group result in specific functional effects more akin to those observed with most other chemical modifications. On the other hand, selective modification of the guanido groups of but two specific arginyl residues apparently affects both activities of the enzyme in an identical and unambigious fashion.

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An Active Proteolytic Fragment of *Gonyaulax* Luciferase†

Neil Krieger,‡ David Njus,§ and J. W. Hastings*

ABSTRACT: Soluble extracts of the bioluminescent dinoflagellate *Gonyaulax polyedra* contain luciferase activity in several molecular weight forms (400,000, 130,000, and 35,000). The active monomeric chain *in vivo* has a molecular weight of about 130,000, as shown by extraction followed by gel filtration, both in 6 M guanidine·HCl. The 400,000 molecular weight form includes at least one chain of the 130,000 molecular weight species, while the 35,000 molecular weight species is a lytic fragment thereof. When extractions are made in buffer at pH 6, all the luciferase is obtained in the lowest molecular weight form due to an endogenous lytic enzyme most active at acid pH's. Digestion of the 400,000 molecular weight species with a low concentration of subtilisin also produces the 35,000 molecular weight form. In both cases the low molecular weight form is heterogeneous, including active species which can be distinguished both by ion-exchange chromatography and by differences in their pH–activity profiles.

Studies concerning the molecular mechanism of circadian rhythms have been hampered by a lack of molecular correlates. In the marine dinoflagellate, *Gonyaulax polyedra,* there is a rhythm of extractable luciferase activity which correlates with a well-defined circadian rhythm of bioluminescence (Hastings and Sweeney, 1957; Hastings and Bode, 1962). Recent experiments concerned with the molecular basis for this activity rhythm were equivocal, partly because the enzymatic activity occurs in several molecular forms whose interrelationships were not well understood (McMurry, 1971; McMurry and Hastings, 1972). The present study clarifies this matter.

The *in vitro* light-producing system from *Gonyaulax* was first characterized by Hastings and Sweeney (1957) in the supernatant from a 36,000 *g* cell extract. The activity was shown to require a dialyzable heat-stable substrate (*Gonyaulax* luciferin), a heat-labile protein (*Gonyaulax* luciferase), and oxy-

† From the Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138. *Received November 20, 1973.* This work was supported by grants from the National Science Foundation (GB 31977X) and the National Institutes of Health (GM 19536).

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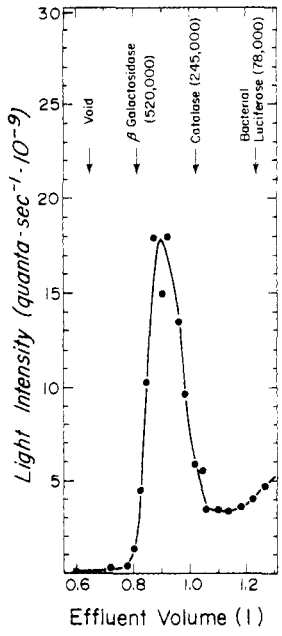

FIGURE 1: Chromatography of crude A-400 luciferase. Cells from 85 l. of culture were extracted at pH 8.4 as described in Methods. The crude luciferase was concentrated to 50 ml by $(NH_4)_2SO_4$ fractionation and applied to a Sephadex G-200 column (5 × 100 cm) equilibrated with 0.025 M Tris-HCl-0.1 M NaCl-10^{-4} M dithiothreitol (pH 8.4) at 4°. Fractions (10 ml) were collected and assayed at pH 6.3 as described in Methods. Enzyme standards were included in the applied sample and were assayed by activity.

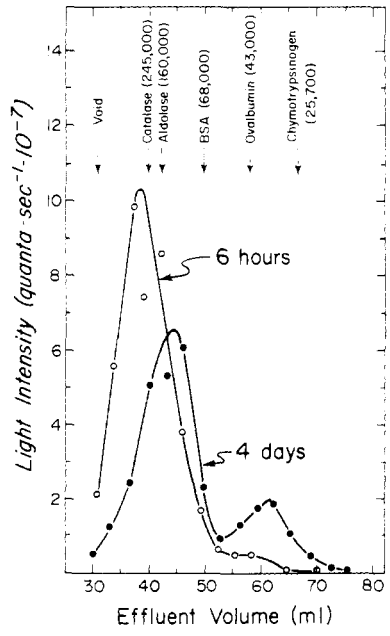

FIGURE 2: Chromatography of partially purified luciferase on Sephadex G-100 showing spontaneous changes in molecular weight. Luciferase was obtained by pooling the A-400 fractions from a Sephadex G-200 purification similar to the one shown in Figure 1. After elution, this luciferase was kept at 4° and pH 8.0. Aliquots were applied to a Sephadex G-100 column (2 × 32.5 cm) after 6 hr and 4 days and eluted with 0.05 M Tris-HCl-5 × 10^{-3} M 2-mercaptoethanol (pH 8) at 4°. About one-third of the applied activity was recovered. Assays were performed at pH 6.3.

gen, with optimal activity at pH 6.6. Later it was reported that luciferase could be obtained in two molecular weight forms, A and B, and that these forms had different pH-activity profiles (Krieger and Hastings, 1968). The larger species (A) was reported to be active only in the acid range, optimal at pH 6.6, while the smaller species (B) was active over a wider range from pH 6.0 to 9.0.

