The Functional Unit of Polyenzymes

DETERMINATION BY RADIATION INACTIVATION

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Recently, target analysis has been re-evaluated as a technique for the determination of molecular sizes (Kempner, E. S. & Schlegel, W. (1979) Anal. Biochem. 92, 2-10). The technique yields the size of the functional unit, i.e. the minimal assembly of structures necessary for a given function such as an enzymatic activity. Using this method, we have now determined the sizes of the functional units for different enzymatic activities on the “arom” conjugate from Euglena, a polyenzyme catalyzing five sequential reactions in the shikimic acid pathway, and on two conjugates from Escherichia coli carrying both aspartokinase and homoserine dehydrogenase activities. In each conjugate, the size for different enzymatic activities was measured and found to be the same. When compared to the molecular weight obtained with other techniques, the target size matched the minimal assembly of substructures which possess a given activity and is called the “functional unit.”

The target sizes of 34 different lyophilized or frozen enzymes reported in earlier studies of radiation inactivation were compared with values obtained in solution by conventional methods (2). The target sizes fell into two clearly distinguishable groups. In one class, the target sizes corresponded to the entire molecule even though these enzymes are known to be composed of one to six subunits. In the other class, the target size was smaller than the entire structure; in almost every case the functional unit corresponded to a single subunit. In these cases, at least, it is clear that radiation destroys entire polypeptide chains; therefore, the method cannot be used to determine functional size smaller than a single subunit. Quantitative comparison with conventional measurements indicates that the radiation molecular weights agreed within 14% on the average. We therefore cautiously assume that molecular weights determined from target analysis in the present study will display an error of less than 20%. Several factors can contribute to this error, such as inaccuracies in radiation dose and variation of enzyme sensitivity with temperature during irradiation (2). In addition, the exclusion of liquid solutions from target analysis studies limits the method, and the possibility of artifacts due to freezing or lyophilization must be considered.

The only radiation study of a protein with supposedly multiple functions is one on xanthine oxidase. It was reported (4) that three different activities, purine oxidation, oxidation of aldehydes, and oxidation of NADH, showed the same radiation sensitivity. The target size was found to be 125,000 for each function compared to the subunit size of 150,000 identified many years later (5).

Other polyenzymic molecules such as fatty acid synthetase, tryptophan synthetase, pyruvate dehydrogenase, and aspartokinase are now known in a variety of biochemical pathways. In all of these there is a biochemical relationship (usually sequential) between catalyzed reactions. In Neurospora and Euglena, there exists a conjugate (1) of five activities in the shikimic acid pathway (6, 7; Fig. 1). The final product is the immediate precursor of chorismic acid, a branch point which leads to the aromatic amino acids, isoprenoid quinones, and folate.

Another unique conjugate is found in the aspartic acid family of Escherichia coli. In this case, enzymes catalyzing

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a. REACTIONS OF THE "arom" COMPLEX

b. INITIAL REACTIONS IN THE ASPARTIC ACID FAMILY

c. REACTIONS OF TRYPTOPHAN SYNTHASE

Fig. 1. Reactions catalyzed by polyenzymes. a, reactions of the "arom" conjugate; b, initial reactions in the aspartic acid family, of which the first and third are catalyzed by a single polyenzyme; c, reactions of tryptophan synthase. The abbreviations used are: DAHP, deoxyarabinoheptulosonic acid 7-phosphate; DHQ, dehydroquinic acid; DHS, dehydroshikimic acid; SA, shikimic acid; SAP, shikimic acid 5-phosphate; PEP, P-enolpyruvate; EPSP, enolpyruvylshikimic acid 5-phosphate; Asp, aspartic acid; βAspPO4, β-aspartyl phosphate; ASA, aspartic semialdehyde; HS, homoserine; InGP, indoleglycerol phosphate; In, indole; Ser, serine; Tryp, tryptophan.

