Probing the *Vibrio harveyi* Luciferase β Subunit Functionality and the Intersubunit Domain by Site-Directed Mutagenesis†

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Received April 19, 1994; Revised Manuscript Received July 13, 1994®

ABSTRACT: While the critical role of the bacterial luciferase α subunit in catalysis has been amply documented, the β subunit was only known to be involved in thermal stability and substrate binding. Two conserved histidyl residues at positions 81 and 82 of the β subunit of *Vibrio harveyi* luciferase were each mutated to an alanine, aspartate, or lysine to probe further the β functionality. These mutations resulted in higher K_m values for reduced riboflavin 5′-phosphate, less efficient oxidations of the aldehyde substrate, and decreased light-emitting activities. βHis82 appears to be significantly more critical than βHis81. For the βHis82-mutated luciferases, the maximal light intensities and total light outputs were reduced to 19–4% of that for the wild-type enzyme, and the values of $V_{max}/K_{m,flavin}$ were decreased by 2–3 orders of magnitude. The reduced light emission activities for these mutated luciferases can be correlated to lower yields of the flavin 4a-hydroperoxide intermediate, reduced productions of the excited flavin emitter, and/or enhanced quenching of the emitter. The β subunit and the conserved βHis82 in particular have thus been shown to be critical not only to flavin binding but also to catalytic characteristics of luciferase. The dimeric structure of luciferase is essential to its high catalytic efficiency. To characterize the intersubunit domain, three sets of single/double mutants were constructed, and the additivities of mutational effects were tested to screen for residues that could interact across the subunit interface. On the bases of relative free energy changes in transition-state activation, and thermal and urea inactivations, effects of the double mutations αE67K/βK67E and αK98E/βE98K were both closely matched by the additive effects of their constituent single mutations. In contrast, significant deviations from the additivity of mutational effects were observed for the αH82D/βH82K and αH82K/βH82D series of mutants. Moreover, with respect to $K_{m,flavin}$ and sensitivities to urea and thermal inactivations, the adverse effects of mutations of βH82K and αH82K were compensated to various degrees by the comutation of αH82D and βH82D, respectively. These results indicate that αHis82 and βHis82 are within a range suitable for interaction and could be converted to interacting sites by mutations.

All known bacterial luciferases are αβ heterodimers. This dimeric structure is essential to the high-efficiency bioluminescence activity (Sinclair *et al.*, 1993; Li *et al.*, 1993). Moreover, the dimeric luciferase apparently has a single active site in catalyzing the bioluminescent oxidation of reduced riboflavin 5′-phosphate ($FMNH_2$)[1] and long-chain aliphatic aldehyde by molecular oxygen with a 1:1:1 stoichiometry (Dunn *et al.*, 1973; Becvar & Hastings, 1975; Hastings & Balny, 1975; Tu, 1982; Suzuki *et al.*, 1983). At present, intriguing questions regarding the nature of the αβ interactions and the specific functional roles of the α and, especially, the β subunit remain largely unresolved.

Several lines of evidence are available to indicate that the α subunit is critical to luciferase catalytic activity. An earlier hybridization study using chemically modified subunit variants showed that only α was involved in catalysis (Meighen *et al.*, 1971a,b). In accord with this, analyses of a large number of luciferase mutants developed by random mutations also revealed that lesions altering the catalytic properties occurred exclusively in the α subunit (Cline & Hastings, 1972; Cline, 1973). The critical role of the α subunit in luciferase catalysis is further indicated by additional subunit hybridization experiments (Ruby & Hastings, 1980) and a series of chemical modification (Nicoli *et al.*, 1974; Cousineau & Meighen, 1976; Welches & Baldwin, 1981; Paquatte & Tu, 1989) and site-specific mutagenesis (Chen & Baldwin, 1989; Baldwin *et al.*, 1989; Xi *et al.*, 1990; Xin *et al.*, 1991) studies.

Much less is known about the structural or functional role of the luciferase β subunit. For quite a number of years, β was only known to be critical in maintaining luciferase in a thermally stable conformation (Meighen *et al.*, 1971b; Cline & Hastings, 1972). Later studies on a limited number of modified luciferase species indicate that β is involved in $FMNH_2$ (Cline, 1973; Meighen & Bartlet, 1980; Welches & Baldwin, 1981; Watanabe *et al.*, 1982) and aldehyde (Tu & Henkin, 1983) binding. As a general pattern, modifications of the luciferase β subunit have been shown to adversely affect only the thermal stability and/or substrate binding but not the subsequent catalytic steps or bioluminescence quantum output.

In the first part of this work, we carried out specific mutations of two of the conserved histidine residues on the β subunit, namely, βHis81 and βHis82, of the *Vibrio harveyi*

† Supported by Grants GM25953 from the National Institutes of Health and E-1030 from The Robert A. Welch Foundation.

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® Abstract published in *Advance ACS Abstracts,* September 15, 1994.

[1] Abbreviation: $FMNH_2$, reduced riboflavin 5′-phosphate.

0006-2960/94/0433-12194$04.50/0

luciferase. Mutations of either of these two histidyl residues resulted in increased K_m values for $FMNH_2$. Moreover, markedly decreased activities in light emission and/or aldehyde monooxygenation have been demonstrated for the first time as a consequence of specific single-residue mutations of the luciferase β subunit.

Individual subunits of bacterial luciferase were reported to be catalytically inactive (Li *et al.*, 1993) or to exhibit extremely low activities (Sinclair *et al.*, 1993). The interaction of α with β is thus essential to the high-efficiency bioluminescence activity of luciferase. Therefore, the delineation of the subunit interface is critical to our understanding of the luciferase structure–function relationship. By the use of bifunctional reagents, the Cys106 residue on the *V. harveyi* luciferase α subunit was shown to be within 8–11 Å from an unidentified cross-linking site on the β subunit (Paquatte *et al.*, 1988). Aside from this single report, domains or amino acid residues that are at or near the subunit interface have thus far remained uncharacterized. In this work, we had examined the effects of single and double mutations of *V. harveyi* luciferase on free energy changes in transition-state activation and enzyme inactivations by heat and urea. An analysis of the additivity of the mutational effects (Wells, 1990) has enabled us to probe the intersubunit domain of luciferase at the level of specific amino acid residues.

