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Catalytic Subunit of Firefly Luciferase*

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ABSTRACT: Using gel filtration chromatography, high-speed sedimentation equilibrium ultracentrifugation, and the combination of the sedimentation and diffusion coefficients the minimum molecular weight of enzymatically active firefly luciferase was calculated to be 50,000. In solvents of relatively low ionic strength the protein aggregates in a rapidly reversible manner as the solubility limit is approached. This aggregation has no effect on the specific activity of the enzyme. Heterogeneity in the purified luciferase preparations was indicated by the observation of only one dehydroluciferyl adenylate binding site per 100,000 molecular weight of protein under conditions in which only a single size species of 50,000 molecular weight was found. Heterogeneity was also suggested by the further resolution of the enzyme into two active fractions of identical specific activity by isoelectric focusing. However, since both fractions retain the ability to make the enzyme–luciferyl adenylate complex, this does not account for the heterogeneity indicated by the binding studies. This suggests the possibility of even greater heterogeneity to the firefly luciferase.

Studies on the substrate binding properties of firefly luciferase (Denburg *et al.*, 1969; J. L. Denburg and R. T. Lee, to be published) have shown that there are two binding sites each for luciferin and ATP per 100,000 molecular weight of enzyme. These results are consistent with the model that luciferase is a dimer of identical 50,000 molecular weight subunits (Travis and McElroy, 1966), each with one binding site for each substrate. However, the enzymatically active substrate, the MgATP complex, is bound to only one site per 100,000 molecular weight of enzyme (J. L. Denburg and R. T. Lee, to be published). Similarly, only one site is found for dehydroluciferyl adenylate (LAMP) formed from the following reaction

$$E + L + MgATP \rightleftharpoons E \cdot LAMP + Mg + PP_i \quad (1)$$

Dehydroluciferin (L) is activated by the enzyme to form dehydroluciferyl adenylate which remains tightly bound to the enzyme with no production of light (Rhodes and McElroy, 1958). In order to have only one binding site per dimer of identical subunits there must be asymmetry in the system. Therefore, the physical properties of firefly luciferase were examined in greater detail. It is reported here that the minimum molecular weight of enzymatically active protein is 50,000 and that this protein aggregates easily.

The reactions catalyzed by firefly luciferase are

$$E + LH_2 + MgATP \rightleftharpoons E \cdot LH_2AMP + Mg + PP_i \quad (2)$$

$$E \cdot LH_2AMP \longrightarrow E + products + CO_2 + AMP + light \quad (3)$$

Materials and Methods

Materials. Luciferase, luciferin, and dehydroluciferin were obtained as previously described (Denburg *et al.*, 1969).

Flash Height Assay. All assays were done as previously described (Denburg *et al.*, 1969). In assaying solutions of high enzyme concentration 6 × 50 mm test tubes were used and the volumes were reduced to one-fourth those usually used.

Binding of Dehydroluciferyl Adenylate. The number of binding sites for LAMP on luciferase was measured by the isolation of the E·LAMP complex by elution from a Sephadex G-25 column, and by fluorescence titration of enzyme and MgATP with dehydroluciferin.

The decrease in fluorescence of dehydroluciferin at 540 mμ has been used to measure the formation of LAMP (Rhodes and McElroy, 1958). Similarly in the presence of

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excess enzyme and MgATP small aliquots of dehydroluciferin can be titrated in and the increase in fluorescence measured. Initially no fluorescence is observed because all the dehydroluciferin is reacting to form LAMP which remains tightly bound to the enzyme. As more aliquots are added, the concentration of unbound dehydroluciferin will increase as will the fluorescence. The increase in fluorescence can be extrapolated back to find the point at which the enzyme is saturated with LAMP. All fluorescence measurements were made on a Turner Model III fluorometer. Exciting light passed through a Turner 110-811(7-60) filter which peaks at 365 mμ, while emitted light passed through a Corning 3-71 filter. Two interference filters were used to decrease the intensity of the emitted light by a factor of 10^2. This enabled the measurements to be done in the proper concentration range of dehydroluciferin. In a typical experiment 4×10^{-6} M luciferase in 0.05 M phosphate, pH 7.8, was incubated with 2×10^{-3} M ATP and 5×10^{-3} M $MgSO_4$ while 10-μl aliquots of a stock dehydroluciferin solution were added. Each aliquot increased the concentration of dehydroluciferin in the assay tube by 5×10^{-7} M.