Steps 1 (aspartokinase) and 3 (homoserine dehydrogenase) of the pathway are in the same molecule; enzyme two is not known to be associated with the conjugate. Furthermore, there are several species of these molecules present simultaneously in this bacterium. Each species is specific for a pathway leading to different products: threonine, methionine, or lysine (8).

The most thoroughly understood complex undoubtedly is the enzyme tryptophan synthase. It is a multisubunit protein or polyenzyme complex with an αβ2 structure; the α and β subunits can be separated and recombined (9). It is therefore possible to irradiate the subunits in both isolated and complexed states. For isolated subunits, assay of residual activity is low, but reconstitution of the intact structure increases enzymatic activity by 1 to 2 orders of magnitude.

The purpose of the present study is to explore the applicability of the radiation technique to polyenzymes with examples with known structure-function relationships (tryptophan synthase, aspartokinase II). The examples include multifunctional proteins where several functions reside in one polypeptide chain and others where the full assembly (aspartokinase) or a partial assembly (tryptophan synthase) is required for the expression of activity. Target theory indicates that only the functional part of a cluster will be seen. In a polyenzyme, each of the several enzymatic activities has a particular functional size and there is no a priori reason to expect that independent activities will have the same structural dimensions unless they are on the same or identical-sized chains. The question can be tested experimentally by simultaneously measuring the surviving functions after radiation exposure.

MATERIALS AND METHODS

Enzyme Preparations—The "arom" conjugate was isolated from Euglena. Forty-three liters of Euglena gracilis strain z (10) culture in late log phase growth in synthetic medium (11) were harvested. Cell breakage by rapid pressure release gave a considerably better yield of enzyme activity than by sonication or grinding with alumina and was used routinely for large batches of cells. Cell extracts were treated with 0.14% protamine sulfate in 0.01 M phosphate buffer, pH 7.5, containing ethylenediaminetetraacetic acid (EDTA, 0.1 mM) and the protease inhibitors phenylmethylsulfonyl fluoride (PMSF, 0.1 mM) and dithiothreitol (DTT, 0.2 mM). After centrifugation (11,000 × g, 20 min, 4°C), 20% Celite (w/w) was added to the supernatant liquid and ammonium sulfate was added to 70% (v/v). The slurry was poured into a column (1.8 × 36.5 cm). A reverse ammonium sulfate gradient (70% to 15%) was used to elute the enzyme conjugate (12). Repetitive dialysis (four times, 200 volumes each, phosphate buffer with inhibitors at 4°C overnight) removed the ammonium sulfate and the sample was then lyophilized. Dehydroshikimic reductase1 and dehydroquinase2 activity were measured by standard methods (6, 13).

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1 EC 1.1.1.25, shikimate:NADP+ 3-oxidoreductase.
2 EC 4.2.1.10, 5-dehydroquinase hydro-lyase.
Enolpyruvylshikimic acid 5-phosphate synthetase\(^1\) was kindly assayed (6) by Dr. Virginia Patel, University of Georgia, Athens, Ga. Purified samples of both aspartokinase I-homoserine dehydrogenase \(^1\) and aspartokinase II-homoserine dehydrogenase II from *E. coli* were kindly supplied by Dr. Georges Cohen, Institut Pasteur, Paris, France. All activities (14) were stable to freezing but not to lyophilization.

Tryptophan synthetase\(^6\) preparations were generously supplied and assayed by Dr. Edith Miles, National Institutes of Health, Bethesda, Md. Lyophilized samples of the \(a\), \(\beta\), and \(a\beta\) subunits retained full activity after resuspension. Irradiated samples were combined with excess matching subunits during assay of the second reaction (Fig. 1). The synthesis of \(\delta\)-tryptophan from indole and \(L\)-serine was assayed by a modification (15) of the spectrophotometric method of Faeder and Hammes (16). A \(K_a\) activity was assayed with excess \(\beta\) subunit; \(\beta\) subunit activity was assayed with excess \(a\) subunit.

