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Random and Site-Directed Mutagenesis of Bacterial Luciferase: Investigation of the Aldehyde Binding Site†

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ABSTRACT: Numerous luciferase structural gene mutants of *Vibrio harveyi* have been generated by random mutagenesis and phenotypically characterized [Cline, T. W., & Hastings, J. W. (1972) *Biochemistry 11*, 3359-3370]. All mutants selected by Cline and Hastings for altered kinetics in the bioluminescence reaction had lesions in the α subunit. One of these mutants, AK-20, has normal or slightly enhanced thermal stability and enhanced $FMNH_2$ binding affinity but a much-reduced quantum yield of bioluminescence and dramatically altered stability of the aldehyde–C4a-peroxydihydroflavin–luciferase intermediate (IIA), with a different aldehyde chain length dependence from that of the wild-type luciferase. To better understand the structural aspects of the aldehyde binding site in bacterial luciferase, we have cloned the *lux*AB genes from the *V. harveyi* mutant AK-20, determined the nucleotide sequence of the entire *lux*A gene, and determined the mutation to be TCT → TTT, resulting in a change of serine → phenylalanine at position 227 of the α subunit. To confirm that this alteration caused the altered kinetic properties of AK-20, we reverted the AK-20 *lux*A gene by oligonucleotide-directed site-specific mutagenesis to the wild-type sequence and found that the resulting enzyme is indistinguishable from the wild-type luciferase with respect to quantum yield, $FMNH_2$ binding affinity, and intermediate IIA decay rates with 1-octanal, 1-decanal, and 1-dodecanal. To investigate the cause of the AK-20 phenotype, i.e., whether the phenotype is due to loss of the seryl residue or to the properties of the phenylalanyl residue, we have constructed mutants with alanine, tyrosine, and tryptophan at α227. The luciferases with α227 Tyr and α227 Trp exhibited kinetic and bioluminescence properties comparable to those of the AK-20 luciferase (α227 Phe) and distinctly different from those of the wild-type luciferase (α227 Ser), whereas luciferase with α227 Ala was similar to the wild-type enzyme in kinetics and quantum yield, suggesting that the properties of AK-20 must be due more to the introduction of the bulky aromatic group at α227 than to the loss of the serine hydroxyl group at that position. The results obtained here do not support a role of αSer_{227} in catalysis, since the αAla_{227} mutant has nearly wild-type activity.

Bacterial luciferase is a flavin monooxygenase that catalyzes the oxidation of a long-chain aliphatic aldehyde to the corresponding carboxylic acid (Scheme I). During the course of this reaction, an enzyme-bound excited state of the flavin is generated that, upon decay, emits blue-green light (λ_{max} = 490 nm). The enzyme is a heterodimer with a single active center thought to reside primarily on the α subunit, although the β subunit is absolutely required for activity [see Ziegler and Baldwin (1981) for a review].

Investigation of the mechanism of action of many enzymes has been enhanced substantially by analysis of mutant forms of the proteins. Although the structural details of proteins provided by high-resolution X-ray crystallographic data are extremely valuable, the data are seldom sufficient to allow one to draw conclusions regarding the mechanisms by which enzymes carry out their functions. To date, we do not have a

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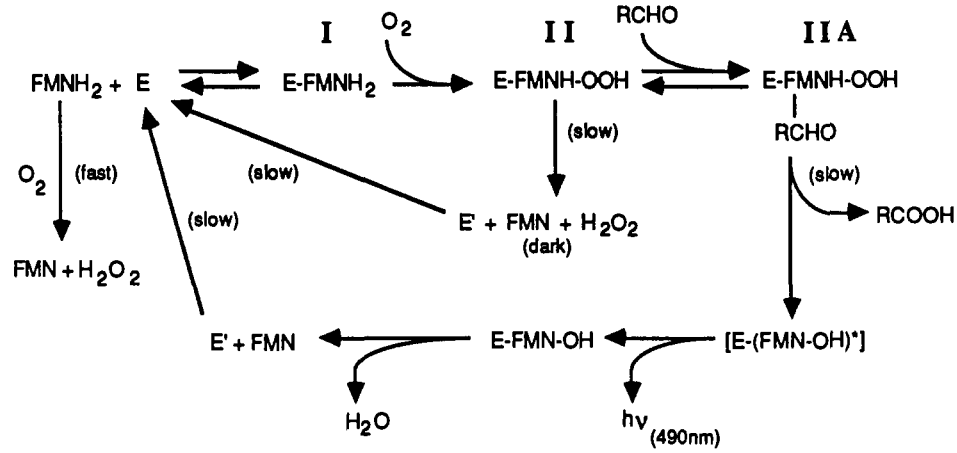
Scheme I