LAMP binds to luciferase with a dissociation constant of 5×10^{-10} M (Rhodes and McElroy, 1958). This tight binding enabled the isolation of the E·LAMP complex by gel filtration chromatography. The reaction mix contained, in 1.2 ml of buffer, 2×10^{-3} M ATP, 5×10^{-3} M $MgSO_4$, 5×10^{-5} M dehydroluciferin, and 6×10^{-6} M enzyme. This is allowed to incubate at 5° for 15 min and then applied to a 1 × 30 cm column of Sephadex G-25 fine. Elution at a rate of approximately 25 ml/hr enabled the complete separation of protein from excess ATP. The excess dehydroluciferin was adsorbed tighter to the column. The amount of dehydroluciferin in each fraction was measured by taking an aliquot and diluting with 0.1 N NaOH. This releases LAMP from the enzyme and hydrolyzes it to form the highly fluorescent dehydroluciferin. The fluorescence of these solutions when compared with a standard curve gave the concentration of dehydroluciferin. In some experiments [^{14}C]ATP was used in the incubation, and aliquots were taken from each fraction and counted in Bray's solution (1960). The concentration of luciferase was determined by the absorbance at 278 mμ, where a solution of 1 mg/ml of enzyme has an optical density of 0.75, after correcting for the absorbance of dehydroluciferin and AMP. These experiments were done either in 0.1 M Tris or 0.1 M cacodylate buffers in a pH range of 6.2–7.5. In phosphate buffers of any pH there was considerable trailing of fluorescent material caused by hydrolysis of the E·LAMP complex during elution.

Renaturation. Luciferase was completely denatured, as measured by the loss of enzymatic activity, within 5 min upon its addition to 6 M guanidine hydrochloride in 0.05 M phosphate, pH 7.0, with 10^{-2} M EDTA at 0°. Aliquots of this solution containing denatured enzyme were diluted 100-fold into 0.1 M phosphate, pH 7.8, and 0°. All solutions contained 10^{-3} M dithiothreitol to prevent any disulfide bonds from forming. The time-dependent renaturation was measured by the recovery of activity in the flash height assay.

G-200 Chromatography. A 3 × 40 cm column of fined Sephadex G-200 was packed and maintained under a pressure head of 10 cm at 5°. The column was calibrated by measuring the elution volumes of yeast alcohol dehydrogenase, *Escherichia coli* isoleucine–tRNA synthetase, bovine plasma albumin, α-amylase, and trypsin. These values were plotted against the log molecular weight of the respective proteins. The protein concentration was measured by the optical density at 278 mμ.

Ultracentrifuge Studies. All sedimentation experiments were done in a Spinco Model E analytical ultracentrifuge with the temperature controlled at 4°. Samples were dialyzed overnight against solvent.

Sedimentation velocity experiments were performed in single-sector cells in the An-D rotor at 50,740 rpm. The rate of migration of the boundary was measured with a Nikon Model 6 comparator after visualization by the schlieren optical system. Sedimentation coefficients were calculated and corrected to standard conditions of water at 20°.

Diffusion measurements were performed in the double-sector synthetic boundary cells with a capillary centerpiece at 5227 rpm. The spreading of the boundary with time was observed by the interference optical system and the fringe shift across the boundary measured. The diffusion coefficients were calculated and also corrected to water at 20°.