In this paper we show that three major molecular weight categories of *Gonyaulax* luciferase (400,000, 130,000, and 35,000) can be distinguished by gel filtration. The two larger have been denoted A-400 and A-130, respectively; the previously reported A luciferase corresponds to these. The active monomeric peptide chain of luciferase, as judged by extraction of the cells in 6 M Gdn·HCl[1] and gel filtration of the extract in 6 M Gdn·HCl, is A-130. The 400,000 molecular weight form includes at least one of the 130,000 molecular weight chains. The lowest molecular weight form, B luciferase, is shown here to be heterogeneous and to be a fragment and not a subunit of the A-130 chain; it arises from the action of an endogenous enzyme, presumably a protease. B can also be obtaind *in vitro* by treatment of A-400 with subtilisin.

Materials and Methods

Cultures of *Gonyaulax* were grown in an enriched sea water medium under conditions of alternating light and dark periods of 12 hr each (Fogel and Hastings, 1971). Cells were harvested by filtration at a specified time during the cycle and resuspended in extraction buffer at 4°. Two different buffers were used, one at pH 8.4 at 4° (0.05 M Tris-HCl-0.01 M EDTA-10^{-3} M dithiothreitol) and the other at pH 6.0 (0.05 M $Na_xK_yPO_4$, 5 × 10^{-4} M dithiothreitol). The cells were passed twice through a hand-operated homogenizer (Fisher Scientific Co.), and cell debris was removed by low-speed centrifugation (2000*g* for 8 min). The particulate bioluminescent activity (DeSa and Hastings, 1968) was sedimented by centrifugation at 27,000*g* for 10 min, and this supernatant provided a crude source of soluble luciferase activity.

Luciferin was prepared from *Pyrocystis lunula (Dissodinium lunula)* from a clone isolated by Dr. Elijah Swift. Cultures were grown on a light-dark cycle as above in *f*/2 medium with silicate omitted (Guillard and Ryther, 1962). These cells were harvested by filtration in the middle of the night phase after exposure to room lights for 1 hr to inhibit flashing. Luciferin was extracted by boiling and purified by chromatography on a DEAE-cellulose column as described by Fogel and Hastings (1971).

Assays for luciferin and luciferase were performed by measuring light emission from the bioluminescent reaction at 24°, using the photometer of Mitchell and Hastings (1971) calibrated with the standard of Hastings and Weber (1963). The complete reaction mixture (2 ml) included (final concentrations) 0.04% bovine serum albumin, 1 M $(NH_4)_2SO_4$, 2.5 × 10^{-4} M EDTA, and 0.1 M Tris-maleic acid-NaOH buffer at the specified pH, either pH 6.3 or 8.0.

Molecular weights of proteins in buffer were estimated according to the method of Andrews (1964) using Sephadex G-100 or G-200 columns standardized with appropriate markers. Molecular weights of proteins in 6 M Gdn·HCl were estimated by gel filtration through a Bio-Gel A-50m agarose column (0.95 × 20 cm) in 6 M Gdn·HCl-0.3 M 2-mercaptoethanol-0.01 M EDTA (pH 8) at 4° (Friedland and Hastings, 1967). Samples of 0.3 ml were applied and 0.3-ml fractions were collected. The standards used were chymotrypsinogen (25,700), bacterial luciferase subunits (39,000), bovine serum albumin (68,000), and β-galactosidase monomer (130,000) (Weber and Osborn, 1969; Hastings *et al.*, 1969). Bacterial luciferase, purified as previously described (Gunsalus-Miguel *et al.*, 1972),

[1] Abbreviations used are: Gdn·HCl, guanidine hydrochloride; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate; DEAE, diethylaminoethyl.

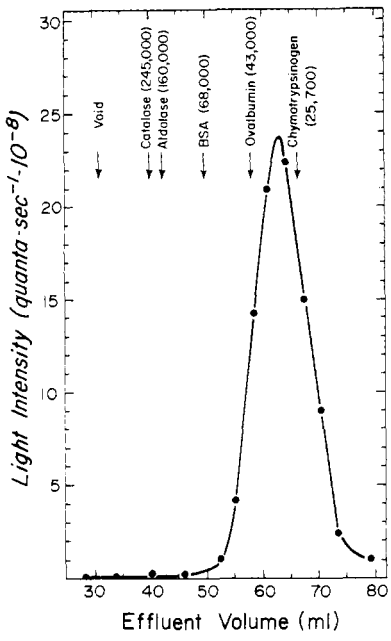

FIGURE 3: Chromatography of B luciferase on Sephadex G-100. Cells were extracted at pH 6.0 as described in Methods. Luciferase was concentrated by fractionation with $(NH_4)_2SO_4$ (35–65% of saturation), resuspended in 0.05 M Tris-HCl–5 × 10^{-3} M 2-mercaptoethanol (pH 8) at 4°, and applied to the Sephadex G-100 column described in Figure 2. The column was eluted and assays were performed as in Figure 2.

