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Formation of Hybrid Luciferases from Subunits of Different Species of *Photobacterium*†

Edward G. Ruby* and J. Woodland Hastings

ABSTRACT: Enzyme divergence within three species of the genus *Photobacterium* (*P. fischeri*, *P. leiognathi*, and *P. phosphoreum*) was studied by comparing the catalytic characteristics and quaternary interactions of bacterial luciferases isolated from each species. Each luciferase was composed of two subunits of different molecular weights as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Subunits were isolated in quantity by DEAE-Sephadex gel filtration in 7 M urea. Isolated subunits had no luciferase activity after renaturation in buffer, but active enzyme could be recovered by renaturation of the heavy and light subunits together. Renaturation of hybrid pairs (containing one subunit from each of two different species) yielded active luciferases, but only in cases where a heavy subunit of one species was combined with a light subunit of another. These hybrids exhibited in vitro catalytic characteristics most like those of the parent luciferase from which the heavy subunit was derived. The light subunit of *P. leiognathi* luciferase conferred an increased thermal stability to all enzymes containing it. The heavy subunit of each of the three *Photobacterium* species was sensitive to trypsinization. Thus, on the basis of structural and functional analogies with the luciferase from *Beneckea harveyi*, the heavy and light subunits of *Photobacterium* species have been designated α and β, respectively.

Bacterial luciferase is an α–β heterodimer catalyzing the mixed-function oxidation by molecular oxygen of reduced flavin mononucleotide and a long-chain aliphatic aldehyde, yielding oxidized flavin, the corresponding acid, water, and a blue-green luminescence (Hastings & Nealson, 1977). The two dissimilar subunits of luciferase can be easily isolated after urea denaturation, and, while the individually renatured subunits have no activity, renaturing them together yields a high recovery of active enzyme (Friedland & Hastings, 1967a).

One of the fundamental questions in luciferase biochemistry has concerned the functions of the two subunits. Studies involving chemical or mutational modifications of one or the other of the subunits isolated from the luminous species *Beneckea harveyi* have indicated that the active center is located on the heavier, or α, subunit, while the lighter, or β, subunit may be structural (Cline & Hastings, 1972; Meighen et al., 1971). Such investigations have not been reported with any of the other species of marine luminous bacteria.

The taxonomic relationships between different strains of luminous bacteria have recently been clarified (Hastings & Nealson, 1977), and five species in two genera, *Beneckea* and *Photobacterium*, have been designated (Reichelt & Baumann, 1973; Reichelt et al., 1976): *B. harveyi*, *B. splendida*, *P. fischeri*, *P. leiognathi*, and *P. phosphoreum*. It was thus of interest to investigate the possibility of producing active hybrid luciferases by combining in vitro a purified subunit from the luciferase of one species with that of another. Using active hybrid luciferases prepared in this manner, it was possible to examine whether the species-specific catalytic and physical properties of each native luciferase (Hastings et al., 1969) were determined primarily by one or both of its subunits.

Materials and Methods

Materials. Trypsin TPCK[1] was purchased from Worthington Biochemical Corp.; nonanal, decanal, and dodecanal were obtained from Aldrich Chemical Co.; tridecanal was provided by T. Nakamura, Department of Biology, Osaka University, Japan. Urea (Ultra-Pure) was a product of Schwarz/Mann.

Bacterial luciferase was isolated as previously described (Gunsalus-Miguel et al., 1972) from *B. harveyi*, M17 (Ulitzur & Hastings, 1978); *P. fischeri*, ATCC 7744; *P. leiognathi*, PL 721; and *P. phosphoreum*, NZ-11-D (Ruby & Morin, 1978).

Luciferase Assay. Luciferase activity was determined at 25 °C in an assay buffer (20 mM potassium phosphate, pH 7.0, and 0.2% BSA) with the addition of 0.2% dodecanal as substrate aldehyde unless otherwise noted. The reaction was initiated by the injection of 1 mL of a solution of 50 μM catalytically reduced flavin mononucleotide, and the light emission was monitored with a photomultiplier–photometer and a strip chart recorder (Hastings et al., 1978). The turnover rate of the reaction was determined from the first-order rate constant (k) for the decay of luminescence intensity observed during the reaction. The relative quantum yield (cq) of the reaction was calculated from the expression $cq = I_0 k^{-1}$, where I_0 is the peak luminescence of the reaction. Both the rate

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* Correspondence should be addressed to this author at Redfield 2-40, Woods Hole Oceanographic Institution, Woods Hole, MA 02543.

