AI-1 Influences the Kinase Activity but Not the Phosphatase Activity of LuxN of Vibrio harveyi *

Received for publication, May 1, 2006, and in revised form, June 27, 2006. Published, JBC Papers in Press, June 28, 2006, DOI 10.1074/jbc.M604108200

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The Gram-negative bacterium Vibrio harveyi produces and responds to three autoinducers, AI-1, AI-2, and CAI-1 to regulate cell density dependent gene expression by a process referred to as quorum sensing. The concentration of the autoinducers is sensed by three cognate hybrid kinases, and information is channeled via the HPT protein LuxU to the response regulator LuxO. Here, a detailed biochemical study on the enzymatic activities of the membrane-integrated hybrid kinase LuxN, the sensor for N-(3-hydroxybutanoyl)homoserine lactone (AI-1), is provided. LuxN was heterologously overproduced as the full-length protein in Escherichia coli. LuxN activities were characterized in vitro and are an autophosphorylation activity with an unusually high ATP turnover rate, stable LuxU phosphorylation, and a slow phosphatase activity with LuxU-P as substrate. The presence of AI-1 affected the kinase but not the phosphatase activity of LuxN. The influence of AI-1 on the LuxN→LuxU signaling step was monitored, and in the presence of AI-1, the kinase activity of LuxN, and hence the amount of LuxU-P produced, were significantly reduced. Half-maximal inhibition of kinase activity by AI-1 occurred at 20 μM. Together, these results indicate that AI-1 directly interacts with LuxN to down-regulate its autokinase activity and suggest that the key regulatory step of the AI-1 quorum sensing system of Vibrio harveyi is AI-1-mediated repression of the LuxN kinase activity.

Vibrio harveyi is a free-living marine bacterium that controls bioluminescence, exopolysaccharide and siderophore production, type III secretion, and other processes through quorum sensing. V. harveyi has three quorum sensing systems. AI-1, an acyl homoserine lactone, sensed by LuxN is employed for interspecies signaling (1). AI-2, a furanosyl borate diester, sensed by LuxN is employed for intraspecies signaling (2). CAI-1, whose chemical nature is unknown, is sensed by CqsS and is proposed to be responsible for Vibrio-specific signaling (3). Information from all three hybrid sensor kinases is transduced via phosphorelay to the response regulator LuxO with the HPT protein LuxU acting as intermediate (Fig. 1). Phosphorylated LuxO, a Ω+-dependent response regulator, activates transcription of genes encoding four small regulatory RNAs, which together with Hfq, destabilize the transcript for the LuxR protein, the master transcriptional regulator required for quorum sensing gene expression (4–6). Bioluminescence is used as the canonical readout for V. harveyi quorum sensing controlled gene expression. Genetic studies indicated that LuxO is phosphorylated at low cell density, causing the luciferase operon to be repressed, and the cells are dark. When a threshold concentration of autoinducer is reached, LuxO is dephosphorylated, leading to induction of the luciferase operon and a bright phenotype (Fig. 1). It has been postulated that the hybrid sensor kinases switch from kinases to phosphatases at high autoinducer concentration and hence high cell density (3–5, 7).

Despite numerous genetic studies of quorum sensing in V. harveyi, biochemical characterization of the corresponding proteins is rare. The solution structure of LuxU was solved by NMR. LuxU contains a four-helix bundle with the active-site histidine residue (His396) exposed to the solution (8). Co-crystallization of LuxP and the periplasmic domain of LuxQ showed that LuxP is bound to LuxQ independent of the presence/absence of AI-2 (9). Amino acid replacements of residues located in the LuxP::LuxQ interface sensitized V. harveyi to AI-2, implying that binding of AI-2 to LuxP causes a conformational change within LuxQ that is transmitted across the membrane, presumably affecting LuxQ enzymatic activities (9).

Here, we characterized LuxN’s enzymatic activities and its phosphotransfer properties to LuxU. LuxN possesses an autokinase activity that has an exceptionally high ATP turnover rate. Moreover, AI-1 has an inhibitory effect on LuxN kinase activity, whereas the LuxN phosphatase activity is unaffected by AI-1.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions—E. coli strains TKR2000 (10) and JM109 (11) were used throughout this study. Cells were grown in KML medium (1% tryptone, 1% KCl, 0.5% yeast extract) and incubated aerobically in Erlenhayn flasks in a rotary shaker at 37 °C. When appropriate, the medium was supplemented with ampicillin (100 μg·ml−1). Overexpression of luxN or luxUl was induced by addition of 0.5 mM isopropyl 1-thio-β-d-galactopyranoside for 3 h.