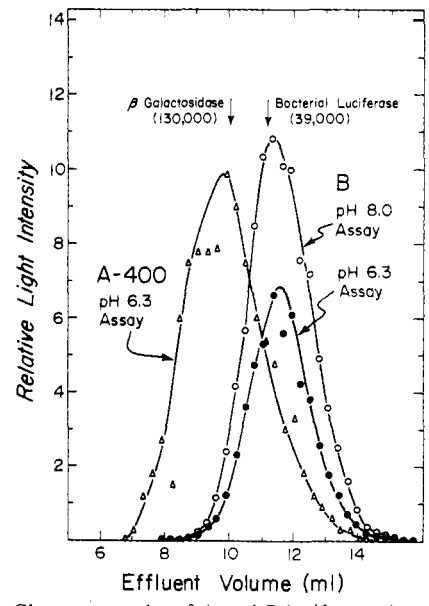

FIGURE 4: Chromatography of A and B luciferases in 6 M Gdn·HCl. Samples of reduced and denatured A and B luciferases were chromatographed on Bio-Gel A-50m in 6 M Gdn·HCl–0.3 M 2-mercaptoethanol–0.01 M EDTA (pH 8) at 4°. Fractions were collected, renatured for 24 hr at 4°, and assayed for luciferase activity at pH 6.3 as described in Methods. The apparent molecular weight of the peptide chain shown by this column chromatography technique was independent of the time allowed for reduction, up to 4 hr at 24°, indicating that any disulfide linkages are broken.

was included as an internal standard in each of the applied samples.

Cells were extracted in 6 M Gdn·HCl by dispersing and homogenizing the cells directly in the above buffer at 4°. Luciferase was renatured by 50-fold dilution into 0.05 M Tris-HCl–0.01 M EDTA–0.1% bovine serum albumin–5 × 10^{-3} M 2-mercaptoethanol (pH 8.0) at 4°. After renaturation for 24 hr at 4°, 0.5-ml aliquots were assayed in the standard assay mix at pH 6.3 or 8.0.

Ultra Pure Gdn·HCl was obtained from Schwarz-Mann. 2% Agarose (Bio-Gel A-50m, 50–100 mesh) was obtained from Bio-Rad Laboratories and was allowed to equilibrate with 6 M Gdn·HCl for about 24 hr at room temperature before packing the column. Enzymes, except bacterial luciferase, were obtained from Sigma. Other reagents were of analytical quality.

Protease assays were performed as previously described (Njus *et al.*, 1974) except that proteolysis was allowed to proceed for 1 hr to provide better resolution.

Results

Figures 1, 2, and 3 illustrate the three molecular weight categories of *Gonyaulax* luciferase observed using gel filtration. A-400 (Figure 1) was obtained by extracting cells during the middle of the night phase with 0.05 M Tris-HCl–0.01 M EDTA–5 × 10^{-3} M 2-mercaptoethanol (pH 8.4) at 4°. In the experiment depicted here, the protein was fractionated by ammonium sulfate precipitation, and the luciferase fraction (precipitating between 35 and 65% of saturation) was applied to a Sephadex G-200 column. A luciferase sample which was not subjected to precipitation by $(NH_4)_2SO_4$ eluted at the same molecular weight position.

The A-130 form first became evident when A-400 eluted from Sephadex G-200 was allowed to stand at 4° and pH 8.0 for 4 days. In Figure 2, this material is compared by chromatography on a Sephadex G-100 column with a sample of the same material which was allowed to stand for only 6 hr before application to the G-100 column. We have not succeeded in stabilizing the A-400 preparation against conversion to the A-130 form, suggesting that the conversion may be the result of a slow dissociation process or limited proteolysis. In addition to the conversion of A-400 to A-130, the appearance of B luciferase is evident.

B luciferase is also obtained when cells (either day or night phase) are extracted at pH 6.0, as described in Methods. Under these conditions, all of the activity occurs in the 35,000 molecular weight range (Figure 3), and is active at both pH 6.3 and 8.0. As will be shown, the difference in the molecular weight of the material obtained is due to the action of an endogenous lytic enzyme which acts on *Gonyaulax* luciferase, but far more slowly at pH 8.0 than at pH 6.0.

