Isolation of Bacterial Luciferases by Affinity Chromatography on 2,2-Diphenylpropylamine–Sepharose: Phosphate-Mediated Binding to an Immobilized Substrate Analogue†

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ABSTRACT: A covalently immobilized form of an inhibitor of bacterial luciferase, 2,2-diphenylpropylamine (DϕPA), was an effective affinity resin for purifying this enzyme from several distinct bacterial species. The inhibitor is competitive with the luciferase aldehyde substrate but enhances binding of the flavin substrate $FMNH_2$ (reduced riboflavin 5′-phosphate); comparable binding interactions occur with luciferase, the immobilized inhibitor DϕPA–Sepharose, and the substrates [Holzman, T. F., & Baldwin, T. O. (1981) *Biochemistry 20*, 5524–5528]. The effect of $FMNH_2$ on the binding of luciferase to DϕPA–Sepharose was mimicked by inorganic phosphate; the luciferase–phosphate complex had a greater affinity for DϕPA–Sepharose than did luciferase. This observation led to the development of a method using DϕPA–Sepharose to purify bacterial luciferase. When crude enzyme in a high-phosphate buffer was applied to a column of the affinity matrix, the luciferase activity was removed from solution. After the column was washed with the same buffer to remove unbound protein, the luciferase was eluted with a non-phosphate cationic buffer. The affinity column has proven useful for rapid purification of luciferase in much greater yield than has been previously possible with standard ion-exchange techniques. This approach has allowed one-step purification of luciferases from ammonium sulfate precipitates of *Vibrio harveyi*, *Vibrio fischeri*, and *Photobacterium phosphoreum*. The dissociation constants in 0.10 M phosphate for the affinity ligand:luciferase complexes were 0.49 μM, 0.28 μM, and 0.15 μM, respectively, for the three species. The dissociation constant for the *V. harveyi* mutant AK-6, which has normal aldehyde binding but greatly reduced affinity for $FMNH_2$, was 0.30 μM, while that for the *V. harveyi* mutant AK-20, which has greatly reduced affinity for aldehyde but a slightly increased affinity for $FMNH_2$, was 1.2 μM. Preliminary experiments indicated that the yellow fluorescence protein (YFP) that participates, through energy transfer, in bioluminescent emission in *V. fischeri* strain Y-1 could be separated from the luciferase in this strain by chromatography on the affinity matrix, whereas other methods of separating luciferase and YFP have had limited success because of the binding of YFP to luciferase.

A successful application of an affinity purification method relies on a suitable immobilized ligand to which a protein of interest will bind. The binding must be of sufficient strength so as to allow chromatographic separation of the protein from other contaminating molecules. The specificity of the interaction between the protein and the immobilized ligand is clearly of central importance. Specificity can be achieved in two ways. The protein may have a high intrinsic specificity for the immobilized ligand, or binding of a second ligand from solution may alter the affinity of the interaction with the immobilized ligand and thereby effect additional specificity. We have used the latter approach to develop an affinity column for the single-step purification of bacterial luciferase.

Bacterial luciferase is an αβ dimer that catalyzes the mixed-function oxidation of $FMNH_2$[1] and a long-chain aldehyde to yield carboxylic acid, FMN, and blue-green light [see the recent review by Ziegler & Baldwin (1981)]. A variety of methods have been described for the purification of this enzyme. Ion-exchange techniques have been successful and are widely used. The most common method was originally described by Gunsalus-Miguel et al. (1972), refined by Baldwin et al. (1975), used by Lee and co-workers (Lee & Murphy, 1975), and has been described in detail by Hastings et al. (1978). A single attempt to utilize affinity chromatography for the purification of bacterial luciferase has been reported (Waters et al., 1974). The affinity ligand chosen, FMN, was a product of the bioluminescence reaction. The data presented by Waters et al. show that luciferase does bind to FMN–Sepharose, but the use of this ligand proved to be ineffective because luciferase eluted from the column in low yield and low purity.

In a previous paper (Holzman & Baldwin, 1981a,b) we showed that the compound 2,2-diphenylpropylamine (DϕPA) is a competitive inhibitor of aldehyde binding to luciferase, while at the same time it enhances the binding of $FMNH_2$ to the enzyme. Similarly, the binding of enzyme to an immobilized derivative of DϕPA, DϕPA–bis[oxirane]–Sepharose, is enhanced in the presence of $FMNH_2$ and reduced in the presence of aldehyde. These data demonstrate cooperativity between the binding of $FMNH_2$ and the binding of DϕPA and, by implication, between $FMNH_2$ binding and aldehyde binding. On the basis of the specificity of the interaction between luciferase and DϕPA–Sepharose, we have successfully utilized this material as an affinity chromatography matrix for the purification of bacterial luciferase.[2] The strategy for the use of DϕPA–Sepharose has taken advantage of conformational alterations in luciferase structure associated with anion binding to enzyme, in particular the phosphate anion (Ziegler & Baldwin, 1981).

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[1] Abbreviations: DϕPA, 2,2-diphenylpropylamine; DϕPA–Sepharose, 2,2-diphenylpropylamine–bis[oxirane]–Sepharose; AH-Sepharose, aminohexyl-Sepharose; FMN, riboflavin 5′-phosphate; $FMNH_2$, reduced FMN; $NaDodSO_4$, sodium dodecyl sulfate; BSA, bovine serum albumin; Tris, tris(hydroxymethyl)aminomethane; DTE, dithioerythritol; LU, light unit; TLU, total light units; Bis-Tris, 2,2-bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol; DEAE, diethylaminoethyl.

[2] The use of DϕPA–Sepharose to purify luciferases from *V. harveyi*, *V. fischeri*, and *P. phosphoreum* was described at the 25th Annual Meeting of the Biophysical Society (Holzman & Baldwin, 1981a).

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The experiments reported here demonstrate that phosphate, like $FMNH_2$, enhances the binding of bacterial luciferase to DϕPA–Sepharose and therefore can be highly useful in an affinity chromatographic method for the purification of this enzyme. The luciferases from three strains of luminous marine bacteria, *Vibrio harveyi*, *Vibrio fischeri*, and *Photobacterium phosphoreum*, bind to the immobilized inhibitor with high affinity in the presence of phosphate. In the absence of phosphate, luciferase affinity for DϕPA–Sepharose is reduced. Studies with mutant enzymes support our proposal that luciferase binding interactions with DϕPA–Sepharose occur at a site on the enzyme normally reserved for the aldehyde substrate. We describe solution conditions useful for one-step purification of luciferases in ammonium sulfate fractions of cell lysates from several strains of luminous marine bacteria.