High-speed sedimentation equilibrium studies were carried out according to the procedure of Yphantis (1964). The attainment of equilibrium was checked by measuring the fringe displacement at several points in the cell, and then observing no further change after 2 more hours of spinning. The fringe displacement, proportional to the protein concentration, was measured throughout the cell and the molecular weight was calculated by use of the following equation.

$$M_{w,app,r} = \frac{2RT}{(1 - \bar{v}\zeta)} \frac{d \ln c(r)}{dr^2} \quad (4)$$

The partial specific volume ($\bar{v}$) was calculated from the amino acid composition and the values of $\bar{v}$ of the individual amino acids (Cohn and Edsall, 1943) and was found to be 0.739. Some of the data were analyzed by the FORTRAN IV high-speed equilibrium ultracentrifugation program of Roark and Yphantis (1969). This produced a value for M_w at each radial point in the cell.

Isoelectric Focusing. The LKB 8100 Ampholine isoelectric focusing equipment was used in all experiments. A pH gradient of 5–8 was made with 1% solutions of LKB ampholines. A voltage of 900 V was applied for 48 hr and the temperature was maintained at 5° by a water-circulating cooling bath. The column was emptied and each fraction was assayed for activity and the pH measured with a Radiometer pH meter.

Results

Binding Sites of Dehydroluciferyl Adenylate. A typical fluorescence titration of luciferase in which increased amounts of dehydroluciferin are added forming E·LAMP is shown in Figure 1. Extrapolation of the lines shows that there was one LAMP binding per 100,000 molecular weight of protein. These experiments were done in 10% $(NH_4)_2SO_4$, 0.1 M Tris, 0.025 M glycylglycine, and 0.05 M phosphate buffers over a pH range of 6.5–8.0. The enzyme concentration was varied from 0.4 mg/ml to 2.0 mg/ml in different experiments. Under all these conditions there was only one LAMP site per 98,000 ± 4000 molecular weight of enzyme.

Isolation of the E·LAMP complex by Sephadex G-25 chromatography produced similar results. Measurements by

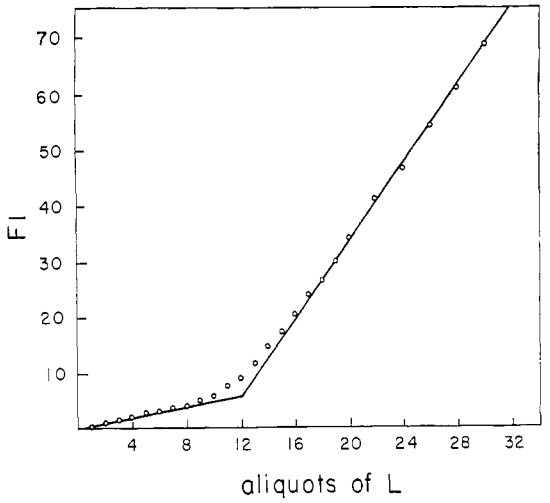

FIGURE 1: Fluorescence titration curve of luciferase with increasing amounts of dehydroluciferin. Each 10-μl aliquot increased the concentration of dehydroluciferin by 1.5×10^{-7} M. The reaction mix contained 2 ml of 0.1 M phosphate, pH 7.0, 1.7×10^{-6} M luciferase, and 2×10^{-3} M MgATP.

fluorescence of the material bound to the protein gave 1.01 ± 0.05 molecules of LAMP per 100,000 molecular weight. Counts of the radioactivity incorporated into the adenylate from [^{14}C]ATP yielded 1.1 ± 0.1 molecules of LAMP per 100,000 molecular weight.