MATERIALS AND METHODS

Materials. A Muta-gene kit including *Escherichia coli* strain MV1190, expression vector pTZ19U, Ultrapure dNTP, T4 polynucleotide kinase, and T7 DNA polymerase were from Bio-Rad Laboratories. Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs. X-Gal and IPTG were from Fisher. [α-^{35}S]dATP (37 TBq/mmol) was a product of Amersham Corp. The DNA Sequencing Kit was from United States Biochemical Corp. Decanal, tetradecanal, and copper(I) bromide were purchased from Aldrich. Sodium hydrosulfite, FMN, dithiothreitol, and ampicillin were from Sigma. [1-^{2}H]Decanal was obtained as described previously (Xin *et al.*, 1991). An Ultrafree-MC filter unit (30 000 NMWL) was obtained from Millipore Corp. Ultrapure urea was from Boehringer Mannheim Biochemicals.

Construction of Single and Double Mutations. Variants of *V. harveyi* luciferase bearing a single mutated amino acid residue at either the α or the β subunit were generated by specific mutations of the recombinant phage MTX1 (Xi *et al.*, 1990) following the general strategy detailed previously (Xin *et al.*, 1991). For these mutations, the wild-type codon GAA for αGlu67 and βGlu98 was mutated to AAA for lysine; AAG for βLys67 was mutated to GAG for glutamate; AAA for αLys98 was mutated to GAA for glutamate; CAT for either αHis82, βHis81, or βHis82 was changed to GCC for alanine, GAT for aspartate, or AAA for lysine. The resulting 12 mutants are identified as αE67K, αH82D, αH82K, αK98E, βK67E, βH81A, βH81D, βH81K, βH82A, βH82D, βH82K, and βE98K. A 3-kb *lux*AB-containing DNA fragment with a single-codon mutation in either the *lux*A or the *lux*B was obtained by *Hin*dIII–*Kpn*I digestion of each of the specifically mutated MTX1 phages. Each fragment was subcloned into a modified pTZ19U vector in which the *Eco*RI site was formerly destroyed. A recombinant plasmid containing the native *lux*AB was similarly constructed. Subsequent screening, *E. coli* MV1190 host cell transformation, and expression were carried out as described previously (Xin *et al.*, 1991).

Four luciferase variants were also generated, each containing two mutated amino acid residues with one in the α and the other in the β subunit. To obtain a double mutation, the *lux*A gene bearing a desired single-codon mutation was first excised with *Eco*RI and *Hin*dIII from an appropriate recombinant plasmid obtained as described above. The native *lux*A gene was also removed by the same treatment from another recombinant plasmid containing a desired single-codon mutation in the *lux*B gene. The excised native *lux*A was replaced by the DNA fragment bearing the mutated *lux*A mentioned above to generate a new recombinant plasmid with modified *lux*A and *lux*B. Specifically, the four mutants so obtained are αE67K/βK67E, αH82D/βH82K, αH82K/βH82D, and αK98E/βE98K. All the single and double-mutations were verified by dideoxy sequencing analyses of double-stranded DNA.

Luciferase Purification and Assays. Wild-type and mutated luciferases were expressed in *E. coli* strain MV1190 harboring the desired plasmid and purified (Hastings *et al.*, 1978) to 85–95% purity on the basis of SDS–polyacrylamide gel electrophoresis. All mutated luciferases showed, discounting a few minor impurity bands in some cases, a single and two protein bands after nondenaturing and denaturing polyacrylamide gel electrophoresis, respectively, with the same migration patterns as those of the wild-type luciferase. Apparently, the wild-type and mutated luciferases were all isolated in dimeric forms. Unless stated otherwise, bioluminescence activities were determined in 0.1 M KP_i, pH 7.0, at 23 °C by the dithionite assay (Hastings *et al.*, 1978) and/or copper(I) assay (Lei & Becvar, 1991). Activities were measured at various concentrations of $FMNH_2$ or aldehyde at a saturating level of the corresponding cosubstrate. The maximal activities and K_m were determined by double-reciprocal plots. The former values are expressed as the peak light intensity (I_o; equivalent to V_{max}) in quanta per second and normalized to per A_{280} per milliliter of enzyme sample. Alternatively, activities were also expressed as total light output in quanta.

Effects of Mutation on Transition-State Activation Energy. The in vitro bioluminescence emissions of wild-type and mutated luciferases in the nonturnover dithionite assay exhibit apparent first-order decay kinetics. This light decay rate constant provides a measure of the k_{cat} of the single-cycle reaction in vitro. Relative changes in the transition-state activation energy resulting from luciferase mutations (defined as $\Delta\Delta G^{\ddagger} = \Delta G^{\ddagger}_{mut} - \Delta G^{\ddagger}_{wt}$ where $\Delta G^{\ddagger}$ is the activation energy for the formation of the transition state from enzyme and free substrate and the subscripts mut and wt correspond to mutant and wild-type, respectively) can be determined according to eq 1 (Wilkinson *et al.*, 1983):

$$\Delta\Delta G^{\ddagger} = -RT \ln[(k_{cat}/K_M)_{mut})/(k_{cat}/K_M)_{wt}] \quad (1)$$

in which K_M is the Michaelis constant for $FMNH_2$.

Thermal Inactivation. Wild-type and mutant luciferases in 0.35 M KP_i, pH 7.0, were each incubated at 52.5 °C. Aliquots (50–100 μL) were withdrawn at different times for measurements of the remaining activities at 23 °C by the Cu(I) assay. The apparent first-order rate constant of thermal inactivation (k_{inact}) was determined by a semilogarithmic plot of the remaining bioluminescence activity versus time. The free energy change of thermal inactivation ($\Delta G^{\ddagger}$) can then be calculated following the relationship of $\ln k_{inact} = \ln(RT/N_o h) - \Delta G^{\ddagger}/RT$ in which R, T, N_o, and h are the gas constant, absolute temperature, Avogadro's number, and Planck constant, respectively, and k_{inact} is expressed in s^{-1}. The relative free energy change for a particular luciferase mutant was finally determined according to $\Delta\Delta G^{\ddagger} = \Delta G^{\ddagger}_{mut} - \Delta G^{\ddagger}_{wt}$.