Materials and Methods

Chemicals. Trizma base, BSA, FMN, and *n*-decanal were obtained from Sigma Chemical Co., DϕPA, 1,4-butanediol diglycidyl ether (95%), *n*-dodecanal, and *n*-tetradecanal from Aldrich Chemical Co., and Sepharoses from Pharmacia Fine Chemicals. All other chemicals were of reagent quality.

Bacterial Cultures. The bacterial strains utilized have been recently reclassified (Baumann et al., 1980). *V. harveyi*, formerly *Beneckea* (strain 392), *V. fischeri*, formerly *Photobacterium* (ATCC 7744), *V. fischeri* strain Y-1 (Ruby & Nealson, 1977), and *P. phosphoreum* (NCMB 844) were grown and harvested as previously described (Hastings et al., 1978). Mutant strains, designated AK-6, AK-20, and M-17, derived from *V. harveyi*, were produced and characterized by Cline (Cline & Hastings, 1972).

Synthesis of DϕPA–Sepharose. Sepharose gel beads were epoxy-activated by the method of Sunderberg & Porath (1974). The affinity ligand was then coupled to the epoxy-activated resin as previously described (Holzman & Baldwin, 1981b). The resulting product was referred to as DϕPA–Sepharose. These previous papers have described in detail the synthesis and proposed structure of the affinity resin.[3]

Luciferase Assays. Luciferases from the three species of bacteria were all assayed by the standard $FMNH_2$ injection assay (Hastings et al., 1978). In this assay catalytically reduced FMN is rapidly injected into a vial containing 1.0 mL of assay buffer [20 mM phosphate (pH 7.0)–0.2% BSA], 10 μL of an aldehyde suspension, and a small aliquot of enzyme. The aldehyde suspensions were prepared by sonication in distilled water of 0.1% (v/v) *n*-decanal for *V. harveyi* and 1% *n*-dodecanal for *V. fischeri* and *P. phosphoreum*. During purification of *P. phosphoreum* luciferase, *n*-tetradecanal was used because the initial maximum light intensity obtained was $\sim$10× that seen with *n*-dodecanal. The *n*-tetradecanal (heated to $\sim$50 °C to melt the aldehyde) was prepared as a 1% (w/v) suspension in distilled water by sonication. Maximum luminescence was obtained with $\sim$25 μL of this suspension. All aldehyde suspensions were prepared immediately before use and were never used longer than 2–3 h to minimize interference from oxidation of the aldehyde.

Electrophoretic Analysis. Protein samples were analyzed for purity by electrophoretic analysis on discontinuous 15% polyacrylamide slab gels prepared by the methods of Davis (1966), Laemmli (1970), and Matsudaira & Burgess (1978). The technique has been described in detail previously (Holzman & Baldwin, 1980a,b). All samples to be analyzed were prepared so that they could be mixed with an equal volume of the 2× $NaDodSO_4$ sample buffer of Matsudaira & Burgess (1978), with 0.5–30 μg of protein/gel well. After electrophoresis the protein bands were stained for several hours with a Coomassie Brilliant Blue R-250 solution [ranging from 0.25 to 0.5% (w/v)] in 25% 2-propanol–10% acetic acid–65% water (fast destain). Destaining was accomplished with the aid of gentle agitation in fast destain for 1–2 h, followed by 7.5% methanol–10% acetic acid in water (slow destain) until the gel background was clear (usually 5–10 h).

Binding Analyses. The samples of highly purified luciferase used for analysis of binding to the affinity matrix were prepared by the standard ion-exchange purification procedure (Hastings et al., 1978) to preclude enzyme contact with the affinity resin. The final purification step for each luciferase sample was chromatography on aminohexyl-Sepharose 4B by a modification of the method of Cousineau & Meighen (1976) (see Results and Discussion).

The binding of samples of wild-type and mutant luciferases to DϕPA–Sepharose was analyzed by titrating the resin with luciferase. Titrations were performed by incubating various amounts of luciferase with constant amounts of the affinity resin. After incubation for $\sim$1 h, the samples were centrifuged to pellet the affinity resin, and the supernatant was assayed for luciferase activity by the standard injection assay.

The apparent K_d for equilibrium binding of each luciferase to the resin and the resin binding capacity for each enzyme were determined based on the following assumptions: (1) a 1:1 binding stoichiometry between each enzyme and affinity ligand molecule and (2) equal accessibility and independence of all resin enzyme binding sites. The following quantities were then defined: $[L_t]$, the total molar concentration of luciferase binding sites on a fixed amount of DϕPA–Sepharose; $[E_t]$, the total molar concentration of luciferase added to solution; [L], the molar concentration of free luciferase binding sites; [E], the molar concentration of free luciferase; [EL], the molar concentration of bound luciferase. Similarly, the equilibrium and mass conservation relations were defined as

$$K_d = [E_i][L_i]/[EL_i] \quad (1)$$

$$[L_t] = [L_i] + [EL_i] \quad (2)$$

where, for a given K_d, the i's denote a series of values in the titration of a fixed amount of affinity resin, $[L_t]$. The equilibrium and conservation relations may be combined to give

$$[E_i] = [E_i]([L_t]/[EL_i]) - K_d \quad (3)$$

A plot of $[E_i]$ vs. $[E_i]/[EL_i]$ should give a straight line with a slope of $[L_t]$ and an intercept of $-K_d$. The values of K_d and $[L_t]$ were calculated from a least-squares fit of the data to eq 3.

Results and Discussion

(I) Binding of Luciferases to DϕPA–Sepharose. The binding of highly purified samples of *V. harveyi*, *V. fischeri*, and *P. phosphoreum* wild-type and mutant luciferases was analyzed by titrating samples of the affinity resin with each luciferase. The data for the binding of wild-type luci se from *V. harveyi* to the affinity resin were representati e binding properties of the different luciferases and ar in Figure 1 according to eq 3 (Materials and Meth values of K_d and $[L_t]$ calculated from the least-squar of binding data for each luciferase are presented

The data demonstrated that the binding was b either there were two classes of available binding luciferases, or the luciferase samples were hetero respect to their ability to bind the resin. While that the apparent heterogeneity is due to binding

[3] U.S. and foreign patent applications on file.