Cloning of luxN and luxUl—The luxN gene was amplified by PCR from purified cosmid DNA using the primers LuxN-
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FIGURE 1. Quorum sensing signal transduction pathway of V. harveyi. H (histidine) and D (aspartate) denote the phosphorylation sites. Arrows indicate the flow of phosphate between the proteins of the cascade. At low cell density, LuxO-P is high. At high cell density, LuxO-P is low. CqsA, LuxS, and LuxM are the synthases of the corresponding autoinducers. PP, periplasm; CM, cytoplasmic membrane; CP, cytoplasm; AI, autoinducer.

BamHI sense (5′-AAAGGGGGATCCATGTTGGATTT-TAGCCTAGAG-3′) and LuxN-PstI antisense (5′-AGGGTG-CCTGCAATCTTCTTCAGCTCCACAAGC-3′). The PCR fragment obtained was digested with BamHI/PstI, gel-purified, and ligated with appropriately digested pTCaiTH (12) to obtain pTLuxN-6His in which luxN is under control of the lac promoter/operator. For cloning of luxN into pKK223-3 (GE Healthcare), a HindIII site in luxN had to be removed. This was accomplished by introducing a silent mutation in the start codon of a DraI site with oligonucleotides LuxN-HindIII sense (5′-GAGGATTTAATCTTCTATT-3′) and LuxN-600 sense (5′-TATTCTATGCGATTGGGC-3′) and applying the PCR overlap extension method (13). The product was digested with XhoI and NsiI, and the resulting 420-bp fragment was introduced into appropriately digested pTlUXN6His resulting in plasmid pTLuxN6His. The mutation was verified by sequencing and detection of a new restriction site for DraI. For construction of pKKLuxN6His a 2646bp fragment of luxN was amplified by PCR from cosmid DNA using the primers LuxU-NdeI sense (5′-AACATTATCGGAGCTTTTTTCAAAGGAGTATAAAT-3′)/LuxU-BamHI antisense (5′-TTGGTT-CCTGCGATCTTATGGTTGTGCAACTGAGC-3′) and LuxU-HindIII antisense (5′-TGAGCTTCAGAAAGCTTGGTTGCTCAAGACAAG-3′) and cloned into plasmid pQE30 (Qiagen) resulting in plasmid pQELuxU6His. As described above, oligonucleotide-directed specific mutagenesis using the PCR overlap extension method was used to introduce codon exchanges corresponding to a replacement of His86 → Glu (13). PCR-generated DNA fragments were verified by sequencing.

Preparation of Inverted Membrane Vesicles—E. coli TKR2000 was transformed with plasmids pKKLuxNb6His, pKKLuxNbH471Q6His, and pKKLuxNbD771N6His, respectively, encoding wild-type LuxN, LuxN-H471Q or LuxN-D771N with a C-terminal His6-tag separated by an amino acid linker consisting of two amino acids. Cells were grown and harvested at late exponential growth phase. Following centrifugation (20 min at 7,000 × g; 4 °C), cells were washed at 4 °C in washing buffer (50 mM Tris/HCl, pH 8.0, 10 mM MgCl2). Subsequently, cells were resuspended and homogenized in lysis buffer (50 mM Tris/HCl, pH 8.0, 10% (v/v) glycerol, 1 mM dithiothreitol (DTT), 2 mM Pefablock SC (Sigma), 30 ng/ml DNase) and disrupted at 4 °C using a cell disruptor (IUL Instruments). Afterward, the suspension obtained was centrifuged (10 min at 11,200 × g; 4 °C) followed by an ultracentrifugation step (1 h at 130,000 × g; 4 °C) to separate soluble proteins from membranes and insoluble proteins. LuxN-containing membrane vesicles were washed in low ionic buffer (1 mM Tris/HCl, pH 8.0, 3 mM EDTA) and resuspended in TG buffer (50 mM Tris/HCl, pH 8.0, 10% (v/v) glycerol).

