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Mutationally Altered Bacterial Luciferase. Implications for Subunit Functions†

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ABSTRACT: In order to determine the functional relationship between the two nonidentical subunits of bacterial luciferase, we have isolated two classes of mutationally altered enzymes and determined the subunit location of the lesions. One class, the temperature-sensitive luciferases, includes those enzymes with lesions primarily altering thermal stability. Various mutants differ considerably with respect to this parameter. Often they also have a decreased ability to recover after treatment with 4 M guanidine hydrochloride. Some of the mutant enzymes are fully active at the low temperature. The second class of mutants, the altered-kinetics luciferases, possess lesions altering a wide assortment of catalytic parameters, usually with no concomitant effect upon thermal stability. Lesions which alter catalytic properties occur exclusively in the α subunit, whereas lesions decreasing thermal stability occur in either of the subunits. We conclude that only the α subunit contributes residues to the active site, but that the β subunit is required to maintain the active conformation of the catalytic subunit, α.

One way to evaluate the contribution of individual subunits, and indeed of individual amino acid residues, to specific functional aspects of proteins is to map the location of lesions responsible for specific types of enzyme defects. Toward this end, we have isolated mutationally altered luciferases by two techniques. One allows us to identify luciferases with lesions primarily affecting the thermal stability of the enzyme. With the other we can isolate luciferases with lesions primarily affecting the enzyme's catalytic parameters. Due to several unique features of the bacterial bioluminescence system, the procedures for mutant selection are simple and provide for the rapid isolation of a large number and variety of altered luciferases.

Among those mutants whose *in vivo* bioluminescence is

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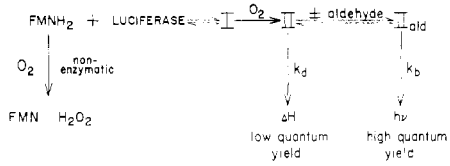

FIGURE 1: The proposed intermediates formed in the reaction catalyzed by bacterial luciferase.

conditional upon temperature, there is a proportion (*ca.* 1–5%) in which this is due to the production of a mutated, temperature-sensitive luciferase (Cline and Hastings, 1971). These may be most easily selected by screening *in vivo* for those bacteria whose luminescence is extinguished rapidly when shifted from the permissive to the restrictive temperature.

Bacteria producing luciferase with lesions affecting catalytic parameters are selected from among those mutants which are darker than the wild type at room temperature. Sufficient luciferase for *in vitro* assays can be obtained by lysis of a small quantity of cells; those in which the kinetics of the decay of luminescence is altered were selected.

The overall luminescence reaction involves the oxidation of reduced flavin mononucleotide ($FMNH_2$) by molecular oxygen; the light-emitting step requires a long-chain aldehyde. The postulated enzyme intermediates are indicated in Figure 1 (Hastings and Gibson, 1963). Rate constants for the decay of II and II_{Ald} are designated as k_d and k_b to indicate dark and bright pathways, respectively. The *in vitro* assay that we used involves the rapid addition of $FMNH_2$ to a solution containing enzyme, aldehyde, and oxygen. Any $FMNH_2$ which has not reacted with enzyme is removed *via* the nonenzymatic (autoxidation) pathway within the first second. Since luminescence continues for a much longer time, the emission must be due to intermediates whose decay (emission) is slow. In this assay a luciferase molecule reacts only once. The rate constant for the decay of luminescence is a sensitive index of the catalytic steps. If the equilibrium between II and II_{Ald} is rapid, and if the decay of II produces no light, the observed decay constant (k_0) will be given by eq 1 where [A] is the free aldehyde concentration

$$k_0 = \frac{k_d + k_b K_A[A]}{1 + K_A[A]} \quad (1)$$

and K_A is the association constant $K_A = [II_{Ald}]/([II][A])$. Mutant luciferase with lesions altering the active site would be expected to differ from the wild type in one or more of the several catalytic parameters, and therefore to exhibit a k_0 different from the wild type under a standard set of assay conditions. A point of important practical consequence is that k_0 is independent of luciferase concentration.

Materials and Methods

Mutant Selection. The parent bacterium used in this work is designated strain MAV (Hastings *et al.*, 1969). Cells were grown using a complete medium containing, per liter, 5 g of Difco Bactotryptone, 3 g of Difco yeast extract, 2 ml of glycerol, 200 ml of distilled water, and 800 ml of sea water, and were aerated by vigorous shaking. Solid medium contained in addition 14 g of Difco Bactoagar.

Mutations were induced by treatment of a suspension of cells (10^{11}/ml of medium) with *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (Aldrich). Aliquots were taken at various times, diluted (10^{-5}) in medium, incubated at 25° for 60 min, and plated at 36° for viable counts. All samples were then stored at 4°, and the one in which there was a survival of about 5% was used to screen for mutants.

Temperature-sensitive luciferase[1] mutants were selected from among those colonies that were dark or dim at 36°, but at least 10% as bright as the wild type at 22° (Cline and Hastings, 1971). TSL mutants can be identified either by an *in vitro* screening procedure for luciferases which are less resistant to thermal inactivation than the wild type (which loses 50% of its activity in 5 min at 45°) or by a simple *in vivo* test: the luminescence of colonies grown at 22° is noted before and 45 min after their transfer to a 36° warm room. Those colonies whose luminescence is greatly diminished following the transfer, and which are not subsequently stimulable by exposure to decanal vapors, invariably possess a more thermally unstable luciferase. TSL-1 through TSL-8H were selected by the first (*in vitro*) procedure; TSL-9 through TSL-20 by the latter (*in vivo*) test.

Altered-kinetics mutants were selected from among those bacteria that were less luminescent (<50%) than the wild type, both at 36° and 22°. Luciferase from these strains was prepared using cells from approximately 14 cm^2 of a lawn grown at 22°, allowing them to lyse overnight at 4° in 4 ml of 0.01 M EDTA (previously adjusted to pH 7), then centrifuging to remove cell debris. Using the *in vitro* assay with decanal (described below), we determined the first-order rate constant for the decay of luminescence of the mutant strains' luciferase. Those with rate constants more than 15% different from the wild type were selected for study as AK mutants.

Luciferase Preparation and Assay. For the characterization of the mutant luciferases, the enzymes were partially purified. TSL mutants were cultured at 22°, AK mutants at 26°. Cells were harvested by centrifugation at 2° within 1 hr after reaching peak *in vivo* luminescence. The cells were frozen and later resuspended, 1 g wet weight per 10 ml of 0.01 M EDTA (previously adjusted to pH 7) at 4°, and allowed to lyse for 8 hr. In some cases lysis was completed by a brief sonication. Cell debris was removed by centrifugation and the luciferase partially purified and concentrated by precipitation between 35 and 80% saturation in ammonium sulfate at 4°. The precipitate was resuspended in a minimal amount of 0.05 M phosphate buffer (pH 7.4)–5 mM dithiothreitol. The soluble protein concentration was usually about 70 mg/ml. Based on the amount of activity present, a wild-type extract prepared in this way contained 3 mg of luciferase/ml. (Extracts of AK-6 and -20 were about 5 times more dilute.) Enzyme suspensions were dialyzed against 0.05 M phosphate buffer (pH 7.4) to remove the ammonium sulfate prior to assay.