[1] Abbreviations used: BSA, bovine serum albumin; DEAE, diethylaminoethyl; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; $NaDodSO_4$, sodium dodecyl sulfate; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone.

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constant and the quantum yield of the reaction differ depending upon the carbon-chain length of the aldehyde used as a substrate (Hastings et al., 1963). Luminescence intensity is reported as quanta per second by reference to the light standard of Hastings & Weber (1963).

Subunit Isolation and Renaturation. Subunits of bacterial luciferases were isolated in quantity by chromatography of the purified enzyme on a DEAE-Sephadex column in potassium phosphate buffer, pH 7.0, 1 mM EDTA, and 7 M urea as previously described (Tu et al., 1977). A linear gradient in phosphate concentration from 40 to 120 mM was used to elute the separated subunits.

The re-formation of active luciferases from combinations of isolated subunits was achieved by a 100-fold dilution of each subunit into the same 1 mL of renaturation buffer containing 100 mM potassium phosphate, pH 7.0, 10 mM DTT, 1 mM EDTA, and 0.2% BSA at 2–4 °C (Tu, 1978). Maximum renatured luciferase activity as determined by the standard assay was recovered within 48 h.

Thermal Denaturation Constant. For each enzyme, eight tubes containing luciferase in 0.1 mL of renaturation buffer (see above) were incubated at 38 °C. At regular intervals, a tube was removed to an ice bath. The luciferase activities of triplicate 10-μL aliquots were determined at 25 °C in 1 mL of assay buffer. Thermal denaturation constants were calculated by linear regression of activity remaining as a function of time at 38 °C.

Electrophoresis. $NaDodSO_4$–polyacrylamide gel electrophoresis was performed by using a modification of the method of Weber & Osborn (1969) in which 1% $NaDodSO_4$ and 5 mM tris(hydroxymethyl)aminomethane–glycine (pH 8.3) instead of phosphate buffer were employed in the separation gel and running buffer. Polypeptides in the gel were visualized after staining for 12 h in Coomassie brilliant blue (Vesterberg, 1972). Apparent molecular weights of the polypeptides were calculated from their mobilities relative to those of protein standards run at both 7.5 and 10% acrylamide gel concentrations.

In experiments in which luciferase activity was to be recovered after electrophoresis, 2-mm sections were excised from an unstained $NaDodSO_4$ gel of luciferase. The gel disks were placed in 1 mL of renaturation buffer at 2–4 °C with or without the addition of purified luciferase subunit isolated by urea chromatography (see above). Proteolyzed samples for electrophoresis were preincubated in 150 mM phosphate buffer (pH 7.0) containing trypsin TPCK (4.2 mg/mL; 20:1 protein to trypsin) at 25 °C until <0.1% of the original luciferase activity remained (typically 30 min).

Results

The luciferases isolated and purified from each of the four species under study were found to possess analogous subunit structures; each was composed of two dissimilar subunits, which were described as relatively heavier and lighter on the basis of their apparent molecular weights from $NaDodSO_4$–polyacrylamide gel electrophoresis. Proteolytic treatment with trypsin of *B. harveyi* luciferase leads to degradation of the heavier (α) but not the lighter (β) subunit (Baldwin et al., 1978; Njus et al., 1974). A similar inactivation by trypsin was found to occur with the luciferases of the three *Photobacterium* species; degradation was also specific for the heavier subunits. Purified native luciferases from four species were proteolyzed with trypsin to <1% of their activity and were subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis. Two major bands were detected with each trypsinized luciferase, one in the same molecular weight region as the native light subunit and the other at a smaller apparent molecular weight (Table I) (Ruby & Hastings, 1979). Renaturation of the posttrypsinization polypeptides with untreated native subunits yielded active luciferase only for those combinations of an untreated heavy subunit with the heavier of the two bands remaining after trypsinization (Table II). This suggested that treatment with trypsin produced at least one cleavage in the heavier subunit of the bacterial luciferase, while the lighter subunit was left relatively unaffected, both with respect to electrophoretic mobility and ability to form active luciferase. Thus, by these structural analogies to the *B. harveyi* enzyme, it is proposed that the heavier and lighter subunits of the *Photobacterium* luciferases be designated α and β, respectively.

