The Reversibility of the Denaturation of Bacterial Luciferase*

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ABSTRACT: Treatment of purified bacterial luciferase with 8 M urea or 5 M guanidine-HCl leads to rapid loss of activity. However, on dilution into phosphate buffer (pH 7.0) containing 1×10^{-3} M dithiothreitol and 0.2% bovine serum albumin, luciferase activity is recovered. The reconstituted protein cannot be distinguished from native luciferase by gel filtration. The recovery of activity is temperature dependent, being more rapid at 23° than at 3°. However, while 100% recovery may be obtained at the lower temperature, less than this is obtained at 23°. The observation that the rate of recovery is dependent upon protein concentration (over the range of 0.18–18 μg ml^{-1}) indicates that a recombination of subunits is a rate-limiting step. The protein in 5 M guanidine is not denatured by heat at temperatures where enzyme activity is characteristically lost rapidly. The activity of luciferase denatured by heat precipitation may, however, be fully recovered.

This is achieved by first dissolving the inactive precipitate in guanidine or urea followed by dilution into phosphate buffer as outlined above.

The experiments reported in this paper were undertaken as a part of a study of the relationship between the structure and function of bacterial luciferase. This enzyme catalyzes the oxidation of reduced flavin mononucleotide ($FMNH_2$)[1] with the concomitant emission of light by a reaction which may be represented in eq 1.

$$FMNH_2 + 0.5O_2 \xrightarrow[RCHO]{\text{luciferase}} FMN + H_2O + h\nu \quad (1)$$

The enzyme may be conveniently assayed by the light emission which occurs when these reactants are mixed. The exact stoichiometry and products formed in the reaction are not established with certainty. Although the long-chain aliphatic aldehyde (RCHO) is required for a high quantum yield, the enzyme catalyzes with equal facility the reaction of $FMNH_2$ with enzyme in the absence of aldehyde, with a photon yield between 10^{-2} and 10^{-3} of that obtained with the aldehyde (Hastings and Gibson, 1963; Hastings *et al.*, 1966). This may be represented as in eq 2.

$$FMNH_2 + 0.5O_2 \xrightarrow{\text{luciferase}} FMN + H_2O + 0.01h\nu \quad (2)$$

$$FMNH_2 + O_2 \xrightarrow{\text{nonenzymatic}} FMN + H_2O_2 \quad (3)$$

A nonenzymatic oxidation of reduced flavin occurs as well (eq 3) with the formation of peroxide (Gibson and Hastings, 1962). Although the enzymatic reaction apparently does not result in peroxide production, the exact fate of the oxygen is not known. Experimental determinations indicate that about three reduced flavins are consumed per photon emitted (Hastings and Gibson, 1963).

Interest in the structure of this protein is centered around gaining more detailed understanding of the steps in this reaction, in which chemical energy is transduced to light energy, the final light-emitting step involving the release of about 60 kcal of energy/mole as radiant energy. In addition, structural information should be of value in connection with the identity of the light-emitting species, the nature of which is still uncertain. Some evidence has indicated that a component in the protein might be involved. Other evidence implicates oxidized flavin, but neither of these possibilities has received any strong experimental support.

In living cells, the reversibility of luciferase denaturation (either by heat or chemical agents) was indicated by the early work of Johnson *et al.* (1945).

In the present study, using purified luciferase, it was observed that virtually 100% of the activity could be recovered following denaturation in 5 M guanidine-HCl or 8 M urea, and that the kinetics of this process could be readily followed over a wide range of activity. It was then found that denaturation by heat and other agents, notably those which cause protein aggregation, could also be reversed. This reversal was accomplished by first dissolving the protein in guanidine-HCl or urea and then renaturing by dilution.

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[1] Abbreviations used: FMN and $FMNH_2$, oxidized and reduced flavin mononucleotides; BSA, bovine serum albumin; NADH, reduced nicotinamide-adenine dinucleotide; DTT, dithiothreitol.