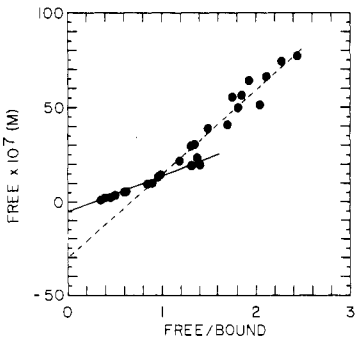

FIGURE 1: Binding analysis of wild-type *V. harveyi* luciferase to DφPA–Sepharose. The titration data were plotted according to eq 3 as described under Materials and Methods. Each data point represents the amount of free luciferase in equilibrium with 5.0 mg (dry weight) of DφPA–Sepharose resuspended in 1.0 mL of 0.10 M phosphate (pH 6.5)–1 mM DTE at 4 °C. In this plot the line slope is equal to the total molar concentration of luciferase binding sites on a fixed amount of resin. The intercept is equal to the apparent dissociation constant for the luciferase:immobilized-inhibitor complex. The solid line is drawn through binding data for the high-affinity binding sites. The dashed line is drawn through the data for the low-affinity binding sites. The biphasic titration behavior observed (see text) for wild-type *V. harveyi* enzyme was representative of all luciferases examined (Holzman, 1982). The apparent dissociation constants and resin capacities for a variety of luciferases are presented in Table I.

Table I: Apparent Dissociation Constants for Binding of Bacterial Luciferases to DφPA-Sepharose[a]

	$K_{d,app}$ (μM)				
luciferase	LAS	HAS	$FMNH_2$	decanal	gel capacity[b]
V. harveyi	2.9	0.49	0.88	6.8	4.6–6.1; 1/2.5
AK-6	3.3	0.30	140	7.0	8.5–11; 1/4.9
AK-20	3.1	1.2	0.15	64	6.0–8.0; 1/1.4
V. fischeri	1.0	0.28	NA[c]	NA	6.0–8.0; 1/1.9
P. phosphoreum	1.0	0.15	NA	NA	4.4–5.8; 1/2.5

[a] Binding studies performed at pH 6.5 in 0.10 M phosphate–1 mM DTE, 4 °C; LAS, low-affinity binding sites; HAS, high-affinity binding sites. [b] Gel capacity for pure luciferase (mg/mL of gel); ratio of HAS capacity to LAS capacity. [c] NA, not available.

of the luciferase samples, this explanation seems unlikely. It is far more likely that the "high" and "low" affinity binding sites reflect the different structures and/or environments of the resin-bound affinity ligand produced during resin synthesis (Holzman & Baldwin, 1981a,b).

The binding of luciferases from wild-type and two mutant strains of *V. harveyi* demonstrated that the mutant having decreased affinity for aldehyde also had decreased affinity for the resin, while the mutant having decreased affinity for $FMNH_2$ had the same affinity for the resin as wild-type enzyme (Table I). Furthermore, the luciferases from *V. fischeri* and *P. phosphoreum*, which have higher affinities than the luciferase from *V. harveyi* for the longer chain aldehydes (C_{12} and C_{14}; Spudich, 1963; Hastings & Nealson, 1977; Watanabe & Nakamura, 1972), also have a higher affinity for the resin.

We have previously shown that anions, in particular the phosphate anion, have a marked effect upon luciferase structure, activity, and its susceptibility to inactivation by a variety of agents (Baldwin & Riley, 1980; Holzman & Baldwin, 1980a,b; Holzman et al., 1980). The effect of phosphate on the binding of luciferase to the affinity resin is shown in Figure 2. Luciferase binding to the immobilized inhibitor was greatly enhanced in the presence of phosphate. $FMNH_2$ enhances the binding of luciferase to the immobilized inhibitor in a similar fashion (Holzman & Baldwin, 1981a,b). It was previously noted by Meighen & MacKenzie (1973) that

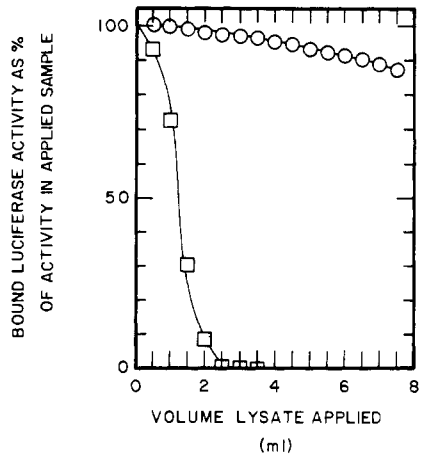

FIGURE 2: Effect of phosphate on binding of luciferase to DφPA–Sepharose 6B. An ammonium sulfate precipitate (40–80% cut) of *V. harveyi* cells was prepared as described under Materials and Methods. Equal amounts of the ammonium sulfate paste were dialyzed into either 100 mM phosphate, pH 7.0–0.50 mM DTE or 10 mM Bis-Tris–90 mM NaCl (pH 7.0)–0.50 mM DTE. After dialysis the samples were diluted with the appropriate buffer to give luciferase activities equivalent to a concentration of pure luciferase of 0.10 mg/mL. The samples were then applied in 0.50-mL aliquots to a 1.0-mL affinity column of DφPA–Sepharose 6B. In each case the column was preequilibrated in the appropriate buffer, and 0.50-mL fractions were collected and assayed for nonbound luciferase activity. On the basis of the activity in the applied lysate, the percent of bound luciferase activity for each fraction collected was calculated: (○) 100 mM phosphate; (□) 10 mM Bis-Tris–90 mM NaCl.

the secondary hydroxyl groups on the flavin side chain affect aldehyde binding affinity. These observations strongly suggest that the binding of phosphate or $FMNH_2$ to luciferase alters the conformation of the aldehyde–inhibitor binding site.