high-resolution crystal structure of bacterial luciferase, but the structural analysis is under way (Swanson et al., 1985). In the meantime, applications of the classical techniques of random mutagenesis and the newer site-directed mutagenesis methods should allow us to probe the distribution of the residues in the primary structure that are critical to the formation of the catalytic center of the enzyme. We have available a collection of more than 20 luciferase structural gene mutants generated by Cline and Hastings (1972), using *N*-methyl-*N*-nitro-*N*-nitrosoguanidine. By screening many of the dim variants of *Vibrio harveyi*, Cline and Hastings (1972) found that mutant forms of luciferase demonstrating specifically altered kinetic properties all had mutations in the α subunit.

For the studies reported here, we investigated the mutant AK-20 from the collection of Cline and Hastings (1972). According to Cline and Hastings, the AK-20 luciferase had an enhanced $FMNH_2$[1] binding affinity relative to the wild type and a much lower affinity for the aldehyde substrate. In addition, the aldehyde chain length dependence for the decay of the enzyme-bound C4a-hydroperoxyflavin–aldehyde complex (intermediate IIA, Scheme I) showed a dramatic alteration relative to that of the wild type. Thus the lesion in the AK-20 luciferase may be in or near the aldehyde binding site. The enzyme had normal thermal stability, suggesting that the alteration had not caused any major perturbation in the tertiary or quaternary structure of the enzyme (Cline & Hastings, 1972).

We report here the cloning of the *lux*AB genes from the *V. harveyi* mutant AK-20, the determination of the complete nucleotide sequence of the mutant *lux*A gene, and the elucidation of the single base change leading to the AK-20 phenotype. We have confirmed that the sole change in the *lux*A gene of AK-20 is TCT → TTT, resulting in Ser → Phe at position 227 of the α subunit, by reversion of the mutant *lux*A gene to the wild-type sequence and analysis of purified enzymes from *Escherichia coli* carrying the cloned wild-type, AK-20, and revertant genes. Furthermore, using site-directed mutagenesis, we have constructed three mutants containing alanine, tyrosine, and tryptophan at the same position in the α subunit.

Experimental Procedures

Materials. Restriction endonucleases were purchased from New England Biolabs and DNA polymerase I (Klenow fragment), T4 DNA polymerase, and DNA ligase were obtained from U.S. Biochemicals. Oligonucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer. NA-45 cellulose filter paper was from Schleicher & Schuell. Radiolabeled deoxynucleoside triphosphates were purchased from New England Nuclear; deoxy- and dideoxynucleotides were from Pharmacia; acrylamide, bis(acrylamide), and urea were from Schwarz/Mann Biotech; 1-octanal, 1-decanal, 1-dodecanal, FMN, and bovine serum albumin were from Sigma Fine Chemicals. All other reagents were of the best quality commercially available.

Bacterial Strains. *V. harveyi* strain B392 (Baumann et al., 1980; ATCC 33843) and the *V. harveyi* mutant AK-20, originally from the laboratory of Prof. J. W. Hastings (Harvard University), have been maintained in this laboratory since 1975. *E. coli* strains used include TB1 (an r^-,m^+ strain generated from JM83 by phage P1 mediated transduction from MM294) (Baldwin et al., 1984), HB101 (Maniatis et al., 1982), JM103Y (strain JM103 cured of P1 phage, courtesy of Dr. Gary Gussin, Massachusetts General Hospital), and CJ236 (Kunkel et al., 1987).

