Calmodulin-activated Cyclic Nucleotide Phosphodiesterase from Brain

RELATIONSHIP OF SUBUNIT STRUCTURE TO ACTIVITY ASSESSED BY RADIATION INACTIVATION

EXPERIMENTAL PROCEDURES

Assay of Enzyme Activity—Enzyme activity was assayed as described (2). Total activity is that assayed in the presence of calmodulin (100 nM) plus calcium (0.4 mM); basal activity is that assayed without these effectors with 0.1 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; and the difference between these values is, by definition, calmodulin-dependent activity.

Preparation of Phosphodiesterase—Bovine brain supernatant was prepared and gel filtration on Sephadex G-200 was carried out as described (1). Active fractions were immediately pooled and concentrated by ultrafiltration (Amicon PM-10, 45/400s.i.) to a protein concentration of 5 mg/ml prior to dilution and freezing of samples for irradiation. Highly purified phosphodiesterase was prepared by a modification of Method 1 (1). The highly purified enzyme was prepared in small quantities (~20 μg of enzyme from a calmodulin-Sepharose column of 300-μl bed volume) just prior to use in the irradiation experiment.

Irradiation Experiments—Enzyme fractions were diluted (1:10 to 1:100) with buffer containing 25 mM 2-(bis-2-hydroxyethyl)amino]ethanesulfonic acid (pH 7.0), 1 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, 5 mM MgCl2, 1 mM NaCl, 250 mM NaCl, 4% glycerol, 1.5 mg/ml ovalbumin, and 4% glycerol was necessary to achieve this degree of enzyme stability. Irradiation with 13 MeV electrons was carried out at the linear accelerator facility of the Armed Forces Radiobiology Research Institute (Bethesda, MD) with samples maintained at temperatures between −110 and −150 °C by a stream of nitrogen gas. Enzymes of known target size were irradiated in each experiment to provide a control for variability in irradiation temperature and data were normalized according to the values obtained. Data were analyzed as previously described (3).

Theoretical Treatment of Radiation Inactivation Data—Equations used for computer-assisted modeling were based on the following assumptions. 1) Before irradiation, a phosphodiesterase preparation contains a certain number of monomers (M) and dimers (D). 2) In the absence of calmodulin, monomers are fully active and dimers are totally inactive. 3) In the presence of calmodulin, a dimer exhibits activity equal to that of two monomers. 4) On radiation destruction of a dimer, an active monomer is generated. This monomer-dimer hypothesis provides a plausible explanation for and definition of basal and calmodulin-dependent phosphodiesterase activity. Based on data from gel filtration and gel electrophoresis (without denaturation), we concluded that the calmodulin-activated phosphodiesterase from brain can exist as interconvertible protomeric and oligomeric species (1). The oligomeric form was activated to a greater degree by calmodulin than was the protomeric form, which is inactive in the presence of calmodulin and dimers which are necessary for catalytic activity. In the presence of calmodulin, a dimer exhibits activity equal to that of two monomers; 3) On radiation destruction of a dimer, an active monomer is generated. This monomer-dimer hypothesis provides a plausible explanation for and definition of basal and calmodulin-dependent phosphodiesterase activity.

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The number of active monomers ($M_{\text{act}}$) consists of those surviving from the initial population ($M = M_0 e^{-2\mu t}$) plus those produced by inactivation of dimers, $M_d = D_2(1 - e^{-2\mu t})/e^{-4\mu t}$. The last term, in brackets, takes into account the radiation inactivation of the monomers derived from dimers. Assuming that the target mass of a dimer is twice that of a monomer, the rate of inactivation of dimer, $k_{dh}$ equals $2k_2$ and the total number of monomers after dose $r$ is

$$M_{\text{act}} = M + M_d = M_0 e^{-2\mu t} + D_2(1 - e^{-2\mu t})/e^{-4\mu t},$$

$$M_{\text{act}} = e^{-2\mu t} [M_0 + D_2(1 - e^{-2\mu t})]$$

(1)