Inactivation by Urea Denaturation. Purified wild-type and mutant luciferases were equilibrated with 0.05 M phosphate buffer, pH 7.0, and the protein concentrations were all standardized to 1 mg/mL. An 100-μL aliquot of enzyme sample was mixed at 20 °C with a denaturation solution to reach a final volume of 1 mL in 4 M urea/0.5 M KP_i, pH 7.0. After different times of incubation at the same temperature, 100-μL aliquots were withdrawn for activity assays as described above. The apparent first-order rate constant of inactivation (k_{inact}), the free energy change of urea inactivation ($\Delta G^{\ddagger}$), and the relative free energy change for a particular luciferase mutant ($\Delta\Delta G^{\ddagger} = \Delta G^{\ddagger}_{mut} - \Delta G^{\ddagger}_{wt}$) were all determined as described above for the thermal inactivation.

Additional Methodologies. For measurements of aldehyde deuterium isotope effects, activities of wild-type and mutated luciferases were determined by the Cu(I) assay using 50 μM $FMNH_2$ and saturating [1-^{2}H]decanal or the control decanal (at about 0.2 mM) as substrates. The deuterium isotope effects (^Dk) were expressed as the ratios of the light decay rate for the control decanal over that for the deuterated decanal. The abilities of various luciferases to convert the tetradecanal substrate to carboxylic acid product were determined as described previously (Xin *et al.*, 1991) with the following modifications: (a) a larger amount of wild-type luciferase (15.6 μM) and a wider range of tetradecanal standard (0–200 nM) were used to construct the standard curve and (b) 50 μM of each luciferase species was tested for its ability to consume 0.5 μM tetradecanal. For measurement of the stability of flavin 4a-hydroperoxide intermediate II, the intermediate was first formed by reacting a wild-type or mutated luciferase with 50 μM $FMNH_2$ (reduced by dithionite) at 23 or 4 °C. Aliquots (20–50 μL) were withdrawn after different times and each was injected into 1 mL of a decanal-saturated buffer to initiate the bioluminescence. The decay rate of II (k_{II}) was determined by monitoring the decrease in the amount of bioluminescence-active II as a function of time. In some cases, intermediate II was isolated at 4 °C by the Sephadex/centrifugation method without any aliphatic stabilization agent, and the yield was determined on the basis of ΔA_{460} between the initial isolate and the same sample upon the completion of decay (Tu, 1986).

RESULTS

Selection of Polypeptide Segments and Amino Acid Residues for Mutation. The elucidation of luciferase structure–function relationships suffers greatly from a lack of detailed structural information by X-ray crystallography or high-resolution NMR. Consequently, it becomes necessary to resort to structural information at lower levels of resolution in guiding the search of structure–function correlations. It has been proposed that, for an evolutionarily related family of enzymes, the structurally and/or functionally important regions are likely to be highly conserved in secondary structures (Cid, 1987). The sequences of the α and β subunits of luciferases from *V. harveyi* (Cohn *et al.*, 1985; Johnston *et al.*, 1986), *Vibrio fischeri* (Baldwin *et al.*, 1987; Foran & Brown, 1988), *Photobacterium leiognathi* (Illarionov *et al.*, 1988), *Photobacterium phosphoreum* (Meighen, 1991), and *Xenorhabdus luminescens* (Johnston *et al.*, 1990; Szittner & Meighen, 1990; Xi *et al.*, 1991) were analyzed using the PeptideStructur program of the WUGCG package to predict the secondary structures and related characteristics (Devereux *et al.*, 1984). On the basis of profiles of α helices, Kyte–Doolittle hydrophilicity, and surface probability, polypeptide segments with significant homologies were identified. Overall, the α subunits are significantly more homologous among themselves than the β subunits are as a group. For the α subunits, residues 63–120 constitute a region of a higher degree of conservation. Correspondingly, residues 64–106 are in a more homologous region of the β subunits. Consequently, sites chosen for specific mutations were all within these two more homologous polypeptide segments.

Table 1: Activities and Kinetic Properties of Wild-Type Luciferase and Variants with Mutations at βHis81 or βHis82[a]

luciferase	rel I_0 (%)	light decay rate (min^{-1})	rel quantum output (%)	K_m (μM)		rel $I_0/K_{m,flavin}$ (%)
				$FMNH_2$	decanal	
wild-type	100	14.4	100	0.4	13.5	100
βH81A	42	13.8	44	0.8	11.2	23
βH81D	13	13.8	14	1.5	15.3	4
βH81K	36	12.6	41	2.2	11.7	7
βH82A	11	18.0	9	0.8	8.2	6
βH82D	17	12.6	19	1.9	10.1	4
βH82K	4	13.8	4	25.6	12.6	0.1

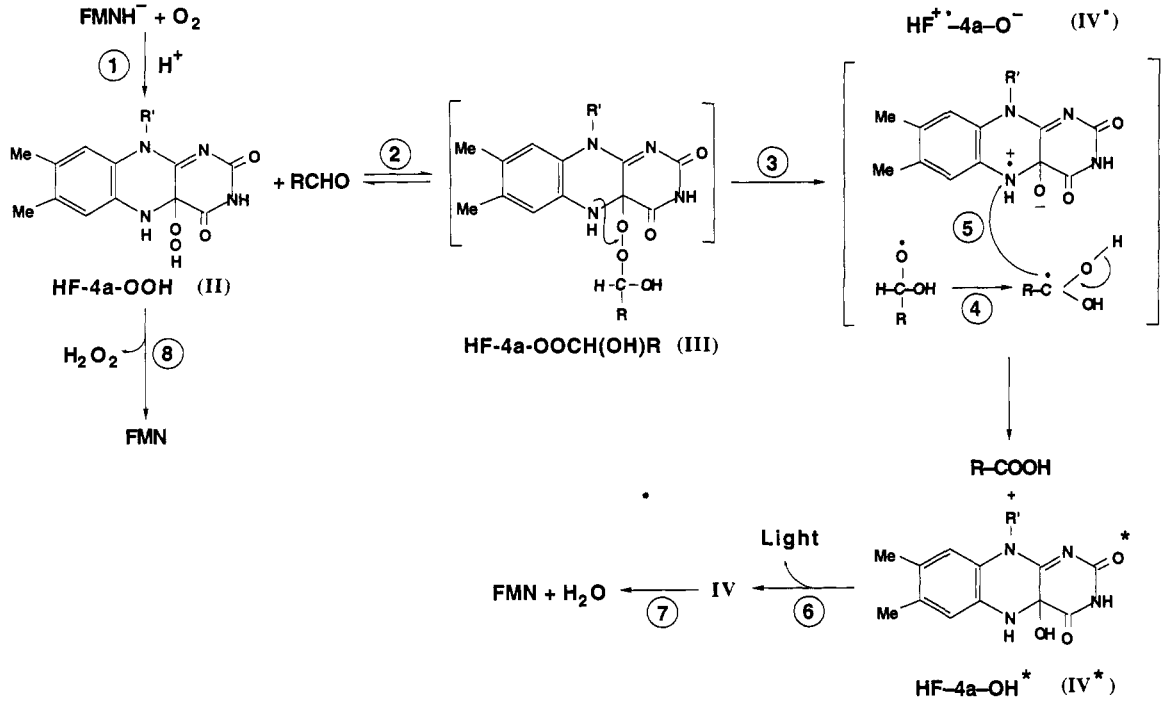
[a] Determined using $FMNH_2$ and decanal as substrates.