Reaction mixtures contained 1 ml of assay buffer (0.1 M phosphate, pH 7.4, with 0.2% w/w bovine serum albumin-Pentex, crystallized) at 21°, 10–20 μl of a 0.1% (v/v) sonicated suspension of decanal (Aldrich) in water, and the enzyme in a volume not exceeding 25 μl. The reaction was initiated by injecting from a syringe 1 ml of catalytically reduced flavin mononucleotide (Sigma). Luciferase activity was measured as the initial maximum light intensity (I_{max}) in quanta per second, using a photomultiplier–photometer (Mitchell and Hastings, 1971). I_{max} is proportional to the amount of luciferase present over an exceedingly wide concentration range;

[1] Abbreviations used are: TSL, temperature-sensitive luciferases; AK, altered kinetics; Gdn·HCl, guanidine hydrochloride.

the amounts of active luciferase assayed in these experiments ranged from 10^{1} to 10^{-5} μg. While decanal was routinely used in measurements of luciferase activity, different aldehydes in different concentrations were used to determine luciferase decay rate constants, as indicated in the footnotes to the tables.

Determination of Substrate Dissociation Constants. All constants were determined at 21°, pH 7.4. Flavin binding was measured using the procedure of Meighen and Hastings (1971) except that a 0.1% (v/v) sonicated suspension of octanal in water was used instead of the 0.005% decanal sonicate. Four assays were performed at each of 12–15 different flavin concentrations ranging from 2.5×10^{-8} to 1.25×10^{-4} M. The dissociation constant was determined using the highest I_{max} at each concentration. Aldehyde dissociation constants were determined from the double-reciprocal plot of $1/v$ (measured as $1/I_{max'}$ where $I_{max'} = I_{max}$ (aldehyde) $- I_{max}$ (no aldehyde)) *vs.* 1/decanal concentration, assuming the stock aldehyde is pure and that all the aldehyde is in solution. Measurement was made over a concentration range from 2.5×10^{-7} to 2.5×10^{-4} M decanal.

Renaturation and Mutant Complementation. Denaturation of luciferase was carried out by diluting extracts 1:2 into 6 M guanidine hydrochloride at 21° to achieve a final concentration of 4 M guanidine–0.08 M dithiothreitol. Undenatured controls were similarly diluted 1:2 into assay buffer at 4°. After at least 30 min, renaturation was accomplished by dilution into buffer at 4° so that the final concentrations (in all cases, including controls) of reagents were as follows: 0.1 M phosphate, 0.1 M guanidine hydrochloride, 0.002 M dithiothreitol, 0.01 M EDTA, and 0.1% bovine serum albumin at a final pH of 6.7. Renaturation mixtures were then incubated at 8° for 72 hr prior to assay. In all cases, no further increase in activity occurred after this time. The concentration of the extract whose renaturability or complementation was being measured (Tables II, IV, and VI) was 5 μl of the denatured enzyme extract per milliliter of renaturation mixture (for the wild type, this represents 5 μg/ml for the partially purified extract, and 0.05 μg/ml for the cell lysates in Tables V and VII). For the titration of TSL-1 and -10 shown in Figure 2, 1.7 μl/ml was present. When TSL-1 or -10 was added during renaturation, 20 μl of the denatured extract per milliliter of renaturation mixture was present. Additional details are provided in the footnotes.

Results

Temperature-Sensitive Mutant Luciferases. The 16 mutant enzymes selected for the production of luciferase with an increased sensitivity to thermal inactivation are listed in Table I. The subunit location of their lesion(s) is indicated (see Table IV). The *in vivo* luminescence of the mutants grown on solid medium at 20° and 36° is given in column 1. Similar relative values were obtained with cells grown in liquid medium (Table V, column 1). All the mutants show a considerable temperature dependence of *in vivo* luminescence. Because their luminescence is also stimulated by aldehyde, strains TSL-8H, -7H, and -3 may be double mutants (Cline and Hastings, 1971).

There is a wide range in the *in vitro* thermal stabilities of the mutant luciferases (column 2). Our listing of the TSL mutants in order of increasing $T_{5m-50\%}$ should not be taken to suggest that this measurement is an unambiguous indication of relative stability. There are differences in the time course, temperature dependence, and reversibility of activity loss that suggest important qualitative differences in the mechanisms of thermal inactivation.

The catalytic properties of these luciferases, as indicated by kinetics of the decay of luminescence in the *in vitro* assay, are given in columns 3 through 6. Only a few of the luciferases have kinetics different from the wild type and the changes are small.

Several of the mutants do not produce wild-type levels of light even at the lower temperature (column 1). This suggests that some intact, thermally sensitive enzymes may not be as active enzymatically as the wild type. This is also indicated by the fact that the mutants that are much less luminescent than the wild type at low temperature (TSL-17, -10) are those whose luciferase has alterations in kinetic parameters.

A property of temperature-sensitive luciferases previously noted (Cline and Hastings, 1971) is their failure to renature after denaturation in 4 M guanidine hydrochloride. Quantitative measurements for all the temperature-sensitive luciferases are presented in Table II. The recovery listed in column 1 is based on the activity of each undenatured enzyme measured at the beginning of the experiment at the dilution ultimately used for renaturation. Some of the undenatured mutant luciferases suffered an activity loss simply by remaining in the renaturation buffer for the 72-hr period of the experiment. The undenatured controls were run in buffer containing 0.1 M guanidine hydrochloride, since 0.1 M guanidine was present during renaturation of the denatured enzymes. Recovery based on the amount of activity remaining in the "undenatured" control after 72 hr is indicated in column 2.

Calculated on the basis of the first control, all except four of the luciferases (TSL-8H, -6H, -7H, and -3) appear to be deficient in renaturation. These four are also the ones whose thermal denaturation occurs only a few degrees below that of the wild type. Luciferases TSL-11 and -20, and to a lesser extent TSL-14, -18, -4H, and -2, lose activity in the recovery buffer. On the basis of the second control, their renaturability after dilution from 4 M guanidine hydrochloride is not much less than wild type (column 2).

TSL-17, -10, -1, -9, and -12 are unambiguously renaturation deficient since the undenatured proteins are stable in recovery buffer at a low temperature but are unable to regain activity after treatment with 4 M guanidine hydrochloride.

Altered-Kinetics Mutant Luciferases. The highest yield of altered-kinetics mutants was among those survivors of the mutagenesis that were dark of very dim and did not respond significantly to decanal vapors. The characteristics of the 13 distinctly different mutant types presented in Tables I and III illustrate the diversity of mutational alterations that are obtained by the selection technique. There is a considerable difference in decay rate constants among the mutants within a column; the values for k_d and k_b range over a factor of 100 (Table I, columns 3–6).

Although, and indeed because, the mutants were selected on the basis of alteration in kinetics of the *in vitro* reaction, neither this nor any other single or simple measurement can provide an index whereby the "activity" of mutant luciferases can be compared to that of the wild type. Mutant enzymes may have alterations in turnover rate, quantum efficiency, and/or substrate binding affinity. However, a good indication of luciferase activity can be obtained by measuring the peak level of the luminescence *in vivo* (Table I, column 1) of mutant cells which produce wild-type amounts of altered enzyme. This provides a direct comparison of the activity of mutant enzymes under conditions not arbitrarily defined by the investigator. The fact that the mutants (except AK-11

TABLE I: Characteristics of the Mutant Luciferases.