Enzyme solutions (30 to 300 pl) were frozen at \(-78^\circ\text{C}\) in 2-ml glass ampoules. Samples of the "arom" complex were also lyophilized as were the tryptophan synthetase samples. Ampoules with frozen or lyophilized samples were sealed in air and stored at \(-80^\circ\text{C}\) for subsequent radiation exposure.

**Irradiation**—Electrons (13 MeV) from a linear accelerator at the Armed Forces Radiobiology Research Institute (Bethesda, Md) were used for most experiments. A few irradiations were performed with 3 MeV electrons from a Van der Graaff accelerator at the National Bureau of Standards (Gaithersburg, Md). Dosimetry was accomplished by thermoluminescent dosimeters as well as radiochromic dye films, with an error of less than 10\%.

Lyophilized samples of the tryptophan synthetase preparations and the *Euglena* "arom" complex were irradiated at 30°C and duplicate experiments were performed with the *Euglena* enzyme at very low temperatures. Enzyme samples frozen in solution were also irradiated under these latter conditions. The temperature was maintained between \(-110^\circ\text{C}\) and \(-150^\circ\text{C}\) by a stream of cold nitrogen gas evaporating from a liquid nitrogen reservoir. Temperature was monitored with a thermometer or platinum probe during exposure.

Target analysis has been described previously (2, 3). At different radiation doses, \(D\), the surviving activity \(A_0\) is determined. From a plot of the logarithm of the fractional surviving activity \((A_0/\bar{A}_0)\) versus dose, a value for \(D_0\) (dose at which \(A_0/\bar{A}_0 = 0.37\)) is derived. For our analysis a least square line constrained to the value \(A_0/\bar{A}_0 = 1.0\) at \(D = 0\) was fitted to the data. The formula of Kepner and Macey (17) was used to calculate the target size in daltons.

**RESULTS**

The "arom" conjugate found in *E. gracilis* has five separate enzymatic activities, catalyzing Reactions 2 through 6 of the shikimic acid pathway (Fig. 1). Samples of this polyenzyme were isolated from rapidly growing *Euglena* cells.

Although the radiation technique does not require pure samples, some separation is useful. It reduces the bulk of material to be irradiated and enriches activity so that the inactivation can be followed over a greater range of radiation exposure. Purification of the "arom" conjugate was monitored by dehydroshikimic reductase activity and protein measurements (Table I). The addition of protamine sulfate removed large amounts of protein, but led to an increase in enzyme activity. A further increase was observed in samples containing ammonium sulfate; this "extra" activity disappeared when the salt was removed by dialysis. The reverse ammonium sulfate gradient further reduced the bulk of the protein. Total purification at this stage was about 15-fold as judged by this enzyme which reached a specific activity of 0.074 pmol/min/mg of protein. Aliquots were stored in phosphate buffer with inhibitors at \(-40^\circ\text{C}\) for more than a year with no appreciable loss of activity. Samples of this preparation were also successfully lyophilized with minimal loss.

The lyophilized conjugate was irradiated both at room temperature and \(<-100^\circ\text{C}\). The surviving activity of the enzymes catalyzing the third, fourth, and sixth steps of the pathway was measured after various radiation exposures. As shown in Fig. 2, dehydroshikimic reductase shows a simple exponential decay with radiation dose at each temperature. Dehydroquinase and enolpyruvylshikimic acid 5-phosphate synthetase activities behaved similarly. The 37\% dose characterizing each inactivation curve is given in Table I. The enzymes are significantly more sensitive when irradiated at room temperature than at \(<-100^\circ\text{C}\). The relative sensitivity

<table>
<thead>
<tr>
<th>Sample</th>
<th>DHS(^{-})reductase activity</th>
<th>Protein (mg)</th>
<th>Specific activity ((\mu)mol/min/mg protein)</th>
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<tbody>
<tr>
<td>Crude</td>
<td>112.2</td>
<td>23,200</td>
<td>0.0048</td>
</tr>
<tr>
<td>Protamine SO(_4)</td>
<td>158.0</td>
<td>5,252</td>
<td>0.030</td>
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<tr>
<td>Pooled (NH(_4))(_2)SO(_4) fractions, dialyzed</td>
<td>59.6</td>
<td>806</td>
<td>0.074</td>
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<tr>
<td>Same, lyophilized, and resuspended</td>
<td>55.3</td>
<td>636</td>
<td>0.087</td>
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<tr>
<td>Pooled (NH(_4))(_2)SO(_4) fractions, dialyzed and stored at (-40^\circ\text{C}) for 446 days</td>
<td>55.8</td>
<td>654</td>
<td>0.085</td>
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* DHS, dehydroshikimic acid.