A comparison of known sequences of the β subunit of bacterial luciferases as described above reveals five conserved histidyl residues at positions 45, 81, 82, 161, and 224. Within this group, only βHis81 and βHis82 are located in the region associated with a more conserved secondary structure. For the elucidation of the functionality of the luciferase β subunit, these two residues were then chosen as the target for site-directed mutagenesis.

Additional single mutations in either the α or the β subunit and the corresponding double mutations were also carried out. The effects of these single and double mutations were compared to screen for residues which are within a range suitable for significant intersubunit interaction. Since the construct of the luciferase subunit interface is totally unknown, there are numerous possibilities in the selection of mutational sites for such tests. Focusing on the more conserved peptide segments of residues 63–120 on the α and residues 64–106 on the β, three combinations of single/double mutations were chosen. The α67/β67 set and the α98/β98 set were selected on the basis of possible charge complementarity. The *V. harveyi* luciferase has an αGlu67 and a βLys67 whereas most other bacterial luciferases have an αLys67 and a βGlu67. Known luciferase sequences also show that the α98 is a lysine whereas the β98 is either an aspartate or a glutamate. The α82/β82 set was chosen because of the conservation of a histidyl residue at these two positions for all bacterial luciferases with known sequences and, as is detailed below, for the important role of the *V. harveyi* βHis82 in the expression of catalytic activity.

Probing β Functionality by Mutations of βHis81 and βHis82. βHis81 and βHis82 were each mutated to an alanine, aspartate, or lysine. Bioluminescence activities and general kinetic properties of such luciferase variants were determined and compared with those of the wild-type luciferase (Table 1). On the basis of peak emission intensities (I_0), these mutated luciferases all exhibited reduced bioluminescence activities at levels of 42–4% of that for the wild-type enzyme. The βHis82 is apparently more critical than βHis81 to the expression of the light-emitting activity. Interestingly, the light decay rates for all six mutated luciferases were similar to that of the wild-type enzyme. Consequently, the reductions of the integrated total light outputs for the luciferase variants parallel their decreases in I_0. Little changes in the K_m for decanal were observed for the mutated luciferases, but all variants showed increased K_m for $FMNH_2$, particularly in the case of βH82K. A comparison was also made in $I_0/K_{m,flavin}$ (equivalent to $V_{max}/$

Scheme 1



K_m). Reductions of roughly 1–2 orders and 2–3 orders of magnitude were detected for the βHis81- and βHis82-mutated luciferases, respectively. As mentioned earlier, all mutated luciferases were isolated in dimeric forms on the basis of patterns of nondenaturing and denaturing polyacrylamide gel electrophoresis. Therefore, the reductions in bioluminescence activities apparently were not a consequence of subunit dissociation.

We have formulated an electron/charge transfer (ECT) mechanism for the luciferase reaction (Scheme 1) (Mager *et al.*, 1988; Cho *et al.*, 1993). The FMN–4a-hydroperoxide intermediate II (HF–4a–OOH) is formed from luciferase-bound N(1)-deprotonated $FMNH^-$ and O_2. The FMN–4a-peroxyhemiacetal intermediate III [HF–4a–OO–CH(OH)R] is then formed from II and aldehyde. Intramolecular electron transfer from N(5) to the O–O bond forms a caged pair of alkylhydroxyoxy radical and the novel FMN–4a-hydroxide radical ($HF^{+\cdot}$ –4a–O^- or intermediate IV·). The alkylhydroxyoxy radical is transformed into a C-centered alkyldihydroxy radical [R–C·$(OH)_2$] which, being a stronger reductant, donates one electron to IV· to form RCOOH and the excited-state FMN–4a-hydroxide (HF–4a–OH* or IV*). The radiative relaxation of IV* leads to luminescence and the formation of ground-state IV. Finally, the dehydration of IV generates FMN and H_2O to complete the overall reaction. In addition to participating in the light-emitting monooxygenation reaction as mentioned above, the key intermediate II also undergoes a dark decay to generate FMN and H_2O_2 with very little or no light emission. The decay rates (step 8) and yields of isolatable intermediate II for the wild-type and βHis81- and βHis82-mutated luciferases were determined (Table 2). In comparison with the wild-type enzyme, the intermediates II derived from βH82D and the other five mutated luciferases exhibited about the same and somewhat higher stabilities, respectively, at 23 °C. For all the mutants tested, βH81D and βH82K are the least active luciferases with mutations at positions 81 and 82, respectively. These two luciferase variants were chosen for additional studies. At 4 °C, the intermediates II from βH81D and βH82K were 2–2.5-fold lower in yields, but both showed higher stabilities than that of the wild-type.

Table 2: Yields and Decay Rates of 4a-Hydroperoxyflavin Intermediate II Formed with Wild-Type Luciferase and Variants with Mutations at βHis81 or βHis82

luciferase	k_{II} at 23 °C (min^{-1})	k_{II} at 4 °C (min^{-1})	$[II]/[II_{WT}]^a$ at 4 °C
wild-type	3.9	0.18	1.0
βH81A	2.2		
βH81D	1.8	0.05	0.5
βH81K	2.5		
βH82A	2.6		
βH82D	3.5		
βH82K	2.4	0.09	0.4

[a] Relative yields, $[II]/[II_{WT}]$, are expressed as the ratios of the yield of the isolatable mutant intermediate II over that of the wild-type intermediate II.

The abilities to consume aldehyde as a substrate (steps 3–5) by the wild-type and the mutated luciferases were compared with their total light outputs (Figure 1). For βH81D and βH82K, the reductions in bioluminescence activities corresponded to that in aldehyde consumptions. However, the other four mutated luciferases showed significantly higher aldehyde consumptions than their corresponding bioluminescence activities.