Purification of LuxU—LuxU-His6 was purified from the cytosolic fraction of E. coli JM109/pQELuxU6His. Cells were grown aerobically in KML medium at 37 °C and induced with isopropyl-1-thio-β-D-galactopyranoside (0.5 mM) during exponential growth (A600 = 0.8) for 3 h. Cells were harvested and lysed as described above. The His-tagged LuxU was purified from the cytosolic fraction by affinity chromatography using nickel-nitrilotriacetic acid-agarose (Qiagen) equilibrated with buffer E (10 mM imidazole, 50 mM Tris/HCl, pH 8.0, 10% (v/v) glycerol, 10 mM 2-mercaptoethanol, 0.4 mM Pefablock SC). His-tagged protein was eluted with buffer E containing 250 mM imidazole. During the entire purification process the temperature was maintained at 4 °C to minimize proteolysis.

Phosphorylation and Dephosphorylation Assays—Autophosphorylation was tested with LuxN in membrane vesicles. A typical reaction mixture (total volume 180 μl) contained 8 mg/ml membrane proteins with LuxN-His6, and when indicated 0.1 mg/ml purified His6-LuxU, in phosphorylation buffer (50 mM Tris/HCl, pH 8.0, 10% (v/v) glycerol, 500 mM KCl, 2 mM DTT). The reaction was started by addition of [γ-32P]ATP (0.94 Ci/mmol) and 110 μM MgCl2. The reaction mixture was incubated at room temperature, and at various time points the reaction was terminated by the addition of SDS loading buffer followed by separation on SDS-polyacrylamide gels. Gels were dried at 80 °C on filter paper or blotted onto nitrocellulose membranes. Dried gels or membranes were exposed to a phosphoscreen for at least 24 h and subsequently scanned using a Phosphoimager SI (Amer sham Biosciences). Signal intensities of phosphorylated proteins were quantified in comparison to an [γ-32P]ATP standard using ImageQuant V5.0.

For dephosphorylation assays, first, LuxU was phosphorylated using LuxN. In this case, the phosphorylation buffer contained 10 mM CaCl2 instead of MgCl2 and twice the amount of
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LuxN and LuxU. After 10-min incubation at room temperature, membrane vesicles were removed by centrifugation (100,000 × g, 15 min, 4 °C), and ATP and CaCl₂ were removed by gel filtration (Sephadex G25 columns, GE Healthcare). Dephosphorylation of LuxU~P (0.6 mg/ml) was initiated by addition of 110 μM MgCl₂, 100 μM ATPγS,² and LuxN in membrane vesicles (8 mg/ml). As described above, samples were subjected to SDS-PAGE and exposed to a phosphoscreen.

Analytical Procedures—Protein was assayed by the method of Peterson (14) using bovine serum albumin as standard. Proteins were separated by SDS-PAGE (15) using 7.5 or 15% acrylamide gels. His-tagged Lux proteins were detected after immunoblotting with monoclonal anti-mouse antibodies against the His-tag (Qiagen) followed by incubation with alkaline phosphatase-conjugated anti-mouse IgG (Rockland) according to the manufacturer’s instructions.