Mutant	Relative Peak Luminescence[a] *in Vivo* 20°	Relative Peak Luminescence[a] *in Vivo* 36°	Thermal Stability,[b] $T_{5m,50\%}$ (°C)	Apparent First-Order Rate Constant for the Decay of Luminescence *in Vitro*[d] (sec^{-1}) No Aldehyde	With $CH_3(CH_2)_xCHO$ $x = 6$	$x = 8$	$x = 10$
	1		2	3	4	5	6
Wild type	100	70	45	0.055	0.025	0.22	0.027
TSL-14(α)	35	0.47	22	0.043	0.025	0.22	0.027
TSL-11(α)	130	0.23	26	0.059	0.027	0.21	0.026
TSL-5H(α)	43	6.3	28	0.042	0.024	0.23	0.027
TSL-18(α)	42	0.11	28	0.060	0.019	0.17	0.029
TSL-17(α)	18	0.0073	33	0.21	0.029	0.18	0.017
TSL-10(α)	15	0.0060	34	0.21	0.027	0.18	0.017
TSL-8H(α)	40[c]	1.7	42	0.052	0.026	0.23	0.027
TSL-4H(β)	100	1.6	24	0.059	0.024	0.21	0.026
TSL-20(β)	125	0.070	25	0.075	0.026	0.21	0.026
TSL-2(β)	105	0.10	26	0.059	0.025	0.21	0.026
TSL-1 (β)	72	0.0083	29	0.062	0.025	0.22	0.026
TSL-9(β)	42	0.012	29	0.059	0.025	0.22	0.025
TSL-12(β)	57	0.072	36	0.079	0.023	0.19	0.022
TSL-6H(β)	80	24	38	0.054	0.026	0.23	0.029
TSL-7H(β)	65[c]	8.7	40	0.051	0.025	0.22	0.027
TSL-3(β)	43[c]	0.012	42	0.049	0.024	0.21	0.027
AK-6(α)	1.3		44	0.0086[e]	0.0041	0.0086	0.0025
AK-20(α)	0.13[c]		45	0.031[e]	0.14[f]	0.033	0.060[f]
AK-16(α)	20		44	0.051	0.020	0.14	0.022
AK-15(α)	43		44	0.073	0.024[f]	0.092	0.021
AK-7(α)	0.62		33	0.086[e]	0.062[f]	0.084	0.037[f]
AK-24A(α)	20		42	0.10	0.055	0.33	0.11
AK-9(α)	1.2		36	0.11[e]	0.036[f]	0.012[f]	0.011[f]
AK-17(α)	1.4		41	0.20[e]	0.023[f]	0.16	0.027
AK-11(α)	0.075		45	0.41[e]	0.042[f]	0.31	0.052
AK-3(α)	0.40		44	0.42	0.037[f]	0.13[f]	0.043[f]
AK-18(α)	0.018[c]		45	0.49	0.018[f]	0.0079[f]	0.022[f]
AK-2H(α)	19		33	0.75	0.092[f]	0.10[f]	0.063[f]
AK-1H(α)	0.78		44	0.96	0.14[f]	0.57	0.081

[a] For a lawn of the bacteria in a 100 × 15 mm petri dish, prepared by spreading 2.5 ml of 0.5% agar culture medium containing 25 μl of an overnight liquid culture. All strains ending in "H" were derived from a mutant of the wild type designated HTB-1. Peak luminescence of HTB-1 at 20° is the same as the wild type, but at 36° it is six times brighter than the wild type. [b] Temperature for 50% inactivation by preincubation in assay buffer for 5 min. [c] The luminescence of these mutants in liquid cultures is stimulable more than 150% by added decanal. [d] As aldehyde concentration is increased, the observed first-order rate constant changes from the endogenous (no aldehyde) value to a value characteristic of the chain length of the aldehyde present. This table lists the maximum or minimum value reached as aldehyde concentration was increased. Usually this value was reached at high concentrations of aldehyde, levels which caused a considerable inhibition of I_{max}. [e] Due to the low activity of these mutant luciferases, the decay rate constant in the absence of aldehyde was determined not by direct observation, but by measurement of the decay of the aldehyde-stimulable level of luminescence when the addition of aldehyde was delayed for varying amounts of time subsequent to the initiation of the assay with $FMNH_2$ (Hastings and Gibson, 1963). [f] In these cases, the decay rate constant continues to diverge from the value without aldehyde even as the maximum (soluble in assay buffer) is reached. Therefore, these reported constants may not represent the true k_b (see eq 1).

TABLE II: Renaturation of Temperature-Sensitive Luciferases after Treatment with 4 M Guanidine Hydrochloride.

Mutant	% Renaturation, Based on Starting Control 1	% Renaturation, Based on 72-hr Control 2
Wild type	45	43
TSL-14	14	53
TSL-11	0.38	33
TSL-5H	31[a]	39
TSL-18	2.5	14
TSL-17	<0.10	<0.10
TSL-10	0.13	0.29
TSL-8H	17	26
TSL-4H	5.9	27
TSL-20	0.94	37
TSL-2	0.42	6.0
TSL-1	0.34	0.58
TSL-9	0.28	0.40
TSL-12	0.052	0.070
TSL-6H	33	40
TSL-7H	55	49
TSL-3	30	31

[a] From Tables IV and V we can determine that the starting control for TSL-5H has already suffered a 62% loss of activity. Based on the theoretical undenatured activity, the % renaturation is 12.

TABLE III: Substrate Dissociation Constants for the Altered-Kinetics Luciferases.

Mutant	Reduced Flavin Mononucleotide (M × 10^4)	*n*-Decylaldehyde[a] (M × 10^4)
Wild type	0.0069	0.068
AK-6	3.2	0.069
AK-20	0.0013	0.51
AK-16	0.0086	0.066
AK-15	0.46	0.040
AK-7	0.083	2.2
AK-24A	0.12	0.086
AK-9	0.0063	0.65
AK-17	0.0072	0.16
AK-11	0.046	0.23
AK-3	0.057	0.41
AK-18	0.080	0.10
AK-2H	0.025	3.8
AK-1H	0.36	0.57

[a] This value should be interpreted only as a relative indication of aldehyde affinity, since it is calculated assuming the stock aldehyde is pure and that all the aldehyde is in aqueous solution.

and -3) do produce wild-type amounts of luciferase will be shown later (Table VII).

In general, those enzymes with more severe kinetic alterations (columns 3–6) tend to be dimmer; however, since we did not attempt to screen bacteria that were as bright or brighter than the wild type, we would not have isolated luciferases with mutations which speed up turnover rate without adversely affecting catalysis.

There is a class of dark mutants which can be made to emit near wild-type levels of luminescence *in vivo* upon the exposure to aldehyde (Rogers and McElroy, 1955; Nealson and Markovitz, 1970; Cline and Hastings, 1971). In our screening for altered-kinetics mutants, 60 members of this class were examined, but none were found to produce kinetically altered luciferase. Although several of the altered-kinetics mutants show a modest stimulation of luminescence *in vivo* by aldehyde, the level reached is well below the wild type.

The AK mutants are listed in order of increasing rate constant for the reaction without added aldehyde (column 3). This same sequence does not hold for the reaction with the aldehydes. In the wild-type (MAV) luciferase there is a characteristic relationship between the decay rates with various aldehydes: decanal > none > octanal = dodecanal (Hastings *et al.*, 1969). This same pattern holds for several mutants, including AK-16 which is slightly slower than wild type and AK-24A which is somewhat faster. But in AK-6 and AK-1H, where the decay rates are very different from the wild type, the relationship is different in that the rate without aldehyde is no longer intermediate between those with decanal and octanal. Nevertheless, the aldehyde patterns remain, in that the rates with decanal are greater than those with octanal and dodecanal. With AK-20 the qualitative fast–slow–slow (C_{10} > $C_8 \cong C_{12}$) relationship no longer holds; decanal gives the slowest rate.

A mutant's kinetics may be different from the wild type with one aldehyde yet not with another (or with none). With AK-17 the rate with dodecanal is the same as with wild type, while with decanal it is lower. Many mutants show a rate without aldehyde faster than that of the wild type but an aldehyde rate slower than normal (*e.g.*, AK-18). An assay of a mixture of two different altered kinetics mutants displays the expected biphasic kinetics.