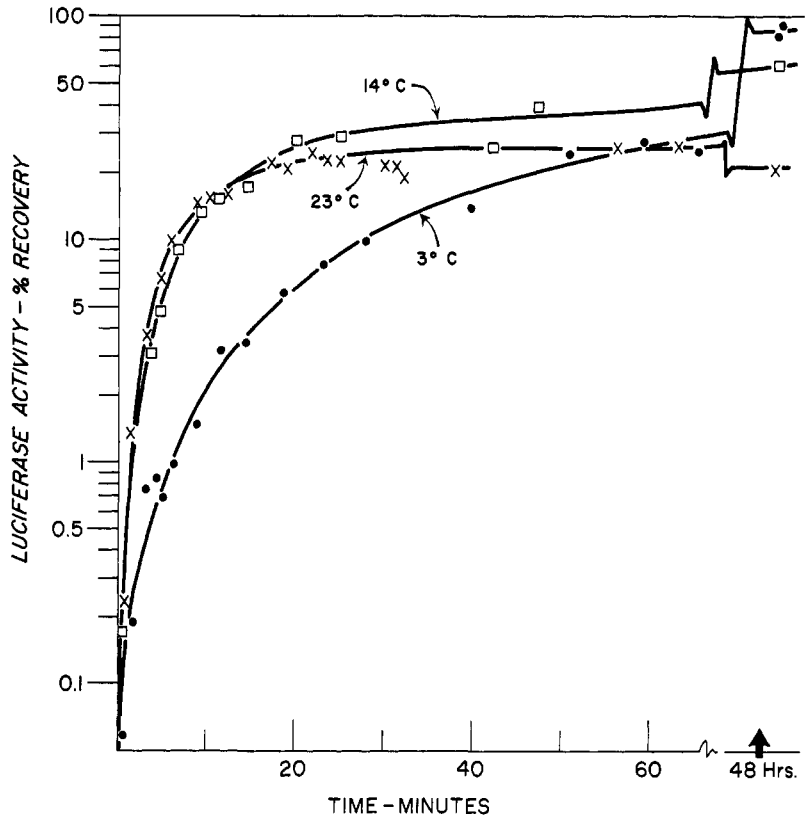

FIGURE 1: Effect of temperature upon the rate of renaturation and yield of luciferase. Ordinate: luciferase activity recovered plotted on a log scale; abscissa: time after 1:50 dilution of the luciferase into buffer, thereby lowering the concentration of guanidine-HCl.

Materials and Methods

Bacterial luciferase was prepared by the method of Hastings *et al.* (1965) and assayed essentially as described there, by measuring the light intensity following the rapid mixing of luciferase and reduced flavin. The reaction mixture contained 0.2 ml of 1.25 M phosphate buffer (pH 7.0), 2×10^{-3} M dithiothreitol, 0.1 ml of a dodecanal suspension prepared by ultrasonic treatment of 20 μl of aldehyde in 10 ml of distilled water, luciferase as specified, and water to a volume of 1.5 ml. In most instances 0.2 ml of a 1% solution of bovine serum albumin (BSA) was also added. The reaction was then initiated by injecting from a syringe 1 ml of 5×10^{-5} M $FMNH_2$, reduced by bubbling hydrogen in the presence of 5% platinized asbestos. The light was detected with a photomultiplier calibrated with the standard of Hastings and Weber (1963), amplified, and recorded graphically. It has been shown (Hastings *et al.*, 1965, 1966) that light intensity is proportional to the amount of enzyme over a range of 10^5. Additional details concerning all of the above procedures are given in the paper by Hastings *et al.* (1965).

Guanidine-HCl was prepared from guanidine carbonate (Eastman) by the method of Anson (1941) and recrystallized from 80% ethanol. Alternatively, the guanidine-HCl was purchased from Eastman, and purified by treatment with activated charcoal. The concentrations of all guanidine-HCl solutions were determined by measuring the refractive index using the formula given by Kielley and Harrington (1960). Urea was purchased from Merck. Cyanate ion was removed from a 10 M solution of urea by stirring with a mixed-bed ion-exchange resin (Bio-Rad AG 501-X8D). Urea (8 M) in formate buffer (pH 3.0) was prepared according to the method of Gotschlich and Edelman (1965). Sephadex G-100 and G-150 and Blue Dextran were purchased from Pharmacia. Mercaptoethanol and dodecyl aldehyde were purchased from Aldrich. BSA and ovalbumin were purchased from Pentex, Kankakee, Ill. Chymotrypsinogen was purchased from Worthington. FMN was a gift of Sigma. Dithiothreitol was obtained from Calbiochem.

Results

Reversible Denaturation from 5 M Guanidine-HCl. The loss of luciferase activity when placed in 5 M guanidine-HCl occurs faster than can be measured by the usual pipetting methods. Measurements show that the enzyme activity, determined immediately after diluting the sample from the 5 M guanidine into the assay mixture, is decreased by a factor greater than ten thousand after only 15 sec in guanidine. This inactiva-