Luciferases A-130 and B both renature readily from 6 M Gdn·HCl recovering between 60 and 90% of their original activity. Therefore, single-strand molecular weights can easily be determined by gel filtration in 6 M Gdn·HCl (Friedland and Hastings, 1967; Fish *et al.*, 1969). In preparation for chromatography in 6 M Gdn·HCl, A-400 (Figure 1) and B luciferase (Figure 3) were extracted and partially purified on Sephadex G-100. These two samples were then denatured in 6 M Gdn·HCl and chromatographed separately on a 2% agarose column in 6 M Gdn·HCl, as described under Methods (Figure 4). The A-400 luciferase activity eluted at a position near the β-galactosidase monomer marker (130,000) with about 60% of the activity being recovered. No activity appears in the molecular weight range above 130,000, and less than 10% of the recovered activity is in the B region. When B luciferase was applied to the column, it eluted at the position of the bacterial luciferase subunit marker (39,000) with about 35% of the activity being recovered. The good recovery of B luciferase at its expected elution volume illustrates that if B had been liberated as a subunit from the A-400 luciferase, there would have been no problem detecting it. The assays at pH 8.0, where A-130 is inactive, verify the identity of B luciferase. We conclude that B luciferase is not a subunit of A-400 or A-130. If B is ever ob-

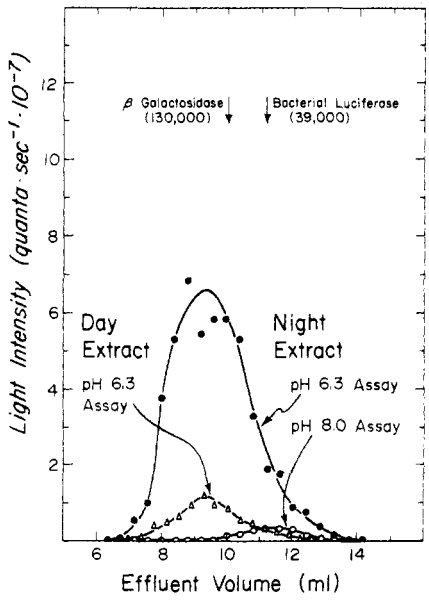

FIGURE 5: Chromatography of luciferase from night and day phase cells in 6 M Gdn·HCl. Cells harvested during the day and the night phase were extracted directly into 6 M Gdn·HCl–0.3 M 2-mercaptoethanol–0.01 M EDTA at pH 8.0 and 4° and then chromatographed as in Figure 5 on Bio-Gel A-50m in the 6 M Gdn·HCl buffer. Fractions were collected, renatured for 24 hr at 4°, and assayed for luciferase activity as described in Methods.

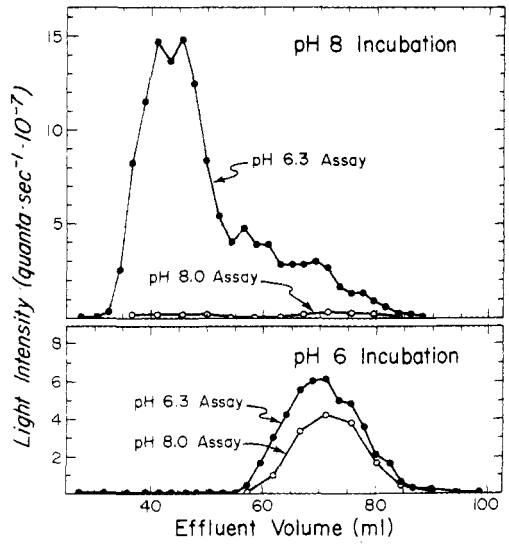

FIGURE 7: Evidence for a soluble endogenous protease. Cells were extracted into 0.05 M Tris-HCl–0.01 M EDTA–10^{-3} M dithiothreitol (pH 8.4) and protein was precipitated with 90% ammonium sulfate with the pH carefully kept near 8. The precipitate was divided into two portions. The control was resuspended in the pH 8.4 extraction buffer; the other sample was resuspended in 0.05 M $Na_xK_yPO_4$–5 × 10^{-4} M dithiothreitol (pH 6.0). After incubation for 2 hr at 4°, both samples were dialyzed against 0.05 M Tris-HCl–5 × 10^{-3} M 2-mercaptoethanol (pH 8.4) and chromatographed on Sephadex G-100. The sample kept at pH 8.4, shown in the top panel, is mostly A luciferase, while the one incubated at pH 6.0 is all B luciferase and has acquired the characteristic activity at pH 8.0.

tained from an A species, it must be through the cleavage of a covalent bond.

To determine the chain length of luciferase under *"in vivo"* conditions, cells (both day and night phase) were extracted directly into 6 M Gdn·HCl and subjected to gel filtration, again in 6 M Gdn·HCl (Figure 5). Such a procedure should minimize the possibility that proteolysis or other lytic processes occur during extraction. In these experiments, we found activity only in the region of the β-galactosidase monomer (130,000) and almost none in the region of mol wt 35,000. Thus the B fragment does not appear to be significant *in vivo*, at least at the two times of day examined here. The possibility that the material in the 130,000 molecular weight peak might be heterogeneous with regard to size was also checked. Samples from the two ends of the peak were rerun on Sephadex G-100 in 0.05 M Tris-HCl–5 × 10^{-3} M 2-mercaptoethanol (pH 8.4); they gave superimposable elution patterns.