(II) General Procedures for Isolating Bacterial Luciferases by Affinity Chromatography. The empirical binding and elution conditions for different luciferases to DφPA–Sepharose were determined. The conditions giving the purest enzyme in maximum yield, in the shortest time, are described in the following purification steps (all performed at 4 °C unless otherwise noted).

(1) Osmotic Lysis. Cell lysis was achieved by the osmotic lysis method (Baldwin et al., 1975; Hastings et al., 1978). Alternatively, the frozen cell mass was chopped into small pieces of ~2–5 g and placed directly into the stirring distilled water. When this method was used, the time required for complete lysis was lengthened somewhat, to 1 or 2 h. In addition, it was observed that, for either method, the ratio of 10 mL of distilled water to 1 g of frozen cells could be reduced to a 5 to 1 ratio without affecting either the time for or extent of lysis. The pH of the lysate was monitored regularly and adjusted to neutrality with 2 M K_2HPO_4.

(2) Ammonium Sulfate Fractionation. The use of this step prior to affinity chromatography was found to be essential. The luciferase concentration of centrifuged cell lysates was low, and the solution was too viscous to flow through the affinity column at a reasonable rate. Furthermore, contaminating protein bound to the affinity resin and reduced its binding capacity for luciferase.

The cell debris and the majority of the detectable proteinases (Holzman, 1982) were removed in the lysate fraction precipitating between 40 and 80% ammonium sulfate (the exact range depending upon the species of bacteria). The ammonium sulfate fractionation provided about a 3-fold purification and resulted in a paste containing 100% of the luciferase (see Tables III–V). When the ammonium sulfate paste was dialyzed into the affinity column application buffer, a highly

Table II: Application and Elution Buffers for Affinity Chromatography of Luciferases from *V. harveyi*, *V. fischeri*, and *P. phosphoreum*

bacterial strain	application buffer	elution buffer
V. harveyi	0.10 M phosphate, pH 8.5, 0.50 M NaCl, 0.50 M KCl, 0.5 mM DTE	25 mM ethanolamine, 5.0 mM Tris, pH 9.1, 0.5 mM DTE
V. fischeri	0.10 M phosphate, pH 7.0, 0.5 mM DTE	0.30 M Tris, pH 8.5, 0.5 mM DTE
P. phosphoreum	0.35 M phosphate, pH 7.0, 0.5 mM DTE	0.10 M Tris, pH 8.1, 0.5 mM DTE

concentrated sample of luciferase was obtained that was suited for application to the affinity column. In the case of the luciferases from *V. harveyi* and *V. fischeri*, once the enzymes were prepared as ammonium sulfate pastes and frozen at −20 °C, they were stable for at least 1 year. The enzyme from *P. phosphoreum* was found to be unstable as a frozen ammonium sulfate paste, and for long-term storage it was dialyzed into phosphate buffer (pH 7.0)–0.5 mM DTE containing 40% glycerol and stored at −20 °C (M. Ziegler, unpublished observation) (see Table IV).

(*3*) *Affinity Chromatography on DφPA–Sepharose.* The ammonium sulfate cuts of lysates to be chromatographed were dialyzed against three changes of a 10-fold volume excess of application buffer (see Table II). The buffer from the last dialysis was used to equilibrate the column before sample application and to elute unbound protein prior to introduction of elution buffer (Table II). The use of this buffer for column equilibration and washing generally resulted in a higher luciferase binding capacity than fresh application buffer. This effect is not understood, but it appears to be related to incomplete dialysis of salts.

Although a thorough analysis of the pH dependency of binding and elution was beyond the scope of this work, preliminary experiments indicated that in anionic buffers the luciferases would bind to the resin anywhere in their pH stability range—roughly pH 6.5–9.5. In cationic buffers the best yields of luciferase upon elution were seen in the alkaline range, pH 8–9.5. However, in these high-pH buffers some of the luciferases tended to spontaneously denature. In the cationic buffers, at pH values ≥9, the $t_{1/2}$ values for inactivation at 4 °C varied from about 6 to 24 h. The luciferase from *P. phosphoreum* was the least stable, and *V. harveyi* luciferase was the most stable. The rate of spontaneous denaturation in high-pH cationic buffer was reduced by placing enough 1.0 M sodium phosphate–potassium phosphate, pH 7.0 (~0.1 mL), in the fraction collection tubes to drop the pH to near neutrality and to provide anion stabilization as the enzyme was eluted from the resin. The column was washed after use with at least three column volumes of a cleaning solution of 25% ethanol–4 M urea–0.18 M phosphate, pH 7.0. For long-term storage, the column was equilibrated in 50% ethanol–water and kept at 4 °C.

For general-purpose purifications, the method outlined above worked well. We did, however, find a variety of conditions that were potentially suitable but less economical, both in terms of time and experimental complexity (Holzman, 1982). With all luciferases tested (*V. harveyi*, *V. fischeri*, and *P. phosphoreum*) the general conditions for binding of luciferase (and some other proteins) required that the application buffer have an anionic buffering species (e.g. phosphate, borate, or arsenate). The best elution conditions required that the buffering species be cationic and/or neutral, for example, ethanolamine, Tris, or Bis-Tris.