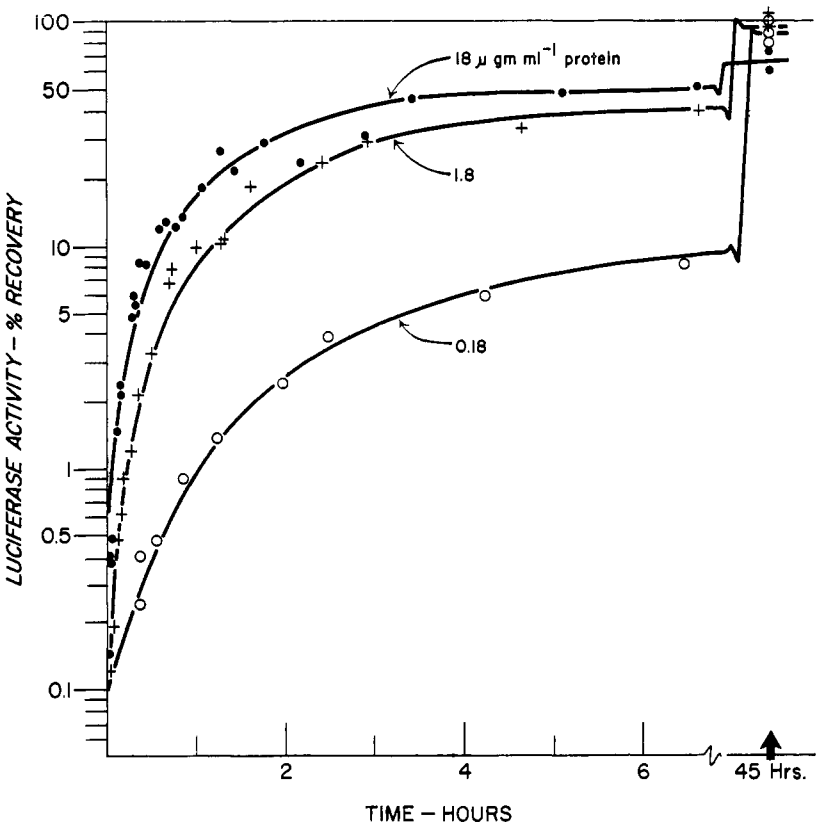

FIGURE 2: Effect of protein concentration present in incubation mixture during renaturation process upon recovery rate. Temperature, 3°; protein concentration as noted.

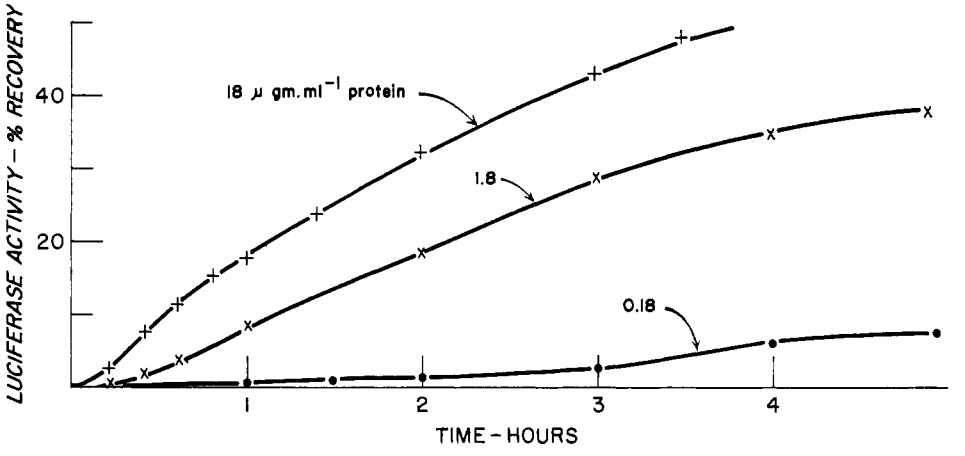

FIGURE 3: Data of Figure 2 plotted on a linear scale.

tion is spontaneously reversed with time in dilute guanidine solutions. When the enzyme in 5 M guanidine was diluted 1:50 into phosphate buffer at pH 7.0 containing 1% BSA and 10^{-3} M dithiothreitol (recovery mixture) and allowed to remain for a while prior to the actual addition of $FMNH_2$ and aldehyde, an appreciable recovery of activity was observed (Figure 1).

This recovery is temperature dependent over the range from 3 to 25°. At lower temperatures the recovery is indeed slow, but ultimately, after 2 or 3 days, it characteristically exceeds that which is obtained at higher temperatures, and is frequently complete or very close to 100%.

Protein Concentration. In view of the facility with which the kinetics of the recovery of activity could be measured, it was of interest to examine its dependence

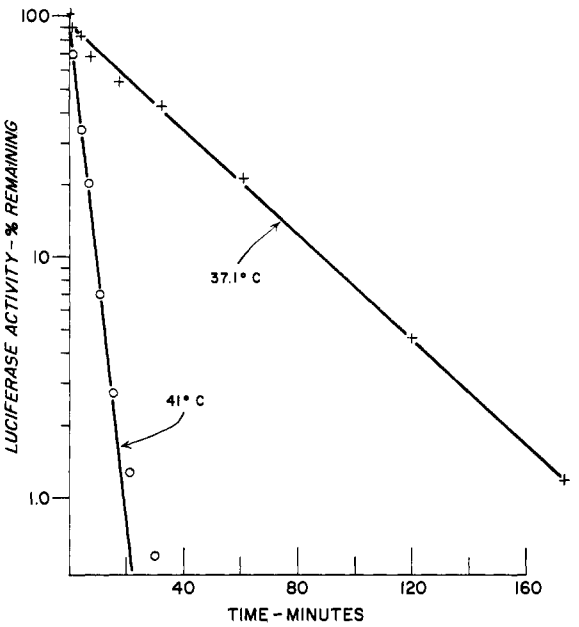

FIGURE 4: Effect of temperature upon the rate of heat denaturation of luciferase in dilute buffer. The activation energy is calculated to be about 110 kcal.