Decanal deuterated at the C1 position is known to exhibit deuterium kinetic isotope effects of 1.4–1.7 on the basis of either the peak light intensity or the rate of light decay of the *V. harveyi* luciferase-catalyzed reaction (Tu *et al.*, 1987). Using the copper(I) assay, deuterium isotope effects of [1-^{2}H]-decanal on the light decay rates were observed to be of 1.5 and 1.7 for βH81A and βH82A, respectively, quite similar to an isotope effect of 1.6 for the wild-type enzyme.

Effects of Single and Double Mutations on Transition-State Activation Energy. This series of experiments and additional studies described below were carried out to examine the additivity of mutational effects on luciferase. In general, effects on a particular functional property were determined for luciferase variants each with a desired single mutation in either the α or the β subunit, and compared with the effects of the corresponding double mutations. Four sets of single/

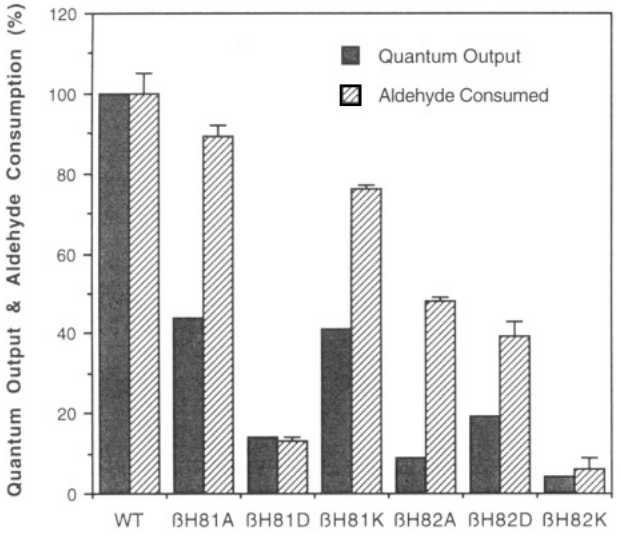


FIGURE 1: Quantum outputs and aldehyde consumptions of wild-type and β-mutated luciferases. Conditions for and methods of measurements are described in the text. Three samples were tested for each luciferase for the tetradecanal consumption. Results are expressed as percents of that for the wild-type enzyme.

Table 3: Bioluminescence Activities, Kinetic Properties, and Inactivation Rate Constants of the Wild-Type Luciferase and Variants with Single or Double Mutations

luciferase	$I_0/I_{0,WT}$[a] (%)	K_m for $FMNH_2$[a]	light decay rate[a] (min^{-1})	k_{inact} (min^{-1}) thermal[b,c]	urea[c,d]
wild-type	100	0.4	14.4	0.67	0.70
αE67K	83	0.8	20.5	0.53	0.67
βK67E	99	0.7	18.0	0.79	0.82
αE67K/βK67E	88	0.7	17.0	0.68	0.78
αK98E	76	0.4	17.2	2.46	2.41
βE98K	91	0.7	19.5	1.50	1.47
αK98E/βE98K	29	0.5	19.3	4.41	4.07
αH82D	28	2.4	16.9	1.52	1.60
βH82K	4	25.6	13.8	2.51	2.80
αH82D/βH82K	0.003	2.4	13.9	0.98	1.24
αH82K	0.7	20.0	16.1	2.92	2.61
βH82D	17	1.9	12.6	0.69	0.87
αH82K/βH82D	0.01	0.4	18.4	1.06	1.37

[a] Determined using $FMNH_2$ and decanal as substrates. I_0 and $I_{0,WT}$ are the peak bioluminescence intensities, normalized to $q \cdot s^{-1} \cdot A_{280}^{-1}\ mL^{-1}$, for the mutated and wild-type luciferases, respectively. [b] At 52.5 °C in 0.35 M KP_i, pH 7.0. [c] Each number is the average of three measurements. For simplicity, the corresponding standard deviations are only shown in Figures 3 and 4. [d] At 20 °C in 0.05 M KP_i, pH 7.0, and 4 M urea.

double mutations were generated. In the order of (α mutant, β mutant, αβ double mutant), the four sets are (αE67K, βK67E, αE67K/βK67E), (αK98E, βE98K, αK98E/βE98K), (αH82D, βH82K, αH82D/βH82K), and (αH82K, βH82D, αH82K/βH82D). The effects of these single and double mutations on the bioluminescence activity, light decay rate, and K_m for $FMNH_2$ are shown in Table 3. Among the single mutants tested, only the mutations at αHis82 or βHis82 showed pronounced decreases (72–99%) in bioluminescence intensities or increases (5–60-fold) in K_m for $FMNH_2$. For the four double mutants, αK98E/βE98K, αH82D/βH82K, and αH82K/βH82D showed lower light emission activities than their respective constituent single mutants whereas αE67K/βK67E exhibited a slight inactivation apparently originated from the αE67K mutation. Very little changes in the K_m for $FMNH_2$ were observed for the double mutants αE67K/βK67E and αK98E/βE98K. On the other hand, significant compensations in the K_m for $FMNH_2$ were detected for the double mutants αH82D/βH82K and αH82K/βH82D in comparison

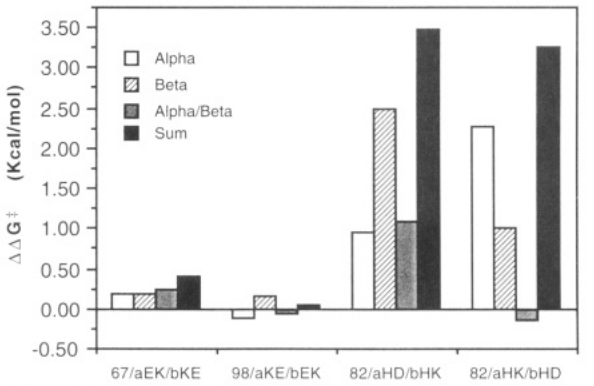


FIGURE 2: Effects of single and double mutations on free energy changes in transition-state activation. Relative free energy changes ($\Delta\Delta G^{\ddagger}$) were determined as described in the text using data shown in Table 3. The abscissa is marked for (the position of mutation in the α and/or β)/(the nature of mutation in the α subunit)/(the nature of mutation in the β subunit). For example, 67/aEK/bKE stands for the (αE67K, βK67E, αE67K/βK67E) set of single and double mutations. Inset: Bar patterns are identified for the subunit(s) that was (were) mutated and the mathematical sum of the effects of individual single mutations.

with their constituent single mutants. All the single and double mutants showed only small changes in the light decay rate of their nonturnover in vitro light emissions. Following eq 1, the effects of single and double mutations on changes in transition-state activation energy ($\Delta\Delta G^{\ddagger}$) were calculated, and the results are shown in Figure 2. For the mutant series (αE67K, βK67E, αE67K/βK67E) and (αK98E, βE98K, αK98E/βE98K), the values of $\Delta\Delta G^{\ddagger}$ for the double mutants were very close to the sum of the $\Delta\Delta G^{\ddagger}$ values of their constituent single mutants. However, marked differences were observed between the $\Delta\Delta G^{\ddagger}$ value for the double mutant and the sum of the $\Delta\Delta G^{\ddagger}$ values of the constituent single mutants for the series (αH82D, βH82K, αH82D/βH82K) and (αH82K, βH82D, αH82K/βH82D).