Cloning of the AK-20 lux Genes. Genomic DNA from AK-20 was isolated as described by Cohn et al. (1983) from cells grown at 30 °C in seawater complete medium (Nealson, 1978). The DNA was digested with *Hin*dIII and *Bgl*II restriction endonucleases to effect the correct orientation upon insertion into the vector. DNA fragments were resolved on a 1% agarose gel. Fragments of three to four kilobases were isolated by electrophoresis onto an NA-45 cellulose membrane (Schleicher & Schuell, Publication 364). The purified fragments were eluted from the NA-45 membrane with a high-salt buffer and ligated at 16 °C into a pUC9 vector cut with *Hin*dIII and *Bam*HI. The recombinant plasmids were used to transform TB1 cells grown on LB/carbenicillin plates at 37 °C; cells carrying the AK-20 *lux*AB genes were detected visually following exposure of the cells to 1-octanal vapor. The plasmid containing the AK-20 *lux*AB genes was designated pLHC1.

Sequencing of the AK-20 luxA Gene. The *lux*A genes from the recombinant plasmids pTB718 (Baldwin et al., 1984) and pLHC1 were removed by digestion with *Hin*dIII and *Eco*RI. The *lux*A-containing fragment from each plasmid was isolated and purified by electrophoresis onto NA-45 cellulose paper as described above and ligated into phage M13 vector (mp19) digested with the same restriction endonucleases. This insertion resulted in an in-frame fusion of the N-terminal end of the β subunit (*lux*B) with the *lacZ'* of the M13mp19, such that the resultant phage would synthesize functional β-ga-

[1] Abbreviations: FMN, riboflavin 5'-phosphate; $FMNH_2$, reduced FMN; LB, Luria–Bertani medium; X-gal, 5-bromo-4-chloro-3-indolyl β-galactoside; SDS, sodium dodecyl sulfate; LU, light unit.

Table I: Message-Complementary Primers Used To Sequence *lux*A from Wild-Type and AK-20 DNA

		PRIMER SEQUENCE		LOCATION [a]
Primer A	3'	C C T A A T A A G A A G G A G	5'	1104-1118
B	3'	G G T C A T C C C T G C G G A		898-912
C	3'	C G G C G T T G A A G A A C C C		728-743
D	3'	C C G G A T G G T T A C T A A		559-573
E	3'	A C C A T A C T G A A C T A C T		391-406
F	3'	C G T C G G C G T G T G G A T		172-186

[a] The location refers to the nucleotide positions designated in Cohn et al. (1985).

Table II: Oligonucleotides Used in the Site-Specific Mutagenesis of *lux*A[a]

base pair number [b]		667 ... 696
AK-20 template [c]	5'	GAC CAC TGT TTG TTT TAC ATC ACC TCC
		D H C L *F*$_{227}$ Y I T S
Phe → Ser Primer	3'	...........CA AAC AGA ATG TAG TGG AGG
Ser → Ala Primer	3'	...........CA AAC CGA ATG TAG TGG AGG
Ser → Tyr Primer	3'	...........CA AAC ATA ATG TAG TGG AGG
Ser → Trp Primer	3'	CTG GTG ACA AAC ACC ATG TAG TGG AGG

[a] The oligonucleotides containing the mismatches to either wild-type or AK-20 *lux*A are shown with the mismatched codons underlined. [b] The base pair location refers to the nucleotide positions designated in Cohn et al. (1985). [c] The amino acids encoded by the *lux*A template are indicated by the single-letter abbreviations, and the wild type → AK-20 mutation is italicized.

lactosidase and appear as blue plaques in the presence of X-gal. JM103Y cells were transfected with these recombinant phage; blue plaques were obtained upon spreading the infected cells on LB medium. The recombinant phage containing the wild-type and AK-20 *lux*A genes were designated M13WT and M13AK20, respectively, and sequenced by the chain termination method of Sanger et al. (1977). Since the sequence of the wild-type *lux*A gene was known (Cohn et al., 1985), oligonucleotides specific for ca. 300-bp intervals of *lux*A were synthesized and used as primers for sequencing the AK-20 *lux*A gene (Table I).