on protein concentration. If the denaturation involves a process involving only individual molecules, as for example, the change from the coiled to the uncoiled state, then the rate of its reversal should be independent of concentration. If, however, molecular interactions are involved, as in the association of subunits, then concentration dependence might be observed. As seen in Figures 2 and 3, this second result was obtained when the protein concentration was varied over a 100-fold range. In that experiment, luciferase whose activity was 1.15×10^{14} quanta sec^{-1}/OD unit at 280 mμ was diluted 1:10 and 1:100 in 0.125 M phosphate buffer at pH 7.0 prior to adding samples of each to 7 M guanidine-HCl. Each of the three samples was then diluted into a recovery mixture; the enzyme concentrations present in these recovery mixtures are indicated, and the enzyme activities, determined at the times shown, were normalized to the theoretical 100% values (Figure 2).

Requirements for Recovery of Enzyme Activity. BSA was used in all the earlier experiments because it had been found to be useful in stabilizing activity at low concentrations of luciferase (Hastings *et al.*, 1965). A similar phenomenon was observed in these experiments; the recovery of activity after treatment with guanidine was enhanced in the presence of BSA, the effect being more pronounced at low luciferase concentrations. BSA is thus not an absolute requirement for renaturation, but it enhances the process.

The presence of a reducing agent is virtually an absolute requirement for recovery (see Table I). The best reagent appears to be dithiothreitol, but 2-mercaptoethanol is also effective, albeit only at higher concentrations (0.05 M). The effect of various buffering agents on the rate or degree of recovery was not investigated.

Effect of Aldehyde upon Recovery of Enzyme Activity. It is to be expected that substrates or other small molecules which bind to the enzyme may have an influence upon the recovery process. Chilson *et al.* (1965) have recently reported that the rate and extent of reactivation of lactic dehydrogenase after denaturation in guanidine-HCl is enhanced in the presence of the coenzyme NADH. The effect of long-chain aldehyde upon luciferase recovery is just the opposite; no recovery whatsoever could be detected in its presence. The experiment was carried out using dodecyl aldehyde purified by vapor phase chromatography added in excess to provide a saturated aqueous solution, about 5×10^{-4} M (Hastings *et al.*, 1963). The recovery mixture contained in addition 2.5 μg ml^{-1} luciferase, 1×10^{-3} M DTT, 0.25 M phosphate buffer, and water to 5 ml.

However, it was observed that native enzyme in buffer can be rapidly inactivated in the presence of dodecyl aldehyde. This suggests that aldehyde promotes the unfolding or dissociation of the enzyme, rather than the opposite. Attempts to verify this hypothesis are not completed; it occurs only with relatively dilute enzyme solutions, meaning that it is difficult to prepare large quantities of aldehyde-denatured enzyme.

Heat Stability of Luciferase. Like most proteins, bacterial luciferase in buffer is readily denatured by heat (Figure 4). At 40° this process takes just a few minutes to occur, is accurately first order over two orders of magnitude, and exhibits a high activation energy (110 kcal). A white insoluble precipitate forms which does not regain activity by incubation in phosphate buffer with DTT and BSA. Since guanidine-HCl was known to dissociate noncovalent bonds and to disrupt inter- and intrachain interactions, it was of interest to determine whether or not luciferase in guanidine-HCl was heat sensitive. This could be conveniently evaluated by testing the ability of luciferase to regain activity after exposure to heat in guanidine-HCl.

In fact, it was found that the luciferase is perfectly stable to heat in 5 M guanidine-HCl at temperatures up to 50 or 60°, which temperatures very rapidly denatured the protein in buffer. At still higher temperatures some irreversible loss of activity in guanidine-HCl could be measured (Figure 5). This loss, however, must be attributed to some reaction different from that which occurs at 40° in buffer (probably a reaction of an amino acid side group) since it exhibits a much lower temperature dependence.

Reversal of the Heat Denaturation. Since the precipitation which occurs upon heat denaturation of proteins is probably due to noncovalent inter- and intrachain associations, and since such interactions are believed to be dissociated by guanidine-HCl, an attempt was made to regain activity from heat-denatured precipitated luciferase by dissolving it in either guanidine-HCl or urea and then removing the latter by dilution. This was successful; in many experiments no adverse effects on activity were found in an enzyme which had been heat denatured as compared to a control which had been only exposed to guanidine-HCl or urea.

