs were taken at an instrumental magnification of 50 000 or 60 000.

Polyacrylamide gel electrophoresis. Gel electrophoresis in the presence of SDS was performed as described by Weber and Osborn [6] using 5% polyacrylamide gels.

Other determinations. All spectrophotometric determinations were carried out in a Hitachi 124 recording spectrophotometer equipped with a constant temperature cell housing. Aspartase concentration was determined using an $E^{1\%}_{1cm}$ value of 5.9 at 280 nm [3]. Protein concentration was determined according to the method of Lowry et al. [7].

The subunit arrangement was classified according to the models described by Klotz et al. [8] as (1) tetrahedron, (2) square (C_4 symmetry), (3) square (D_2 symmetry).

Results

Cross-linking with bifunctional reagent

Cross-linking of aspartase with dimethyl suberimidate and subsequent SDS-polyacrylamide gel electrophoresis yielded four discrete bands. Fig. 1A shows the results obtained under various reaction conditions. The four bands were assigned as the monomer, dimer, trimer and tetramer in this order from the bottom towards the top, as judged from their relative mobilities on the electrophoretogram. The bands corresponding to the molecular weight of dimer and tetramer were more intensive than that of trimer. Fig. 1B shows the results of similar experiments with rabbit muscle aldolase. Its subunit structure was already elucidated by X-ray crystallography [9,10] as possessing tetrahedral symmetry (222 symmetry) and each subunit was found to locate at the vertices of a regular tetrahedron. As the cross-linking reaction proceeded, the proportion of the monomer decreased, while those of the dimer, trimer and tetramer increased. However, the mode of influence of the reaction conditions on the properties of aldolase subunits appears to be considerably different from those of aspartase.

The reactivity towards the cross-linking reagent is designated as P, Q and R in a tetramer (Fig. 2) *. The relationship between k_p, k_q and k_r is considered to reflect the spacial arrangement of the subunits. Fig. 3 shows the simulated compositions of cross-linked subunits taking relative amounts of each molecular species, C_1, C_2, C_3 and C_4 as the ordinate and the degree of cross-linking ($1-C_1$) as the abscissa. The molecular species which are not cross-linked are expected to behave as the monomer on gel electrophoresis in the presence of SDS.

In order to correlate the results of densitometric patterns of cross-linked aspartase and aldolase to the

* See footnote p. 261.