Back-Mutation of the AK-20 luxA to Wild Type and Generation of Site-Directed Mutants S227A, S227Y, and S227W. CJ236 cells (*dut*$^-$ *ung*$^-$), kindly provided by Dr. T. A. Kunkel, were infected with M13WT and M13AK20. A plaque of each type was picked and restreaked in LB/streptomycin medium, thus generating phage with uracil-containing DNA (Kunkel, 1985; Kunkel et al., 1987). Single-stranded DNA template was isolated from M13AK20 and hybridized to a 20-residue oligonucleotide that spanned the point of the mutation in the *lux*A gene. This oligonucleotide contained a mismatch (TTT → TCT) that would allow reversion of Phe → Ser at α position 227. The oligonucleotides used in making S227A, S227Y, and S227W were of similar length, except for the oligomer used in the design of S227W, which contained 30 residues (Table II). Extension of the hybrid was carried out at room temperature by T4 DNA polymerase, and ligation was carried out at 16 °C by DNA ligase. The covalently closed circular DNA was used to transfect JM103Y cells. Single-stranded template DNA was isolated from the resultant phage and screened by DNA sequencing. The mutant *lux*A gene was excised from the RF form of the phage DNA and inserted into a vector (pLAV11) containing only the wild-type *lux*B gene (L. J. Chlumsky and T. O. Baldwin, unpublished results). These recombinant plasmids were used to transform TB1 cells to growth on LB/carbenicillin plates. The initial screen for cells encoding the altered enzymes was by light emission upon exposure to 1-octanal and 1-decanal vapor. The DNA sequences of the plasmids that showed bioluminescence activity were then confirmed by sequencing of the *lux*A gene in the recombinant plasmids (Chen & Seeburg, 1985; Sequenase Protocol, 3rd ed., U.S. Biochemical Corp.).

Luciferase Isolation and Purification. The cells harboring the cloned luciferases were grown in LB/carbenicillin medium at 37 °C and harvested at the end of exponential-growth phase. The cells were subjected to French pressure cell lysis followed by a 40%–70% ammonium sulfate fractionation step. The pellet was dialyzed against 0.1 M phosphate buffer and solubilized protein chromatographed on a luciferase affinity resin as described by Holzman and Baldwin (1982). Analysis of the pooled fractions on SDS gels indicated greater than 95% purity.

Luciferase Assays and Data Acquisition. Unless otherwise indicated, bioluminescence activity assays were performed at 25 °C by the standard $FMNH_2$ injection assay in which catalytically reduced FMN is injected into a vial containing luciferase, aldehyde, and dissolved O_2 in 1 mL of BSA-containing buffer (Hastings et al., 1978). For certain experiments, the "dithionite assay" of Meighen and Hastings (1971) was used as modified by Meighen and MacKenzie (1973). In the dithionite assay, a minimal amount of sodium dithionite is added to a vial containing luciferase and FMN in air-equilibrated buffer. The dithionite reduces the FMN and removes the O_2 from the solution. The bioluminescence reaction is initiated by injection of an air-equilibrated sonicated suspension of aldehyde. Bioluminescence was monitored on a photomultiplier photometer (Mitchell & Hastings, 1971) calibrated with the radioactive liquid standard of Hastings and Weber (1963) (1 LU = 9.5×10^9 quanta s^{-1}). Because of the rapid nonenzymatic oxidation of excess $FMNH_2$ in the reaction mixture, both assays involve a single turnover of the enzyme; at saturating flavin and aldehyde concentrations, the peak luminescence emission (I_{max}) is proportional to the enzyme concentration, and the bioluminescence decay rate (k_b) reflects the decay of intermediate IIA in Scheme I (Hastings & Gibson, 1963; Cline & Hastings, 1972). Measurement of decay rates in these experiments entailed acquisition and processing of data from the photomultiplier–photometer with a dedicated IBM/AT computer. Programs for the acquisition and analyses of the data were written in this laboratory by Joseph Chambley and Dr. Stan Swanson. Data were taken at 1–8-ms intervals over the first 4.5 s, at 8-ms intervals over the next 4 s, and at 16-ms intervals for the next 8 s, covering a total of 16.5 s from the time of initiation of the reaction.

Results and Discussion

Cloning of the AK-20 lux Genes. When the bacterial colonies harboring the recombinant plasmids, generated as described under Experimental Procedures, were screened for light emission in the presence of 1-decanal, very low light levels were observed from several of the ca. 800 colonies. However, in the presence of 1-octanal, 24 of the colonies examined exhibited substantially increased bioluminescence activity. That these colonies emitted a higher steady-state light intensity in the presence of 1-octanal than in the presence of 1-decanal was very interesting and was probably due to the unique aldehyde chain length dependence characteristic of the AK-20 luciferase (Cline & Hastings, 1972). One glowing colony was isolated and restreaked on LB/carbenicillin plates several times to ensure that the eventual population of cells was homogeneous. Restriction endonuclease mapping revealed that the