The eight subunits from the four luciferases were prepared in quantity by DEAE-Sephadex gel filtration in 7 M urea. Each isolated subunit was renatured in the presence of one of each of the other seven subunits in attempts to produce hybrid luciferases. Of the 28 possible combinations of subunit pairs, active luciferases were formed only between the four natural pairs and between five hybrid pairs (Figure 1). Two qualitative observations could be made: (1) all active combinations involved an α and a β subunit (no α_x–α_y or β_x–β_y pairings were active), and (2) neither subunit of *B. harveyi* luciferase formed detectably active hybrids with any of the *Photobacterium* subunits. (The limits of detectability were <1% of the native pair levels.)

Table I: Apparent Molecular Weights of Subunits of Luciferase before and after Trypsin Inactivation

species	subunit	apparent M_r[a] native	apparent M_r[a] + trypsin[b]
P. leiognathi	α	44000 ± 1000 (7)	33000 ± 500 (3)
	β	39000 ± 2000 (7)	39000 ± 2000 (3)
P. phosphoreum	α	44000 ± 1000 (6)	33000 ± 500 (3)
	β	37000 ± 2000 (6)	37000 ± 1000 (3)
P. fischeri	α	39000 ± 1000 (5)	31000 ± 500 (2)
	β	37000 ± 1000 (5)	37000 ± 1000 (3)
B. harveyi	α	43000 ± 1500 (2)	13000 ± 500 (2)
	β	33000 ± 1000 (2)	34000 ± 500 (2)

[a] Determined by the relative mobility during $NaDodSO_4$ electrophoresis of the luciferases in both 7.5 and 10% acrylamide gel concentrations. Values are the mean (± 2 standard deviations) of the number of determinations indicated in parentheses. [b] Prior to electrophoresis luciferases were treated with 4.2 mg/mL trypsin TPCK (molar ratio of luciferase to trypsin = 450:1 in 250 mM sodium phosphate buffer (pH 7.0) and 10 mM DTT for 40 min at 25 °C.

Table II: Luciferase Activity of Posttrypsinization Polypeptide Bands When Renatured with Intact α or β Subunit[a]

polypeptide band[d]	native luciferase subunits added[b] α (44000)[c]	β (39000)
band 1 (39000)	61[e]	2.6
band 2 (33000)	3.3	3.3

[a] Expressed in quanta per second × 10^{-9} after subtraction of the small background activity (less than 1.5×10^9 q s^{-1}) of individually renatured subunits. Experiment utilized luciferase from *P. leiognathi*; similar results were obtained with *P. fischeri* and *P. phosphoreum* luciferases. [b] Purified subunits prepared by column chromatography (see Materials and Methods). [c] Apparent molecular weight as determined by $NaDodSO_4$ gel electrophoresis. [d] From $NaDodSO_4$ gel of luciferase after trypsinization. Only two polypeptide bands were visible on stained gels. [e] Equivalent to 43% of the activity obtained when the β subunit from an equivalent amount of (untrypsinized) electrophoresed luciferase is renatured with column-purified α subunit.