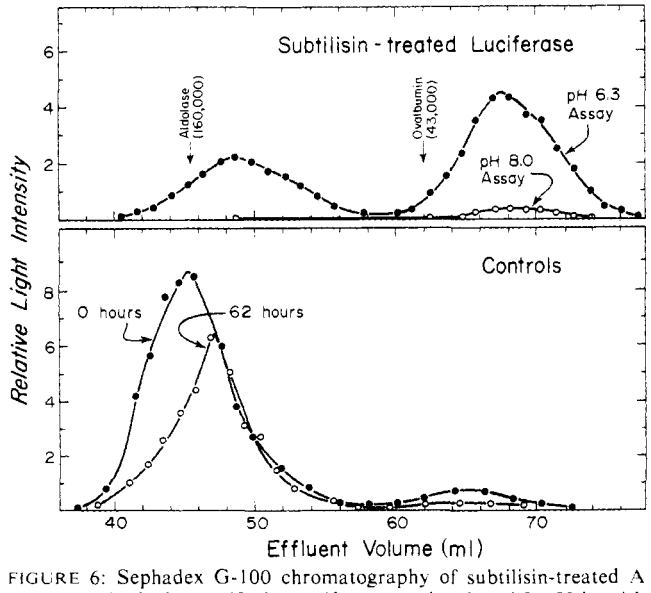

FIGURE 6: Sephadex G-100 chromatography of subtilisin-treated A luciferase. Sephadex-purified A luciferase was incubated for 52 hr with subtilisin (10^{-5} mg/ml) in 0.05 M Tris-HCl–0.01 M EDTA–5 × 10^{-3} M 2-mercaptoethanol at pH 8.0 and 4° and chromatographed on Sephadex G-100 in the same buffer (top panel). The controls without subtilisin at zero time and 62 hr are shown in the bottom panel. Assays of the controls were performed at pH 6.3; the subtilisin-treated material declined less than 20% during the experiment.

In the experiment of Figure 5, the amount of luciferase activity recovered from the column was seven times as high for the night extract as for the day extract. This is similar to the extractability ratios found by both Hastings and Bode (1962) and McMurry and Hastings (1972). One possible explanation for the rise and fall of extractable luciferase activity from *Gonyaulax* is that there is a reversible association of a second molecule with luciferase. Chromatography in 6 M Gdn·HCl would have separated any such molecule from luciferase unless, of course, it also had a molecular weight near 130,000.

The possibility that an activating ion, especially calcium, might be involved in luciferase activity was suggested by results with other bioluminescent systems (Lynch *et al.*, 1972; Shimomura *et al.*, 1962; Cormier, 1962). The fact that full activity was obtained when renaturation was carried out in the presence of 0.01 M EDTA indicates that calcium is not involved. Moreover there was no difference in the amount of renatured activity when Ca^{2+} was added to the renaturation mixture in the absence of EDTA.

A-400 can be converted to B luciferase with small amounts of added subtilisin (Figure 6). Material pooled from the A-400 peak of a Sephadex G-100 column was allowed to stand at 4°, pH 8.0, with 10^{-5} mg/ml of subtilisin. After 52-hr subtilisin treatment, a 1-ml aliquot was rechromatographed on the Sephadex G-100 column, and 40% of the applied luciferase activity (65% of that recovered) was found in the B peak. In a control lacking subtilisin kept at 4° for 62 hr, this number was only 6%. The conversion of A-400 to A-130 described in Figure 2 has occurred here as well.

Fogel and Hastings (1971) have shown that all of the lucif-

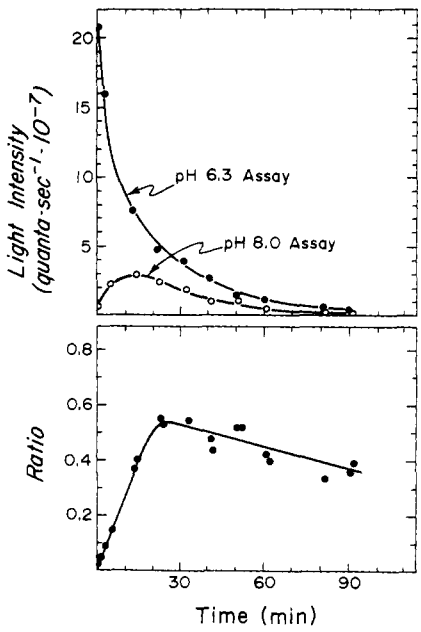

FIGURE 8: Treatment of B luciferase with subtilisin. Sephadex-purified B luciferase was allowed to stand with subtilisin (10^{-4} mg/ml) at 24° at pH 8.0. At the indicated times, 0.1-ml aliquots were withdrawn and assayed at pH 6.3 and 8.0. The lower panel shows the ratio of the activity at pH 8 to that at pH 6.3. A control sample showed no change.