Table III: Comparison of *V. harveyi* AK-20 Mutant Luciferase with Cloned AK-20 Luciferase Expressed in *E. coli*

enzyme source[d]	K_d for $FMNH_2$ (μM)[a]		k_b (s^{-1})[b]			cq (quanta/mg of luciferase) $\times 10^{-14}$[c]		
	1-octanal	1-decanal	1-octanal	1-decanal	1-dodecanal	1-octanal	1-decanal	1-dodecanal
AK20	0.67	0.49	0.277	0.109	0.157	1.70	0.47	1.34
LHC1	0.57	0.38	0.278	0.112	0.159	1.69	0.46	1.32

[a] $FMNH_2$ binding studies were carried out at 25 °C with the dithionite assay: the enzyme was mixed with the flavin solution containing 25 mM β-mercaptoethanol and reduced with sodium dithionite. The reaction was initiated by the vigorous injection of 1 mL of a 0.05% sonicated aldehyde suspension at 25 °C; the [$FMNH_2$] range was from 3.0 to 0.05 μM; final [E] = 12.7 nM. [b] Rate of intermediate IIA decay, as measured by the rate of bioluminescence decay. The decay was monitored for 16 s, as described under Experimental Procedures. The decay rate was then determined for the period 2–6 s after peak bioluminescence emission. [c] Total quanta per turnover per milligram of the enzyme with the designated aldehyde, calculated as I_{max} (Q/s) ÷ k_b. I_{max} is the maximum light output under saturating aldehyde and $FMNH_2$ conditions. [d] Source of luciferase: AK-20, luciferase isolated from the *V. harveyi* mutant AK-20; LHC1, cloned AK-20 luciferase.

plasmid construction was similar to that generated by Baldwin et al. (1984) and Waddle et al. (1987). This recombinant plasmid containing the AK-20 *lux*AB genes was designated pLHC1.

Sequencing of the AK-20 luxA Gene. The entire *lux*A gene of the AK-20 luciferase was sequenced as described under Experimental Procedures. In comparison to the wild-type *lux*A sequence, we found only one base change (C → T) in the entire *lux*A gene, resulting in substitution of a phenylalanyl residue for serine at position 227 of the amino acid sequence of the α subunit. The DNA sequencing gel showing this mutation is shown in Figure 1.

The amino acid sequence of the residues in the immediate vicinity of the mutation is -His_{224}-Cys_{225}-Leu_{226}-Ser_{227}-Tyr_{228}-. The serine to phenylalanine change in this region of the α subunit is significant in several respects. These results give the first indication that this region of the α subunit affects the structure of the active center of luciferase. Previous studies on luciferase utilizing chemical modification have shown that modification of the α-amino group of either subunit (Welches & Baldwin, 1981), Cys_{106} of the α subunit (Ziegler-Nicoli et al., 1974), or a single histidinyl residue on the α subunit (Cousineau & Meighen, 1976) results in inactivation of the enzyme. Limited proteolysis studies have indicated that the regions of the α subunit near amino acid residues 280 and 115 are particularly susceptible to proteolytic digestion by trypsin and chymotrypsin and are probably in or near the active center (Rausch, 1983). The region near Ser_{227} has not been implicated with respect to trypsin- and chymotrypsin-labile bonds or by chemical modification to contribute to the active center.

Back-Mutation of AK-20 Luciferase to Wild Type. To show that the AK-20 phenotype was due solely to the Ser → Phe substitution at position 227 of the α subunit, we sought to revert the luciferase from pLHC1 to the wild type. The altered *lux*A gene was excised from phage M13mp19 and cloned into the pLAV11 construct with the *lux*A deleted (L. J. Chlumsky and T. O. Baldwin, unpublished results). This construct was designed such that the encoded luciferase contained the revertant α subunit and wild-type β subunit. The resultant colonies were screened for bioluminescence in the presence of 1-octanal and 1-decanal and were found to have wild-type chain length dependence with the two aldehydes [brighter with 1-decanal than with 1-octanal, whereas the cells bearing the parent plasmid (pLHC1 with the AK-20 mutation) were brighter with 1-octanal than with 1-decanal]. The revertant plasmid, apparently identical with wild type, was designated pLHC2; its *lux*AB gene product is designated αF227S.