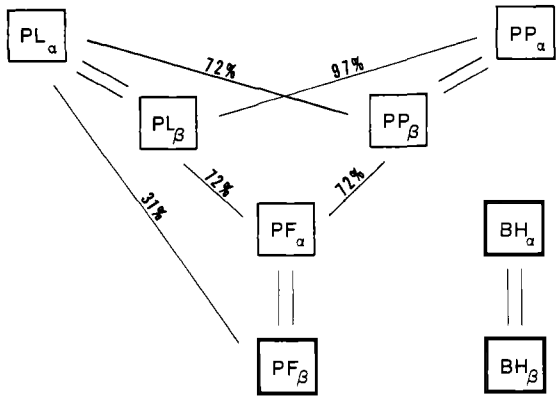

FIGURE 1: Pairs of renatured luciferase subunits that yield active enzyme. Isolated α and β subunits of the luciferases of four species of luminous bacteria [*P. leiognathi* (PL), *P. phosphoreum* (PP), *P. fischeri* (PF), and *B. harveyi* (BH)] were renatured in pairs. All 28 possible combinations were tried and only those pairs connected by lines yielded detectably active luciferase upon renaturation. Double lines connect natural pairs, and single lines connect pairs forming active hybrid enzymes. Percentages indicate the activity of the hybrid pair relative to the natural pair contributing the α subunit; values are the mean of four separate determinations.

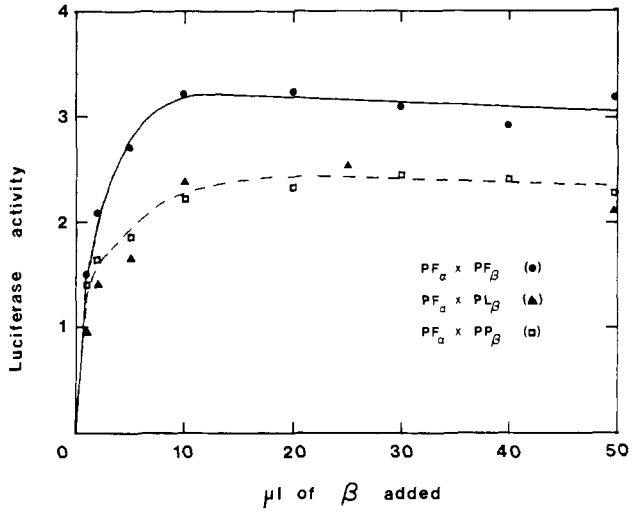

FIGURE 2: Luciferase activity after renaturation of a limiting amount of *P. fischeri* α subunit with increasing amounts of β subunit from three *Photobacterium* species: *P. fischeri* (PF), *P. leiognathi* (PL), and *P. phosphoreum* (PP). One activity unit is equal to 3.7×10^{10} quanta s^{-1}.

The amount of activity possessed by these hybrids (relative to the natural subunit pairs) was determined by titrating an aliquot of a given α subunit with increasing amounts of β subunit (Figure 2). Thus, the maximum activity of the luciferases could be quantitatively compared on a per α-subunit basis. The percent activity of each of the five hybrids (Figure 1) was then calculated relative to the activity of the natural enzyme pair that contributed the α subunit.

The aldehyde-specific catalytic properties of these five hybrid luciferases were determined by using aliphatic aldehydes of different carbon-chain lengths as substrate. The turnover rate constants and relative quantum yields were calculated and compared to those of luciferases reconstituted from natural pairs (Table III). In all cases, hybrid luciferases exhibited catalytic properties most like those of the natural luciferase contributing the α subunit to the hybrid pair.

Thermal denaturation rates of luciferase composed of *Photobacterium* subunits were determined at 38 °C. In most cases the stabilities of the hybrid enzymes were intermediate to those of the two native pairs of subunits (Figure 3). Hybrids formed between the α subunits of either *P. fischeri* or *P. phosphoreum* and the β subunit of *P. leiognathi* were

Table III: Catalytic Properties of Hybrid Luciferases as a Function of Substrate Aldehyde Carbon-Chain Length

subunits combined[a]		% act. rel to homologous pair	rate constant at 25 °C (s^{-1})		rel quantum yield	
α	β		C-12[b]	C-13	C-13/ C-9	C-12/ C-10
PL	PL	100	0.39	0.88	1.6	1.4
PL	PP	72 ± 2[c]	0.35	0.80	1.3	1.3
PL	PF	31 ± 6	0.40	0.89	1.5	1.5
PF	PF	100	0.48	1.30	0.9	1.1
PF	PL	72 ± 8	0.46	1.35	0.8	1.0
PF	PP	72 ± 18	0.42	1.30	0.8	1.0
PP	PP	100	0.19	0.39	17.8	4.2
PP	PL	97 ± 16	0.19	0.39	15.0	3.9
PP	PF	<1				

[a] Pairs of subunits were renatured together, including the heavy (α) and the light (β) subunit from either *P. leiognathi* (PL), *P. fischeri* (PF), or *P. phosphoreum* (PP). [b] C-12, dodecanal; C-13, tridecanal; C-9, nonanal; C-10, decanal. [c] Average of four determinations (±1 standard deviation).