RESULTS

Characterization of the in Vitro Autophosphorylation Activity of LuxN—Full-length LuxN was overproduced in the F₁/F₀ ATPase negative E. coli strain TKR2000. In vitro phosphorylation of LuxN was examined in inverted membrane vesicles. Incubation of these vesicles in the presence of [γ-³²P]ATP led to very rapid, but short-lived autophosphorylation of LuxN (Fig. 2, A and E). The maximal phosphorylation level was reached in less than 1 min, after which the amount of LuxN~P decreased. LuxN autophosphorylation activity was maximal in the presence of K⁺ ions (0.5 M) and under reducing conditions, whereas NaCl, even at low concentration, prevented autophosphorylation completely (data not shown). The turnover rate of LuxN~P was unusually high. No phosphorylation of LuxN was detected using low concentrations of radiolabeled [γ-³²P]ATP with the highest specific radioactivity (3000 Ci/mmol) commercially available (data not shown). An increase of the ATP concentration led to an increase in LuxN autophosphorylation, which was maximal at about 2.5 mM ATP. Thus, for the kinase activity, a Vₘₐₓ of 27 pmol/min/mg of protein, and an apparent Kₘ for ATP of 555 μM were determined. It should be noted that all phosphorylation experiments were performed with 20–100 μM [γ-³²P]ATP, which enabled significant labeling with reasonable levels of radioactivity. Consequently, the rates of LuxN phosphorylation measured were far below the Vₘₐₓ determined for the autokinase activity. LuxN derivatives containing amino acid replacements of the two phosphorylated phosphohistidine sites, LuxN-H₄₇₁Q (Fig. 2B), and LuxN-D₇₇₁N (Fig. 2C) were tested for autokinase activity. Replacement of the conserved histidine residue (His₇₇₁) abolished autophosphorylation activity (Fig. 2B). The weakly phosphorylated protein observed in the figure is unrelated to LuxN. It runs at a slightly higher molecular mass on the gel than does LuxN. Moreover, in control vesicles lacking LuxN, a weakly phosphorylated protein of the same size was observed (Fig. 2D). In contrast, LuxN-D₇₇₁N exhibited 2–3-fold higher phosphorylation than wild-type LuxN (Fig. 2, C and E). Western blots show that this difference cannot be attributed to higher production of the mutant LuxN protein (Fig. 2F), so we interpret this result to be a consequence of the increased stability of the phosphoamidate (His~P) than the mixed acid ester (Asp~P) under the alkaline conditions of an SDS gel (16, 17). Furthermore, an anhydride bond has a higher energy state compared with a phosphoamidate bond. LuxN-D₇₇₁N lacks this high energy phosphorylation site, and thus phosphorylation at His₇₇₁ seems to have increased stability. The autophosphorylation activity of LuxN-D₇₇₁N combined with the lack of activity of LuxN-H₄₇₁Q indicated that His₇₇₁ is phosphorylated by ATP in the transmitter domain before the phosphoryl group is transferred to Asp₇₇₁ located in the receiver domain.

The Hybrid Sensor Kinase LuxN Transfers the Phosphoryl Group to the Histidine Phosphotransfer Protein LuxU—Incubation of purified His₄₇₁-LuxU with radiolabeled ATP did not result in phosphorylation of the protein (data not shown). In contrast, the phosphoryl group was transferred very fast from phosphorylated LuxN to LuxU resulting in accumulation of LuxU~P (Fig. 3, A and C). As judged by Western blot (Fig. 3B), 50 times

² The abbreviation used is: ATPγS, adenosine 5’-O-(thiotriphosphate).
more LuxU was present than LuxN in this experiment, which is consistent with the typical low levels of integral membrane histidine kinases found in cells compared with their soluble partners (18). Whereas phosphorylated LuxN was barely detectable, LuxU \(_{\text{H11011}\text{P}}\) was stable and increased constantly for 10 min. This result indicates that LuxU can be regarded as a sink for phosphate from LuxN. Using 100 \(\mu\text{M}\) \([\gamma^{32}\text{P}]\text{ATP}\) as substrate, about 1% of the LuxU molecules were labeled at a rate of 0.83 pmol/min/mg protein in a LuxN-specific manner. No phosphotransfer was observed when LuxN-H471Q or LuxN-D771N was used (data not shown). Furthermore, replacement of His58 with Gln in LuxU also abolished the phosphorylation of the protein (data not shown), confirming the proposed phosphorylation sites in both LuxN and LuxU.

**Reconstitution of LuxN \(\rightarrow\) LuxU Signal Transduction in Vitro**

To mimic the *in vivo* situation, LuxN and LuxU were incubated together, and the reaction was initiated by addition of radiolabeled ATP. The time course of LuxU phosphorylation was followed for 60 min (Fig. 4, A and C). Following addition of ATP, phosphorylated LuxU was detected within 1 min, and it reached a maximum level after 10 min and declined thereafter. Phosphorylated LuxN was hardly detectable (data not shown). The identical experiment was performed following preincubation of the membrane vesicles containing LuxN with synthetically prepared AI-1 (50 \(\mu\text{M}\)). \(^3\text{H}\)-Labeled homoserine lactone is freely diffusible through the cytoplasmic membrane, and following exogenous addition it is found within 20 s in the cytoplasm (19). Nevertheless, to ensure that AI-1 reached the periplasmic domain in inside-out membrane vesicles, vesicles were preincubated (5 min) with AI-1. Preliminary tests revealed that an additional sonication step did not improve the results. The time course for LuxN-dependent phosphorylation of LuxU was essentially identical in the presence and absence of AI-1, how-
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**Figure 5. Influence of AI-1 on the LuxN kinase activity.** LuxN in membrane vesicles was preincubated with the indicated concentrations of AI-1 for 5 min, and the autophosphorylation reaction was initiated by adding 100 μM [γ-32P]ATP. Samples were withdrawn, and the initial rates of autophosphorylation were determined.