Comparison of the Functional Properties of the Luciferases Encoded by the Wild-Type, AK-20, and Revertant luxAB Genes. Luciferases from both *E. coli* (Baldwin et al., 1984) and *V. harveyi* were isolated and purified as described under Experimental Procedures. A comparison of several properties

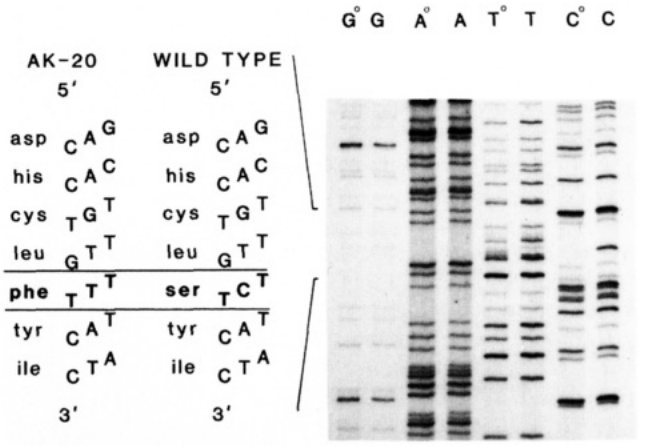


FIGURE 1: Autoradiograph of *lux*A DNA sequencing ladder depicting the base change (C → T) resulting in Ser → Phe mutation at position 227 of the α subunit. The sequencing ladder was generated by the dideoxy chain termination method (Sanger et al., 1977), and samples were loaded such that the corresponding lanes of the mutant (G°, A°, T°, C°) and wild-type (G, A, T, C) sequences could be read side by side. The amino acids corresponding to the appropriate codons are also shown.

of the AK-20 luciferase with those of the cloned AK-20 luciferase isolated from *E. coli* cells bearing pLHC1 (Table III) shows that the cloned luciferase and the *V. harveyi* AK-20 luciferase possess the same kinetic properties. The aldehyde chain length dependence for the decay of intermediate IIA and $FMNH_2$ binding affinity are significantly different from those of the wild-type enzyme.

That we have reverted the AK-20 luciferase to the wild type by oligonucleotide-directed mutagenesis is demonstrated by a comparison of the properties of the revertant luciferase, αF227S, with those of *V. harveyi* (wild type) and cloned wild-type luciferase (Table IV). The kinetic parameters of these latter three luciferases are similar, demonstrating a successful reversion of the AK-20 luciferase to wild type by a single base change.

We have also constructed the three mutants αS227A, αS227Y, and αS227W containing alanine, tyrosine, and tryptophan, respectively, at the site of the AK-20 mutation. These mutants were generated in an attempt to correlate the AK-20 phenotype with either the loss of the serine hydroxyl group or the incorporation of the bulky, aromatic group of phenylalanine. Comparisons of the site-specific mutant enzyme activities with those of wild-type and AK-20 luciferases are shown in Table V. The tyrosine and tryptophan mutants show an even lower affinity for $FMNH_2$ than the wild type. The decay rates of intermediates IIA (k_b) and the total light yields from the tyrosine and tryptophan mutants are only slightly different from those of the AK-20 luciferase, but definitely unlike the those of wild type. The maximum light intensity (I_{max}) from one turnover of the AK-20 luciferase with

Table IV: Comparison of Wild-Type *V. harveyi* Luciferase with Cloned Wild-Type and Revertant Enzymes Expressed in *E. coli*

	K_d for $FMNH_2$ (μM)[a]		k_b (s^{-1})[b]			cq (quanta/mg of luciferase) $\times 10^{-14}$[c]		
enzyme source[d]	1-octanal	1-decanal	1-octanal	1-decanal	1-dodecanal	1-octanal	1-decanal	1-dodecanal
LHC2	1.8	1.0	0.085	0.470	0.074	5.18	5.32	5.95
JH1	1.6	0.89	0.085	0.467	0.068	5.18	5.35	6.47
WT	1.6	0.87	0.085	0.443	0.064	5.18	5.64	6.87

