Phosphotransfer Reactions of the CbbRRS Three-Protein Two-Component System from *Rhodopseudomonas palustris* CGA010 Appear To Be Controlled by an Internal Molecular Switch on the Sensor Kinase[▽]

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The CbbRRS system is an atypical three-protein two-component system that modulates the expression of the *cbb*$_I$ CO$_2$ fixation operon of *Rhodopseudomonas palustris*, possibly in response to a redox signal. It consists of a membrane-bound hybrid sensor kinase, CbbSR, with a transmitter and receiver domain, and two response regulator proteins, CbbRR1 and CbbRR2. No detectable helix-turn-helix DNA binding domain is associated with either response regulator, but an HPt domain and a second receiver domain are predicted at the C-terminal region of CbbRR1 and CbbRR2, respectively. The abundance of conserved residues predicted to participate in a His-Asp phosphorelay raised the question of their de facto involvement. In this study, the role of the multiple receiver domains was elucidated in vitro by generating site-directed mutants of the putative conserved residues. Distinct phosphorylation patterns were obtained with two truncated versions of the hybrid sensor kinase, CbbSR^{T189} and CbbSR^{R96} (CbbSR beginning at residues T189 and R96, respectively). These constructs also exhibited substantially different affinities for ATP and phosphorylation stability, which was found to be dependent on a conserved Asp residue (Asp-696) within the kinase receiver domain. Asp-696 also played an important role in defining the specificity of phosphorylation for response regulators CbbRR1 or CbbRR2, and this residue appeared to act in conjunction with residues within the region from Arg-96 to Thr-189 at the N terminus of the sensor kinase. The net effect of concerted interactions at these distinct regions of CbbSR created an internal molecular switch that appears to coordinate a unique branched phosphorelay system.

Two-component signal transduction systems regulate the expression of genes important for a myriad of physiological processes common to prokaryotes (8); such systems have also been recently identified to function in eukaryotes as well (5, 9, 13). In its most simple iteration, two-component systems consist of a membrane-bound sensor histidine kinase protein that receives an environmental cue and then transfers the signal to a single soluble response regulator protein. The sensor histidine kinase protein has a nonconserved N-terminal region, dedicated to sensing a particular signal, attached to a conserved kinase domain. The response regulator protein typically consists of a conserved receiver domain and a variable effector domain. The general mechanism of signal transduction involves a linear pattern of events whereby autophosphorylation of the kinase precedes phosphorylation of the cognate response regulator protein, ultimately leading to a change in behavior determined by the nature of the effector domain. Thus, one important manifestation is the regulation of gene expression; alternatively, the signal transduction process might lead to modification of protein-protein interactions or the modulation of enzymatic activity (13). Numerous variations of the basic modular units have been described over the years; e.g., where the kinase core might stand alone as a sensor

protein (2). Alternatively, the kinase domain may be associated with one or more receiver domains on the same protein to form a hybrid sensor kinase molecule (3, 10, 14). In the latter instance, an HPt domain is often interposed to facilitate phosphotransfer reactions between separate receiver domains. By contrast, receiver domains and HPt domains, where required, may be localized on completely separate proteins (2, 7, 9).