Binding of Dehydroluciferin to E·LAMP. Denburg *et al.* (1969) had shown that there are two binding sites for dehydroluciferin per 100,000 molecular weight. Since only one LAMP is bound to the enzyme an attempt was made to demonstrate further binding of dehydroluciferin to the E·LAMP complex. The E·LAMP complex was isolated by chromatography on a G-25 column in 0.1 M Tris, pH 8.0. The increase in fluorescence at 440 mμ when dehydroluciferin is bound to the enzyme was used to measure binding (Denburg *et al.*, 1969). When dehydroluciferin was titrated into a solution of the E·LAMP complex no increase in the 440-mμ fluorescence was observed. This technique is sensitive enough to detect a tenfold increase in the affinity of the enzyme for dehydroluciferin. Therefore, when one LAMP molecule was bound to the enzyme two dehydroluciferin sites were no longer accessible. The possibility that dehydroluciferin can still bind to E·LAMP but without the increase in fluorescence at 440 mμ seemed unlikely since this fluorescence change arises from putting the molecule in a hydrophobic environment. Most of the energy for binding comes from such hydrophobic interactions and without them binding could not take place.

Renaturation. The first indication that the smallest active species of the enzyme was not a dimer came from measurements of the rate of renaturation of guanidine hydrochloride denatured luciferase. After optimizing the pH, temperature, and buffer composition of the renaturation mix the maximal amount of enzymatic activity recovered was only 30% that of the control. This yield was observed to be independent of the time of incubation (from 15 min to 24 hr) in 6 M guanindine hydrochloride. Therefore, either the irreversible denaturation was occurring very rapidly in the guanidine hydrochloride and affecting only 70% of the molecules or else an inactive species is being formed in the renaturation mix. A renaturation curve is shown in Figure 2. It is seen that the rate of renaturation was independent of the protein concentration over a range of 2–20 μg/ml. At higher concentrations of protein precipitates are formed and the per cent of the activity recovered is lower.

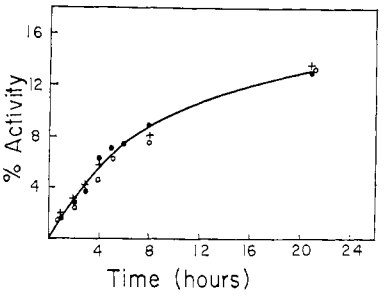

FIGURE 2: Renaturation curve of guanidine hydrochloride denatured luciferase in 0.1 M phosphate, pH 7.8, 0° with 10^{-3} M dithiothreitol. The concentration of enzyme was (●) 2 μg/ml; (○) 10 μg/ml; (+) 20 μg/ml.

Luciferase was shown to exist as a single polypeptide chain of 50,000 molecular weight in 6 M guanidine hydrochloride (Travis and McElroy, 1966). At the dilute concentration of protein used in the renaturation mixes it is expected that the rate-limiting step would be the recombination of subunits (if it were required) which should be dependent on the concentration of protein. This independence of the renaturation rate of the concentration of protein suggested that the rate-limiting step was a reordering of the polypeptide chain and did not require any collision among the molecules.

Sedimentation Velocity Measurements. The variation of the sedimentation coefficient with initial concentration of protein is shown in Figure 3. It was observed that in 0.1 M phosphate with 10^{-3} M dithiothreitol at pH 7.8 and 5° the sedimentation coefficient was increasing as the enzyme concentration was increased to its solubility limit of 10 mg/ml. At all points except the most concentrated a single symmet-

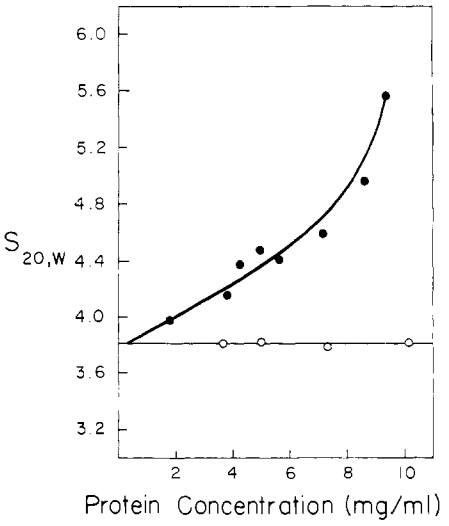

FIGURE 3: The variation of sedimentation coefficient with initial protein concentration in (●) 0.1 M phosphate, pH 7.8, and (○) 10% $(NH_4)_2SO_4$, pH 7.8.