In many cases it was not possible to determine accurately the value of k_b. It can be seen from eq 1 that if k_d is large and k_b is small or if K_A is decreased, concentrations of aldehyde above the limit of solubility will be required for k_0 to reach k_b.

The thermal stability of the luciferase from the AK mutants was investigated and found (column 2) to be very similar to the wild type for 8 of the 13 mutants, including those showing the most severe kinetic alterations (AK-6, -20, -1H). However, in some cases there was an effect on thermal stability. Mutants AK-7 and -2H both have a $T_{5m-50\%}$ 12° below that for the wild type (and, incidentally, atypical kinetics of thermal inactivation). They are also the two mutants with the highest apparent decanal dissociation constants (Table III, column 2). These two mutants which, by kinetic criteria are clearly distinguishable, may nevertheless contain similar kinds of structural lesions.

The lesions which result in altered kinetics may cause, at the same time, very large differences in the enzymes' binding properties for one or both of the substrates, as indicated in Table III, columns 1 and 2. There appear to be no generalizations between kinetic and substrate binding properties. Both the slowest (AK-6) and the fastest (AK-1H) mutants show a large decrease in flavin affinity, but AK-9 and -17 have unaltered flavin affinities even though they have substantial ki-

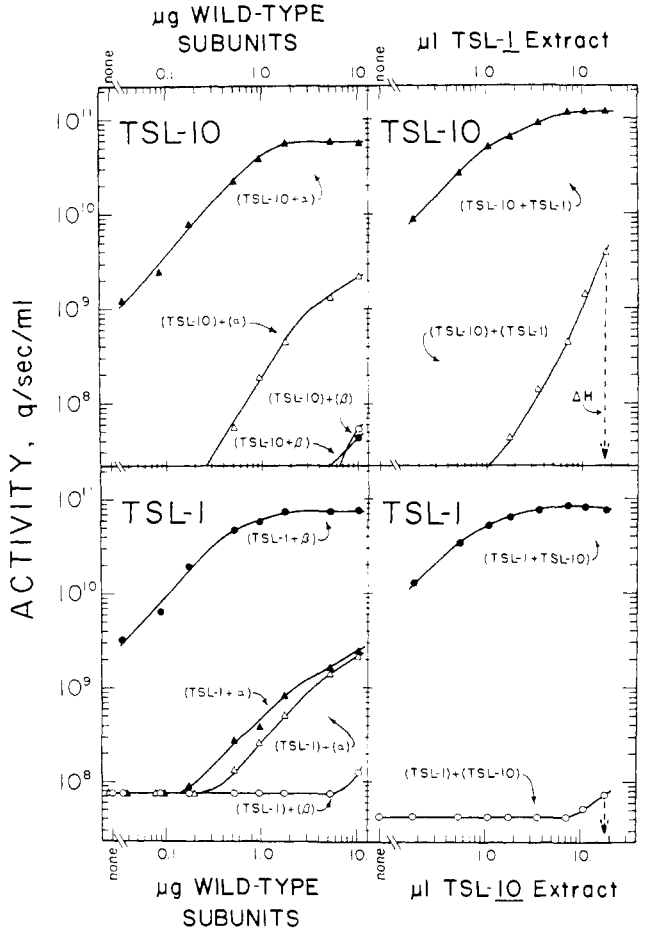

FIGURE 2: Renaturation of an α-defective and a β-defective TSL mutant luciferase following treatment with 4 M guanidine hydrochloride. All quantities are described per ml of the renaturation mixture (see Materials and Methods); 1.7 μl of a guanidine-denatured extract of either TSL-10 (top) or TSL-1 (bottom) was titrated by either purified wild-type subunits (left) or guanidine-denatured TSL mutant extract (right). Each point represents a different renaturation mixture containing the materials indicated: △, ○ designate the experimental control, the sum of the maximum activity recovered by renaturation of the mutant and the titrating material *separately;* ▲, ● designate the maximum activity recovered by renaturation of the same concentrations of materials *together*. The dashed lines indicate that the activity can be greatly reduced by a mild heat treatment that does not affect the wild-type luciferase activity.

netic alterations. AK-20 is the only mutant which has a greater affinity for $FMNH_2$ than does the wild type (at this pH). Where the flavin concentration required is very high (AK-6), extrapolation to V_{max} was required because of self-absorption of light. It should be noted that this method of determining the flavin dissociation constant assumes that the rate of formation of intermediate II from I (Figure 1) is rapid compared to the rate of dissociation of I into luciferase and $FMNH_2$. This assumption may not be valid for all the AK mutants.

Although determinations of aldehyde binding constants were made difficult by several factors, the data indicate that aldehyde affinities of most of the kinetic mutants differ from wild-type luciferase (Table III, column 2). One difficulty relates to the problem of determining the free aldehyde concentration. Aldehyde is relatively insoluble in aqueous solution and is subject to autoxidation (Hastings *et al.*, 1963). Also, aldehyde may bind to other components, especially bovine serum albumin, which was present in all assays. Kinetic factors may also cause difficulties. The determination of aldehyde binding from measurements of the initial maximum velocity (I_{max}) assumes that the equilibrium between II and II_{Ald} is rapid compared to k_d and k_b. This assumption may not be valid for some of the mutants with greatly altered values of k_d or k_b. Also, steps leading to the formation of II are inhibited at high aldehyde concentrations, so an extrapolation to V_{max} must be made with some mutants, adding uncertainty to the determination. Luciferase of some mutants (AK-7) may be even more susceptible than the wild type to this inhibitory effect of high aldehyde.

The mutant with the largest alteration of flavin binding (and severe kinetic alterations as well), AK-6, has a wild-type aldehyde binding constant. In contrast, AK-9 has a greatly increased aldehyde dissociation constant, yet is like wild type in flavin binding. Many mutants show a substantial alteration of both binding constants, but one (AK-16) shows significant alterations in neither. These qualitative differences suggest that mutants selected on the basis of their altered kinetics possess lesions in a variety of residues at the active site.

Mutant Subunit Complementation. A first step in mapping the lesions which are associated with a particular defect is to determine their subunit distribution; that is, whether in a given class they occur in α or β or both. This determination was made by a complementation test. Native luciferase can be dissociated into its subunits by 4 M Gdn·HCl; upon dilution, reassociation occurs, forming native enzyme. If a luciferase possesses a lesion in only one of the subunits, only the addition of a wild-type subunit corresponding to the defective one will result in the renaturation of wild-type luciferase.

The properties of the TSL mutants suggested that these luciferases could serve as a convenient source of one or the other wild-type subunit for use in complementation studies. Such a usage is critically dependent upon the assumption that their altered properties are due to lesions in only one of the two polypeptide chains, and that the presence of the mutant subunit will not interfere with the association between wild-type subunits during renaturation.

The experiments of Figure 2 show that this is the case for TSL-10 and TSL-1. TSL-10 renaturation is stimulated only by the addition of wild-type α subunit (top left), whereas with TSL-1 only the wild-type β subunit results in a high recovery of activity (bottom left). In both cases the (high) activity recovered with the complementing subunit possesses wild-type thermal stability and kinetics. The lesion causing renaturation deficiency must therefore be the same as, or at least in the same subunit as, the lesion causing thermal and kinetic alterations, indicating that the complementary mutant subunit is structurally unaltered. In the case of TSL-1, the small amount of stimulation over the control which occurs with added α is the result of the formation of additional TSL-1 luciferase; it is as thermally unstable as TSL-1.