Table IV: Thermal Stability of Hybrid Luciferases

subunits combined[a]		thermal denaturation constant (× 10^2) (min^{-1})
β	α	
PL	PL	1.8
PL	PP	5.3
PL	PF	2.0
PP	PP	46
PP	PL	11
PP	PF	26
PF	PF	41
PF	PL	63
PF	PP	nd[b]

[a] For explanation of notation, see Table III, footnote *a*. [b] Not determinable.

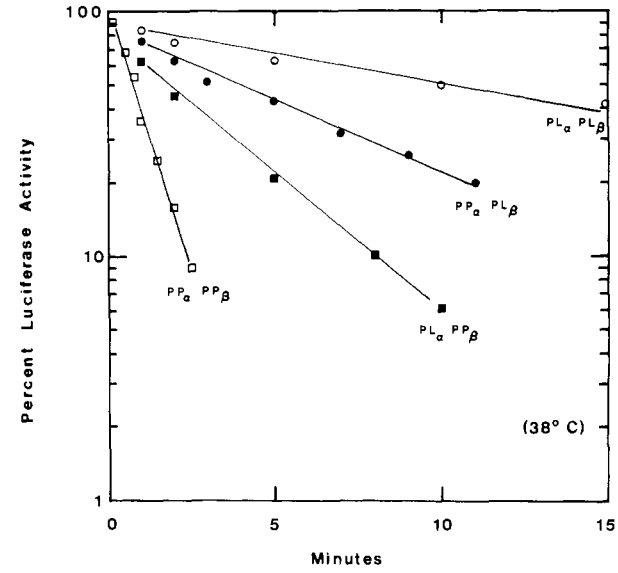

FIGURE 3: Rate of loss of enzymatic activity of renatured luciferases incubated at 38 °C. Luciferases are composed of α and β subunits from *P. leiognathi* (PL) and *P. phosphoreum* (PP). One activity unit is equal to 3.7×10^{10} quanta s^{-1}. The data for the hybrid luciferases (PLα + PPβ and PPα + PLβ) extrapolate to <90% activity at time zero due to contamination by whole *P. phosphoreum* enzyme (PPα + PPβ) in the individual PPα and PPβ preparations used to produce the hybrids. Because *P. phosphoreum* luciferase is rapidly deactivated by the heat treatment this contaminating activity disppears within the first 30 s. The denaturation of the hybrid luciferases is described by the more gradual rate of inactivation of the bulk of the enzyme activity.

substantially more stable than the native *P. fischeri* or *P. phosphoreum* enzymes (Table IV). In contrast, hybrids consisting of β subunits of these latter two species were only slightly more (1.6 times) or considerably less (6–35 times) stable to thermal denaturation. This implicates some feature of the β subunit of *P. leiognathi* in the relatively high thermal stability of enzymes containing it.

Discussion

It has been known for several years that both *B. harveyi* and *P. fischeri* luciferases are composed of two structurally dissimilar subunits (Friedland & Hastings, 1967b; Hastings et al., 1969). For *B. harveyi*, it is known that the subunits are also not functionally equivalent; only the larger or α subunit has been implicated in the catalysis (Hastings & Nealson, 1977). Two lines of evidence have suggested this conclusion: (1) chemical modification of individual subunits (Meighen et al., 1971) and (2) localization of lesions in altered-kinetics mutants to the α subunit (Cline & Hastings, 1972). The subunits of *P. fischeri* luciferase have properties that suggest functional analogies to those of *B. harveyi* (Meighen et al., 1970). However, attempts to produce active heterospecific hybrid luciferases between these two species were unsuccessful (Gunsalus-Miguel et al., 1972; Hastings et al., 1969).