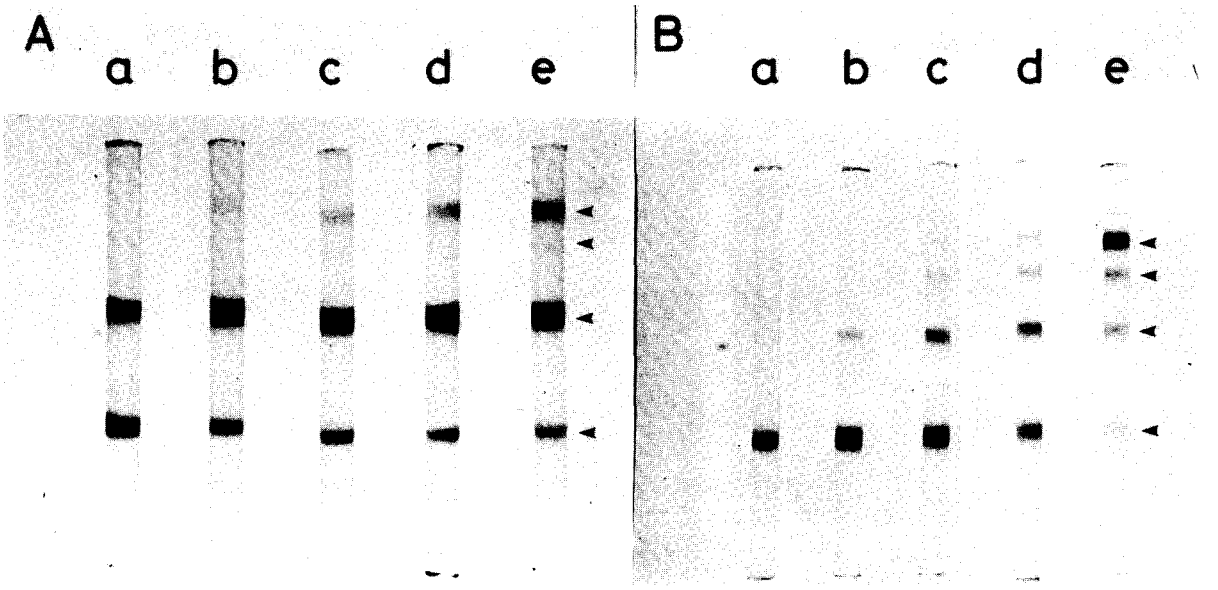

Fig. 1. SDS-polyacrylamide gel electrophoresis. The cross-linking reactions were conducted under the designated concentrations of dimethyl suberimidate and reaction time as given. A. Aspartase: a, 1 mM, 1 h; b, 10 mM, 20 min; c, 5 mM, 1 h; d, 10 mM, 1 h; e, 15 mM, 1 h. Protein concentration, 1.0 mg/ml. Each tube contained 25 μg enzyme protein. Electrophoresis was conducted with a 3 mA current for the first 1 h and 6 mA for the succeeding 12 h. The direction was from the top towards down. After the electrophoresis proteins were stained with Coomassie brilliant blue. B. Aldolase: protein used: a, 12.5 μg; b–c, 25 μg. Electrophoresis was carried out as described for aspartase. The dimethyl suberimidate concentration and the reaction times were: a, 0 (native); b, 0.5 mM, 1 h; c, 5 mM, 20 min; d, 5 mM, 1 h; e, 20 mM, 1 h.

simulated curves, enzyme preparations were treated with dimethyl suberimidate under various conditions and then analyzed by SDS-polyacrylamide gel electrophoresis. After the gels were stained with Coomassie brilliant blue, the concentration of each band was determined densitometrically. A good linearity was demonstrated between the degree of staining and the amount of proteins used under the experimental conditions (data not shown). Each peak in the densitogram was cut and weighed. The results are shown in Fig. 4. Here, the ordinate indicates the relative amount of each molecular species and the abscissa the degree of cross-linking taking the amount of the monomer as a standard. The best-fitted simulation curves were chosen from Fig. 3 and superimposed on Fig. 4. As to c of $k_p = ck_q$, $k_r = 0$, the values of 1, 1/2, 1/4, 1/6, 1/8 and 1/10 were examined. As seen from the results, aspartase appears to have a characteristic of $k_p = 1/8\, k_q$, $k_r = 0$. This strongly indicates that the cross-linkages were easily formed within dimers and that the structure of the dimer of dimers is most likely. These features are compatible with the possibility that the quaternary structure of aspartase is arranged with D_2 symmetry. In contrast, aldolase has a characteristic of $k_p = k_q = k_r$. This feature coin-

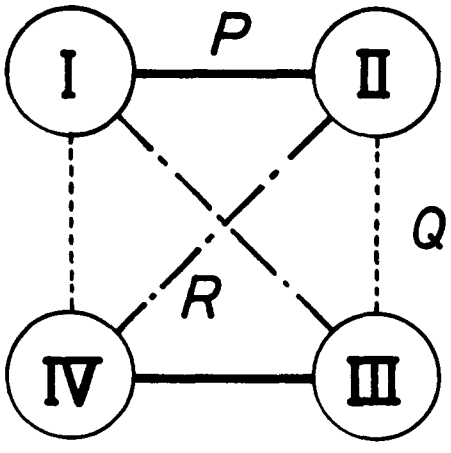

Fig. 2. Possible cross-linkages in the tetramer. I, II, III and IV represent each subunit and P, Q and R represent the cross-linkages among the subunits.