The equivalence of purified wild-type subunits and subunits derived from TSL mutants for complementation can be seen by comparing the right and left of Figure 2. For a fixed amount of TSL-1, the maximum activity recovered was the same with either purified β or TSL-10 subunits. With TSL-10, however, more activity was recovered when TSL-1 was used as the source of α; a possible explanation is suggested below.

Although the control backgrounds with TSL-10 (top panels) are apparently the same in the right and left, there is a significant difference between them. The background activity [(TSL-10) + (α)] is largely due to the fact that α subunit

TABLE IV: Mutant Subunit Complementation—Subunit Location of Lesions Decreasing Thermal Stability.

Mutant	Undenatured[d] Control 1	Renatured with[e] α (TSL-1) 2	Renatured with[f] β (TSL-10) 3
Wild Type			
Activity before ΔH[a]	9.8	6.6	5.0
Remaining after $\Delta H1$	99%	109%	92%
Remaining after $\Delta H2$	47%	53%	55%
None Added			
Activity before ΔH		0.081	0.0011
Remaining after $\Delta H1$		1%	<10%
TSL-14			
Activity before ΔH	0.56	7.1	0.43
Remaining after $\Delta H1$	6%	75%	10%
TSL-11			
Activity before ΔH	0.061	5.7	0.032
Remaining after $\Delta H1$	2%	88%	3%
TSL-5H			
Activity before ΔH	0.75	6.5	0.40
Remaining after $\Delta H1$	5%	94%	8%
TSL-18			
Activity before ΔH	0.11	5.1	0.022
Remaining after $\Delta H1$	1%	59%	1%
TSL-17			
Activity before ΔH	0.14	4.6	<0.0011
Remaining after $\Delta H1$	1%	106%	<10%
TSL-10			
Activity before ΔH	0.072	4.3	
Remaining after $\Delta H1$	2%	86%	
TSL-8H			
Activity before ΔH	7.6	5.9	3.8
Remaining after $\Delta H2$	4%	51%	5%
TSL-4H			
Activity before ΔH	0.62	0.63	2.2
Remaining after $\Delta H1$	<1%	<1%	86%
TSL-20			
Activity before ΔH	0.21	0.41	5.3
Remaining after $\Delta H1$	<1%	<1%	87%
TSL-2			
Activity before ΔH	0.57	0.22	4.5
Remaining after $\Delta H1$	<1%	<1	91%
TSL-1			
Activity before ΔH	0.57		2.9
Remaining after $\Delta H1$	<1%		97%
TSL-9			
Activity before ΔH	4.8	0.20	5.5
Remaining after $\Delta H1$	<1%	1%	87%
TSL-12			
Activity before ΔH	2.3	0.14	5.1
Remaining after $\Delta H1$	3%	1%	78%
TSL-6H			
Activity before ΔH	4.3	2.7	3.8
Remaining after $\Delta H1$	15%	19%	82%
TSL-7H			
Activity before ΔH	7.5	4.7	7.3
Remaining after $\Delta H2$	3%	6%	37%
TSL-3			
Activity before ΔH	5.5	3.7	4.2
Remaining after $\Delta H2$	11%	20%	41%

[a] Activity is expressed as q/sec per ml of the renaturation mixture $\times 10^{-11}$. [b] $\Delta H1$: First heating treatment: 2.0 ml of the sample in a 12 $\times$ 75 mm test tube (initially at 8°) was set into a water bath at 37.5°, incubated for 20 min without shaking, then placed into ice. [c] $\Delta H2$: Second heating treatment: same as footnote *b* but subsequently incubated for 12 min in a water bath at 42°. [d] This is the 72-hr control of Table II. [e] Based on the data from Figure 2 the amount of nondefective α subunit present during these renaturations could potentially yield 9.5 $\times 10^{11}$ q/sec per ml if fully complemented with nondefective β. [f] The nondefective β present could potentially yield 13.8 $\times 10^{11}$ q/sec per ml if combined with nondefective α.

is not completely free of β, so that some authentic wild-type luciferase activity is formed. The background activities in the right panel, obtained when TSL-1 and TSL-10 were renatured independently [(TSL-1) + (TSL-10)], are due to the small but detectable quantities of renatured mutant luciferases. These, however, are heat labile (indicated by the dashed line) and can therefore be distinguished from the wild-type (heat stable) activity which is obtained when the two mutants are renatured together (TSL-10 + TSL-1).

In Figure 2 (right panels) the level of activity obtained and the absence of inhibition at higher concentrations of subunits indicate that the defective subunits do not interfere with the association between the two nondefective subunits. Using crude extracts of cells, one can therefore obtain a quantitative value for the amount of nondefective subunit for either the wild type or mutants.

These points suggest some of the advantages of using mutants rather than purified wild-type proteins as the source of subunits. First, separation of wild-type subunits involves chromatography in urea (Gunsalus-Miguel *et al.*, 1972). The α subunit is kept in urea prior to use, where it suffers considerable inactivation. The possibility that the inactivated α subunits may form inactive luciferase with good β is suggested as an explanation for the relatively low plateau in the titration of TSL-10 (top left). These problems do not arise with the mutants, since the subunits need not be separated, thereby avoiding urea at that step. Second, the contaminating β that is present in purified wild-type α preparations is an unavoidable consequence of the fact that α elutes after β in the urea–DEAE-Sephadex chromatography step.

Complementation with Temperature-Sensitive Luciferases. Knowing the location of the lesions in TSL-1 and TSL-10, we used these two mutants as sources of one or the other wild-type subunit for complementation tests on the rest of the TSL mutant isolates. If a TSL mutant forms material of wild-type heat stability and renaturability with TSL-1 but not TSL-10, the unknown must possess a defect only in the α subunit; conversely, if it forms a good yield of wild-type material only with TSL-10, it can be designated a β mutant.

For those enzymes that are renaturation deficient, the

TABLE V: Relative Synthesis and *in Vitro* Specific Activity of Temperature-Sensitive Luciferases.

Mutant	*In Vivo* Activity[a] when Harvested, Mutant/Wild Type 1	*In Vitro* Activity[b] Extracted, Mutant/Wild Type 2	Mutant Luciferase[c] Synthesis, Measured by Subunit Complementation, Mutant/Wild Type 3	Relative *in Vitro* Specific Activity, Mutant/Wild Type 4
TSL-14	0.49	0.41	1.1	0.39
TSL-11	1.12	0.95	1.1	0.91
TSL-5H	0.40	0.39	1.1	0.38
TSL-18	0.39	0.041	0.60(0.95)[d]	0.07(0.04)[d]
TSL-17	0.11	0.020	0.76	0.027
TSL-10	0.12	0.026	0.76	0.035
TSL-8H	0.24/1.12	0.95	0.93	1.0
TSL-4H	0.69	0.75	1.2	0.65
TSL-20	1.12	0.84	1.1	0.79
TSL-2	0.98	0.81	1.1	0.76
TSL-1	0.40	1.0	0.96	1.1
TSL-9	0.54	0.91	1.0	0.88
TSL-12	0.92	0.63	1.6	0.40
TSL-6H	0.62	0.71	0.96	0.74
TSL-7H	0.46/0.90	1.1	1.3	0.90
TSL-3	0.20/0.74	0.73	0.96	0.76