Our results with the three *Photobacterium* species have shown that the heavy or α subunits of the luciferases have analogous structural and functional characteristics. The aldehyde-specific catalytic properties of turnover rate and quantum yield appear to be associated with the α subunit, with no discernible effect of the source of the β subunit involved in the dimer (Table III). The α subunit of *B. harveyi* luciferase has similarly been observed to control the enzyme's turnover rate (Cline & Hastings, 1972), and, due to homologies in tryptic peptides, the heavier subunit of *P. fischeri* luciferase was termed the α subunit as well (Meighen et al., 1970). It is proposed here that the heavier subunits of three luminous *Photobacterium* species (*P. fischeri*, *P. leiognathi*, and *P. phosphoreum*) be designated α subunits on the basis of functional analogies to the *B. harveyi* α subunit.

In their native state, all three *Photobacterium* luciferases were readily inactivated by trypsin. In each case the α subunit was cleaved to yield a smaller but still major peptide with an apparent molecular weight of 31 000–33 000 (Table I). The α subunit of *B. harveyi* was similarly attacked although a smaller major fragment was produced. The size(s) of the minor fragment(s) produced from the trypsinization of each of these luciferases is unknown. In addition, all three *Photobacterium* luciferases had β subunits that remained functionally active after trypsinization of the enzyme (Ruby & Hastings, 1979); no α-subunit activity was detected in any of the protein fragments remaining after trypsin treatment.

The degree of hybridization between subunits of *P. leiognathi* and *P. phosphoreum* was high (72–97% of the activity of the renatured natural pairs). Taxonomically these are the most closely related luminous species of *Photobacterium* and exhibit distinct but related genomic sequences as determined by DNA–DNA hybridization studies (Reichelt et al., 1976). The successful luciferase subunit hybridization in these bacteria is reminiscent of the situation observed among another family of heteromers, the tryptophan synthetases of different species of enteric bacteria (Balbinder, 1964). In this group of enzymes, small differences in primary structure were not sufficient to prevent effective heterospecific subunit interactions that restored catalytic activity.

P. fischeri exhibits a more complex pattern of luciferase subunit hybridization. Although the α subunit hybridizes well (72%) with β subunits of either *P. leiognathi* or *P. phosphoreum*, the β subunit of *P. fischeri* forms active hybrids to a smaller (31%) or undetectable (<1%) degree with the other *Photobacterium* species. Due to uncertainty concerning what factors of subunit structure are critical to the formation of active, renatured luciferase, it is difficult to assess the significance of these results. However, it does appear that during the speciation of the genus *Photobacterium*, divergence in those factors responsible for successful dimer formation has been greater between *P. fischeri* and the other two species than between *P. leiognathi* and *P. phosphoreum* themselves.[2] In addition, divergence between *B. harveyi* and the *Photobacterium* species has, as previously noted, been sufficient to preclude successful hybridization between these genera (Hastings et al., 1969). It should be mentioned, however, that there is evidence for intergeneric homologies as judged by competition in renaturation and immunological criteria (Gunsalus-Miguel et al., 1972).

Perhaps as an adaptation related to its tropical occurrence (Reichelt et al., 1977), *P. leiognathi* contains a luciferase that is considerably more stable to heat inactivation than those of other *Photobacterium* species (Table IV). The thermal denaturation constants of hybrid luciferases composed of *P. leiognathi* subunits are not easily interpreted, but it is clear that the β subunit of *P. leiognathi* increased the thermal stability of hybrid luciferases made with any of the *Photobacterium* species α subunits (Table IV). Single amino acid substitutions in the α subunit of *Escherichia coli* tryptophan synthetase have been reported to alter the thermal stability of the enzyme without grossly affecting its ability to form an active conformation (Yutani et al., 1977). Differences between the primary sequences of the β subunits of *P. leiognathi* and the other *Photobacterium* species may, in an analogous way, account for the increased thermal stability of hybrids made with *P. leiognathi* β subunit, as well as their high specific activity.

In addition to furthering our understanding of speciation within this family of enzymes, our findings suggest that bacterial luciferases provide a potential model system for the study of quaternary interactions of multimeric protein complexes in general. Bacterial luciferases are simple heterodimers which are easily obtainable in quantity and occur in evolutionarily related forms exhibiting distinct catalytic and structural properties (Tables III and IV). Thus, elucidation of such fundamental phenomena of protein chemistry as subunit interactions and mechanisms of catalysis can be approached through the continued study of hybrid luciferases.