![Graph showing autophosphorylation of LuxN as a function of AI-1 concentration](Image 342x619 to 407x655)

However, strikingly lower LuxU-P was produced (60% reduction) when AI-1 was present compared with when it was absent (Fig. 4, B and C). Thus, the presence of AI-1 does not prevent LuxN autophosphorylation or phosphotransfer from LuxN to LuxU. Rather, AI-1 significantly reduces LuxU-P production. Addition of AI-1-containing conditioned cell-free culture fluids produced comparable effects (data not shown). The concentration-dependent influence of AI-1 on LuxN→LuxU signaling is shown in Fig. 4D. Half-maximal inhibition of LuxU phosphorylation was determined with 5 μM AI-1. At concentrations higher than 20 μM, a constant level of phosphorylated LuxU-P was observed.

Autoinducer-1 Inhibits the LuxN Kinase Activity—We considered two possibilities for the effects of AI-1 on the levels of LuxU phosphorylation; AI-1 could affect LuxN kinase activity or LuxN phosphatase activity. To examine the effects of AI-1 on LuxN kinase activity, various concentrations of AI-1 were incubated with LuxN and the initial rates of autophosphorylation determined. Because of the weak phosphorylation signal observed with wild-type LuxN (see Fig. 2), which is likely further reduced in the presence of AI-1, the LuxN-D771N derivative was used for this series of experiments to provide us a method with which to reliably quantify our data. The kinase activity of LuxN-D771N was significantly affected by AI-1 (Fig. 5). Increasing AI-1 concentrations successively decreased the rate of autophosphorylation of LuxN-D771N. The concentration-dependent influence of AI-1 followed the same course as was observed for the LuxN→LuxU phosphorylation. The half-maximal inhibition was determined to be 20 μM AI-1. Despite the difficulties associated with the wild-type protein, we could in fact observe that AI-1 affected the wild-type LuxN kinase activity (Fig. 5). The phosphorylation signal for the wild-type was only slightly above background, which could account for the observed weak effects of AI-1 on wild-type autophosphorylation activity. Nevertheless, even the highest AI-1 concentration did not completely abolish the autophosphorylation activity of LuxN wild-type or LuxN-D771N.

LuxN Phosphatase Activity Is Not Regulated by AI-1—To determine whether the decline of LuxU-P observed in the reconstituted cascade (see Fig. 4A) was due to effects of AI-1 on phosphatase activity of LuxN, we examined this activity. Purified LuxU-P was incubated with LuxN at a ratio 50:1, and the time-dependent LuxU-P phosphatase activity was 0.33 pmol/min/mg protein (Fig. 6, B and G). This result shows that indeed, LuxN has phosphatase activity on LuxU-P because LuxU-P alone was stable for at least 2 h (Fig. 6, A and G). LuxN phosphatase activity was independent of the presence of ATP (data not shown). The significance of the phosphorylation sites in LuxN were examined in the phosphatase assay. The LuxN-H471Q phosphatase activity was indistinguishable from the wild type (Fig. 6, D and G). However, no LuxU→P phosphatase was observed when LuxN-D771N was used (Fig. 6, E and F) indicating that this aspartate residue is essential for the phosphatase activity of LuxN. Surprisingly, AI-1 had no effect on the phosphatase activity of LuxN. In all cases, the phosphorylation rates in the presence of AI-1 were nearly identical to the rate determined in its absence (Fig. 6, B, C, E, and F).
DISCUSSION