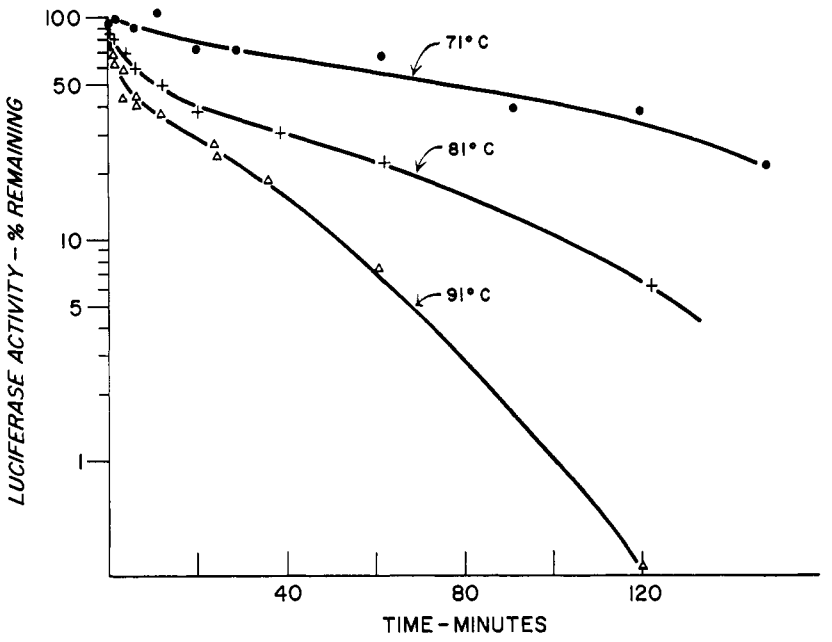

FIGURE 5: Effect of temperature upon the rate of inactivation of luciferase in 5 M guanidine-HCl. The activation energy for this process is much lower, being in the vicinity of 15 kcal.

Experiments illustrating this result are presented in Table I. A 1.5-ml sample of luciferase (optical density at 280 mμ, 1.65; sp act. 1.7×10^{14} quanta sec^{-1} mg) was denatured by heating at 42° for 36 min in 0.1 M phosphate buffer (pH 7) with 1.8×10^{-3} M DTT present. The enzyme solution was clear and colorless; upon heating a white precipitate formed which was dispersed and suspended to take samples. Less than 10^{-3} of the activity originally present remained. Samples (0.1 ml) of the denatured enzyme were added either to 0.9 ml of 6 M guanidine-HCl or 0.9 ml of 9 M urea in 0.1 M phosphate buffer (pH 7) (no. 4 and 8, respectively), with 2 mg of DTT added to each. Unheated enzyme was treated similarly for comparison (no. 2 and 7). After 2 hr 0.1 ml of these solutions was each diluted to a volume of 5 ml with 0.2% BSA in 0.2 M phosphate at pH 7. As a large amount of DTT was already present, no additional quantity was added in the recovery mix; its concentration there was calculated to be 2.6×10^{-4} M. In an experiment in which DTT was omitted altogether, virtually no activity was recovered (no. 3).

TABLE I: Recovery of Activity of Denatured Luciferase.[a]

Expt No.	Treatment	Enzyme Act	% Recov
1	Control, phosphate buffer, pH 7	2.75	100
2	No heat, 5 M guanidine	2.0	71
3	Same, no DTT added	0.01	<1
4	Heat denatured, 5 M guanidine	1.6	58
5	Heat denatured, precipitate, 5 M guanidine	0.98	35
6	Heat denatured, supernatant, 5 M guanidine	0.02	<1
7	No heat, 8 M urea	1.7	61
8	Heat denatured, 8 M urea	1.73	63

[a] The enzyme activity is quoted in terms of the initial light intensity in quanta $sec^{-1} \times 10^{-10}$/0.05 ml of the sample after the several dilution steps ($^1/_{500}$).

The preparations were allowed to remain in the recovery mixtures for 28 hr at 4°, at which time the activities were determined. Although less than 100% recovery was achieved in this time in the samples treated only with guanidine-HCl or urea (no. 2 and 7), the added heat step was virtually without effect in each case. By separating the precipitate and supernatant by centrifugation (no. 5 and 6) it was further shown that the recoverable activity was confined to the precipitate in the heated enzyme; thus no soluble factor in the supernatant is needed for the recovery process.

This type of experiment has been repeated on several occasions with similar results. In addition, this result has been obtained with an enzyme having a specific activity at least ten times lower than the material used in Table I, indicating that the presence of large amounts of other proteins does not drastically interfere with the process.