**TABLE I**

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FIG. 2. Inactivation of dehydroshikimic reductase activity by high energy electron irradiation. Lyophilized samples of a partially purified enzyme preparation from *E. gracilis* were irradiated at either 30°C or \(<-100^\circ\text{C}\). Fitted line is from constrained least squares analysis.
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TABLE I

<table>
<thead>
<tr>
<th>Radiation sensitivity of enzymes in polyenzyme conjugates</th>
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<td>Conjugate</td>
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<td>&quot;Arom&quot; conjugate (E. gracilis)</td>
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<td>Aspartic acid family (E. coli)</td>
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<td>Tryptophan synthase (E. coli)</td>
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- It has been shown (2) that the radiation sensitivity of enzymes changes with temperature in a predictable fashion. Analysis of published temperature studies of seven different enzymes showed that

\[
\frac{D_0(-100^\circ C)}{D_0(30^\circ C)} = 2.57 \pm 0.29
\]

This factor was used to adjust the present data to 30°C sensitivity (18).

- lyo., lyophilized; fro., frozen.
- EPSP, enolpyruvylshikimic acid 5-phosphate.

of lyophilized samples at the two temperatures is the same as that observed for simple enzymes (2). Using a factor of 2.57 (see Table II), the 37% dose observed in lyophilized samples at <-100°C could be adjusted to compare with the value obtained at 30°C. The physical or chemical basis for this factor is not yet known. Molecular weights of 1.3 to 1.9 x 10^5 were calculated for the three activities.

Purified samples of aspartokinase I-homoserine dehydrogenase I (threonine) and aspartokinase II-homoserine dehydrogenase II (methionine) survived freezing, but attempts to lyophilize samples led to substantial losses in activity. Frozen solutions of both enzymes in buffer were irradiated at <-100°C as described. In addition, samples of aspartokinase I-homoserine dehydrogenase I were frozen as an ammonium sulfate precipitate and were also irradiated. After exposure, the thawed samples were diluted with buffer before assay. In the case of the precipitated enzyme, this dilution reduced the ammonium sulfate concentration by at least a factor of 40 and the enzyme redissolved. Both aspartokinase and homoserine dehydrogenase activities in each species were found to follow a simple exponential inactivation to as low as 2% survival. The inactivation of homoserine dehydrogenase II in a typical experiment is shown in Fig. 3. The 37% dose was corrected for the temperature effect by assuming the same factor as determined for lyophilized enzymes (2) and confirmed for frozen solutions of 5'-nucleotidase (18). The target molecular weights are listed in Table II. No significant differences were found for the independent activities in each molecule when irradiated under the same conditions. The two molecular species

FIG. 3. Inactivation of homoserine dehydrogenase activity by high energy electron irradiation. Frozen solutions of purified homoserine dehydrogenase II from E. coli were irradiated at <-100°C. Theoretical inactivation curves are drawn for a target of 176,000 daltons (——) and 88,000 daltons (—-).
are known to have different molecular weights (19, 20), which is also observed here by radiation inactivation. It is clear that the entire molecule is needed for function in both cases, although the subunit structure is known to be tetrameric (subunit \( M_2 = 56,000 \) for Species I and dimeric (subunit \( M_2 = 88,000 \) for Species II (20). In the cases of Species I as an ammonium sulfate precipitate, a dimeric target was observed. The radiation sensitivity of Species II was unaltered when irradiated as an ammonium sulfate precipitate (data not shown).