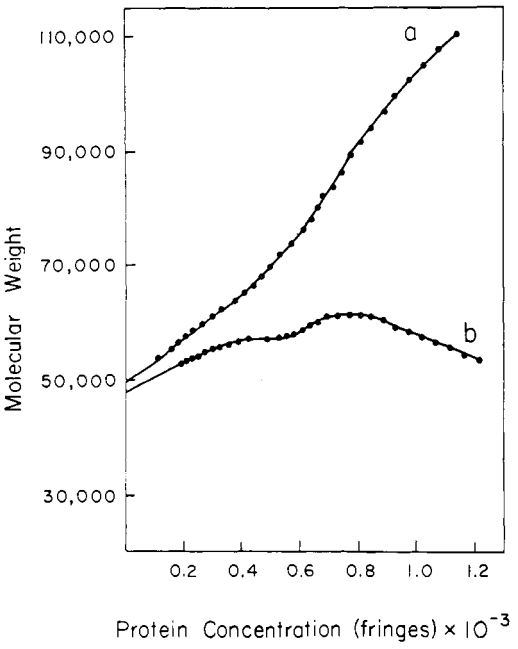

FIGURE 4: The variation of molecular weight with protein concentration in (a) 0.01 M phosphate, pH 7.8, and (b) the presence of saturating amounts of dehydroluciferin and MgATP in 0.1 M phosphate, pH 7.8.

rical schlieren pattern was observed throughout the course of sedimentation. At the nearly saturating concentration there was again a single peak in the schlieren pattern but it was definitely asymmetrical. This apparent aggregation of the enzyme was rapidly reversible. If a concentrated solution of enzyme was dialyzed overnight against buffer and diluted immediately before placing in the analytical ultracentrifuge cell, a sedimentation coefficient was calculated that was identical with that obtained from a run with a solution of identical protein concentration that also had been dialyzed overnight, but at this more dilute concentration. The presence of the dithiothreitol prevented disulfide bonds from forming.

In solutions of higher ionic strength, where the solubility of the protein is greater, no such aggregation was observed over the same range of protein concentrations. The sedimentation coefficients were independent of the initial concentration of enzyme in 10% $(NH_4)_2SO_4$ at pH 7.8 with 10^{-3} M dithiothreitol at 5° (Figure 3) and in 0.4 M phosphate buffer under the same conditions. The values of $s_{20,w}$ extrapolated to zero concentration of protein were the same in all buffers, 3.8 S.

The diffusion coefficients were also measured in 10% $(NH_4)_2SO_4$ and shown to be independent of the enzyme concentration. The value of $d_{20,w}$ 6.6×10^{-7} cm^2/sec was obtained and combined with the sedimentation coefficient to calculate the molecular weight to be 53,600.

Gel Filtration Chromatography. Luciferase at a concentration of 3 mg/ml in 0.1 M phosphate, pH 7.8, was applied to a Sephadex G-200 column and eluted with the same buffer. The elution pattern was a single symmetrical peak of constant specific activity that eluted at a position corresponding to a protein of 53,000 ± 3000 molecular weight. The peak tube was a tenfold dilution of the sample applied. An identical elution pattern was observed when the luciferase was applied at a concentration of 0.3 mg/ml and the protein measured by the optical density at 220 mμ.

Ten tails picked from live fireflies were homogenized in the presence of 0.1 M phosphate, pH 7.8, at 5°. The supernatant from a 20 min, 30,000*g* spin was applied to this G-200 column and all the tubes of eluate were assayed for flash-height activity. The enzymatic activity eluted nearly at the same position as the purified luciferase, corresponding to a molecular weight of 48,000. This indicated that no artifacts affecting the size of the active enzyme were introduced during usual purification procedures.