LuxN belongs to the family of unorthodox sensor kinases that are equipped with two phosphorylation sites. Sequence alignment studies suggested His^{471} and Asp^{771} as the phosphorylation sites of LuxN. Consistent with this, genetic analyses of LuxN derivatives with point mutations at the putative sites of phosphorylation provided evidence for the important roles of the conserved amino acid residues His^{471} in the transmitter domain and Asp^{771} in the receiver domain (20). Moreover, investigation of the bioluminescence phenotypes of these and other lux mutants of *V. harveyi* led to the conclusion that LuxN catalyzes three enzymatic activities within a phosphorelay: (i) autophosphorylation and intramolecular phosphotransfer from His^{471} to Asp^{771}; (ii) phosphotransfer from LuxN to LuxU, a histidine phosphotransfer protein; and (iii) dephosphorylation of LuxU. In the present report these proposed enzymatic activities of LuxN and the effect of AI-1 on them were investigated *in vitro*.

luxN was cloned and overexpressed in *E. coli* as a full-length protein including the N-terminal membrane-spanning domain. For *in vitro* phosphorylation assays, inverted membrane vesicles containing LuxN were incubated with radiolabeled [γ-^{32}P]ATP. Autophosphorylation of LuxN was found to be an unusually fast reaction and the labeling of LuxN was extremely short-lived. Moreover, autophosphorylation was only detectable at relatively high ATP concentrations (>20 μM). All of these results indicate that the equilibrium favors the reactants ATP and unlabeled LuxN and not the products ADP and phosphorylated LuxN. No sensor kinase or hybrid sensor kinase characterized to date (21–23) exhibits such unusual kinetic behavior. The unusualLuxN autophosphorylation kinetics might be a specific requirement for executing the all-or-nothing quorum sensing transition in *V. harveyi*, whereas other behaviors controlled by sensor kinases do not require such rapid/extreme kinetics. *In vitro* studies with derivatives bearing amino acid replacements at the sites proposed for phosphorylation from genetic studies, His^{471} and Asp^{771} in LuxN and His^{58} in LuxU (20, 24), confirmed the phosphorelay consisting of LuxN-His^{471} → LuxN-Asp^{771} → LuxU-His^{58}.

In contrast to the weak and unstable autophosphorylation of LuxN, the equilibrium is shifted toward the phosphorylated proteins in the presence of LuxU. Although the measured phosphorylation degree of LuxU was determined to be only 1%, this is in the range of the values observed for other sensor kinase/response regulator proteins (18). The low degree of phosphorylation is largely due to the low ATP concentration used in *in vitro* experiments. For example, similar phosphorylation levels were obtained *in vitro* for the KdpD/KdpE system of *E. coli*, however, mathematical simulations predicted that a much higher phosphorylation level of the proteins would occur at physiological ATP concentrations (18). LuxU seems to act as a phosphate sink scavenging phosphoryl groups from LuxN (see further discussion below). Although we made numerous attempts to obtain LuxO (the final member of the Lux phosphorelay) as a soluble protein, we were unsuccessful. Therefore, it remains unclear whether LuxU retains its exquisite ability to scavenge phosphoryl groups in the presence of LuxO.

In the reconstituted system, in which reactions were initiated upon addition of ATP to LuxN and LuxU, rapid phosphorylation of LuxU was observed and declined afterward. The degree of LuxU phosphorylation in a reconstituted system is the result of all LuxN enzymatic activities, which are equilibrium reactions. Therefore, it is conceivable that the ratio of kinase/phosphatase activity changes over time due to the consumption of ATP and/or the accumulation of ADP. However, this rapid time course of phosphorylation is unusual in comparison with other two-component systems, like KdpD/KdpE (18) or EnvZ/OmpR (22), which were similarly tested. However, one important difference between the LuxN/LuxU experiments and those with other two-component systems such as KdpD/KdpE and EnvZ/OmpR is that the latter were performed in the presence of the corresponding response regulators and the cognate DNA to which the response regulators bind. It has been shown that the presence of DNA shifts the equilibrium toward the phosphorylated response regulator (25). Despite the lack of a response regulator to test in the LuxN/LuxU signaling cascade, there are other explanations for the rapid decline of LuxU–P. Because of the unusually high turnover rate of LuxN kinase activity, the substrate ATP might be rapidly exhausted *in vitro*. Alternatively, LuxU could have a high LuxU–P phosphatase activity. Although our experiments revealed a phosphatase activity of LuxN with LuxU–P as substrate, its activity was low compared with autokinase activity (see discussion below).