[a] Luminescence (per ml) at the time of harvest of cells grown at 20° in vigorously shaken liquid cultures. Cultures were harvested at an optical density of 2.1 ± 0.2 measured at 660 nm on a Coleman Jr. spectrophotometer equipped with a red filter. At this stage, the cultures have reached approximately one-fourth of their peak *in vivo* luminescence (the wild type gave 8.9×10^{11} q/sec per ml). For strains whose luminescence is stimulable by added aldehyde the value to the left of the slash is without decanal, to the right is with decanal (see Cline and Hastings, 1971). [b] Samples (8 ml) of cells were centrifuged at 2°. The pellets were resuspended in 2 ml of distilled water and lysed by sonication at 4°. The lysate (10 μl) was assayed, with decanal. The wild type gave 2.0×10^{12} q/sec per ml of culture. [c] Determined by renaturation with a very large excess of the complementing subunit (TSL-1 for α-defective luciferases, to measure the amount of β; TSL-10 for β-defective enzymes to measure the amount of α). The wild type gave 2.6×10^{12} q/sec per ml renatured with TSL-10, 2.1×10^{12} q/sec per ml renatured with TSL-1. The first figure was used for calculation of the amount of luciferase in α-defective mutants (−14 through −8H), the second for β-defective (−4H through −3). The theoretical maximum amount of nondefective α subunit that could be assayed (calculated to the same concentration as in footnote *b*) was 2.2×10^{14} q/sec, of nondefective β, 1.5×10^{14}. The activity recovered in the absence of added mutant was $<4 \times 10^{10}$ q/sec per ml. This low background is due to the fact that the renaturation mixtures (and controls) were heated as indicated in footnote *b* of Table IV prior to the assay; only heat-stable material will remain. [d] Since the material recovered following renaturation of TSL-18 with excess α is not of wild-type heat stability, this strain will appear low in luciferase content by the assay described in footnote *c*. A value (in parentheses) for the amount of material present in the unheated renaturation mixture due to the combination of TSL-18 β with TSL-1 α is calculated by subtracting the average loss in activity as a consequence of heating the mixtures (*i.e.*, the average background due to TSL-1 renaturation) from the activity of the extract prior to heating.

subunit location of the lesion can be determined on the basis of the activity recovered in mixed renaturations with TSL-1 or TSL-10 (Table IV). The property of renaturation deficiency is not confined to lesions in only one of the two subunits. With the others (TSL-8H, -6H, -7H, and -3), the assignment of the defective subunit must be made on some other basis, since they renature as well as the wild type with or without additions.

Column 1 (Table IV) serves as a control to record the temperature sensitivity of the uncomplemented mutant enzymes under the buffer conditions and heating procedures used to distinguish mutant from wild-type activity. A mild heating procedure (ΔH1) that has no effect on wild-type activity greatly inactivates most TSL enzymes. TSL-8H, -3, and -7H were inactivated only slightly by this mild heating so a more severe treatment (Δ*H*2, Table IV, footnote *c*) was utilized.

The effect of added subunits on mutant renaturation is best described by reference to TSL-11 and -9. TSL-11 renaturation is stimulated appreciably only by α, and the activity recovered is essentially as stable as wild type. While there is a modest stimulation of activity by β (see Table II, column 2 for control) the material recovered is clearly mutant, not wild type, in its heat stability. With TSL-9, on the other hand, only the addition of β (TSL-10) results in a large stimulation of activity, forming material of wild-type stability. The assignment of the defective subunit for mutants which renature well (TSL-8H, -6H, -7H, and -3) was made on the basis of the thermal sensitivity of the material recovered after denaturation.

If the mutant luciferases are defective in only one subunit, the complemented material should be as stable as the wild type. While this was so in most cases, TSL-7H and TSL-18 were exceptions. For TSL-7H (which is not renaturation deficient), the explanation may be that the defective subunit

TABLE VI: Mutant Subunit Complementation—Subunit Location of Lesions Causing Altered Kinetics.

	Effect on the Initial Maximum Light Intensity[a]			Effect on the Observed Rate Constant for the[a] Decay of Luminescence		
Mutant	Undenatured Control, Mutant/Wild Type 1	Renatured with β (TSL-10), Renatured/ Undenatured Control 2	Renatured with α (TSL-1), Renatured/ Undenatured Control 3	Undenatured Control, Mutant/ Wild Type 4	Renatured with β (TSL-10), Mutant/ Wild Type 5	Renatured with α (TSL-1), Mutant/ Wild Type 6
Wild type	1.0	0.45	0.76	1.0	1.0	1.0
AK-6	0.000062	0.20	3800	*b*	*b*	1.0
AK-20	0.0011	0.49	250	0.17[b]	0.21[b]	1.0
AK-16	0.21	0.036	2.6	0.67	0.75	1.0
AK-15	0.052	0.57	6.1	0.41	0.42	0.95
AK-7	0.0048	0.44	88	0.43	0.47	1.0
AK-24A	0.52	0.59	1.0	1.4	1.5	1.0
AK-9	0.0036	0.21	150	0.52	0.55[b]	1.05
AK-17	0.0016	0.038	230	0.076	*b*	1.05
AK-11	0.00038	0.66	110	1.7[b]	*b*	1.0
AK-3	0.0073	0.74	3.1	1.3	1.4	1.05
AK-18	0.0018	0.43	320	0.76	0.85	1.05
AK-2H	0.010	0.56	41	2.6	2.7	1.0
AK-1H	0.0088	0.60	51	3.4	3.4	1.05

[a] All assays were with decanal (see text). Wild-type undenatured control $I_{max} = 8.5 \times 10^{11}$ quanta/sec per ml and $k = 0.21$ sec^{-1} (0.20 for columns 5 and 6). Controls for columns 2 and 3 (*i.e.*, no AK mutant enzyme added) were 2.1×10^7 and 6.3×10^9 q/sec per ml, respectively. [b] In these cases there was insufficient activity for (an accurate) determination.

competes effectively with the wild-type, thereby producing a mixture of renatured wild-type and mutant luciferases. In this experiment TSL-10 was present in only a twofold excess over TSL-7H. In the case of TSL-18 it is unlikely that the result was due to competition of TSL-18 α with the wild-type α subunit, since the mutant is renaturation deficient. Even with a 30-fold excess of wild-type α the recovered material, though clearly far more stable than parent TSL-18, was not as stable as wild-type (Table V, column 3). Consequently, although the lesion in α is primarily responsible for the mutant phenotype, the enzyme may also possess a lesion in β.

In every case the heat-stable (wild type) material recovered with the complementing subunit is kinetically indistinguishable from the wild type in the assay with decanal (Table I). We can conclude that the lesions causing the enzymatic differences are either the same, or at least in the same subunit as those affecting thermal stability.

Although some temperature-sensitive mutants produce wild-type levels of light at low temperature, we included in our selection several that do not (Table V, column 1). There are at least three explanations for this which relate to the luciferase itself: (1) the mutants might not produce wild-type amounts of the enzyme, (2) the enzyme might be produced in normal amounts but be catalytically defective, or (3) the enzyme might be so unstable that a considerable amount of inactivation occurs *in vivo* even at the apparently permissive temperature.

To determine whether the TSL mutants are synthesizing wild-type amounts of luciferase and whether the mutant luciferases are fully active *in vitro*, we prepared extracts from equal numbers of cells grown under identical conditions (at 20°). In order to minimize inactivation of the luciferases, cells were lysed immediately after harvesting; enzyme assays were carried out promptly without further purification. Column 2 of Table V reports the *in vitro* activity of these extracts. All the β-defective mutants appear to have near wild-type levels of activity (>0.5). Of the seven α mutants, this is true for only two, TSL-11 and -8H.