Effects of Single and Double Mutations on Thermal Inactivation. Thermal inactivations of wild-type and mutated luciferases were carried out in 0.35 M KP_i, pH 7.0, at 52.5 °C, and the apparent first-order rate constants of inactivation (k_{inact}) are shown in Table 3. While αE67K showed a slightly better thermal stability than the wild-type enzyme, all the other single and double mutants shown in Table 3 exhibited either no significant changes or relatively small increases in their thermal inactivation rates. From the thermal inactivation rate constants, relative free energy changes ($\Delta\Delta G^{\ddagger}$) were calculated (Figure 3). For mutations at positions α67 and/or β67, the effect of the double mutation is similar to the additive effect of the constituent single mutations. The same characteristic was also observed for mutations at positions α98 and/or β98. In contrast, large differences between the $\Delta\Delta G^{\ddagger}$ of the double mutation and the sum of the $\Delta\Delta G^{\ddagger}$ values of corresponding single mutations were detected for the mutant series (αH82D, βH82K, αH82D/βH82K) and (αH82K, βH82D, αH82K/βH82D).

Effects of Single and Double Mutations on Urea Denaturation. In the presence of 4 M urea and at 20 °C, inactivations of the wil- type and mutated luciferases occurred following apparent first-order kinetics with rate constants shown in Table 3. The general patterns of effects of single and double mutations on urea inactivation closely parallel those of thermal inactivation (Table 3, Figures 3 and 4). In comparison with the wild-type luciferase, αE67K was marginally more resistant to urea inactivation whereas all other mutants showed somewhat decreased stabilities (Table 3). In

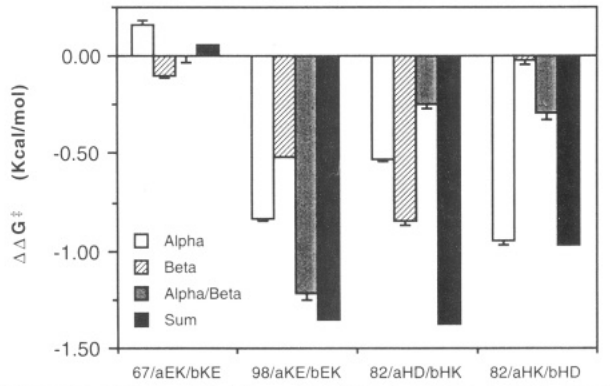


FIGURE 3: Effects of single and double mutations on free energy changes in luciferase thermal inactivation. Relative free energy changes ($\Delta\Delta G^{\ddagger}$) were determined as described in the text using data shown in Table 3. The abscissa and the inset are marked as described in Figure 2.

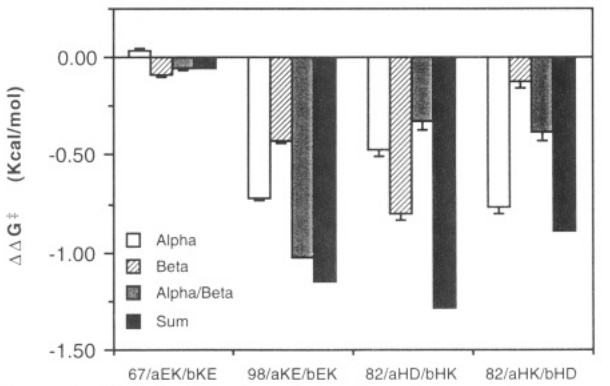


FIGURE 4: Effects of single and double mutations on free energy changes in luciferase urea inactivation. Relative free energy changes ($\Delta\Delta G^{\ddagger}$) were determined as described in the text using data shown in Table 3. The abscissa and the inset are marked as described in Figure 2.

terms of $\Delta\Delta G^{\ddagger}$, the effect of double mutation of αE67K/βK67E and that of αK98E/βE98K were each closely matched by the additive effect of the respective constituent single mutations. On the other hand, the values of $\Delta\Delta G^{\ddagger}$ for the double mutants αH82D/βH82K and αH82K/βH82D were each significantly different from the sum of the $\Delta\Delta G^{\ddagger}$ values of the corresponding single mutants (Figure 4).

DISCUSSION

Mutations of βHis81 and βHis82 resulted in decreased affinities for $FMNH_2$ (Table 1), an effect similar to those observed with a few β-modified variants in earlier investigations (Meighen & Bartlet, 1980; Welches & Baldwin, 1981). However, a truly distinct feature revealed by the present study is that single-residue mutations of the β subunit can result in marked reductions in bioluminescence activities. In comparison with the wild-type luciferase and on the basis of peak emission intensity (I_o, equivalent to V_{max}) or total light output, activities for the βHis81-mutated luciferases were reduced to 13–44%. The βHis82 exhibited an even more critical role; the three luciferases mutated at this position were only associated with 4–19% activities. In contrast, all β-mutated luciferases examined earlier retained much higher levels of activity, ranging from 40% for a single mutant to as high as 65–110% for all other β mutants (Cline & Hastings, 1972; Cline, 1973). On the basis of $I_o/K_{m,flavin}$, up to 2 and 3 orders of magnitude of activity reductions were associated with the βHis81- and βHis82-mutated luciferases, respectively. To our knowledge, marked reductions in the luciferase light emission activity as a result of specific single-residue mutations on the β subunit were demonstrated for the first time. As mentioned under Results, the substantial inactivations of luciferase by mutations of βHis81 or βHis82 were not a consequence of subunit dissociation.