Sephadex Analyses. Information concerning the size of protein molecules may, in favorable instances, be ob-

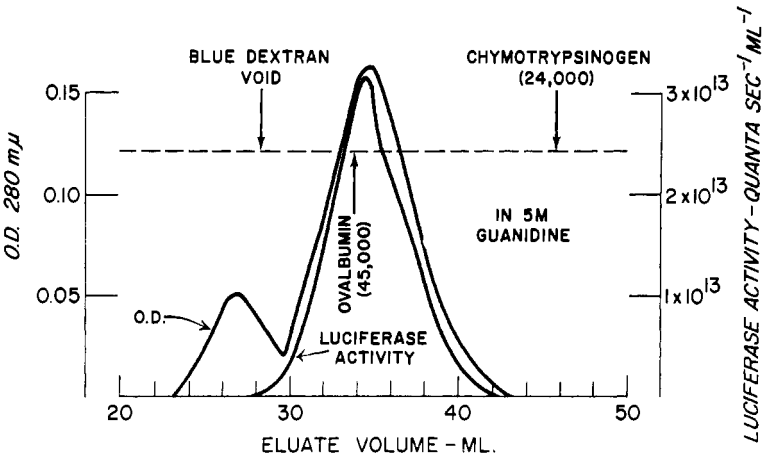

FIGURE 6 Elution pattern of luciferase on Sephadex G-150, with all experiments being run in the presence of 5 M guanidine-HCl. Luciferase activity was determined by allowing a small aliquot from each tube to renature following dilution. The higher molecular weight material failed to exhibit any recoverable activity. The peaks for the elution of some of the other marker compounds are indicated, these being measured by their absorption. The following values were obtained, in milliliters: luciferase activity, 34.6; luciferase optical density, 35.0; Blue Dextran, 28.4; ovalbumin, 33.6; chymotrypsinogen, 46.2; and riboflavin 5′-phosphate, 76.4. The values in parentheses refer to the assumed molecular weights.

tained by filtration techniques with gels such as Sephadex or Bio-Gel (Andrews, 1964). This technique is most certainly sensitive to the shape of the molecule, as well as its molecular weight.

Measurements were first made to determine the size of the enzyme reconstituted after exposure to guanidine-HCl. A column (107 × 1 cm) packed with Sephadex G-100 was equilibrated with 0.05 M phosphate buffer (pH 7) with 1×10^{-4} M DTT and calibrated with several different compounds. Luciferase was inactivated by dialysis against 5 M guanidine-HCl in the presence of 5×10^{-4} M DTT. It was then diluted 333-fold in 1×10^{-3} M DTT and 0.05 M phosphate buffer (pH 7). After 15 hr at 4° followed by 2 hr at 15° about 15% of the expected activity had been recovered. The low recovery was attributed to the fact that BSA was omitted, and to the relatively short time allowed for recovery. The protein was then precipitated by the addition of $(NH_4)_2SO_4$. The pellet was dissolved, and insoluble material was removed by centrifugation and then placed on the calibrated Sephadex column. Its elution position and pattern was indistinguishable from native luciferase.

Attempts were next made to estimate the size of the luciferase subunits by passing it through G-150 Sephadex in the presence of 5 M guanidine-HCl (Figure 6). From the experiment of Figure 2, which indicates that the molecule is composed of subunits, it is to be expected that the apparent size in guanidine-HCl would be less than in buffer. However, if a normally compact molecule unfolds in the presence of guanidine-HCl, then its apparent size by the gel filtration method would be greater than the same molecule in the compact form. The estimation of size therefore presumably depends heavily upon the behavior of the molecules used to calibrate the column compared to that of the molecule being studied. A column (110 × 1 cm) was equilibrated with 5 M guanidine-HCl with 5×10^{-4} M DTT added and calibrated with markers; luciferase was dialyzed against the guanidine-HCl–DTT prior to being placed on the column. The luciferase activity of each fraction was determined by adding 0.1 ml to 5 ml of the standard recovery mixture and incubating for 23 hr at 15°. The specific activity of the recovered enzyme was very good, being slightly higher than that of the starting material. From the positions of the marker proteins, one can see that the apparent size of the luciferase in guanidine-HCl is near a molecular weight of 40,000 (Figure 6).

A similar Sephadex filtration was carried out with luciferase in the presence of 8 M urea–formate, calibrated with the same markers. Similar results were obtained, the luciferase eluting slightly after ovalbumin, and well ahead of chymotrypsinogen.

Sodium dodecyl sulfate was also observed to inactivate luciferase. Following the removal of dodecyl sulfate, only part of the luciferase activity was recovered. The denatured protein appeared to be a high molecular weight aggregate which, like heat-denatured luciferase, could be renatured by treatment with guanidine-HCl.

The experiments were carried out by dialyzing enzyme (optical density at 280 mμ, 18.4) against 0.01 M sodium dodecyl sulfate and 10^{-3} M DTT for 6 hr at 22° followed by 50-min dialysis against 0.05 M phosphate buffer (pH 7.0) and 10^{-2} M DTT. A 0.3-ml sample was removed and placed on the G-100 Sephadex column previously used and calibrated.