Assuming steady state kinetics, with $M$ as the enzymatically active monomer, $S$ as substrate, and $A$ as activity assayed without calmodulin (i.e. basal)

$$A = [M]k_d[S]/K_m + [S]$$

where $K_m = k_{-1} + k_2/k_1$. After radiation dose $r$, therefore, basal activity is

$$A_r = e^{-2\mu t} [M_0 + D_2(1 - e^{-2\mu t})]k_d[S]/K_m + [S]$$

(2a)

and when expressed relative to that initially present

$$A_r/A_0 = e^{-2\mu t} [1 + \frac{D_2}{M_0}(1 - e^{-2\mu t})]$$

(2b)

The activity assayed in the presence of calmodulin represents that of the monomers (see above) plus that contributed by dimers. It is assumed that under these conditions the activity of a dimer is equal to that of two monomers. Thus, the total number of active units after radiation dose $r$ can be considered equal to $M_{\text{act}}$ plus $2D_2$.

$$M_{\text{act}} + 2D_2 = e^{-2\mu t} [M_0 + D_2(1 - e^{-2\mu t})] + 2D_2e^{-2\mu t},$$

$$M_{\text{act}} + 2D_2 = e^{-2\mu t} [M_0 + D_2(1 - e^{-2\mu t} + 2e^{-2\mu t})]$$

(3)

Again assuming steady state kinetics, the total activity (assayed in the presence of calmodulin) after radiation dose $r$ is

$$A_{\text{act}} = (e^{-2\mu t} [M_0 + D_2(1 - e^{-2\mu t})] + 2D_2e^{-2\mu t})k_d[S]/K_m + [S]$$

$$A_{\text{act}} = e^{-2\mu t} [M_0 + D_2(1 - e^{-2\mu t})] + 2D_2e^{-2\mu t}$$

(4a)

and when expressed relative to that initially present

$$A_{\text{act}}/A_{0\text{act}} = e^{-2\mu t} [(M_{\text{act}} + 2D_2) + [M_0 + 2D_2]],$$

$$A_{\text{act}}/A_{0\text{act}} = e^{-2\mu t} [M_0 + D_2(1 - e^{-2\mu t}) + 2D_2e^{-2\mu t}]$$

(4b)

From Equations 4a and 2a, the total activity relative to basal activity after radiation dose $r$ is

$$A_{\text{act}}/A_r = 1 + \frac{2D_2e^{-2\mu t}}{e^{-2\mu t}[M_0 + D_2(1 - e^{-2\mu t})]}$$

(5)

In Table II, this is expressed slightly differently, as percentage stimulation of basal activity by calmodulin, $100 \times (A_{\text{act}} - A_r)/A_r$ or $100 \times$ calmodulin-dependent activity / basal activity).

**RESULTS**

**Estimation of Functional Size of Basal and Calmodulin-dependent Activities by Target Theory Analysis**—Fig. 1 presents data from a representative experiment in which the basal activity of a highly purified phosphodiesterase preparation was measured after different doses of radiation. Following low doses of radiation, instead of the expected loss of activity, there was actually an increase of 10 to 15%. With further irradiation, an apparent exponential loss of activity was observed. The estimated molecular mass of the decaying component in this experiment was ~62,000 daltons. The increase in basal activity produced by low doses of radiation, which is an unusual finding, was observed with both crude and highly purified enzyme preparations.

Calmodulin-dependent phosphodiesterase activity (total activity minus basal) of the same samples shown in Fig. 1 was not increased by low doses of radiation and decayed in an exponential fashion (Fig. 2). The loss of activity was substantially more rapid than the concomitant loss of basal activity, suggesting a larger radiation-sensitive target; calculation of the apparent target size yielded a molecular mass of 104,000 daltons. Total phosphodiesterase activity (basal plus calmodulin-dependent) was also not increased by radiation (Fig. 3), and the rate of loss was intermediate between those for basal and calmodulin-dependent activities, corresponding to an "average" molecular mass of 76,000 daltons.