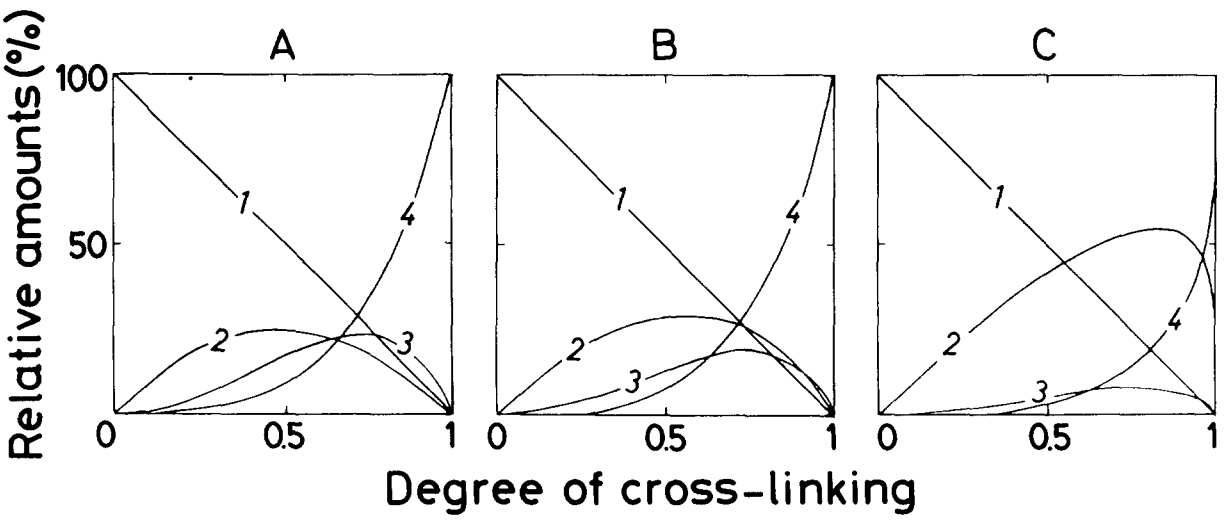

Fig. 3. Simulated composition of cross-linked subunits. Typical examples for the correlation among k_p, k_q and k_r are shown in A, B and C. The numbers 1, 2, 3 and 4 represent the proportions of the monomer, dimer, trimer and tetramer, respectively. A, $k_p = k_q = k_r$; B, $k_p = k_q$, $k_r = 0$; C, $k_p = 1/8\ k_q$, $k_r = 0$.

cides with the tetrahedral structure.

It is of interest that even after the cross-linking reaction proceeded to a large extent, most of the aspartate activity was retained. This fact indicates that neither the modification of the target amino groups of the lysine and arginine residues nor the constrained linking of the subunits interferes with the catalytic function of the enzyme.

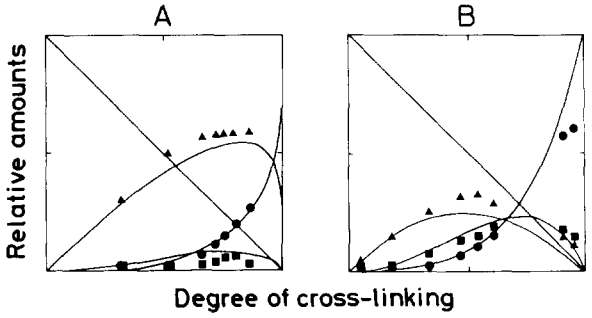

Fig. 4. Composition of cross-linked subunits. The abscissa was expressed taking the amount of the monomer as the standard. ▲, dimer; ■, trimer; ●, tetramer. The solid lines represent best-fitted simulation curves in Fig. 3. A, aspartase; B, aldolase. The experiments were conducted under such conditions that the reagent concentration was 0.5–20 mM, the reaction time was 20 min–3 h, and the protein concentration was 1 mg/ml.