Sedimentation Equilibrium Measurements. Molecular weights were measured by the high-speed sedimentation equilibrium method of Yphantis (1964). In 0.1 M phosphate buffer, pH 7.8, and in 0.1 M Tris–0.1 M NaCl, pH 7.1, plots of log concentration against r^2 were straight lines, indicating homogeneity in the size of the protein. The values of the weight-average molecular weight extrapolated to zero protein concentration were 48,000–52,000.

However, *N*-ethylmaleimide-treated enzyme in 0.1 M Tris–0.1 M NaCl, pH 7.1, and native enzyme in 0.01 M phosphate buffer, pH 7.8, with 10^{-3} M dithiothreitol produced plots of log concentration against r^2 with definite curvature. These data were analyzed by use of the computer program of Roark and Yphantis (1969). Figure 4 shows the variation of the molecular weight of luciferase as a function of the concentration of protein across the cell with 0.01 M phosphate buffer, pH 7.8, as solvent. In this buffer the saturating concentration of enzyme is about 0.7 mg/ml. If it is true that aggregation occurs as the solubility limit is approached, there should be a significant amount of aggregated protein present. This was observed in Figure 4.

Luciferase at a concentration of 1 mg/ml was dialyzed overnight against 0.1 M phosphate, pH 7.8, in the presence of 5×10^{-5} M dehydroluciferin and 2×10^{-3} M MgATP. Under these conditions the enzyme had been shown to have one LAMP molecule bound per 100,000 molecular weight of protein. When this sample was analyzed in the ultracentrifuge by high-speed sedimentation equilibrium, an apparently homogeneous in size protein of 50,000 molecular weight was observed (Figure 4).

Specific Activity of Aggregated Protein. In 0.01 M phosphate, pH 7.8, the maximum solubility of luciferase was only 0.7 mg/ml. As the saturation point was reached the protein aggregates as seen in Figure 4a. The enzyme was assayed for activity at concentrations from 0.04 mg/ml to 0.6 mg/ml. It was observed that there was no change in specific activity over this concentration range, while the amount of aggregated protein was increasing as the concentration increased.

Heterogeneity of Luciferase. Further fractionation of the purified luciferase by chromatography on several ion-exchange resins proved unsuccessful. Polyacrylamide gel electrophoresis was impossible because of precipitation of the enzyme. However, isoelectric focusing enabled further resolution of the luciferase into two precipitated bands of protein with isoelectric points of 5.7 and 6.4. It is very difficult to empty the column without breaking up these bands. A typical pattern is shown in Figure 5. The little peak in the middle was caused by the breaking up of the first band. Both peaks were observed to be enzymatically active and to have the same specific activity. This specific activity was lower than that of na-

tive enzyme. This was presumably due to the inactivation of the enzyme in the solutions of low pH. Assays in which aliquots from both peaks were mixed together did not increase the specific activity. Electrofocusing experiments done in the presence of 6 M urea, 5 M formamide, or 1% Tween "80" all failed in keeping the protein bands in solution at the isoelectric points. However, in each case the similar pattern of two bands was observed.

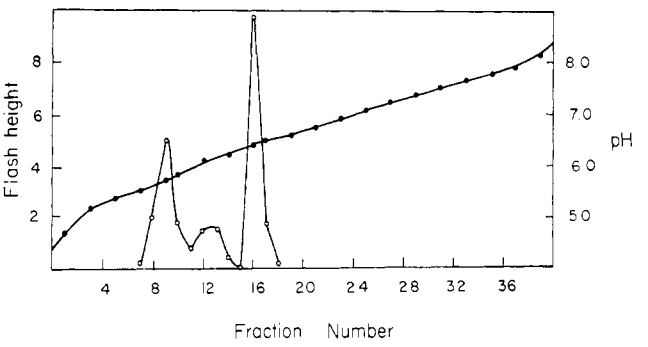

FIGURE 5: Elution pattern from an isoelectric focusing experiment.