The eluate from the Sephadex column (Figure 7) exhibited two major protein peaks but only a single activity peak. The activity was found to correspond to the elution position of native luciferase, while the other protein peak came out in the void volume, indicating that it

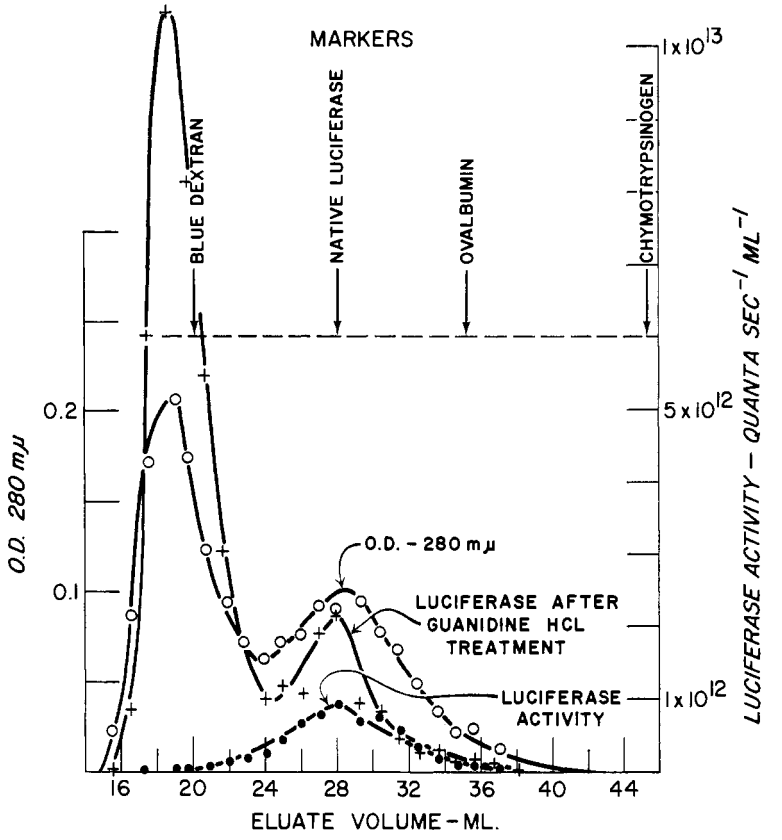

FIGURE 7: Elution patterns on Sephadex G-100 in 0.05 M phosphate buffer (pH 7.0) with 5×10^{-4} M DTT. The markers include native luciferase, which eluted with a peak at 28.0 ml; renatured luciferase after denaturation in guanidine-HCl, 28.6 ml; Blue Dextran, 20.2 ml; ovalbumin, 35.4 ml; and chymotrypsinogen, 45.2 ml. Luciferase after treatment with dodecyl sulfate exhibits two protein bands as indicated by the optical density at 280 mμ (○), but only a single activity band at 28.0 ml (●). After exposing these fractions individually to 5 M guanidine-HCl, followed by dilution into recovery mix luciferase activity was recovered from the formerly inactive high molecular weight species at 19.0 ml (+).

was high molecular weight material. However, the activity of the high molecular weight component could be recovered by treatment with guanidine-HCl, as was done with heat-denatured luciferase.

Discussion

The recovery of activity of enzymes after denaturation in guanidine or urea has been previously demonstrated with a number of enzymes including β-galactosidase (Zipser, 1963), enolase (Westhead, 1964), several dehydrogenases (Epstein *et al.*, 1964; Chilson *et al.*, 1966), and fumarase (Hill and Kanarek, 1964). In extending this observation to bacterial luciferase, it has been possible to follow in detail the kinetics of the renaturation process, and its dependence on temperature and protein concentration. The latter data provide very strong evidence that the restoration of enzyme activity involves a protein–protein collisional process, which is presumed to be involved in the reaggregation of protein subunits to form the active enzyme.

The molecular structure of luciferase has not been fully established, but it is known from other studies to have a molecular weight of about 60,000 in its active form (Hastings *et al.*, 1965). From amino acid analyses and from sedimentation both in 6 M guanidine-HCl (G. M. Edelman, E. Gall, J. Friedland, and J. W. Hastings, to be published) and in dodecyl sulfate (Kuwabara *et al.*, 1965), the molecule is believed to comprise four subunits. From the experiments described in the present paper, in which the protein was passed through Sephadex in the presence of 5 M guanidine-HCl (Figure 6), it may be concluded that the subunits are equal or very nearly equal in molecular weight, irrespective of their absolute molecular weight.