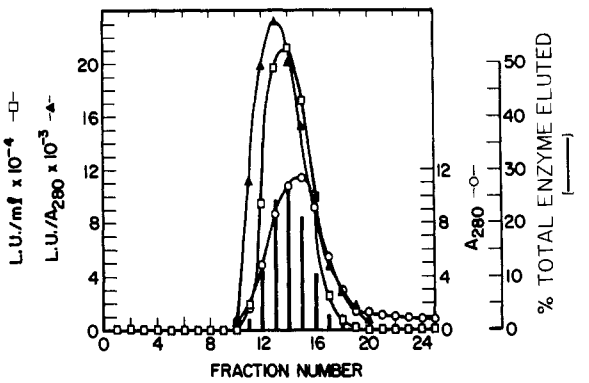

FIGURE 3: Elution profile of a sample of *V. harveyi* luciferase from a 15-mL DφPA–Sepharose 6B column. The luciferase sample, from a 40–80% ammonium sulfate cut of lysed cells, was dialyzed into sample application buffer: 0.10 M phosphate (pH 8.5)–0.50 NaCl–0.50 M KCl–1 mM DTE. Sample and/or application buffer flow rate was ~40 mL/h and was maintained with a peristaltic pump. An 86-mL aliquot of sample with a luciferase activity of ~31 000 LU/mL and an A_{280} of ~42.5 OD was pumped onto the column. Sample application was halted, and the application buffer wash was started when the luciferase activity eluting was 5–10% of that being applied. The total activity recovered in the eluted sample and application buffer wash (254-mL total) was 250 000 LU. Based on these data, about 91% of the applied activity was bound to the resin during sample application. After the eluted A_{280} and activity were back to near-base-line values, the elution buffer was started and its flow maintained at 5–10 mL/h. Fractions of ~2.5 mL were then collected: (○) A_{280}; (□) LU/mL; (▲) LU/A_{280}. The total activity recovered in the pooled column fractions was 2.26×10^6 LU or about 85% of applied activity and >95% of bound activity. A second affinity column was run (data not shown) with the remaining fraction of the dialyzed 40–80% ammonium sulfate cut (Table III). The recovery of the applied activity from the pooled samples from the second column was >90%.

(*4*) *Aminohexyl-Sepharose Chromatography.* Affinity chromatography was useful for purifying a substantial portion of the luciferase bound to the affinity column to near homogeneity (see Figures 6, 7, and 9). In those cases where the entire eluate from the affinity column was pooled (see Figures 3 and 4) or if one wished to purify affinity column side cuts, then a final purification step of ion-exchange chromatography on aminohexyl-Sepharose was used to remove the remaining contaminants.

(*III*) *Purification of Luciferase from V. harveyi.* The affinity column purification of the luciferase from *V. harveyi* by the simple "on–off" strategy discussed above is presented in Figure 3. The large amount of unbound (non-luciferase) protein eluted from the column was collected in large fractions that were monitored for absorbance at 280 nm and luciferase activity (data not shown in figure). The activity (pooled for maximum yield) from the column presented in Figure 3, and another exactly like it, was chromatographed on AH-Sepharose 4B (Figure 4). In Table III the yields at the various stages of the purification procedure are presented. The purity of the luciferase at each stage of the procedure may be seen in the $NaDodSO_4$ slab gel presented in Figure 5. On the basis of the data in Table III and the gel in Figure 5 it was evident that the simple column technique for affinity purification of the luciferase from *V. harveyi* was quite effective. Comparison of the affinity technique with the standard purification procedure of Hastings et al. (1978), based on the pools of luciferase taken at the affinity column (Table III) and DEAE-Sephadex stages, revealed that the yield of the affinity procedure was 5 times that of the standard method for fractions of similar purity (specific activity). Furthermore, if the fractions from the affinity column had been pooled conservatively for high specific activity, it was evident that the affinity

Table III: Purification of *V. harveyi* Luciferase on DϕPA-Sepharose 6B

step or fraction	vol (mL)	A_{280}	total A_{280}	act.[a] (LU/mL × 10^{-3})	total act. (LU × 10^{-6})	sp act. (LU/A_{280})	x-fold purification (previous step)	time/step	yield (% initial total act.)
lysate[b]	1100	28.0	30 800	5.3	5.85	189		~1 h	100
40% ammonium sulfate supernatant	1100			5.3	5.85			~2-h centrifugation	
80% ammonium sulfate precipitate after dialysis	245	42.5	10 412	23.9	5.85	562	2.98	~2-h centrifugation, 12-h dialysis	
affinity column pool[c]	55.2	4.82	266	93.5	5.16	19 400	34.5	~32 h[c,d]	88
AH-Sepharose column pool[c]	76.6	1.58	121	50.2	3.84	32 000	1.65	~6 h[d]	66

[a] Activity measurements made with *n*-decanal. [b] From 100 g of frozen cells. [c] Dialyzed ammonium sulfate precipitate split into two samples, each was separately chromatographed (~16 h/run) on the affinity column (Figure 4). [d] The columns were preequilibrated in the appropriate buffer. AH-Sepharose column profile is presented in Figure 5.

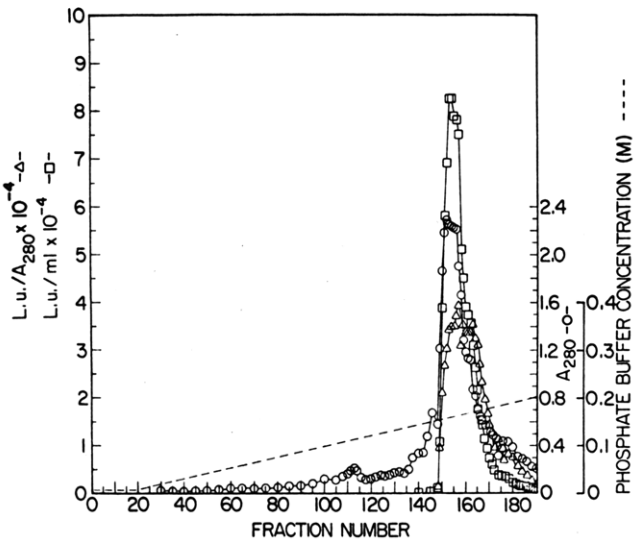

FIGURE 4: AH-Sepharose 4B chromatography of affinity-purified *V. harveyi* luciferase. The samples of luciferase from the affinity column in Figure 3 (see also Table III) were pooled and dialyzed against the AH-Sepharose column application buffer: 5.0 mM phosphate, pH 7.0–0.50 mM DTE. The sample was then pumped (~60 mL/h) on to the column bed (1.2 × 90 cm, ~100 mL), which was preequilibrated in application buffer. The luciferase was then eluted with a linear gradient from 5.0 to 350 mM phosphate (400 mL each) (pH 7.0)–0.50 mM DTE: (○) A_{280}; (□) LU/mL; (△) LU/A_{280}. Fractions 150–168 were pooled, giving a total recovery of 3.84 × 10^6 LU with a specific activity of ~32000 LU/A_{280} in 76.6 mL or a 66% yield from the initial cell lysate (see Table III).