[a] $FMNH_2$ binding studies were carried out at 25 °C with the dithionite assay: the enzyme was mixed with the flavin solution containing 25 mM β-mercaptoethanol and reduced with sodium dithionite. The reaction was initiated by the vigorous injection of 1 mL of a 0.05% sonicated aldehyde suspension at 25 °C; the $[FMNH]_2$ range was from 3.0 to 0.05 μM; final [E] = 12.7 nM. [b] Rate of intermediate IIA decay, as measured by the rate of bioluminescence decay. The decay was monitored for 16 s, as described under Experimental Procedures. The decay rate was then determined for the period 2–6 s after peak bioluminescence emission. [c] Total quanta per turnover per milligram of the enzyme with the designated aldehyde, calculated as I_{max} (Q/s) ÷ k_b. I_{max} is the maximum light output under saturating aldehyde and $FMNH_2$ conditions. [d] Source of luciferase: WT, luciferase isolated from *V. harveyi*; JH1, cloned *V. harveyi* luciferase isolated from *E. coli*; LHC2, AK-20 luciferase reverted to wild type and isolated from *E. coli*.

Table V: Comparison of AK-20, Wild-Type, and Altered Luciferases Constructed by Site-Directed Mutagenesis[a]

enzyme source[b]	K_d for $FMNH_2$ (μM) (1-decanal)	k_b (s^{-1})			cq (quanta/mg of luciferase) $\times 10^{-14}$		
		1-octanal	1-decanal	1-dodecanal	1-octanal	1-decanal	1-dodecanal
AK-20	0.49	0.277	0.109	0.157	1.70	0.47	1.34
WT	0.87	0.085	0.443	0.064	5.18	5.35	6.87
αS227A	1.1	0.081	0.469	0.070	5.22	5.67	5.38
αS227Y	1.5	0.500	0.162	0.226	2.00	0.51	1.64
αS227W	6.8	0.422	0.190	0.376	2.67	1.07	0.21

[a] Experimental conditions are the same as for Tables III and IV. [b] AK-20, luciferase isolated from the *V. harveyi* mutant AK-20; WT, luciferase isolated from *V. harveyi*; αS227A, luciferase mutant containing a Ser → Ala substitution at 227 of the α subunit; αS227W, luciferase mutant containing a Ser → Trp substitution at 227 of the α subunit; αS227Y, luciferase mutant containing a Ser → Tyr substitution at 227 of the α subunit.

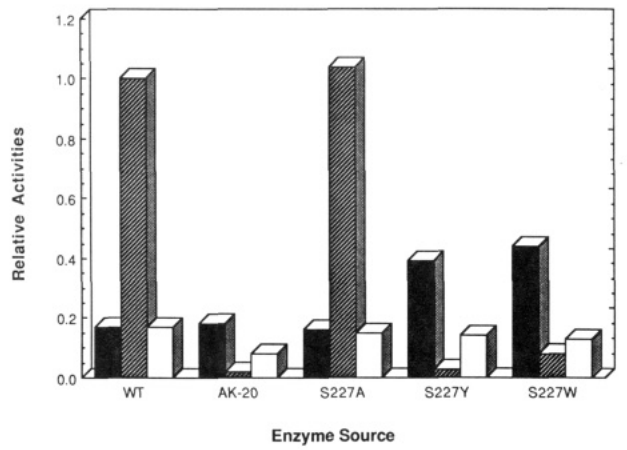


FIGURE 2: Relative maximum activities (I_{max}) of luciferases from wild type, AK-20, and mutants generated by site-directed mutagenesis with three different aldehyde substrates. Bioluminescence was measured by the dithionite method at saturating levels of substrates as described under Experimental Procedures. The aldehydes used in these experiments were 1-octanal (solid bars), 1-decanal (hatched bars), and 1-dodecanal (open bars).

1-decanal is only about 2% of the wild-type intensity, while I_{max} values for the tyrosine and tryptophan mutants are about 3% and 8%, respectively (Figure 2). Finally, the decay rates of the intermediates IIA from these mutants show aldehyde alkyl chain length dependence similar to that of AK-20 and unlike the wild-type pattern. Wild-type intermediate IIA decays faster with 1-decanal than with 1-octanal or 1-dodecanal, whereas IIA from AK-20 and the tyrosine and tryptophan mutants decays more rapidly with 1-octanal. The site-directed mutant with alanine at position α227, however, is very similar to the wild-type enzyme with respect to $FMNH_2$ binding affinity, intermediate IIA decay rates with the different aldehydes, I_{max}, and total bioluminescence yield.