To assay the amount of the nondefective mutant subunit, the extracts were denatured with Gdn·HCl, then renatured with a very large excess of the complementing subunit (column 3). The excess subunits do not inhibit the recovery of the wild-type enzyme. The wild-type control (see Table V, footnotes *b* and *c*) serves to indicate that the α and β subunits are present in equal amounts *in vivo*, so that an assay for either subunit should indicate the degree to which the gene for luciferase is expressed.

All the mutants produce wild-type amounts of luciferase (column 3). With this information we can establish a value for the *in vitro* specific activity of the mutant enzymes relative to the wild type (column 4). Every α mutant, except TSL-11 and -8H, is significantly less active (<0.5) than the wild-type. The only β mutant with a low *in vitro* specific activity is TSL-12. The low *in vitro* specific activities cannot be attributed entirely to *in vitro* inactivation since the "*in vivo* specific activities" are low as well, *i.e.*, the *in vivo* luminescence of these mutants is below that expected based on the amount of luciferase they contain.

By analogy with the altered kinetics mutants, we feel it is likely that the low activity of TSL-14, -5H, -18, -17, -10, and -12 is due at least in part to reduced catalytic activity of the intact mutant luciferases, since the kinetic parameters of the luminescence reaction catalyzed by these luciferases differ somewhat from those of the wild type.

TABLE VII: Amount of Luciferase Synthesized by Altered-Kinetics Mutants.

Mutant	Luciferase Synthesis, as[a] Measured by Complementation with α Subunit (TSL-1)
AK-6	1.1
AK-20	0.5
AK-16	0.9
AK-15	1.4
AK-7	0.9
AK-24A	1.5
AK-9	0.7
AK-17	1.1
AK-11	0.1
AK-3	0.2
AK-18	0.9
AK-2H	0.8
AK-1H	0.5

[a] Cells were grown at 26° in liquid cultures and harvested 1 hr after reaching peak luminescence. See footnotes *b* and *c* of Table V for additional information. For this table, the wild-type value for the amount of β subunit is 7.5×10^{12} quanta/sec per ml of culture harvested.

The fact that the values in column 3 of Table V are so similar for the wild type and the mutants indicates that the complemented material is fully active, supporting our contention that the lesion causing the mutant phenotype is in only one of the two luciferase subunits of each mutant enzyme.

Complementation with Altered-Kinetics Luciferase. Table VI reports on the effect of renaturation with wild-type subunits on the initial maximum intensity and the decay rate observed using the standard luciferase assay. As with the TSL mutants discussed in the previous section, wild-type luciferase should be formed by renaturation of luciferase from an altered-kinetics mutant with the wild-type subunit corresponding to the defective peptide chain of the mutant. We confirmed that the apparent wild-type material formed did in fact result from a recombination of TSL and AK mutant subunits by subjecting the material in column 3 to the mild heating procedure ($\Delta H1$) used in Table IV. In no case was there a significant loss of activity.

In every case, only the addition of wild-type α subunit (TSL-1) will greatly stimulate the level of activity recovered (column 3). Except for AK-11 and -3, the level reached with added α suggests that the material formed is as active as wild-type enzyme.

As judged by the kinetics of the renatured luciferase, again only the addition of wild-type α to the AK enzyme produces wild-type luciferase (Table VI, column 6). AK mutant luciferases continue to display their altered decay rates even after guanidine denaturation and 600-fold dilution (column 5). The apparent discrepancy between the values for decay rate constants in Table VI and those in Table I (column 5) is a consequence of the fact that the decanal concentration optimum for wild-type luciferase was used for the assays in Table VI, while a much larger aldehyde concentration was often required to determine the k_b values in Table I.

Since most of the AK mutants renature well, the defective AK subunit should compete with the added wild-type subunit. However, since the AK luciferases are less active than the wild type only the wild-type decay rate will be observed (column 6).

Both for the purification and characterization of the enzymes and for the comparison of mutant and wild-type properties *in vivo*, the most useful mutants are those in which the genes for the luminescent system are expressed to the same extent as in the wild type.

In order to obtain a quantitative measurement of the amount of luciferase synthesized by the AK mutants, AK cultures were harvested 1 hr after reaching peak luminescence and cell lysates of each were prepared at the same dilution. The extracts, as in Table V, were then denatured and renatured in the presence of a large excess of TSL-1 (α). As before, the renaturation mix was heated prior to assaying in order to remove any activity due to renatured TSL-1. All mutants except AK-11 and AK-3 appear to produce near wild-type levels of luciferase (>0.5; Table VII). Two other observations indicate that AK-11 and -3 produce less luciferase than the wild type: β is not defective (as measured by complementation with α), and there is less antigenically cross-reacting material, as judged by Ouchterlony double diffusion gels using antibody prepared against pure, wild-type luciferase (Gunsalus-Miguel *et al.*, 1972).

Of the 706 mutants (selected for being less bright than the wild type) that were originally screened for altered-kinetics luciferase, only nine had activity in crude extracts too low (*ca.* 6×10^{-4} of the wild type) to permit a determination of the decay rate. In order to establish that our failure to isolate β-defective altered-kinetics mutants was not due to their possibly low activity, we reexamined the five of these nine mutants that were still available. The mutants were grown in liquid culture (22°), and their luciferase was partially purified. We determined the kinetics of the mutant luciferases (one possessed only 3×10^{-6} of the wild-type level of activity) and tested the enzymes for subunit complementation. All five possessed lesions in their α subunits. Three of the five mutant enzymes displayed altered kinetics, in each case due to their defective α. Only one of the five mutants (an AK) produced wild-type amounts of luciferase as measured by our complementation test. These results bring to 20 the number of altered-kinetics luciferases isolated, every one caused by a lesion(s) in the α subunit.

Discussion

For a number of heteropolymeric proteins, the determination of the functional relationship between the subunits has been aided by the fact that at least one of the isolated subunits has some activity (Ginsberg and Stadtman, 1970). In contrast, neither of the subunits of bacterial luciferase appears to have activity by itself (Friedland and Hastings, 1967; Gunsalus-Miguel *et al.*, 1972). A concurrent study in this laboratory of the effects of chemical modification (succinylation) on luciferase has indicated that the subunits are not functionally equivalent and that the α subunit is involved in catalysis (Meighen *et al.*, 1971; Meighen and Hastings, 1971). Taking this into account, we can suggest several possible explanations for the apparent inactivity of the isolated subunits; (1) the different subunits might be responsible for catalyzing different steps in the reaction sequence leading to light emission; (2) residues from both subunits might be involved simultaneously in the necessary substrate binding and

catalysis for one or more steps in the reaction; (3) the entire reaction sequence might be mediated by functional groups from only one subunit, but the stable, catalytically active, three-dimensional arrangement of these groups might require the presence of a second peptide chain, a noncatalytic subunit. We believe that this last alternative is the correct explanation.

In order to distinguish between the alternative functional relationships that might exist between the subunits of bacterial luciferase, one must be able to obtain proteins with specific alterations in a large number and variety of amino acid residues. From the limited number of enzyme structures that have been determined to date through the use of X-ray crystallography, it has become apparent that a considerable number of different amino acids are involved in substrate (or prosthetic group) binding and catalysis (Dickerson and Geis, 1969); certainly a considerable number of residues must also be required for the proper functioning of luciferase. In addition, it seems that a large percentage of the amino acids of an enzyme are involved in directing and maintaining the proper pattern of folding of the peptide chain, through hydrophobic and hydrogen bonds. While these residues are not directly involved in reactions with the substrate, they may nevertheless be essential for enzyme activity.