Discussion

The mimimum enzymatically active molecular weight of firefly luciferase was shown here to be 50,000. The strong tendency of this protein to aggregate under certain conditions was probably responsible for the previously reported value of 100,000 for the molecular weight (Travis and McElroy, 1966). The aggregation was observed in solvents of relatively low ionic strength as the solubility limit of the protein was reached. The data were not sufficient to decide whether there was a monomer–dimer or monomer–dimer–trimer equilibrium occurring or to calculate the equilibrium constants. The single, symmetrical peak observed in the schlieren patterns of experiments in which the sedimentation coefficient was increasing as the protein concentration was increased suggested a rapidly reversible monomer–dimer system (Gilbert, 1955). However, the asymmetry observed in the schlieren pattern at saturating concentrations of enzyme indicated the presence of polymers greater than dimer. Since no change in the specific activity of the protein was observed as the molecular weight of the enzyme increased, this aggregation plays no physiological role in the regulation of the enzymatic activity and may be fairly nonspecific.

The tendency to aggregate and the poor solubility properties of luciferase may be expected in light of the very high percentage of nonpolar amino acids found in the enzyme. Many parameters suggested to be a measure of the hydrophobicity of proteins have been reported in the literature (Waugh, 1954; Fisher, 1964; Bigelow, 1967). The average hydrophobicity, as defined by Bigelow (1967), of luciferase was calculated to be 1240 cal/residue from the amino acid composition as reported by DeLuca *et al.* (1964). This makes luciferase one of the most hydrophobic proteins in the literature. The high percentage of nonpolar amino acids necessitates that some of them be on the external surface of luciferase. The possibility of hydrophobic intermolecular interactions in luciferase helps to explain its physical properties.

Heterogeneity in the purified luciferase preparations of maximal specific activity was indicated by the results of the binding studies. One LAMP binding site per 100,000 molecular weight of protein was observed under conditions in which only a single size species of 50,000 molecular weight was present. In addition, the active substrate, MgATP, had only one binding site per 100,000 molecular weight while there were two sites for ATP (J. L. Denburg and R. T. Lee, to be published).

The differences in the various forms of the enzyme must be very small. A single N-terminal serine was found, and the number of peptides from a tryptic digest agreed with the theoretical number predicted on the basis of identical subunits of 50,000 molecular weight (Travis and McElroy, 1966). However, recent evidence for heterogeneity was the observation of two different C-terminal amino acids, leucine and serine (R. T. Lee, unpublished results).

One of the more appealing explanations for the above observations is that each 50,000 molecular weight subunit has one luciferin and one ATP binding site. However, the ATP binding site of one of the subunits has been modified in such a manner that it can no longer bind the active substrate, MgATP. Therefore, only one of the two subunits can react to synthesize adenylate and form an enzyme–LAMP complex. This model predicts that luciferase completely in the form of the E·LAMP complex should still retain the ability to bind one molecule of dehydroluciferin per 100,000 molecular weight. However, this result was not observed. No further binding of dehydroluciferin to E·LAMP could be measured. Therefore, this suggests that the two luciferin binding sites are on the same subunit as the active site at which the adenylate is being synthesized. If this is the case, then the two essential sulfhydryl groups that were shown to be located at the luciferin binding sites (Denburg *et al.*, 1969) must also be on the same subunit.

The further resolution of the enzyme by isoelectric focusing produced two enzymatically active fractions of identical specific activity. These two precipitated bands do not represent different conformations of the same polypeptide chain because identical patterns were observed in 6 M urea, 5 M formamide, and 1% Tween "80", conditions in which the protein was completely denatured. The extreme insolubility of the protein at the isoelectric points made the emptying of the column without breaking up the bands very difficult. This has greatly hindered further studies on the characterization of these two fractions. The observation of enzymatic activity in both fractions was totally unexpected in the light of all previously proposed models. This also does not help in any way to account for the heterogeneity indicated by the binding studies. Perhaps, this suggests the possibility of even greater heterogeneity in firefly luciferase.

The absence of any significant fraction of enzymatically inactive protein eliminates the obvious explanation for the binding studies data presented here that half the protein in the pure luciferase preparations of maximal specific activity is inactive. Other explanations have considered the possibility that catalysis required momentary interactions between subunits resulting from random collisions can also be eliminated because there is no decrease in the specific activity at very low concentrations of enzyme.