The CbbRRS (*C*alvin *B*enson *B*assham *r*esponse *r*egulators *s*ensor kinase) of *Rhodopseudomonas palustris* is a three-protein two-component system (11) consisting of a membrane-bound sensor kinase protein (CbbSR) and two response regulator proteins (CbbRR1 and CbbRR2) (Fig. 1). CbbSR is a large hybrid sensor kinase protein that, in addition to possessing two canonical transmembrane regions and a transmitter domain, also contains a C-terminal receiver domain. Two potential PAS motifs, predicted by PFAM analysis (http://www.ncbi.nlm.nih.gov/BLAST), are present in the N-terminal region (Fig. 1B). Response regulator 1, CbbRR1, has an HPt domain and a receiver domain. Response regulator 2, CbbRR2, has two receiver domains (Fig. 1B). Besides this modular organization, common among two-component signal transduction pathways, the components of the CbbRRS system have no homology to other known proteins (11). Collectively, the CbbRRS system was recently shown to regulate expression of the form I ribulose 1,5-bisphosphate carboxylase/oxygenase or *cbbLS* genes (Fig. 1A) of the *cbb*$_I$ operon of *R. palustris* (11). As the two response regulators, CbbRR1 and CbbRR2, presumably do not interact with DNA directly, due to the absence of a discernible helix-turn-helix motif, it was proposed that the
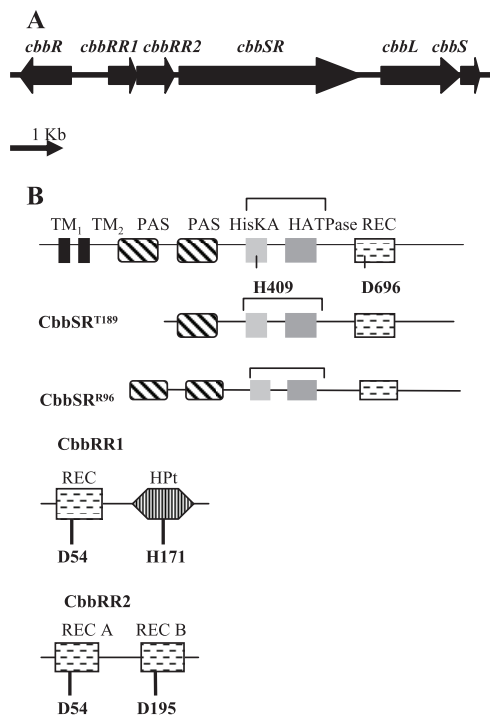

FIG. 1. Gene organization (A) and potential protein domains (B) found on the CbbRRS system proteins CbbSR, CbbRR1, and CbbRR2. The predicted conserved residues involved in phosphotransfer reactions are indicated. A schematic representation of the two truncated sensor kinase proteins (CbbSR[T189] and CbbSR[R96]) is also shown.

response regulators posttranslationally modulate the CbbR transcription factor (encoded by the *cbbR* gene) (Fig. 1A), which is itself indispensable for the expression of the *cbbLS* genes (11). In a previous investigation, it was also shown that the full-length membrane-bound kinase, CbbSR, catalyzes phosphorylation of both response regulators (CbbRR1 and CbbRR2) independently, ruling out any absolute requirement for a multistep phosphorelay between the kinase and the two response regulator proteins. Clearly, a single sensor kinase has the potential to interact with four distinct receiver domains on three different proteins, including two different response regulator proteins. The four different receiver domains show limited amino acid identity (Fig. 2), and yet this system plays an

important role with regard to the control of *cbbLS* transcription in *R. palustris* (11). To better understand what appears to be a complex signal transduction mechanism for modulating expression of genes required for CO_2 assimilation under changing environmental conditions, we dissected the phosphorelay reactions in vitro and determined the role of each of the distinct functional domains of the three proteins. The likely route of the phosphoryl group following autophosphorylation of the histidine residue (His-409) on the sensor kinase was traced; also the roles of specific residues important for phosphotransfer within the receiver domain of the hybrid sensor kinase and each of the two response regulator proteins were determined. As a result of these studies, a potential internal molecular switch was identified within the sensor kinase that appeared to direct the route and specificity of phosphotransfer to the response regulator proteins.

MATERIALS AND METHODS

Strains, growth conditions, and plasmids. Strains and plasmids used in this study are reported in Table 1. *Escherichia coli* DH5α was used for general cloning purposes as well as production of recombinant proteins. Cultures were grown at 37°C with constant shaking (200 rpm) in Luria-Bertani broth. Antibiotics were added at the following final concentrations: kanamycin, 50 μg ml^{-1}; ampicillin, 100 μg ml^{-1}. Standard molecular biology techniques were used for chromosomal DNA purification, restriction digests, and cloning (1). A QIAGEN Mini Prep kit was routinely employed to prepare plasmid DNA. When necessary, DNA fragments were purified from agarose gels using the Gel Purification Kit from QIAGEN. Plasmids pQE8027, pQE8024, pQE8740, pQE8797, and pQE8798 have been described elsewhere (11). The coding region corresponding to the predicted whole-cytoplasmic portion of sensor kinase CbbSR was PCR amplified by using the primers 5′-GGGGGTACCCGGCGCAACGCGCACAAG-3′ and 5′-CCCAAGCTTTCAGGTCAGCTCGTG-3′ (restriction sites are underlined), in the presence of 1 U of *Pfx Platinum* DNA polymerase (Invitrogen), and directly cloned into pCR-Blunt II TOPO to generate plasmid pSR128. After sequencing ensured the absence of mutations, a KpnI/BamHI fragment of approximately 950 bp was ligated in frame into vector pQE8740 (11) by exploiting a unique BamHI site within the *cbbSR* gene to generate plasmid pQE12840 (CbbSR[R96], indicating CbbSR starting from residue R96).