Data like those shown in Figs 1 and 2 were also analyzed by considering the effect of radiation on the magnitude of calmodulin activation of the phosphodiesterase. As shown in Table I, the percentage activation by calmodulin decreased with increasing radiation from >200% to about 50%.

The stage of purity of the enzyme did not influence the apparent target sizes for basal or calmodulin-dependent activity (Table II). Whether crude supernatant or enzyme preparations that behaved as larger or smaller molecules on gel filtration were used, radiation experiments yielded results comparable to those obtained with highly purified preparations like that shown in Fig. 4. Thus, the characteristics of their radiation inactivation appear to reflect intrinsic properties of the basal and calmodulin-dependent forms of phosphodiesterase and are not related to the purity or previous treatment of the samples irradiated. When all data from the experiments shown in Table II were normalized (temperature coefficient of 2.63) and plotted together, average target sizes were 60,000 and 103,000 for basal and calmodulin-dependent activity, respectively.

**Predictions for Radiation Inactivation of Phosphodiesterase Based on Computer-assisted Modeling**—To explain the radiation inactivation data, we postulated that: 1) phosphodiesterase monomers are fully active in the presence of absence of calmodulin, i.e. are responsible for basal activity; 2) dimers are inactive in the absence of calmodulin and in its presence express activity equal to that of two monomers, i.e. are responsible for calmodulin-dependent activity; 3) radiation
Ca\textsuperscript{2+}-activated Phosphodiesterase Activity and Subunit Structure

**Fig. 2.** Calmodulin-dependent phosphodiesterase activity after different doses of radiation. Enzyme samples were from the experiment shown in Fig. 1.

**Fig. 3.** Total activity of phosphodiesterase after different doses of radiation. Enzyme samples were from the experiment shown in Fig. 1.

**Table I**

| Dose (Mrads) | Experimental | Calculated |
|--------------|--------------|------------|
| 0            | 215          | 200        |
| 1            | 190          | 186        |
| 3            | 130          | 151        |
| 6            | 117          | 120        |
| 9            | 98           | 98         |
| 12           | 105          | 82         |
| 18           | 78           | 61         |
| 36           | 54           | 29         |

\( ^a \) 100 \times \text{calmodulin-dependent activity + basal activity.}

**Table II**

| Experiment | Enzyme preparation | n \( ^a \) | Basal | Total \( ^b \) | Calmodulin-dependent |
|------------|--------------------|------------|-------|----------------|---------------------|
| 1 Fresh supernatant | 1 68 83 136 |
| "Small" enzyme from S-300 | 1 66 87 127 |
| "Large" enzyme from S-300 | 1 72 91 136 |
| 2 "Small" enzyme from S-300 | 3 51 66 89 |
| 3 "Large" enzyme from S-300 | 2 70 89 106 |
| 4 Enzyme from Method 1 | 1 76 86 114 |
| 5 Enzyme from Method 1\( ^d \) | 4 61 73 101 |
| 6 Enzyme from Method 1\( ^d \) | 3 62 76 104 |

\( ^a \) Number of preparations irradiated. The mean of percentage of original activity of each preparation remaining was plotted versus radiation dose to estimate the apparent target size as shown in Figs. 1 to 3.

\( ^b \) Based on the analysis presented here, these values obviously represent an hypothetical molecular mass, an "average" molecular mass for the monomer-dimer population. They are presented here only to show the magnitude and reproducibility of the difference between the apparent masses for basal and total activity.

\( ^c \) "Small" and "large" enzyme represent fractions from supernatant that on gel filtration (Sephacryl S-300) behaved as molecules of \( M_r \approx 60,000 \) and 135,000, respectively (see Ref. 1).

\( ^d \) One preparation of the enzyme was further purified by chromatography on phenyl-Sepharose prior to affinity chromatography.

The three preparations irradiated were the same enzyme diluted to relative concentrations of 1.0, 0.3, and 0.1. Total protein concentration was maintained at 1.5 mg/ml with ovalbumin.