No consistent model has been found yet to explain all the experimental results reported here. It is hoped that more

work will help to further elucidate the relationships between the substrate binding sites and the heterogeneity of firefly luciferase.

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Site of Reaction of a Specific Diazo Inactivator of Pepsin*

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ABSTRACT: At pH 5.5 in the presence of Cu(II), 1-diazo-4-phenyl-2-butanone reacts with swine pepsin in 1:1 molar stoichiometry to give a catalytically inactive enzyme. The inactivator reacts with pepsin to give an ester of 1-hydroxy-4-phenyl-2-butanone with the β-carboxyl group of a uniquely reactive aspartyl residue which is believed to be at the active site. This ester as well as a related model compound hydrolyze readily at pH 8 and room temperature. Following digestion of the inactivated pepsin by swine pepsin at pH 2 several peptides containing the reacted aspartyl residue were isolated. From the sequences of these peptides the sequence around the reactive aspartyl residue is indicated to be: Ile-Val-Asp-Thr-(Gly,Thr)-Ser-Leu. This sequence is similar to that containing the aspartyl group which reacts with *N*-diazoacetyl-L-phenylalanine methyl ester (Bayliss, R. S., Knowles, J. R., and Wybrandt, G. B. (1969), *Biochem. J. 113*, 377).

Kinetic studies with pepsin indicate that at least two carboxyl groups on the enzyme are involved in catalysis (Jackson *et al.*, 1965, 1969; Clement *et al.*, 1968; Zeffren and Kaiser, 1967; Lutsenko *et al.*, 1967; Denberg *et al.*, 1968; Hollands and Fruton, 1968; Cornish-Bowden and Knowles, 1969; Lundblad and Stein, 1969). Stein and Fahrney (1968) have shown that a carboxyl group is directly involved in the pepsin-catalyzed hydrolysis of sulfite esters, and Shkarenkova *et al.* (1968) have observed that oxygen from $H_2{}^{18}O$ is rapidly incorporated into two carboxyl groups of pepsin. Erlanger and his coworkers (1965, 1966; see also Gross and Morell, 1966) have identified one uniquely reactive carboxyl group using a phenacyl halide inhibitor. However, this carboxyl is apparently not catalytically active but rather is involved in substrate binding because the modified enzyme still retains considerable catalytic activity toward hemoglobin as substrate (Erlanger *et al.*, 1967).

In an attempt to identify a carboxyl group at the active site of pepsin by means of a carboxyl-reactive reagent we chose the readily synthesized diazoketone 1-diazo-4-phenyl-2-butanone (DPB)[1] since it mimics partially the structure of phenylalanine amides which are good substrates for pepsin. DPB, in the presence of Cu(II) as catalyst, was found to react with pepsin in a 1:1 molar ratio to give a completely inactive enzyme (Hamilton *et al.*, 1967). After the work with DPB was begun, Delpierre and Fruton (1965) reported the inhibition of pepsin activity by diphenyldiazomethane. This inactivation is apparently not specific since more than 1 mole of the inhibitor reacts per mole of enzyme. Independent of our work several other groups of investigators (Rajagopalan *et al.*, 1966; Delpierre and Fruton, 1966; Ong and Perlmann, 1967; Erlanger *et al.*, 1967; Bayliss and Knowles, 1968; Kozlov *et al.*, 1967; Stepanov and Vagonova, 1968; Lundblad and Stein, 1969) have found that other diazocarbonyl compounds which have structures related to substrates of the enzymes will also inactivate pepsin.

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[1] Abbreviations used are: 1-diazo-4-phenyl-2-butanone, DPB; 1-hydroxy-4-phenyl-2-butanone, HPB; 1-acetoxy-4-phenyl-2-butanone, AcHPB; 5-dimethylaminonaphthalene-1-sulfonyl, dansyl.