Electron microscopic analysis

As reported previously [3], the negatively stained electron microscopic images of aspartase appear to be tetrameric with a diameter of 7.5–10 nm. In the present work, a number of images were classified into several groups according to their characteristics and the subunit structure was estimated from their distributions. As shown in Fig. 5, essentially five types of the arrangement should be possible for a tetrameric protein, without considering the polarity in each subunit. Since it is possible to anticipate what kinds of image are to be obtained in each case, the possible subunit structure can also be assigned by classification of the images. The electron microscopic photographs obtained with a direct magnification of 60 000-fold were further enlarged on photo-printing paper at a final magnification of 1 000 000-fold.

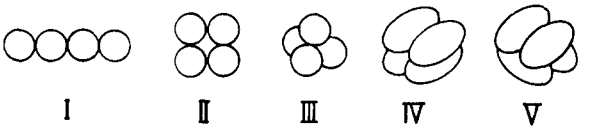

Fig. 5. Possible subunit structures for tetrameric enzymes. The models are confined to those composed of identical polypeptide chains. The monomers in I–III are spherical and those in IV and V are ellipsoidal.

Fig. 6 shows the results of classification of 172 images. Here, the images belonging to B were most dominant and those belonging to D, E and C were the next in this order. The model I in Fig. 5 should be excluded for functional proteins. Furthermore, images belonging to F could not be found however images belonging to B were plentiful. Therefore, the model II is also excluded. Likewise, III should be excluded, since no image appears to be triangular. Although IV could be a candidate based on the images belonging to A and D, the possibility seems to be rather small, because B occupies a large proportion and C and E were also plentiful. In the case of IV, the distribution should follow $k_p = k_q$, $k_r = 0$, when

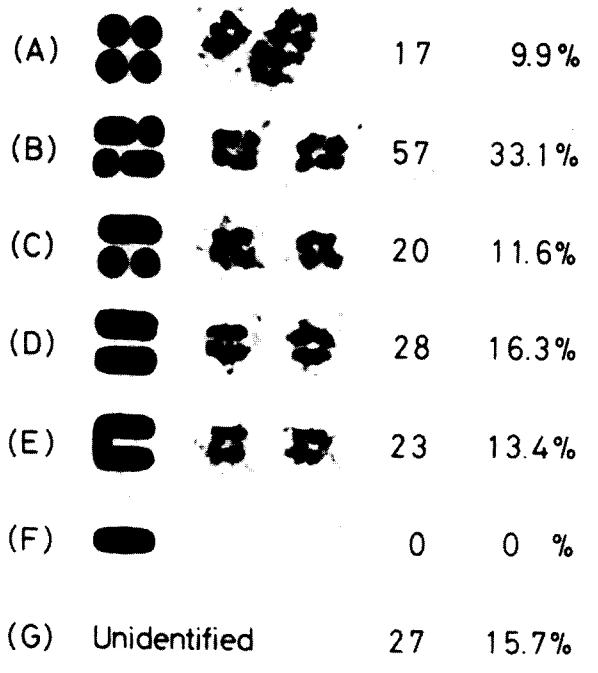

Fig. 6. Distribution of the electron microscopic images of aspartase. The electron microscopic images obtained with a direct magnification of 50 000 were further enlarged on photoprinting paper up to 1 000 000-fold magnification. A total of 172 images were classified according to their characteristics. The group G contains those which can not be included in groups A–F. The numbers in the second column from the right indicate the counted numbers of the images and those in the rightest column relative percentage of the numbers.

reacted with the cross-linking reagent. However, this was not the case. Based on the above results, it is reasonable to conclude that aspartase has a structure of V, namely, D_2 symmetry.