Construction of site-directed mutants. Mutagenic PCR was performed to generate site-directed mutants of the sensor kinase and two response regulators. One specific residue was substituted at a time, using conditions recommended by the manufacturer (Quick Change site-directed mutagenic kit; Stratagene). All substitutions were verified after sequencing the mutagenic PCR products at the Plant-Microbe Genomic Facility at The Ohio State University before the products were subcloned into the pQE80 expression vector at appropriate restriction sites. Double site-directed mutants were generated by performing mutagenic PCRs on templates previously mutagenized. In particular, plasmids pQE8055 (D54NCbbRR1) and pQE8057 (H171DCbbRR1) were generated using the following mutagenic primer pairs (mutated codons are underlined): the pair 5′-C
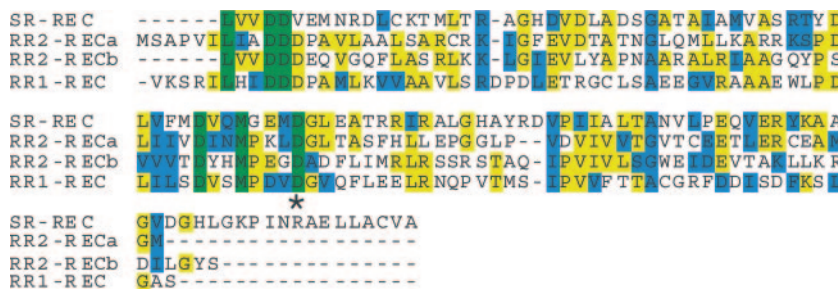

FIG. 2. Sequence alignment of the receiver domains present on proteins of the CbbRRS system (11). SR-REC, sensor kinase CbbSR; RR2-REC A, first receiver domain of response regulator CbbRR2; RR2-REC B, second receiver domain of response regulator CbbRR2; RR1-REC, receiver domain of response regulator CbbRR1. The asterisk indicates the conserved phosphorylation site. Conserved residues are shown in green, and identical residues are shown in yellow, whereas similar residues are shown in cyan.

TABLE 1. Strain and plasmids used in this study

Strain or plasmid	Description[a]	Source or reference
E. coli DH5α	General cloning strain and recombinant protein expression host	Invitrogen
Plasmids		
pCR-Blunt II TOPO	General cloning plasmid for direct insertion of blunt-ended PCR products; Km[r]	Invitrogen
pSR128	Cytoplasmic region of the sensor kinase *cbbSR* starting from residue R96 cloned into pCR-Blunt II TOPO; Km[r]	This study
pQE80	*cis*-repressed, IPTG-inducible, N-terminal His$_6$-tagged recombinant protein overexpression vector; Amp[r]	QIAGEN
pQE8027	*cbbRR1* coding region cloned into the BamHI/HindIII sites of pQE80; Amp[r]	11
pQE8055	D54N *cbbRR1* site-directed mutant cloned into the BamHI/HindIII sites of pQE80; Amp[r]	This study
pQE8057	H171D *cbbRR1* site-directed mutant cloned into the BamHI/HindIII sites of pQE80; Amp[r]	This study
pQE8073	D54N H171D *cbbRR1* site-directed double mutant cloned into the BamHI/HindIII sites of pQE80; Amp[r]	This study
pQE8024	*cbbRR2* coding region cloned into the BamHI/HindIII sites of pQE80; Amp[r]	11
pQE8060	D54N *cbbRR2* site-directed mutant cloned into the BamHI/HindIII sites of pQE80; Amp[r]	This study
pQE8061	D195N *cbbRR2* site-directed mutant cloned into the BamHI/HindIII sites of pQE80; Amp[r]	This study
pQE8075	D54N D195N *cbbRR2* site-directed double mutant cloned into the BamHI/HindIII sites of pQE80; Amp[r]	This study
pQE8740	Truncated *cbbSR* coding region comprising residues 189 to 903 cloned into the KpnI/HindIII sites of pQE80; Amp[r]	11
pQE8797	Truncated D696N *cbbSR*, starting from T189, cloned into the KpnI/HindIII sites of pQE80; Amp[r]	11
pQE8798	Truncated H409D *cbbSR*, starting from residue T189, cloned into the KpnI/HindIII sites of pQE80; Amp[r]	11
pQE12840	Truncated *cbb*SR coding region comprising residues 96 to 903 cloned into the KpnI/HindIII sites of pQE80; Amp[r]	This study
pQE12897	Truncated D696N *cbbSR*, starting from residue R96, cloned into the KpnI/HindIII sites of pQE80; Amp[r]	This study
pQE12898	Truncated H409D *cbbSR*, starting from residue R96, cloned into the KpnI/HindIII sites of pQE80; Amp[r]	This study