These results demonstrate that the AK-20 phenotype is due more to the incorporation of the bulky phenylalanine than to the loss of the serine hydroxyl group. However, there may be other factors involved, as evidenced by the decreased $FMNH_2$ affinity and intermediate IIA stability, especially in the case of the tryptophan mutant. Overall, these α227 mutants suggest very strongly that this region of the protein affects the structure of the active center. While α227 may comprise a part of the active center, it is also possible that this region does not contribute directly to the active center but that the insertion of large hydrophobic groups at α227 affects the tertiary structure such that changes are transmitted to the active center. We do not favor this interpretation; if the effect is transmitted from a distant location to the active center, it is not manifested in thermal instability or more global effects on the active centers of these enzymes.

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Registry No. Ser, 56-45-1; Phe, 63-91-2; Ala, 56-41-7; Tyr, 60-18-4; Trp, 73-22-3; $FMNH_2$, 5666-16-0; octanal, 124-13-0; decanal, 112-31-2; dodecanal, 112-54-9; luciferase, 9014-00-0.

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Identification of Electron-Transfer Reactions Involving the Acceptor A_1 of Photosystem I at Room Temperature

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ABSTRACT: The electron-transfer reactions of photosystem I (PS I) were investigated by flash-absorption spectroscopy at room temperature under highly reducing conditions in spinach and in the cyanobacterium *Synechocystis* 6803. Different conditions of background illumination were used in the presence of the reductant dithionite to generate two different redox states of the reaction center by photoaccumulating electrons on the PS I acceptors. When the iron–sulfur center Fe-S_X is prereduced by a weak illumination, the relaxation of flash-induced absorption changes exhibits two decay phases with $t_{1/2} \approx 750$ ns (in *Synechocystis*) and a few microseconds. These two phases can be ascribed respectively to the back-reaction between the oxidized primary donor P-700$^+$ and the reduced secondary acceptor A_1^- and to the decay of the P-700 triplet state, which is formed during this back-reaction with an efficiency larger than 90%. In contrast, at 10 K, the radical pair (P-700$^+$–A_1^-) decays directly to the ground state with $t_{1/2} \approx 20$–25 μs under the same conditions (Fe-S_X prereduced). Under illumination with a strong background light, the recombination kinetics of PS I are characteristic of the decay of the primary radical pair (P-700$^+$–A_0^-) ($t_{1/2} \approx 25$–30 ns) and of the decay of the P-700 triplet state which is formed during the back-reaction. This kinetic behavior is conserved when PS I reaction centers that have been submitted to strong illumination conditions are kept afterward in the dark. This indicates that the electron transfer is blocked at the level of A_0 although Fe-S_X is reoxidized during the dark adaptation. The results show that this block is probably due to the fact that A_1 stays reduced in the dark although no radical EPR signal is observable. To explain these observations, a process of A_1 double reduction is proposed that supports the identification of A_1 with a quinone molecule (vitamin K_1).

The reaction center of photosystem I (PS I)[1] in oxygen-evolving organisms is known to include the primary electron donor P-700 and five electron acceptors. One or possibly several of the iron–sulfur centers Fe-S_A, Fe-S_B, and Fe-S_X reduce soluble ferredoxin, which in turn is thought to be involved in cyclic electron flow around PS I as well as to engage the reduction of NADP$^+$. The primary acceptor A_0 is presumably made of chlorophyll molecule(s) and absorbs around 690–694 nm (Nuijs et al., 1986; Shuvalov et al., 1986; Wasielewski et al., 1987; Mathis et al., 1988). An acceptor named A_1 is generally thought to mediate electron transfer between the primary acceptor A_0 and the iron–sulfur center Fe-S_X (Bonnerjea & Evans, 1982; Gast et al., 1983).

In accordance with the presence of vitamin K_1 (phylloquinone) in PS I reaction centers (Interschick-Niebler & Lichtenthaller, 1981; Takahashi et al., 1985; Schoeder &

[1] Abbreviations: PS I, photosystem I; DPIP, dichlorophenolindophenol; chl, chlorophyll; EPR, electron paramagnetic resonance; CIDEP, chemically induced dynamic electron polarization.

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