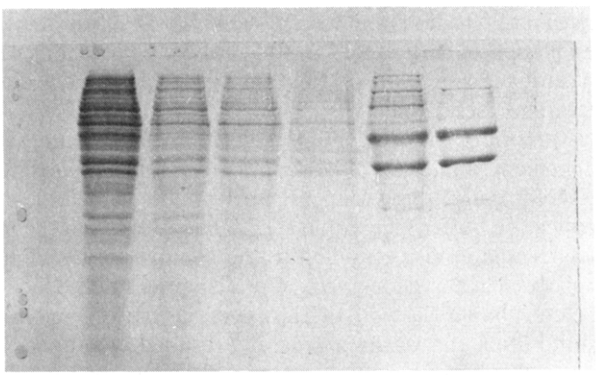

FIGURE 5: $NaDodSO_4$–polyacrylamide gel electrophoresis of *V. harveyi* bacterial luciferase at various stages in affinity purification scheme. Samples were prepared for electrophoresis as discussed under Materials and Methods and were loaded on the gel so as to give 5–10 μg of protein/well. Left to right: (lane 1) dialyzed 40–80% ammonium sulfate cut; (lanes 2–4) pools of the 1st, 2nd, and 3rd application buffer washes of the affinity resin containing nonbound protein; (lane 5) pooled affinity column eluate (fractions 11–19, Figure 3); (lane 6) pooled AH-Sepharose column fractions (fractions 150–168, Figure 4).

technique was capable of producing nearly homogeneous *V. harveyi* luciferase (Figure 6).

(IV) Purification of Luciferase from V. fischeri. The luciferase from *V. fischeri* was purified by a method similar to that used for the enzyme from *V. harveyi*. In order to recover the maximum amount of luciferase activity from the cell lysate, it was necessary to perform a 35–80% ammonium sulfate cut. The affinity column application and elution buffers found to be most effective for purifying the *V. fischeri* luciferase are presented in Table II, and the column elution profile is shown in Figure 7. The purity of the luciferase after affinity chromatography was analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis and is shown in the inset in Figure 7. In Table IV the yields from the affinity procedure are presented. It was evident from the data that fraction 22 from the affinity column contained ~55% of the applied luciferase activity (or ~65% of bound) and had undergone ~110-fold purification from the applied sample. Thus the affinity technique appeared to be quite useful for rapid purification of the luciferase from *V. fischeri* in high yield. Finally, if a greater yield of highly purified enzyme was desired, a broad cut of activity from the affinity column was chromatographed on AH-Sepharose (data not shown) in exactly the same manner as that described for the *V. harveyi* luciferase (Figure 4). The elution profile of the luciferase from *V. fischeri* on AH-Sepharose was the same as that seen for the *V. harveyi* enzyme.

(V) Chromatographic Behavior of Luciferase and Yellow Fluorescence Protein from V. fischeri Strain Y-1. The *V. fischeri* strain Y-1 has a yellow bioluminescence emission (λ_{max} at 545 nm and a shoulder at 490 nm) in comparison to the wild-type strain (λ_{max} at 490 nm) (Ruby & Nealson, 1977). Extracts of the strain have been found to have, in addition to luciferase, a yellow fluorescent protein (YFP) that is required for the 545-nm emission and participates in emission through an energy transfer mechanism (Leisman & Nealson, 1981). In attempting to purify this protein, Leisman found that in the standard purification techniques of ion-exchange and/or gel-permeation chromatography, the YFP copurified with the luciferase, making it difficult to study each protein independently (G. Leisman, personal communication). Thus, a preliminary examination of the chromatographic behavior of the mixture of luciferase and YFP, present in the dialyzed 35–80% ammonium sulfate cut of a Y-1 cell lysate, was un-

Table IV: Purification of *V. fischeri* Luciferase on DφPA–Sepharose 6B

step or fraction	vol (mL)	A_{280}	total A_{280}	act.[a] (LU/mL × 10^{-3})	total act. (LU × 10^{-3})	sp act. (LU/A_{280})	x-fold purification (previous step)	time/step	yield (% initial total act.)
lysate[b]	55.5	27.4	1520	5.75	319	210		~1 h	100
35–80% ammonium sulfate precipitate after dialysis	13	38.8	504	24.5	318	631	3.0	~2-h centrifugation	100
affinity column, analytical scale, 1.0-mL bed volume[c]								<2 h for entire process	
(I) breakthrough and wash (fractions 1–20)	20	~25	~500	2.3	45.5	91			14.3
(II) affinity eluate									
fraction 21	1.0	2.16	2.16	32.7	32.7	15 140	24		10.3
fraction 22	1.0	2.50	2.50	173	173	69 100	110		54.1
fraction 23	1.0	2.32	2.32	48.5	48.5	21 000	33		15.2

[a] Activity measurements made with *n*-dodecanal. [b] From 5.1 g of frozen cells. [c] The column profile is presented in Figure 8.