No strong conclusions can be drawn from a study of the kinetics of the renaturation process, which indicate a reaction order between 1 and 2. The complicated kinetic picture is undoubtedly due to the fact that the rate of the renaturation is governed by several processes, including both refolding and reassociation. Recovery of activity following guanidine treatment is absolutely dependent upon keeping the SH groups reduced; but there are evidently no disulfides involved, since none are believed to be present in native luciferase (J. Friedland and J. W. Hastings, unpublished).

An interesting aspect of these observations, which has also been evident in other studies of this type (Dawson *et al.*, 1967) is that the individual subunits manage to reassemble with their specific partners mid what must be a large number of unprofitable collisional encounters, this being especially remarkable in crude extracts. A high affinity must therefore exist for the "correct" association. The nature of the specificity in this binding phenomenon is a matter of great interest.

The studies concerned with temperature effects confirm two generalizations. The first is that proteins in the presence of a denaturing agent such as guanidine-HCl are not susceptible to inactivation by heat aggregation. This is an expected result, assuming that these agents act by breaking noncovalent bonds. Proteins in the presence of agents such as guanidine-HCl are thus to be considered as highly stable compounds at room temperature, whose native configuration may be regained at will by simply removing the guanidine.

The second point relates to the ability of the denaturing reagents such as guanidine-HCl to restore activity to proteins which have been denatured by other means. This was shown some years ago by Perrin and Monod (1963) who demonstrated the renaturation of heat-denatured β-galactosidase by treatment with urea. Similar conclusions have come from the more recent work of Westhead (1964). Many kinds of denaturation appear to involve the aggregation of protein to form inactive high molecular weight species without, in many cases, any effects upon the primary structure of the protein. In these cases it is evident that one has only to dissociate these aggregates to a stage from which refolding to the active native state can occur. The experiments with enzyme inactivated in dodecyl sulfate illustrate this point well, and it is to be expected that proteins denatured by a variety of means can be renatured by appropriate treatment with urea or guanidine. Indeed it seems reasonable to hypothesize that unless there is a covalent change, all denaturation processes are, in principle, reversible.

The results also merit a comment concerning their implications for the bioluminescent reaction. As indicated in the beginning of this paper, a number of the features of the reaction remain unresolved; the present results demonstrate that the reaction does not involve some small molecules noncovalently bound to protein. Nor is there any other special feature of the secondary or tertiary structure of the enzyme which is not fully and spontaneously reversible. Although these and other similar considerations had seemed only remotely tenable, their exclusion provides a firm basis for future studies of this reaction.

Acknowledgment

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References

Andrews, P. (1964), *Biochem. J. 91*, 222.
Anson, M. L. (1941), *J. Gen. Physiol. 24*, 399.
Chilson O. P., Kitto, G. B., and Kaplan, N. O. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 1006.
Chilson, O. P., Kitto, G. B., Pudles, J., and Kaplan, N. O. (1966), *J. Biol. Chem. 241*, 2431.
Dawson, D. M., Eppenberger, H. M., and Kaplan, N. O. (1967), *J. Biol. Chem. 242*, 210.
Epstein, C. J., Carter, M. M., and Goldberger, R. F. (1964), *Biochim. Biophys. Acta 92*, 391.
Gibson, Q. H., and Hastings, J. W. (1962), *Biochem. J. 83*, 368.
Gotschlich, E. C., and Edelman, G. M. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 558.
Hastings, J. W., and Gibson, Q. H. (1963), *J. Biol. Chem. 238*, 2537.
Hastings, J. W., Gibson, Q. H., Friedland, J., and Spudich, J. (1966), *in* Bioluminescence in Progress, Johnson, F. H., and Haneda, Y., Ed., Princeton, N. J., Princeton University, pp 151–186.
Hastings, J. W., Riley, W. H., and Massa, J. (1965), *J. Biol. Chem. 240*, 1473.
Hastings, J. W., Spudich, J. A., and Malnic, G. (1963), *J. Biol. Chem. 238*, 3100.
Hastings, J. W., and Weber, G. (1963), *J. Opt. Soc. Am. 53*, 1410.
Hill, R. L., and Kanarek, L. (1964), Subunit Structure of Proteins: Biochemical and Genetic Aspects, Upton, N. Y., Brookhaven National Laboratory, pp 80–97.
Johnson, F. H., Eyring, R., Steblay, H., Chaplin, C. H., and Gerhardi, G. (1945), *J. Gen. Physiol. 28*, 463.
Kielley, W. W., and Harrington, W. F. (1960), *Biochim. Biophys. Acta 41*, 401.
Kuwabara, S., Cormier, M. J., Dure, L. S., Kriess, P., and Pfuderer, P. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 822.
Perrin, D., and Monod, J. (1963), *Biochem. Biophys. Res. Commun. 12*, 425.
Westhead, E. W. (1964), *Biochemistry 3*, 1062.
Zipser, D. (1963), *J. Mol. Biol. 7*, 113.