Following Scheme 1, adverse effects on the light emission activity can be a consequence of reduced yields of certain reaction intermediates (due to competing dark pathways such as step 8), a lower efficiency for the formation of excited flavin 4a-hydroxide emitter (steps 3–5, particularly step 5), and/or enhanced quenching of the emitter (step 6). For the native luciferase, the formation of excited intermediate IV* is coupled to the monooxygenation of aldehyde (steps 3–5) with the attacking activated oxygen derived from an O–O bond scission of the flavin 4a-peroxyhemiacetal intermediate III (Hastings & Balny, 1975). Alternatively, a C(4a)–O bond scission of intermediate II (step 8) would lead to a dark decay. For mutated luciferases, one way to reduce the bioluminescence activity could be an enhancement of such a dark decay for intermediate II . This is indeed the case for 10 α-mutated luciferases which showed a correlation between the decreases in bioluminescence activity and the dark decay rates of the intermediate II (Xin *et al.*, 1991). However, the βHis81- and βHis82-mutated luciferases do not follow this pattern; the stabilities of their intermediate II species were better than or, in the case of βH82D, about the same as that of the native enzyme. On the other hand, reductions in the yield of II were about 2-fold for βH81D and βH82K, hence partially accounting for the 7- and 25-fold decreases in total light output, respectively.

We have also demonstrated that single-residue mutations of the β subunit significantly decreased the ability of luciferase to consume aldehyde (Scheme 1, steps 3–5) as a substrate (Figure 1). The reduced aldehyde consumptions by βH81D and βH82K corresponded quantitatively to their decreases in total light output. Such an observation indicates that the chemiexcitation of the emitter (step 5) is efficiently coupled to the aldehyde monooxygenation (steps 3–5), and the main cause for the reduced light emission by these two luciferase mutants is the decreased production of the excited flavin 4a-hydroxide emitter. Once their excited emitters are formed, their emission efficiencies associated with the step 6 are similar to that of the native enzyme emitter. For the other four luciferase variants, their aldehyde consumptions were lower than that of the wild-type enzyme but all were higher than their respective light emission activities. The reduced capacity for aldehyde monooxygenation is apparently a contributing factor but not the sole cause for the low bioluminescence activities of these mutants. To account for the additional reductions in light output, either the excited emitters have lower emission efficiencies (step 6) or the chemical event of aldehyde monooxygenation is not efficiently coupled to the excitation event of intermediate IV* (step 5).

The question as to whether βHis81 or βHis82 is directly involved in the abstraction of the aldehyde C1-hydrogen (step 4) was investigated. The native luciferase showed a [1-^{2}H]-decanal deuterium kinetic effect of 1.6 on light emission. If either βHis81 or βHis82 is directly involved in the abstraction of the aldehyde C1-hydrogen, their mutations to a poor catalytic base such as alanine are expected to substantially reduce the rate of C1-hydrogen abstraction and, correspondingly, increase the magnitude of the deuterium kinetic effect. However, neither βH81A nor βH82A showed any significant changes in the [1-^{2}H]decanal deuterium kinetic effect, thus providing no support for such a catalytic role of βHis81 or

βHis82. Moreover, the light decay rates for all six luciferase variants were about the same as that for the wild-type luciferase (Table 1). Since the radiative relaxation of the excited emitter is believed to proceed on a nanosecond time scale, the much slower light decay of the in vitro nonturnover bioluminescence reaction thus reflects the kinetics for the formation of the excited emitter and the coupling aldehyde monooxygenation. Therefore, the lack of any pronounced effect on the light decay rate by mutations of βHis81 or βHis82 further supports the conclusion that neither histidyl residue directly participates in the oxidation of aldehyde.

The results discussed thus far indicate that adverse effects on catalytic (i.e., I_o, total quanta output, $I_o/K_{m,flavin}$, and aldehyde consumption) and kinetic (K_m for $FMNH_2$) properties of luciferase can result from mutations of the conserved βHis81 or βHis82, with the latter being significantly more critical. Using these two histidyl residues as sites of structural perturbation, it is thus demonstrated that the β subunit is important not only to flavin binding but also to subsequent catalytic steps and bioluminescence quantum output. A direct role in the aldehyde C1-hydrogen abstraction has not been identified for βHis81 or βHis82. However, the reduced bioluminescence activities of the β-mutated luciferases studied in this work can be correlated to decreased yields of intermediate II, diminished aldehyde monooxygenation, partial uncoupling of aldehyde oxidation from emitter formation, and/or enhanced quenching of the excited emitter.

Wells (1990) has demonstrated that the additivity of mutational effects can be used as a criterion to evaluate the interactions between specific sites in proteins. If two sites are devoid of any significant interaction, the effect on the free energy change of a particular function for a double mutation of these sites should be the sum of the effects of the corresponding single mutations. The lack of such an additivity would indicate either a significant interaction between these two sites or a change in the reaction mechanism or rate-limiting step. The two sites under test either could be within the same protein subunit or be located in two different protein molecules (Empie & Laskowski, 1982). This principle has now been applied to luciferase in search of specific amino acid residues that are within the intersubunit domain.

Mutational effects of single/double mutations were determined with respect to transition-state activation (Figure 2), thermal inactivation (Figure 3), and urea inactivation (Figure 4). In all three tests, a general pattern emerged. For the α67/β67 and α98/β98 mutant sets, the effects of double mutations were all closely matched by the sum of the effects of the corresponding constituent single mutations. In contrast, the effects of single mutations were clearly not additive for the (αH82D, βH82K, αH82D/βH82K) and (αH82K, βH82D, αH82K/βH82D) series of mutations. All mutants were active in bioluminescence, and their rate constants for the decays of light emission and intermediate II were not markedly different from those of the wild-type luciferase. It is thus unlikely that the lack of additive mutational effects is due to a change in the reaction mechanism or rate-limiting step. Therefore, the present study indicates that αHis82 and βHis82 in the *V. harveyi* luciferase are within a range suitable for significant interaction, and could be converted to interacting sites by mutations. However, these results do not necessarily imply that αHis82 and βHis82 are directly involved in the specific binding of α and β in the wild-type luciferase. Our previous work indicates that αCys106 is close to the β subunit (Paquatte *et al.*, 1988). The present study reveals new information regarding the intersubunit domain of luciferase at the level of specific amino acid residues at α and β, and provides a useful reference point for the design of future investigations.