[a] IPTG, isopropyl-β-ᴅ-thiogalactopyranoside.

TGATCCTTAGCAACGTTTCGATGCCG-3′ and 5′-CGGCATCGAAACGT TGCTAAGG ATCAG-3′ (D54N) and the pair 5′-CGCCATGTGGCTGACA AGCTGGTCGGC-3′ and 5′-GCCGACCAGCTTGTCAGCCACATGGCG-3′ (H171D), respectively. The expression vector pQE8073 was used for the isolation of the recombinant CbbRR1 double mutant protein D54NH171DCbbRR1. Plasmids pQE8060 (D54NCbbRR2) and pQE8061 (D195NCbbRR2) were generated by using the following mutagenic primer pairs (mutated codons are underlined): the pair 5′-CTGATCATCGTCAACATCAACATGCCG-3′ and 5′-CGG CATGTTGATGTTGACGATG ATCAG-3′ (D54N) and the pair 5′-GTGGTG GTCACCAACTATCACATGCCG-3′ and 5′-CGGCATGTGATAGTTGGTG ACCACCAC-3′ (D195N). The expression vector pQE8075 was used for the isolation of the CbbRR2 double mutant protein (D54ND195NCbbRR2). Plasmid pQE12897 (D696NCbbSR^R96) and pQE12898 (H409NCbbSR^R96) were derived from in-frame ligation of the KpnI/BamHI fragment from pSR128 into plasmids pQE8797 and pQE8798, respectively (11), as described above for the construction of pQE12840.

Purification of recombinant proteins. N-terminal His$_6$-tagged recombinant proteins, hereafter simply referred to as CbbSR, CbbRR1, and CbbRR2, were synthesized in *E. coli* and purified by nickel column chromatography as previously described (11).

Phosphorylation reactions. Phosphorylation reactions were performed in 50 mM Tris-Cl, pH 8.0, 5 mM MgCl$_2$, 50 mM KCl, and 0.1 mM dithiothreitol. The final concentration of [γ-^{32}P]ATP (specific activity of 10 mCi/mmol; 1.7 μM stock solution) (Perkin Elmer) varied depending on the type of reaction being examined and is specified in the figure legends. The phosphorylation reactions were carried out at room temperature for the desired time and were terminated by adding 3× sample buffer (37.5 mM Tris-Cl, pH 6.8, 30% glycerol, 10% sodium dodecyl sulfate [SDS; wt/vol], 0.6% β-mercaptoethanol). The reaction products were separated by electrophoresis on 8% or 12% SDS-polyacrylamide gel electrophoresis (PAGE) gels (17 by 16.5 by 0.3 cm) and were run at 4°C at 200 V constant voltage for 2 h. Gels were dried, exposed to a high-resolution phosphor screen (Kodak), and analyzed using a PhosphorImager with ImageQuant software (Molecular Dynamics 5.0; Amersham Biosciences, Pittsburgh, PA). For the label decay experiments, an excess of nonradioactive ATP (100 mM) was added to the reaction mixture following a 20-min preincubation with [γ-^{32}P]ATP. Aliquots were taken prior to the addition of unlabeled ATP (time zero) and at various time points after the addition of cold ATP. Different dilutions of [γ-^{32}P]ATP were used to generate, via ImageQuant, a calibration curve to quantify the label associated with the products of the phosphorylation reactions. All time-dependent quantitation results were expressed as the average of at least three independent experiments. The gel figures shown are representative of each set of experiments.