**Fig. 4.** Electrophoresis of purified phosphodiesterase. 4 \( \mu g \) of the enzyme preparation used in the experiment shown in Figs. 1 to 3 were subjected to gel electrophoresis under denaturing conditions (11). Standards (STD) (\( \sim 5 \mu g \) each) are shown on the right.

destruction of a dimer results in inactivation of one of its subunits and liberation of an active monomer (indistinguishable in catalytic properties and target size from the initially present monomers). This hypothesis was evaluated using computer-assisted modeling with the equations outlined under "Experimental Procedures."
activity instead of twice (see Table 11). It may be that the effective differences between the true and assumed activities over many experiments was approximately dimers with and/or without calmodulin.

The target size of a dimer is not, in fact, precisely twice that of a monomer. Equation of dimer/monomer molar ratios are greater than of monomer to dimer. A monomer target size of 60,000 daltons and an irradiation temperature coefficient of 2.60 were assumed. Monomer/dimer (M:D) molar ratios were 1:0 ( ), 1:0.5 (C), 1:1 ( ), 1:2 ( ), or 1:4 ( ). Equation 4b ("Materials and Methods") was used in the computation of these values.

The model predicts that, when initial dimer/monomer molar ratios are greater than 1:1 (calmodulin-dependent activity twice basal), an increase in basal activity should result from the ratio of dimers to monomers, i.e. the ratio of calmodulin-dependent to basal activity. For calmodulin-dependent phosphodiesterase activity, the calculated decay rates for different ratios of dimer/monomer all correspond to that of a dimer (data not shown), in accordance with our hypothesis. In addition, as shown in Table I, our observations on the effects of radiation on percentage activation of the enzyme by calmodulin are in close agreement with those predicted by the model.

For total activity (Fig. 6), the apparent slopes of the theoretical curves with ratios of dimer/monomer greater than or equal to one correspond to a target size 10,000–12,000 daltons greater than that for monomer. It should be noted that such equations are quite complex (see "Experimental Procedures") and the lines generated possess slight curvature. The magnitude of the deviation from an apparent exponential decay is such that it would not be demonstrable using experimental data; hence, the observed apparent target size of ~75,000 daltons for total activity (average of all experiments) is in good agreement with the prediction of the model for the "average" target size of the monomer-dimer population.

**DISCUSSION**

When a peptide is irradiated, its native structure is destroyed, as is the biological activity dependent on that structure. If the biological function requires an association of the peptide with other native peptides (an oligomeric structure), radiation destruction of any part of the complex will eliminate this activity. Thus, in studies of many different proteins, two distinct classes of inactivation have been observed (4). In one case, the target size is that of a single subunit, regardless of the ability of peptide to form oligomeric structures. In the other, the target size is that of a composite structure. As reported here, radiation inactivation of the calmodulin-stimulated phosphodiesterase yielded complex data with the apparent target size dependent on the conditions under which the irradiated samples were assayed.

Basal phosphodiesterase activity was invariably increased by low doses of radiation whether crude or highly purified preparations of the phosphodiesterase were used. Such effects of radiation have never been reported for a soluble enzyme, although radiation inactivation studies of the insulin receptor in rat liver yielded somewhat similar findings (5). Destruction of a large inhibitory component was postulated to account for the increase in receptor affinity produced by low doses of radiation. This seems an improbable explanation for the observations reported here, especially those with highly purified phosphodiesterase preparations. The increase in basal phosphodiesterase activity resulting from low doses of radiation could reflect the presence of a potential protease or a large inhibitory component. It has been shown with tryptophan synthetase, which has the oligomeric structure $a_2b_2$, that destruction of the $a_2$ portion of the complex does not destroy the $b_2$ structure necessary for catalytic function (4). In this and other systems, the function of structures that are associated noncovalently is retained after destruction of other components of the complex. The calmodulin-dependent phosphodiesterase activity was destroyed by irradiation in an apparent monoeponential fashion corresponding to a target size roughly twice that estimated for basal activity. Such a structure with its latent (masked) activity obviously might represent a potential source of active monomers.