Discussion

As long as stable crystals are available, X-ray crystallography should be the most powerful means for elucidation of the quaternary structure of enzymes. In contrast, it is quite difficult to find effective means for elucidation of the quaternary structure of enzymes, when stable crystals are not available. In this investigation, two distinct methods were successfully employed for this purpose, namely, the cross-linking with a bifunctional reagent and analysis of electron microscopic images. Similar studies were reported several years ago from two laboratories. Hucho et al. [11] carried out studies on several tetrameric enzymes composed of identical subunits with an aim to elucidate the basic correlation between the degree of cross-linking reaction and the theoretical distribution of the reaction products. At almost the same time Hajdu et al. [12] conducted studies from a different standpoint. For example, Hucho et al. [11] conducted the calculations based on the probabilities of cross-linking (p, q and r), whereas Hajdu et al. [12] employed k_p, k_q and k_r. As the latter authors pointed out in their paper, the $p/q/r$ ratio is inadequate to describe the relative propensities for cross-linking of the subunits, as it changes with time, while the $k_p/k_q/k_r$ ratio has a physical meaning independent of time. In the present investigation, the quantity of $k_p/k_q/k_r$ was adopted in the first place. In the second place, however, the degree of cross-linking (actually $1-C_1$) was taken in the abscissa and the quantity of each molecular species was taken in the ordinate, unlike the above cited two groups who took p or $k_p\tau$ in the abscissa and the quantity of the monomer, dimer, trimer and tetramer in the ordinate. Since the values for p and $k_p\tau$ can not directly be obtained from experimental results, it is quite difficult to compare the actual data with theoretical curves. In contrast, our system made the comparison of the experimental data with theoretical curves feasible.

Bifunctional imidates are known to react specifically with protein amino groups. Therefore, as long as a large number of lysine or arginine residues are

present on the surface of proteins polarized reactions of the cross-linking can be minimized to a large extent. Aspartase has as many as 95 and 66 residues of lysine and arginine, respectivley, and the distribution of the molecular species formed by the cross-linking reaction appears to reflect precisely the geometric arrangement of the subunits. As described in a preceding section, Klotz et al. [8] classified the subunit arrangement in tetrameric proteins composed of identical subunits into the following three groups; (1) tetrahedron, (2) square (C_4 symmetry), (3) square (D_2 symmetry). The theoretical distribution of the cross-linking reaction corresponds to (1) $k_p = k_q = k_r$, (2) $k_p = k_q$, $k_r = 0$ and (3) $k_p = ck_q$ $(0 < c < 1)$, $k_r = 0$. Although their classification is valid mainly for spherical proteins, the theoretical distribution can be extended to the models IV and V in Fig. 5. In these two models, the structural distribution should follow (2) $k_p = k_q$, $k_r = 0$ and (3) $k_p = ck_q$ $(0 < c < 1)$, $k_r = 0$, respectively. By the cross-linking experiments, the structure of dimer of dimers with $k_p = 1/8\ k_q$, $k_r = 0$ was demonstrated.

As long as the electron microscopic images appear to be simple, the assignment of the subunit arrangement can be attained rather easily. In fact, the subunit structures of tryptophanase (EC 4.1.99.1) [13] and L-arabinose isomerase (EC 5.3.1.4) [14] were successfully determined by electron microscopic techniques. In contrast, the quaternary structure of aspartase could not easily be determined, because the appearance and shape of the images were quite complex. In this investigation, careful classification of a large number of images made the analysis possible. Thus, we were able to estimate the subunit arrangement of aspartase with reasonable accuracy by examination of the results from both cross-linking experiments and electron microscopy. This approach provides different useful information on the subunit arrangement in oligomeric proteins, as does the X-ray analysis. The results obtained with the two methods revealed that aspartase has a tetrameric structure consisting of two pairs of dimers, in which the two pairs of rod-shape subunits meet perpendicularly, being typical of D_2 symmetry.

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