RESULTS

Phosphotransfer activities of the truncated wild-type sensor kinase. Comparisons of deduced amino acid sequences of CbbRR1, CbbRR2, and sensor kinase CbbSR to well-charac-
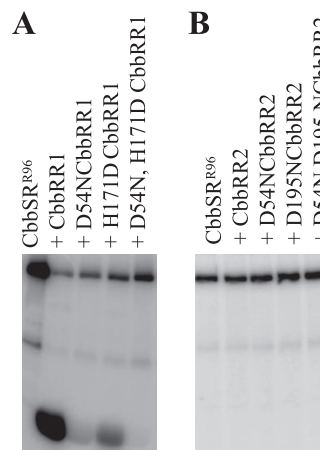

FIG. 3. Phosphotransfer between sensor kinase CbbSR^R96 and response regulator CbbRR1 (wild-type and wild-type plus D54N, H171D, and D54NH171D mutant CbbRR1 proteins) (A) and response regulator CbbRR2 (wild-type and wild-type plus D54N, D195N, and D54ND195N mutant CbbRR2) (B). A total of 380 pmol of sensor kinase was preincubated with 20 μM [γ-^{32}P]ATP for 20 min (far left lanes) before the addition of approximately 1 nmol of each response regulator, and the reaction mixture was incubated at room temperature for 1 h.
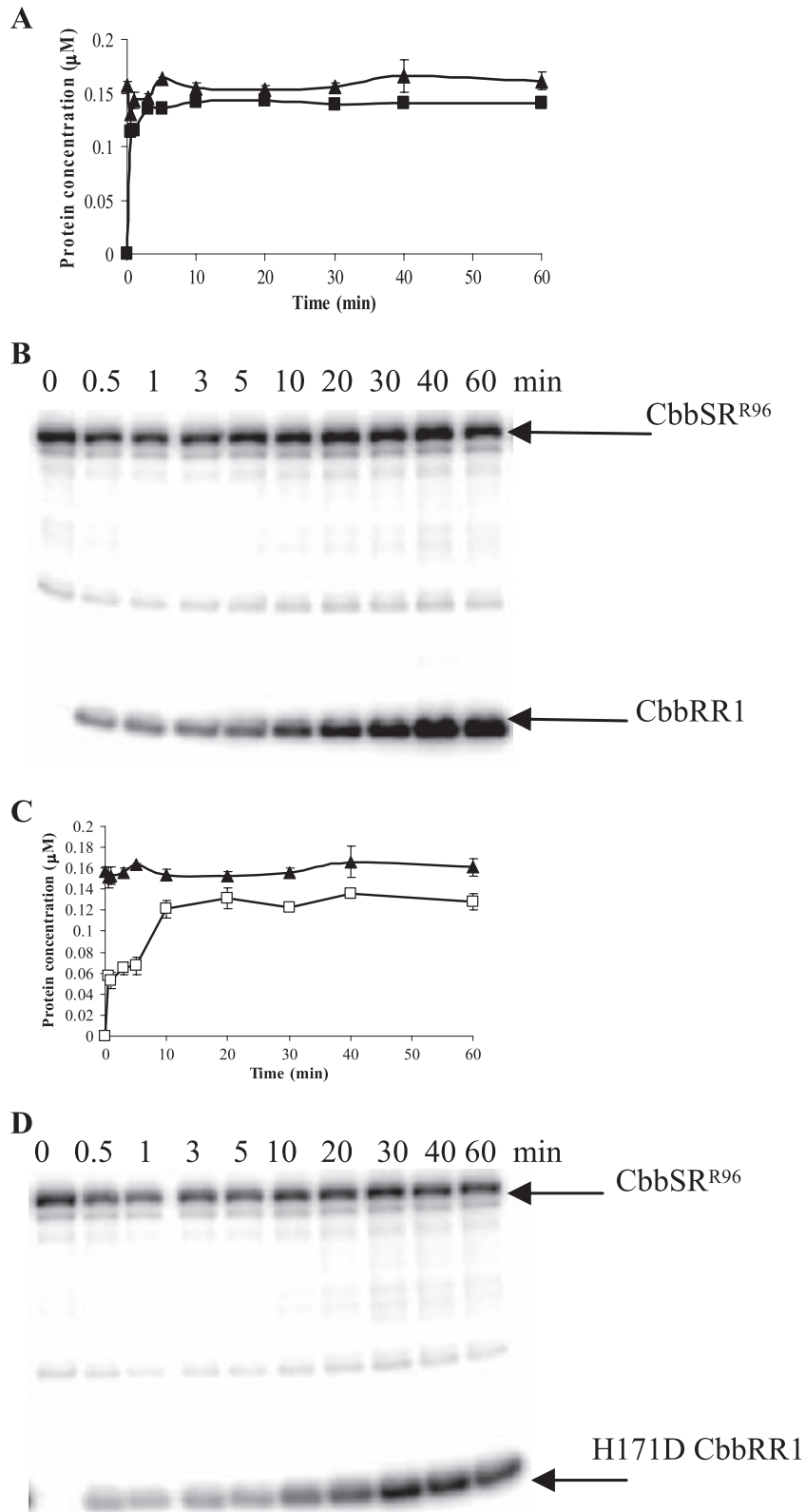
FIG. 4. Time course of phosphorylation between CbbSR^{R96} and CbbRR1 (A and B) or H171DCbbRR1 (C and D). The concentration of phosphorylated products obtained at various times was determined (A and C) for CbbSR^{R96} (▲), CbbRR1 (■), and H171DCbbRR1 (□). A total of 380 pmol of sensor kinase was preincubated with 20 μM [γ-^{32}P]ATP for 20 min (time zero) before the addition of approximately 1 nmol of response regulator. Ten-milliliter aliquots were then removed at the time points indicated; the reactions were stopped by adding 3× SDS loading buffer and stored on ice until the end of the experiment.