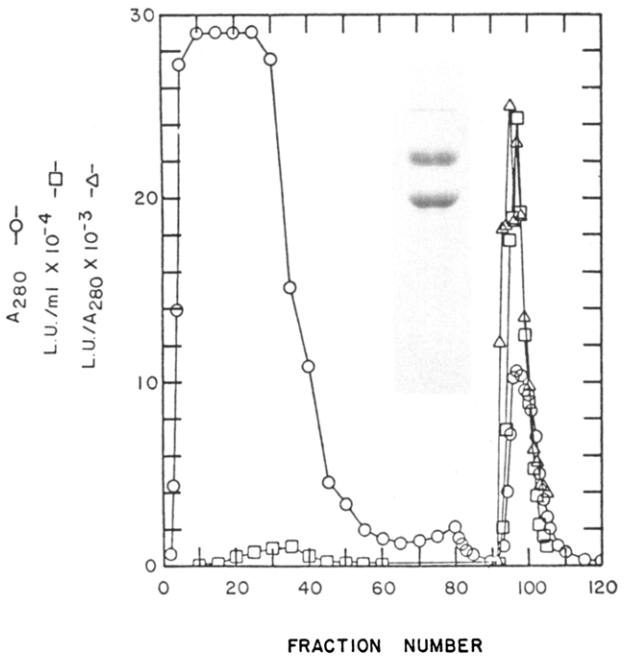

FIGURE 6: Affinity chromatography of wild-type *V. harveyi* luciferase on DφPA–Sepharose 6B. Eluted enzyme was pooled for maximum specific activity. The enzyme sample was from a 40–80% ammonium sulfate cut of a cell lysate (see Materials and Methods). Chromatographic profiles: (○) A_{280}; (□) LU/mL; (△) LU/A_{280}. The sample was applied in the affinity column application buffer (see Table II), beginning at fraction 1, with a peristaltic pump at ~40 mL/h. The 30-mL column was preequilibrated in application buffer. The applied sample contained luciferase at an activity of ~53 000 LU/mL. The sample application was halted and an application buffer wash was started when the eluted luciferase activity was 3–5% of that being applied (at fraction 20). A total of 74.2 mL of sample (3.94×10^6 TLU) was applied. After the A_{280} and activity in the wash were back to near-base-line values, the elution buffer (see Table II) was started at fraction 63. Its flow was maintained at 5–10 mL/h. The total activity recovered in a conservative high specific activity pool (fractions 93–98) was 2.24×10^6 LU or a 57% yield of applied activity. The pooled fractions were analyzed for purity by $NaDodSO_4$–polyacrylamide gel electrophoresis (inset). The sample for electrophoresis was prepared as discussed in Materials and Methods and was loaded so as to give ~15 μg of protein per well.

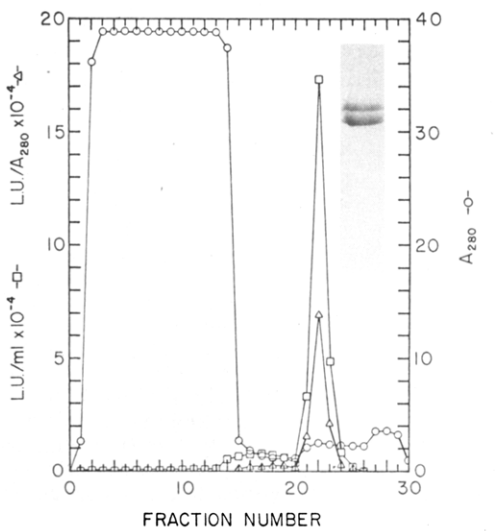

FIGURE 7: Chromatography of an aliquot of a 35–80% ammonium sulfate cut of wild-type *V. fischeri* cell lysate on DφPA–Sepharose 6B. Chromatographic profiles: (○) A_{280}; (□) LU/mL; (△) LU/A_{280}. The sample was applied in the *V. fischeri* luciferase application buffer (Table II) and was loaded on the column (beginning at fraction 1) with a volumetric pipet. The 1.0-mL column was preequilibrated in application buffer and was run as fast as the resin bed would permit (40–60 mL/h). The sample contained luciferase at an activity of ~24 500 LU/mL. The sample application was halted and an application buffer wash was started when the eluted luciferase activity was 3–5% of that being applied (at fraction 13). A total of 13 mL of sample was applied. The resin bed was then washed with seven 1.0-mL aliquots of application buffer. Elution buffer (see Materials and Methods) was then applied (beginning at fraction 21), in 1.0-mL aliquots. Comparative recoveries of the eluted luciferase fractions are presented in Table IV. At fraction 27 the column cleaning buffer was applied. The peak specific activity fraction (22) was examined for purity by $NaDodSO_4$–polyacrylamide gel electrophoresis (inset) in the standard fashion discussed under Materials and Methods and was loaded on the gel so as to give ~10 μg of protein/well.

dertaken to determine the effectiveness of the affinity matrix in separating the two proteins. In Figure 8 the chromatographic profiles of the luciferase activity and the yellow fluorescence are presented. Visual inspection of the material eluting in the application buffer wash and elution buffer revealed no yellow fluorescence in the elution buffer wash. It was evident from the trailing of the fluorescence during the application buffer wash that the fluorescent material was retained on the column to some extent. Presumably this effect was due to the interaction of the YFP with the luciferase bound to the affinity matrix. This experiment indicated that the affinity resin could be useful for separating YFP from luciferase.

(*VI*) *Purification of Luciferase from P. phosphoreum.* The luciferase from *P. phosphoreum* was purified by the same

Table V: Purification of *P. phosphoreum* Luciferase on DϕPA-Sepharose 6B

step or fraction	vol (mL)	A_{280}	total A_{280}	act.[a] (LU/mL × 10^{-4})	total act. (LU × 10^{-5})	sp act. (LU/A_{280})	x-fold purification (previous step)	time/step	yield (% initial total act.)
lysate[b]	13.7	29.1	399	6.23	8.53	2141		~1 h	100
35–80% ammonium sulfate precipitate after dialysis	7	18.4	129	12.1	8.52	6020	3.1	~2-h centrifugation	100
affinity column, analytical scale, 1.0-mL bed volume[c]								<2 h for entire process	
(I) breakthrough and wash (fractions 1–15)	15	8.2	123	0.394	0.59	480			6.9
(II) affinity eluate (pooled fractions 18–22)[d]	5	1.31	6.56	9.66	4.83	73700	12		57

[a] Activity measurements made with *n*-tetradecanal. [b] From 1.4 g of frozen cells. [c] The column profile is presented in Figure 10. [d] For long-term storage the enzyme was dialyzed into 0.1 M sodium phosphate–potassium phosphate buffer with 40% glycerol; the enzyme loses specific activity if frozen in aqueous solution or as an ammonium sulfate paste.

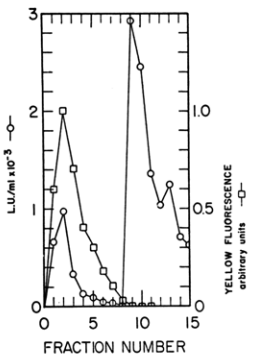

FIGURE 8: Chromatographic behavior of luciferase and yellow fluorescence protein from *V. fischeri* strain Y-1 on DϕPA–Sepharose 6B. Profiles of luciferase activity (○) (LU/mL) and yellow fluorescence (□). A 1.0-mL sample from a 35–80% ammonium sulfate cut of a Y-1 cell lysate was applied in the appropriate application buffer (Table II) at fraction 1 with a volumetric pipet. The 1.0-mL column was preequilibrated in application buffer and was run as fast as the resin bed would permit (40–60 mL/h). The sample contained luciferase at an activity of ~16 000 LU/mL. The resin bed was then washed with seven 1.0-mL aliquots of application buffer. Elution buffer (Table II) was then applied (beginning at fraction 9) in 1.0-mL aliquots. The elution buffer flow was halted after the majority of the luciferase was recovered (~13 000 LU). It was visually evident (using a long-wave UV hand lamp) that the yellow fluorescent material had eluted in the application buffer wash and not with the luciferase activity.