The differences in the patterns of radiation inactivation of basal and calmodulin-dependent activity clearly cannot be

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1 The experimental target size for the calmodulin-dependent form over many experiments was approximately 1.7 times that for the basal activity instead of twice (see Table II). It may be that the effective target size of a dimer is not, in fact, precisely twice that of a monomer. On the other hand, this discrepancy could be related to small differences between the true and assumed activities of monomers and dimers with and/or without calmodulin.

2 The percentage stimulation by calmodulin after high doses (36–75 Mrads) of radiation plateaued at 40–50%. It is possible that this difference between the experimental observations and the continuing decline in calmodulin stimulation predicted by the model is that in samples containing a very large fraction of monomers after irradiation some self-association (dimer formation) tends to occur under assay conditions.
reconciled with the existence of a homogeneous population of phosphodiesterase molecules, each of which possesses the same intrinsic (basal) catalytic activity that is increased to the same maximum on interaction with calmodulin. If this were the case, i.e., if all phosphodiesterase molecules were identical, the degree of calmodulin stimulation should not change with radiation inactivation of the enzyme unless, for example, some component that modifies calmodulin activation were generated during irradiation. We found no evidence for the presence of such a hypothetical effector when samples in which 50 to 70% of total activity had been inactivated by irradiation were added to fresh enzyme.

All of our observations are consistent with the hypothesis that basal phosphodiesterase activity resides in a population of monomers (which can interact with, but are not further activated by, calmodulin) and that radiation inactivation of the dimeric form, which is inactive in the absence of calmodulin, can produce active monomers (accounting for the increase in basal activity following low doses of radiation). If, as postulated here, the phosphodiesterase (under conditions of assay or irradiation) consists of two populations of molecules, one inactive (calmodulin-dependent) and the other fully active (basal activity) without calmodulin, the size of the first population relative to the second determines the degree of activity of the preparation by calmodulin. It is possible, of course, that the activity of a monomer is slightly less than that of a monomer associated with calmodulin, that dimers possess some finite but very low activity in the absence of calmodulin, or that the presence of associated forms larger than dimers may influence the data obtained. Nevertheless, the experimental data agree remarkably well with the results predicted by the simple two-component mathematical model of the proposed subunit relationships.

The existence of the phosphodiesterase as interconvertible monomers and dimers that differ in catalytic activity would obviously provide the possibility for regulation of its function by effectors that influence subunit self-association. Some of the known "physiological" inhibitors, e.g., polyamines (2), K⁺ (6), and activators, e.g., calmodulin, phospholipids (7-10), of basal phosphodiesterase activity, as well as other effectors, could act by altering the monomer-dimer distribution. Information concerning this possibility may be obtained from further studies using radiation inactivation and/or gel filtration.

Using gel filtration and electrophoresis (under nondenaturing conditions), evidence for the existence of the phosphodiesterase as monomers and dimers (and larger associated forms) was obtained and the transition from one form to another during purification or storage of the enzyme was documented (1). Within the framework of our hypothesis we must assume that, following isolation of phosphodiesterase monomers or dimers by gel filtration, some monomer/dimer distribution tends to be established during assay, as such fractions always displayed both basal and calmodulin-dependent activity (1). Similarly, the radiation inactivation data reported here are consistent with the presence of both monomers and dimers whether the preparations irradiated were isolated as "large" or "small" molecules by gel filtration. Without being able to maintain the physically separated forms in the monomeric or dimeric state during assay and/or irradiation, our hypothesis cannot be directly proven. It does, however, provide, in terms of molecular mechanism, a plausible explanation for all of the available information and a definition of basal and calmodulin-dependent phosphodiesterase activity (degree of calmodulin stimulation).

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*The activity of fractions isolated as dimers was, however, stimulated to a greater degree by calmodulin than was the activity of those isolated as monomers (1).