Tests of wild-type LuxN and the two derivatives of LuxN in which the amino acids corresponding to the phosphorylation sites are replaced showed that Asp^{771} in LuxN is essential for the dephosphorylation of LuxU–P. These biochemical data are in agreement with the phenotypic studies of the *V. harveyi* luxN-D771A mutant (20). Phospho-LuxO represses bioluminescence expression in *V. harveyi* and *V. harveyi luxN* D771A produced bioluminescence in a density dependent manner, but no stimulation of light production was observed in the presence of AI-1 suggesting that the luxN D771A mutant lacks a phosphatase activity that can remove phosphate from LuxO (via LuxU) to induce bioluminescence expression (1, 20). An important role for the analogous aspartate residues in both kinase and phosphatase activity was also reported for the hybrid sensor kinases ArcB and BvgS (26, 27). These earlier studies provided the first evidence that the aspartate residue on sensor kinases is re-phosphorylated during dephosphorylation (17). Our investigations support this possibility, because we detected faint amounts of LuxN–P in LuxU–P dephosphorylation assays (data not shown).

The studies presented here were performed with full-length LuxN protein, which included all transmembrane helices and their corresponding intervening periplasmic regions. To mimic the endogenous environment of LuxN (the cytoplasmic membrane), the protein was embedded in vesicles for all of the assays in this work. This strategy allowed us to examine the different enzymatic activities of LuxN *in vitro* and most importantly to examine the influence the AI-1 signal molecule has on each of the signaling functions of LuxN. In contrast to earlier assumptions, we demonstrated here that the phosphatase activity of
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LuxN is independent of AI-1. In addition, AI-1 had no effect on the dephosphorylation rate of any tested LuxN derivative. Nonetheless, the presence of AI-1 significantly lowered the amount of LuxU→P in the LuxN→LuxU phosphorelay, which is in agreement with the general model suggesting that the concentration of LuxU→P and subsequently that of LuxO→P decreases upon accumulation of AI-1 (Fig. 1). A half-maximal reduction in the amount of LuxU→P in the presence of 5 μM AI-1 matches well with the estimated AI-1 concentration in cell-free culture fluids of bright V. harveyi strains (4). Our experiments also clearly demonstrate that the kinase activity of LuxN was reduced as the AI-1 concentration was increased. Since the rate of kinase activity was affected to the same extent as the rate of LuxU→P phosphorylation in a LuxN→LuxU phosphorylation assay (data not shown), we conclude that the LuxN phosphotransfer activity was not affected by AI-1.

The results presented here reveal an AI-1 regulated kinase and an AI-1 independent phosphatase activity of the hybrid sensor kinase LuxN. The kinetics of these two opposing enzymatic activities are quite different. The rate of autophosphorylation was found to be very high, whereas the rate of dephosphorylation was slow. Furthermore, phosphorylated LuxN was barely detectable, whereas LuxU→P accumulated. Regulation of the kinase activity with these observed properties has the advantage that the cells possess a fast reacting tool to respond to AI-1. Furthermore, the high energy state of the anhydride bond might trigger conformational changes at a distal signaling surface like the LuxU protein, which, in turn, shifts the chemical equilibrium within the phosphorelay toward the phosphorylation of LuxU. Conformational coupling was also observed for the chemotaxis response regulator CheY (28). In addition, because of the lability of phosphorylated LuxN, the amount of phosphorylated substrate is rate-limiting for the subsequent phosphorylation reactions. Moreover, a low, but constitutive, phosphatase activity further influences the phosphorylation degree of LuxU and consequently the activation of the response regulator LuxO. All these parameters raise the possibility for fine tuning of AI-dependent gene expression in V. harveyi. It should be taken into account that bioluminescence in V. harveyi is also regulated by two other sensor kinases, LuxQ and CqsS. While the same enzyme activities are postulated for LuxQ and CqsS, it was also suggested that the three systems do not operate equally (1, 29). It was concluded from deletion mutants that signaling strength follows: LuxN > LuxQ > CqsS (3, 20, 29). An analogous in vitro characterization of the LuxQ kinase and phosphatase activities is currently underway. Direct comparison of the enzymatic activities of all the sensors in the presence of their cognate AI molecules will enable an understanding of signal integration and relay in the V. harveyi quorum sensing network.

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