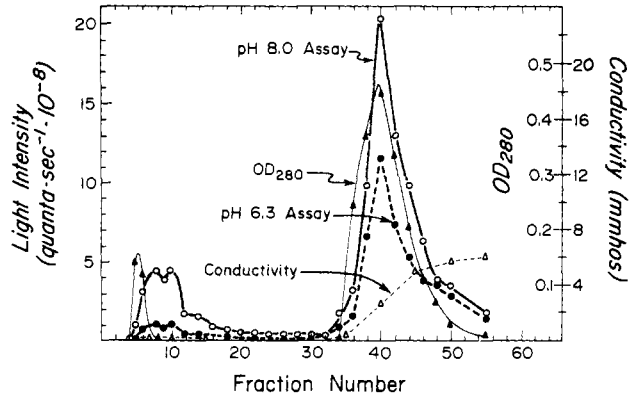

FIGURE 9: QAE-Sephadex chromatography of B luciferase. B luciferase from a pH 6.0 extract was concentrated by $(NH_4)_2SO_4$ fractionation followed by dialysis against 0.001 M Tris-HCl (pH 7.8)–0.014 M 2-mercaptoethanol at 4°. To a 1.5 × 6 cm QAE-Sephadex column, equilibrated with the above buffer, a 0.5-ml sample was applied, followed by a wash of 30 ml of the same buffer. A linear gradient of NaCl (0–0.35 M) in this buffer was then applied. Fractions of about 1 ml were collected and 5-μl samples were assayed at both pH 6.3 and 8.0; 55% of the activity at pH 6.3 was recovered.

erase may be extracted in the B form when the extraction buffer is at pH 6.0. In conjunction with the above results, this suggests the presence of an endogenous protease which degrades luciferase more rapidly at pH 6 than at pH 8. An experiment which indicates the presence of such an enzyme is illustrated in Figure 7. Cells were extracted at pH 8.4 and protein was precipitated with 90% ammonium sulfate. Half of the precipitate was resuspended in buffer at pH 6.0 and the other half, as a control, was resuspended in the extraction buffer at pH 8.4. After 2 hr at 4°, both samples were returned to pH 8.4 and chromatographed on Sephadex G-100. Almost all of the material kept at pH 8.4 eluted in the high molecular weight region, while the material incubated at pH 6.0 was all in the 35,000 molecular weight form and exhibited the pH 8.0 activity characteristic of the B form. Thus a soluble luciferase-degrading enzyme is evidently present in the crude extract, is precipitable with 90% ammonium sulfate, and is active at a slightly acidic pH. The acidic pH itself is not responsible for cleavage, since luciferase partially purified on Sephadex is degraded much more slowly than the crude enzyme. The endogenous lytic enzyme is equivalent to roughly 1 μg/ml of subtilisin in its ability to convert A-400 luciferase to B.

The 35,000 molecular weight luciferase category, B luciferase, is itself heterogeneous. B was originally described both in terms of its 35,000 molecular weight and its broad pH range for activity, which extended from pH 5.5 to 9. This is in contrast to the higher molecular weight form recognized at that time which lacked activity above pH 7. While the B material of Figure 3 is true to this description, the low molecular weight luciferases of Figures 1, 2, and 6 are not; in these cases, pH 8 activity is less than 10% of the pH 6.6 activity. But this B material will rapidly develop substantial activity at pH 8 upon further incubation with subtilisin at 24°. Figure 8 illustrates this for the B material of Figure 2.

The B luciferase extracted as described in Figure 3 can be demonstrated to be heterogeneous by chromatography on QAE-Sephadex (Figure 9). Activity elutes in two peaks, indicating that more than one species of B occurs under these conditions. This confirms a similar earlier experiment (Fogel and Hastings, 1971). The first peak elutes before applying the salt gradient, and the second elutes at roughly 0.2 M NaCl. The first peak was rerun on a second QAE-Sephadex column to be certain that it was distinct in its behavior from the second peak, and it again eluted before any salt gradient was applied. A portion from each of the two peaks eluted from QAE-Sephadex was applied to Sephadex G-100 and each eluted at a volume corresponding to 35,000 molecular weight. Both differ from the B obtained as described in Figure 2, in that their activity when assayed at pH 8 was *greater* than at pH 6.3.

In summary, we can distinguish several different species of B luciferase, all of which are in the molecular weight range of 35,000. We presume that they differ with respect to the specific peptide bond cleaved. The fact that the pH–activity profile can be so different in enzyme species which are apparently so closely related is truly striking and a description of the molecular basis for this will be of considerable interest.