One additional point should be noted. The adverse effects of mutations of βH82K and αH82K on the K_m for $FMNH_2$ were compensated by the comutation of αH82D and βH82D, respectively (Table 3). To lesser degrees, such compensational effects of the double mutations were also observed in thermal and urea inactivations (Table 3). Compensational effects of the modification of one subunit on compromised properties resulting from the modification of the other luciferase subunit have not been demonstrated previously. The αHis82 and βHis82 sites provide an interesting system for further studies on the intersubunit communication of luciferase.

REFERENCES

Baldwin, T. O., Chen, L. H., Chlumsky, L. J., Devine, J. H., Johnston, T. C., Lin, J.-W., Sugihara, J., Waddle, J. J., & Ziegler, M. M. (1987) in *Flavins and Flavoproteins* (Edmondson, D. E., & McCormick, D. B., Eds.) pp 621–631, Water de Gruyter and Co., Berlin.

Baldwin, T. O., Chen, L. H., Chlumsky, L. J., Devine, J. H., & Ziegler, M. M. (1989) *J. Biolumin. Chemilumin. 4,* 40–48.

Becvar, J. E., & Hastings, J. W. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72,* 3374–3376.

Chen, L. H., & Baldwin, T. O. (1989) *Biochemistry 28,* 2684–2689.

Cho, K. W., Tu, S.-C., & Shao, R. (1993) *Photochem. Photobiol. 57,* 396–402.

Cid, H. (1987) in *Chemical Modification of Enzymes: Active Site Studies* (Eyzaguirre, J., Ed.) pp 97–118, Ellis Horwood Limited, Chichester, U.K.

Cline, T. W. (1973) Ph.D. Thesis, Harvard University, Cambridge, MA.

Cline, T. W., & Hastings, J. W. (1972) *Biochemistry 11,* 3359–3370.

Cohn, D. H., Mileham, A. J., Simon, M. I., Nealson, K. H., Rausch, S. K., Bonam, D., & Baldwin, T. O. (1985) *J. Biol. Chem. 260,* 6139–6146.

Cousineau, J., & Meighen, E. A. (1976) *Biochemistry 15,* 4992–5000.

Devereux, J., Haeberli, P., & Smithies, O. (1984) *Nucleic Acids Res. 12,* 387–395.

Dunn, D. K., Michaliszyn, G. A., Bogacki, I. G., & Meighen, E. A. (1973) *Biochemistry 12,* 4911–4918.

Empie, M. W., & Laskowski, M. (1982) *Biochemistry 21,* 2274–2284.

Foran, D. R., & Brown, W. M. (1988) *Nucleic Acids Res. 16,* 777.

Hastings, J. W., & Balny, C. (1975) *J. Biol. Chem. 250,* 7288–7293.

Hastings, J. W., Baldwin, T. O., & Nicoli, M. Z. (1978) *Methods Enzymol. 57,* 135–152.

Illarionov, B. A., Protopopova, M. V., Karginov, V. A., Mertvetsov, N. P., & Gitelson, J. I. (1988) *Nucleic Acids Res. 16,* 9855.

Johnston, T. C., Thompson, R. B., & Baldwin, T. O. (1986) *J. Biol. Chem. 261,* 4805–4811.

Johnston, T. C., Rucker, E. B., Cochrum, L., Hruska, K. S., & Vandegrift, V. (1990) *Biochem. Biophys. Res. Commun. 170,* 407–415.

Lei, B.-F., & Becvar, J. E. (1991) *Photochem. Photobiol. 54,* 473–476.

Li, Z., Szittner, R., & Meighen, E. A. (1993) *Biochim. Biophys. Acta 1158,* 137–145.

Mager, H. I. X., Sazou, D., Liu, Y. H., Tu, S.-C., & Kadish, K. M. (1988) *J. Am. Chem. Soc. 110,* 3759–3762.

Meighen, E. A. (1991) *Microbiol. Rev. 55,* 123–142.

Meighen, E. A., & Bartlet, I. (1980) *J. Biol. Chem. 255,* 11181–11187.

Meighen, E. A., Nicoli, M. Z., & Hastings, J. W. (1971a) *Biochemistry 10,* 4062–4068.

Meighen, E. A., Nicoli, M. Z., & Hastings, J. W. (1971b) *Biochemistry 10,* 4069–4073.
Nicoli, M. Z., Meighen, E. A., & Hastings, J. W. (1974) *J. Biol. Chem. 249,* 2385–2392.
Paquatte, O., & Tu, S.-C. (1989) *Photochem. Photobiol. 50,* 817–825.
Paquatte, O., Fried, A., & Tu, S.-C. (1988) *Arch. Biochem. Biophys. 264,* 392–399.
Ruby, E. G., & Hastings, J. W. (1980) *Biochemistry 19,* 4989–4993.
Sinclair, J. F., Waddle, J. J., Waddill, E. F., & Baldwin, T. O. (1993) *Biochemistry 32,* 5036–5044.
Suzuki, K., Kaidoh, T., Katagiri, M., & Tsuchiya, T. (1983) *Biochim. Biophys. Acta 722,* 297–301.
Szittner, R., & Meighen, E. (1990) *J. Biol. Chem. 265,* 16581–16587.
Tu, S.-C. (1982) *J. Biol. Chem. 257,* 3719–3725.
Tu, S.-C. (1986) *Methods Enzymol. 133,* 128–139.
Tu, S.-C., & Henkin, J. (1983) *Biochemistry 22,* 519–523.
Tu, S.-C., Wang, L.-H., & Yu, Y. (1987) in *Flavins and Flavoproteins* (Edmondson, D. E., & McCormick, D. B., Eds.) pp 539–548, Water de Gruyter and Co., Berlin.
Watanabe, H., Hastings, J. W., & Tu, S.-C. (1982) *Arch. Biochem. Biophys. 215,* 405–413.
Welches, W. R., & Baldwin, T. O. (1981) *Biochemistry 20,* 512–517.
Wells, J. A. (1990) *Biochemistry 29,* 8509–8517.
Wilkinson, A. J., Fersht, A. R., Blow, D. M., & Winter, G. (1983) *Biochemistry 22,* 3581–3586.
Xi, L., Cho, K. W., Herndon, M. E., & Tu, S.-C. (1990) *J. Biol. Chem. 265,* 4200–4203.
Xi, L., Cho, K.-W., & Tu, S.-C. (1991) *J. Bacteriol. 173,* 1399–1405.
Xin, X., Xi, L., & Tu, S.-C. (1991) *Biochemistry 30,* 11255–11262.