After irradiation of the individual tryptophan synthetase subunits, the \( \alpha_2\beta_2 \) structure was reconstituted with excess matching subunit in the assay mixture. Similarly, the irradiated \( \alpha_2\beta_2 \) form was assayed in the presence of excess \( \alpha \) or excess \( \beta_2 \). Assay of the second reaction (Fig. 1) led to the target sizes listed in Table II. The larger subunit is isolated as the dimer \( \beta_2 \) which appears to be the functional unit for the conversion of indole to tryptophan, whether irradiated in the isolated or native state (with either subunit added subsequently). Irradiation of the isolated \( \alpha \) subunit leads to a target size of 69,000 which may not be different from the \( \alpha_2 \) molecular weight of 58,000.

**DISCUSSION**

The \( arom \) conjugate of Euglena is not as well known as the similar polyenzyme found in Neurospora. Both catalyze all five reactions comprising Steps 2 through 6 from 3-deoxy-t-arabino-heptulosonic acid 7-phosphate to 5-enolpyruvylshi-kimate 3-phosphate (Fig. 1). Both have a heat-labile activity in Step 3. The Neurospora protein has a molecular weight of 270,000 (21), although values of 230,000 (22) and 300,000 (23) have also been reported. These determinations are especially difficult because of the presence of specific proteases (24). They may also be present in Euglena where a slightly smaller molecular weight of 249,000 has recently been reported (25).

The radiation inactivation of three enzymes in the Euglena conjugate leads to much smaller molecular weight values. Many enzyme target sizes have been found to be considerably smaller than the accepted molecular weight, but these have invariably been found to correspond to subunit polypeptides (2). The subunit structure of the Euglena polypynzyme is not known, but the Neurospora conjugate is known to be a dimer of 150,000 to 165,000 molecular weight (21, 24). It is reasonable to suggest that the Euglena enzyme will also be found to have a dimeric structure, and that each unit is independently functional.

The aspartokinase-homoserine dehydrogenase reactions are catalyzed by different proteins in different pathways. The proteins differ in genetics, structure, regulation, and immunology (8). The threonine-specific aspartokinase I-homoserine dehydrogenase I is a tetramer whose entire structure is necessary for both functions. Similarly, the methionine-specific aspartokinase II-homoserine dehydrogenase II protein is a dimer; both chains are needed for the two enzymatic activities. The experimental points for homoserine dehydrogenase II inactivation shown in Fig. 3 are plotted with two theoretical inactivation curves: the dashed line corresponds to an 88,000-dalton target (the monomer size), while the solid line is the inactivation curve expected for a 176,000-dalton target. The experimental distinction between the two is obvious. It has recently been suggested (26) on the basis of renaturation kinetics that the monomer is inactive and that the functional unit is the dimer, a conclusion confirmed by the present study.

In the case where an enzyme cluster is irradiated in a state other than the active form, target analysis may be complicated if recombination of structural elements occurs during rearrangement to the active form. From target analysis of aspartokinase I-homoserine dehydrogenase I frozen in buffer, the functional unit was found to be the tetrameric structure. We also irradiated the complex inactivated by 50% saturated ammonium sulfate (>80% inhibition of homoserine dehydrogenase and >98% inhibition of aspartokinase activities). Target analysis yielded the size of the dimer. If we assume that ammonium sulfate allows the tetramer to dissociate into dimers, then irradiation of \( N \) dimers with a radiation dose \( D \) will inactivate a fraction, \( a \). The surviving fraction is \( 1 - a = e^{-kd} \) where \( k \) is the dimer target size. Subsequent dilution redissolves the precipitate and allows the tetramers to reform. If only undamaged dimers can form tetramers, the number of active tetramers will be \((1-a)N/2\). The inactivation of enzyme activity will be described by

\[
N \frac{e^{-kd}}{2} = \frac{N}{2} \frac{1-a}{N} = \frac{N}{2} e^{-kd/2}
\]

so that \( V = k \), i.e. the observed target for enzyme inactivation is that of the dimer. On the other hand, if all dimers (damaged and undamaged) form tetramers but only tetramers consisting of two intact dimers are active, then the number of active tetramers will be \((1-a)^2N/2\). Then

\[
N \frac{e^{-kd}}{4} = \frac{N}{2} (1-a)^2 = \frac{N}{2} e^{-kd/2}
\]

and \( W = 2k \). The observed target will be twice that of the dimer.