We have shown by gel filtration in 6 M Gdn·HCl that B luciferase is a fragment of the A-130 luciferase chain obtained through the cleavage of a covalent bond. The fact that several forms of the B fragment are found makes it unlikely that the cleavage occurs at some unusual bond, but we do not know enough about luciferase structure to rule this out. However, the fact that subtilisin mimics the conversion of A-400 to B suggests that this is caused by an endogenous protease. This conversion is effected by a soluble endogenous material which precipitates with 90% ammonium sulfate, is most active at acid pH's, and at pH 6.0 is equivalent to roughly 1 μg/ml of subtilisin in its ability to cut luciferase. The ammonium sulfate precipitated extract does indeed possess this amount of proteolytic activity. Using heat denatured casein as a substrate, proteolytic activity at pH 6 equivalent to 1.6 μg/ml of subtilisin can be demonstrated. Using Azocoll as the substrate, proteolytic activity equivalent to 0.07 μg/ml of subtilisin is found. Thus, while the activity depends greatly on the substrate and the specificity of the protease, the necessary activity seems to be present. Furthermore, the activity at pH 6.0 (as assayed with Azocoll) was about ten times greater than the activity at pH 8.0. It is likely, therefore, that the generation of B luciferase is due to this proteolytic activity in the crude extract.

Luciferase cleavage by the endogenous activity is not inhibited by phenylmethanesulfonyl fluoride, suggesting that the de-

grading enzyme is not a serine protease (Fahrney and Gold, 1963). Inhibitors of cysteine proteases cannot be tested because they also inactivate luciferase, thus making the luciferase degradation assay impractical. To demonstrate unequivocally that the endogenous enzyme corresponds to the observed protease activity, it is necessary to isolate the enzyme. We have found that the luciferase-degrading activity can be recovered from a Sephadex G-200 column from the region corresponding to a molecular weight of 60,000–190,000. This material also possesses proteolytic activity as assayed on casein and Azocoll. Further investigation should clarify the nature of this enzyme.

Discussion

Spurious activities arising from proteolysis during extraction and purification of proteins have now been observed in many systems (Pringle, 1970; Brutlag *et al.*, 1969; Gardner *et al.*, 1971; Fall and Vagelos, 1973; Colombo and Marcus, 1973). In one sense, this is a nuisance that must be minimized because it hinders purification and experimental work. Since the cleavage of luciferase occurs rapidly at acid pH's but only very slowly at pH 8, degradation can be minimized simply by keeping the extracted luciferase at or above pH 8.0.

The ability to generate specific fragments through proteolysis presents some interesting opportunities as well. Brutlag *et al.* (1969) have separated functions of DNA polymerase in this way. Proteolysis of fructose 1,6-diphosphatase changes the pH–activity profile and diminishes inhibition by AMP and activation by potassium ions (Pontremoli *et al.*, 1973; Colombo and Marcus, 1973). We have already noted that proteolysis of *Gonyaulax* luciferase broadens the pH–activity profile to include alkaline pH's. Apparently, this difference is attributable to a small region of the peptide chain near the point of cleavage since low molecular weight fragments can be obtained which have little activity at pH 8, and this activity increases markedly upon further proteolysis.

The observation that low molecular weight B fragments renature properly indicates that the information for correct folding, as well as activity, is present within this fragment representing only 25% of the original chain length. Platt *et al.* (1973) have reported a similar situation in that the 28,000 molecular weight tryptic core of lac repressor protein (native mol wt 38,000) refolds and assumes its regular tetrameric structure when renatured from Gdn·HCl.

The major advantage of the luminescent activity assay is that it can be performed with impure preparations at very low luciferase concentrations. We routinely perform activity experiments with an estimated 0.1 ng of enzyme. With such small quantities of an only partially purified enzyme, we can offer no independent physical measurements concerning the configuration of the enzyme in either Tris buffer or 6 M Gdn·HCl. However, its elution volume in gel filtration studies in relation to a series of protein markers is consistent under both conditions, *i.e.*, it seems to move as a globular protein upon gel filtration in Tris buffer and as a random coil upon gel filtration in a denaturing solvent.

The relationship between A-130 and A-400 is not yet known. Although A-400 may be a multimeric form of A-130, it is also possible that A-400 consists of one or two A-130 chains combined with other protein or non-protein components. This is especially pertinent since luciferase also occurs in a membrane-bound form as part of the particulate bioluminescent structure, the scintillon (Henry and Hastings, 1974; Fuller *et al.*, 1972). Thus, extraction may result in the liberation of A-130 molecules associated perhaps nonspecifically with membrane proteins and lipids as the A-400 species.

There is a circadian rhythm in extractable luciferase activity and we have shown that this variation is apparent even when the extract is made and chromatographed in 6 M Gdn·HCl. Therefore, the rhythm cannot be due to noncovalently bound activators or inhibitors. The luciferase chain must either be covalently modified or it must be synthesized and degraded every day. We wish to establish whether or not the observed proteolytic activity and its active product, B luciferase, have a functional significance in this regard.