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[2] A new species of luminous *Photobacterium*, *P. logei*, has been recently described (Bang et al., 1978). This taxon appears to be closely allied to *P. fischeri* and thus it would be of interest to examine the hybridization of subunits of this species' luciferase with those of its congeners.

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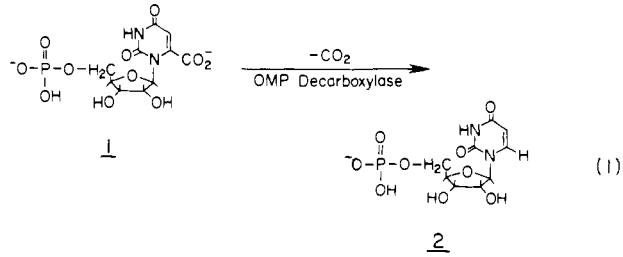
Inhibition of Orotidine-5′-phosphate Decarboxylase by 1-(5′-Phospho-β-D-ribofuranosyl)barbituric Acid, 6-Azauridine 5′-Phosphate, and Uridine 5′-Phosphate†

Howard L. Levine, Richard S. Brody,‡ and F. H. Westheimer*

ABSTRACT: 1-(5′-Phospho-β-D-ribofuranosyl)barbituric acid, an analogue of orotidylic acid, binds to orotidine-5′-phosphate decarboxylase about 100 000 times as strongly as does the substrate. The K_i at pH 6 is 9×10^{-12} M and the half-time for dissociation at 4 °C is about 10 h. The binding of the barbiturate analogue to the enzyme is thus one of the strongest interactions between small molecules and proteins that have been measured. The possibility that the inhibitor is a transition-state analogue is discussed.

Orotidine-5′-phosphate decarboxylase (OMP decarboxylase,[1] EC 4.1.1.23) catalyzes the conversion of orotidine 5′-phosphate (**1**) to uridine 5′-phosphate (**2**) (eq 1), in the de



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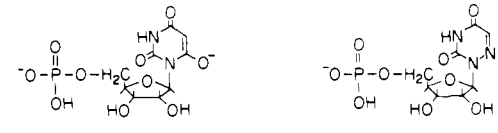
novo biosynthesis of pyrimidines (Lieberman et al., 1955). The enzyme from yeast has recently been purified to homogeneity by affinity chromatography (Brody & Westheimer, 1979; Reyes & Sandquist, 1978). In the course of the development of our affinity column (Brody & Westheimer, 1979), 1-(5′-phospho-β-D-ribofuranosyl)barbituric acid (**3**) was prepared.



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This compound has been shown to be a potent inhibitor of OMP decarboxylase from rat brain with a K_i of 4.1×10^{-9} M at pH 7.4 (Potvin et al., 1978). We now report that BMP is an extraordinarily powerful inhibitor of the yeast enzyme, binding so tightly that the stoichiometric enzyme–inhibitor complex can be purified by gel filtration with no hint of dissociation. The dissociation constant which we have measured for the binding of this inhibitor to yeast OMP decarboxylase is 9×10^{-12} M at pH 6 and 4 °C. Although the constant is much too small to be measured directly, it could be determined by a series of relays. The barbiturate inhibitor was equilibrated

† From the James Bryant Conant Laboratory of the Chemistry Department of Harvard University, Cambridge, Massachsetts 02138. *Received March 5, 1980.* This work was supported by National Institutes of Health Grant GM 04712-24.

‡ Present address: Department of Chemistry, Ohio State University, Columbus, OH 43210.

[1] Abbreviations used: OMP decarboxylase, orotidine-5′-phosphate decarboxylase; OMP, orotidine 5′-phosphate; UMP, uridine 5′-phosphate; BMP, 1-(5′-phospho-β-D-ribofuranosyl)barbituric acid; azaUMP, 6-azauridine 5′-phosphate; $NaDodSO_4$, sodium dodecyl sulfate.

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