It is already known that normal dimers spontaneously form the tetrameric structure (8). However, since radiation causes extensive damage to individual molecules (2), any protein damaged sufficiently to destroy enzymatic activity would probably also have lost other properties including the ability to form tetramers. The explanation for the observed target size is therefore both mathematically and biochemically consistent.

In the case of tryptophan synthetase, a similar situation was observed. Irradiation of the intact \( \alpha_2\beta_2 \) structure or the isolated \( \beta_2 \) subunit led to the identification of \( \beta_2 \) as the functional unit for the conversion of indole to tryptophan, in agreement with the same conclusion derived from conventional methods of analysis (9). In addition, the \( \beta_2 \) target size observed in the intact complex allows a further conclusion: a “hit” in a subunits does not destroy the \( \beta_2 \) structure. This is consistent with radiation inactivation of monofunctional, multi-subunit proteins in which a target size of a single chain was observed (2), and the present results of the Euglena “arom” complex in which destruction of one chain is without effect on others.

Experiments with the isolated \( \alpha \) subunit indicate a situation comparable to that in the analysis given above. The \( \alpha \) subunit alone is inactive in the second reaction (9). However, in its presence, the \( \alpha_2\beta_2 \) structure can re-form and increase the activity of the \( \beta_2 \) subunit by 2 orders of magnitude. It is known further that the isolated \( \alpha \) subunit in solution exists as the 29,000-dalton monomer, but the nature of the lyophilized form is of course unknown. If it is assumed that the \( \alpha_2 \) dimer forms during lyophilization, then the present data can be simply explained. Although the assay is for the \( \beta_2 \) enzymatic activity, the irradiation of the isolated \( \alpha \) subunit effectively shifts the measurement to that of recombination. Thus, the radiation analysis shows that the functional unit for reconstitution of the intact enzyme is \( \alpha_2 \).

Multifunctional enzyme clusters play a fundamental role in cellular homeostasis and cellular responses. The interconnection of different catalytic activities offers the cell the advantages of “aberrant” kinetic features, compartmentalization, and a possibility for redundancy in the regulation of catalytic
activities. The understanding of more complicated aspects of polyenzymes requires the knowledge of the fundamental structure-activity relations in a cluster, i.e. if the structures which are minimally required for catalytic activity are not known, the molecular mechanisms of their regulation cannot be studied. As mentioned previously, this information is sometimes difficult to obtain for technical reasons. Target analysis yields this information with minimal perturbation of the original system. The present study clearly shows the applicability of the radiation approach to polyenzyme conjugates. It is possible to determine the size of the subunit assembly required for a given activity in an intact complex (aspartokinase I). Our study also shows the potential for determining structural requirements for the assembly of complexes (aspartokinase I, tryptophan synthetase). However, note that target analysis only yields the size of the functional unit in lyophilized or frozen enzymes. This feature may not always be sufficient for identification of structures. In the examples in this study, the sizes obtained (even with the relatively large error margin (20%)) appear relevant to the molecular weight of the structural components in solution.

In each polyenzyme, the same target size was observed for all the measured activities. If this proves to be a general characteristic, there will be significant implications for the evolution and function of polyenzymes. For example, the juxtaposition of sequential enzymes could be accomplished genetically only by association in the same polypeptide strand.

Acknowledgments—We thank Dr. Virginia Patel for her help with the “arom” conjugate, especially for the measurement of enolpyruvylshikimic acid 5-phosphate synthetase. We are grateful to Drs. Georges Cohen and Alice Daubry for supplying the aspartokinase enzymes. We are deeply indebted to Dr. Edith Miles for supplying and measuring tryptophan synthase.

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