Acknowledgment

The assistance of Ann Gunsalus Miguel with some of these experiments is gratefully acknowledged.

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Relaxation Spectra of Proteinases. Isomerizations of Carboxypeptidase A (Cox) and (Anson)†

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ABSTRACT: Carboxypeptidase A (Cox) isomerizes at 25° and at neutral pH values in the absence of substrates. The relaxation effect of largest amplitude (τ_1), detected with proton indicators by the equilibrium temperature-jump method, is characterized by pH-dependent rate constants varying from 12 $msec^{-1}$ in acid to 6 $msec^{-1}$ in alkaline solution, and by a single pK_a value near 6. The τ_1 effect is also present in the commercial Worthington enzyme and in the isomers A_γ^{Leu}, A_γ^{Val}, and A_β^{Leu} The pH-dependent behavior of the isomerization can be interpreted in terms of the three-protonation-state model for the enzyme (Auld, D. S., and Vallee, B. L. (1970), *Biochemistry 9,* 4352) and therefore may be linked to the ionization of a group important to the catalytic step of peptide hydrolysis. The transient-state behavior of noncompetitive inhibitors of peptide hydrolysis is correlated with the analogous steady-state behavior. The τ_1 effect is not observed at inhibitor concentrations sufficient to saturate the enzyme under steady-state conditions. Rate constants greater than 12 $msec^{-1}$ are observed in the presence of saturating concentrations of glycyl-L-tyrosine, a pseudosubstrate, and L-phenylalanine, a product and competitive inhibitor of peptide hydrolysis, whereas no change in the relaxation time or amplitude occurs upon addition of L-phenyllactate and L-mandelate, products and competitive inhibitors of ester hydrolysis. The relevance of these results to a proposal of nonidentical binding sites for peptide and ester substrates of carboxypeptidase (Vallee *et al.* (1968), *Biochemistry 7,* 3547) is discussed.

Extensive steady-state kinetic studies of the carboxypeptidase A (Cox)[1] catalyzed hydrolysis of tripeptides over the pH range 4.5–10 and temperature range 5–35° have revealed three protonated forms of the enzyme important to the hydrolysis of peptides ($EH_2 \rightleftarrows EH \rightleftarrows E$). Deprotonation of EH_2 is required for catalysis whereas deprotonation of EH prevents peptide binding (Auld and Vallee, 1970b, 1971). In addition, the use of N-dansylated peptide substrates has allowed studies of enzyme–substrate complexes by stopped-flow fluorescence (Latt *et al.,* 1970, 1972). By this means the mode of action of inhibitors has been assigned directly. Mixed inhibition by a number of agents has been resolved into noncompetitive and competitive components and their pH dependence was determined (Auld *et al.,* 1972).

Any isomerizations of the holoenzyme, as well as studies of transient-state kinetics of the action of an enzyme on substrates, pseudosubstrates, and inhibitors, are essential for a description of a detailed mechanism of an enzyme, as was first fully demonstrated for ribonuclease (Hammes, 1968). In the work reported here, a sensitive temperature-jump apparatus (French and Elwood, in preparation) has been used to detect relaxation effects in carboxypeptidase A (Cox) and (Anson) and the isomers A_γ^{Leu}, A_γ^{Val}, and A_β^{Leu} obtained from Anson enzyme. The behavior of the two main isomerizations, τ_1 and τ_2, that occur near neutrality in the Anson enzyme has been explored as a function of pH and after addition of inhibitors, and a pseudosubstrate, glycyl-L-tyrosine. The pH dependence of the τ_1 isomerization of carboxypeptidase A (Cox) was also determined.

Material and Methods

Apparatus. The detector, a combination photodiode-operational amplifier, of the temperature-jump apparatus (French and Elwood, paper in preparation) was adjusted to give a frequency response (0–20 kHz) and signal-to-noise ratio (typically, 6000) that were adequate for study of the relaxation behavior reported here. The precision of the rate constants fell off above 15 $msec^{-1}$ because of the 20-kHz setting of the amplifier. During alignment of the apparatus care was taken to reduce the amplitude of the only artifact that was observed with unbuffered indicator–salt solutions (rate constant *ca.* 100 $msec^{-1}$) to the broad band noise level of the apparatus (*ca.* 5 mV or 0.00003 *A* unit, 0–30 kHz). Relaxation effects that were used for quantitative calculations were obtained with 4.0° temperature jumps; they varied in amplitude from about 50 to 500 mV, corresponding to transmittance changes less than 0.006*T*.

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[1] The nomenclature used in the text for the various preparations and isomers of carboxypeptidases is that of Petra and Neurath (1969). Mes is an abbreviation for 2-(*N*-morpholino)ethanesulfonic acid.