Although chemical modification provides a valuable technique for studying luciferase, mutational alteration has a number of advantages for the investigation of the subunits' functions. Using mutational modification, one selects the desired altered enzyme phenotype without regard to, and hence not limited by, the accessibility or reactivity of the amino acid whose modification produces the change. One can obtain large quantities of a highly homogeneous preparation of a protein in which, in principle, the charge, size, and/or hydrophobicity of any particular residue is changed. Furthermore, it is possible to precisely characterize the mutant enzymes and thereby to correlate alterations in various aspects of enzyme function with specific changes in subunit functional groups.

A feature that makes luciferase particularly amenable to study by mutational modification is the ease with which one can specifically select for alterations which decrease luciferase activity by reducing the catalytic capacity of each enzyme molecule, rather than by simply destabilizing the wild-type confirmation and thereby reducing the concentration of fully active luciferase (which would result in a low I_{max} but wild-type kinetics). While there may be some lesions which reduce the effectiveness of each luciferase molecule without altering the decay rate constants, the absence of wild-type activity in luciferases which do display altered kinetics indicates that their low activity is not simply due to low stability. A screening based on decay kinetics is aided by the sensitivity of the assay, and can be done without regard to the concentration of luciferase.

The results of the AK luciferase characterization demonstrate that the selection for altered kinetics is a powerful technique for isolating luciferases containing modifications which affect different aspects of the luminescence reaction to various degrees. The lifetimes of mutant intermediates II and/or II_{Ald} may be more than ten times longer or ten times shorter than for the wild type. Flavin and aldehyde binding affinities may be altered individually or simultaneously over a wide range. The relationship between enzyme turnover rate and aldehyde chain length can be reversed. Mutational modification of luciferase has proven to be far more effective than chemical modification, both in the degree of alteration possible for any parameter, and in the variety of catalytic parameters that can be varied.

Using subunit complementation, we have determined that the mutant properties of all the AK luciferases so far isolated (20) are attributable to lesions in only the α subunit. In view of the wide diversity of mutant types in this class, this makes a catalytic role for the β subunit unlikely. The isolation of larger numbers of AK mutants will more firmly establish the conclusion.

The large number of β-defective, temperature-sensitive mutants isolated serves to demonstrate that our failure to find β-defective AK mutants is not due to a much lower mutability (relative to that for α) of the genetic element for the β subunit. The fact that we determined the kinetics of every mutant with less than 50% of the wild-type level of luminescence (see Results) makes it unlikely that we missed β-defective AK mutants on account of their possibly low enzymatic activity.

Although β does not seem to be directly involved in catalysis, it is nevertheless essential for enzyme activity. Neither of the subunits separated by chromatography in urea will reform active material by itself. Our analysis of temperature-sensitive luciferases shows that the thermal stability and renaturability of bacterial luciferase can be altered over a wide range by lesions in β. This suggests that β is required to stabilize the catalytically active conformation of residues on α, not only for refolding from urea, but for the intact luciferase molecule as well. The hypothesis that α contains the active site while β is required for conformational stability is further supported by the fact that mutations which decrease thermal stability without at the same time decreasing the activity of the enzyme occur predominantly in the β subunit. Since β is required for activity, it could serve a negative controlling function; however, there is no data at present to support such a role *in vivo* (see Nealson *et al.*, 1970).

The fact that the mutant TSL-12 exhibits minor kinetic alterations due to a lesion in β is not difficult to reconcile with our model. In view of the hypothesized conformational dependence of α on β, we would expect that structural changes in the β subunit could have a small effect on catalytic parameters. With TSL-12 kinetic alterations are far smaller than for any of the AK mutants; they are smaller even than those for several of the α-defective TSL mutants. The enzyme has a high specific activity, however, TSL-12 is one of the more thermally sensitive and one of the most renaturation-deficient, temperature-sensitive luciferases.

The specific nature of many of the changes occurring in these mutationally altered enzymes indicates that they will be useful for far more than simply indicating subunit function. A majority of the altered-kinetics mutants, including those with the most aberrant catalytic properties, are essentially as stable as the wild type to thermal inactivation and are not renaturation deficient. This suggests that alterations in catalytically important functional groups can occur without causing substantial changes in the conformation of the peptide chain. Since they are stable, the purification of these AK enzymes should be straightforward. Determination of the precise changes in functional groups that have occurred in these proteins should enable us to evaluate the role of individual amino acids in the luminescence reaction.

On the other hand, because we can directly assess the activity of luciferase *in vivo* we can easily identify thermally unstable mutant luciferases which show no alterations in catalytic parameters. These fully active TSL's will permit us to investigate that aspect of protein structure concerned with

maintaining the active enzyme conformation separately from that concerned directly with shaping the active site.

Luciferases from AK-6, -20, -18, and -1H have been purified without difficulty. In the purification of luciferases from TSL-11, -4H, -9, and -12 losses have occurred, but they have not been prohibitive. The properties of the purified mutant enzymes appear not to differ from those in crude extracts.

Many features of the luciferase system, including the ease with which a large number and variety of mutants can be generated, and the ability to follow enzyme activity *in vivo* at the permissive and restrictive temperatures, make it ideally suited as a system for investigation of the mode of action of enzymatic lesions that cause increased thermal sensitivity. However, if the potential advantages of this system are to be realized, we must understand the chemistry of the luminescence reaction. The altered-kinetics mutants should be especially useful in this latter problem.

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Nucleoside Diphosphokinase from Beef Heart Cytosol. I. Physical and Kinetic Properties†

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ABSTRACT: Nucleoside diphosphokinase (NDP-kinase) from beef heart cytosol has been purified about 200-fold. With this preparation the following molecular characteristics were assessed by sucrose gradient centrifugation, gel filtration on Bio-Gel P300, and electrofocusing: s_{20} = 5.9 S, mol wt 108,000, and isoelectric point of a major component = 8.5. The kinetic properties of beef heart cytosol NDP-kinase were consistent with a Ping-Pong mechanism. The ATP–ADP exchange catalyzed by the enzyme was three times faster than the UTP–ADP exchange and five to seven times faster than the CTP–ADP and GTP–ADP exchange. The rate of exchange depended essentially on the nature of the nucleoside triphosphate, but not on that of the nucleoside diphosphate. Also, the affinity was found to vary according to the nucleoside triphosphate; for instance, the K_M for UTP was three times as high as the K_M for ATP. By contrast, the K_M for various nucleoside diphosphates, ADP, UDP, CDP, GDP, were in the same range from 0.04 to 0.06 mM. The K_M for ATP was roughly six times as high as the K_M for ADP. Mg-ATP and Mg-ADP, and not the free nucleotides, were the true substrates; this contrasts with the mitochondrial beef heart NDP-kinase for which free ADP was preferred to Mg-ADP. Excess Mg-ADP was inhibitory and the K_i for excess Mg-ADP acting as inhibitor was only twice as high as the K_M for Mg-ADP acting as substrate. AMP was a competitive inhibitor both with respect to ADP and ATP. The K_i for AMP was 2.5 times higher than the K_M for ATP and 100 times higher than the K_M for ADP. The possible involvement of NDP-kinase in the regulation of nucleoside triphosphate synthesis is discussed.

The attention which has been paid in the past few years to the mitochondrial nucleoside diphosphokinase,[1] an enzyme catalyzing an ADP–ATP exchange, stems mainly from data (for review, *cf.* Lehninger and Wadkins, 1962) relating the mitochondrial ADP–ATP-exchange reaction to the mechanism of oxidative phosphorylation. In this laboratory, prop-

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[1] Abbreviations used are: NDP-kinase, nucleoside diphosphokinase or nucleoside triphosphate:nucleoside diphosphate transphosphorylase, EC 2.7.4.6; (Mg), free magnesium concentration; AOPCP, adenosine 5′-methylenediphosphonate.