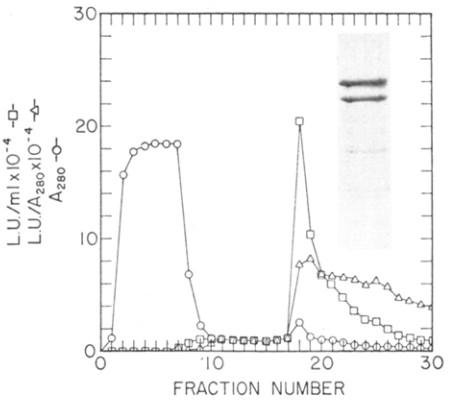

FIGURE 9: Chromatography of an aliquot of a 35–80% ammonium sulfate cut of wild-type *P. phosphoreum* cell lysate on DϕPA–Sepharose 6B. Chromatographic profiles: (○) A_{280}; (□) LU/mL; (△) LU/A_{280}. The sample was applied in the appropriate application buffer (Table II) after dialysis out of the storage buffer [0.21 M phosphate (pH 7.1)–0.50 mM DTE–40% glycerol]. The sample was loaded on the column in 1.0-mL aliquots beginning at fraction 1. The 1.0-mL column was preequilibrated in application buffer and was run as fast as the resin bed would permit (40–60 mL/h). The sample contained luciferase at an activity of 121 800 LU/mL. The sample application was halted (at fraction 7) and application buffer was started when the eluted luciferase activity was ~1% of that being applied. A total of 7.0 mL of sample was applied. The resin bed was then washed with eight 1.0-mL aliquots of application buffer, and the elution buffer (see Materials and Methods) was then applied, beginning at fraction 16, in 1.0-mL aliquots. The tubes into which the eluate was collected were previously spiked with ~50 μL of 1.0 M sodium phosphate–potassium phosphate, pH 7.1. Thus, as the luciferase eluted, it was immediately brought into contact with the phosphate to aid in stabilizing the enzyme (see Materials and Methods). The purification is summarized in Table V. The eluted fractions 18–22 were pooled and analyzed by NaDodSO$_4$–polyacrylamide gel electrophoresis (inset) in the standard fashion discussed under Materials and Methods. The sample was loaded so as to give ~15 μg of protein/well.

strategy used for the luciferases from *V. harveyi* and *V. fisheri*; the details are summarized in Table II, the column elution profile is presented in Figure 9, and the results are summarized in Table V. It was evident from these data that the pooled fractions (18–22) contained ~57% of the applied luciferase activity and had undergone ~12-fold purification from the sample applied to the affinity column. A comparison of the required fold purification of the *P. phosphoreum* enzyme with that of the *V. harveyi* enzyme appears to be a reflection of the elevated levels of luciferase present in *P. phosphoreum* (M. Ziegler, personal communication). If a greater yield of highly purified enzyme was desired, a broader pool of activity from the affinity column was chromatographed on AH-Sepharose (data not shown) in exactly the same manner as that described for the *V. harveyi* luciferase.

In summary, these data demonstrate the affinity technique to be a fast, efficient method for producing highly purified luciferase in high yield. The useful yields from conservative affinity column pools for all three luciferases were all greater than 50% of the activity applied (Tables III–V). NaDodSO$_4$–polyacrylamide gel electrophoretic analysis of the luciferase from these pools demonstrated each was nearly homogeneous (Figures 6, 7, and 9). Broader, less conservative, pools of luciferase activity could also be taken at the affinity column step, with a corresponding decrease in purity (Figures 3 and 5). The material was then chromatographed on a final column of AH-Sepharose (Figures 4 and 5). In comparison, the standard purification scheme (Gunsalus-Miguel et al., 1972; Hastings et al., 1978) takes much longer to perform,

and a conservative cut of luciferase activity at the DEAE-Sephadex column step gives material having about half the specific activity of affinity purified enzyme (cut conservatively) with only a 17.5% yield from the initial lysate (Hastings et al., 1978). The affinity method was therefore judged to be of greater utility for small- or large-scale luciferase purification than the standard batch elution and ion-exchange methods.

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Raman Spectroscopy of Avidin: Secondary Structure, Disulfide Conformation, and the Environment of Tyrosine†

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ABSTRACT: An analysis of the amide I region of Raman spectra indicates that avidin has 10 ± 5% and 55 ± 4% of its residues in helical and β-strand conformations, respectively. Predictions of secondary structure on the basis of the sequence of avidin are consistent with the high percentage of residues in the β conformation. We observe no differences between the spectra of avidin in solution and in crystals nor is there a significant difference between the secondary structures of avidin and the complex of avidin with biotin. In addition, the ratio of the intensities of the tyrosine doublet at 826 and 855 cm^{-1} indicates the lone tyrosine side chain of an avidin subunit is in a strong hydrogen bond as a proton acceptor. The Raman data also indicate the single disulfide of an avidin subunit has dihedral angles of 0–50° for each of its two C_β–S bonds and a dihedral angle of 85 ± 20° for its disulfide bond. We discuss the significance of these results in relation to findings of earlier work on avidin.

Avidin from the whites of hen eggs is a tetramer composed of monomers of M_r 15700. Each subunit of avidin binds one molecule of D-biotin with an affinity (pK_d = 15) among the highest of all associations between naturally occurring ligands and biological macromolecules (Green, 1963). The biological role of avidin in the egg is not clear. Green (1975), however, has suggested an antibacterial function for avidin; by sequestering D-biotin, avidin inhibits bacterial growth in the egg. On the basis of binding studies of biotin analogues, the principal interaction of the biotin molecule with avidin involves only the ureido portion of the ligand. In addition, the property of D-biotin to protect tryptophan residues from oxidizing agents suggests the proximity of indole side chains to the binding

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