terized bacterial response regulators (data not shown) resulted in the identification of residues Asp-54 and His-171 on CbbRR1 and Asp-54 and Asp-195 on CbbRR2 as potential specific sites of phosphorylation. The importance and role of these residues on each response regulator protein during phosphotransfer, along with the conserved functional residues identified on the sensor kinase (11), were assessed after purified site-directed mutant proteins were prepared. Truncated soluble His-tagged recombinant sensor kinase constructs CbbSR^{R96} and CbbSR^{T189} (CbbSR starting from residue T189) were prepared and purified using constructs that provided the cytoplasmic (but not membrane-spanning) portion of the protein (Fig. 1). As expected, CbbSR^{R96} catalyzed an ATP-dependent auto-phosphorylation reaction, followed by subsequent phosphotransfer to wild-type CbbRR1 (11) (Fig. 3A). Alteration of both Asp-54 and His-171 of CbbRR1 prevented label incorporation/phosphorylation of CbbRR1 (Fig. 3A). By contrast, considerable phosphotransfer to the H171DCbbRR1 mutant protein occurred, while the D54N construct exhibited only weak phosphorylation. These results showed that Asp-54 is the major phosphoacceptor of CbbRR1; however, the data also suggested possible phosphotransfer between residues Asp-696 from CbbSR to His-171 of CbbRR1 (further described below). No phosphorylation of either wild-type or site-directed mutant constructs of CbbRR2 by CbbSR^{R96} was observed (Fig. 3B).

To further define phosphotransfer from the sensor kinase to CbbRR1, both the kinetics and specificity of these reactions were examined. Time course experiments indicated that CbbSR^{R96} catalyzed rapid transfer of γ-^{32}P to CbbRR1 (Fig. 4A and B). Similarly, phosphotransfer from CbbSR^{R96} to the mutant H171DCbbRR1 protein occurred, however at a reduced initial rate (Fig. 4C and D). Both the wild-type and mutant CbbRR1 proteins reached a consistent level of phosphorylation in equilibrium with the sensor kinase. Since unincorporated [γ-^{32}P]ATP was not removed from the reaction mixture, no significant dephosphorylation of the kinase protein was observed concomitant with the phosphorylation of CbbRR1. Analogous results were obtained with the shorter truncated kinase, CbbSR^{T189} (data not shown).

Considerable differences in both the kinetics and specificity of phosphotransfer to the CbbRR1 constructs were observed with the kinase mutant D696NCbbSR^{R96} (Fig. 5A and B) and its more truncated homolog D696NCbbSR^{T189} (Fig. 5C and D). Wild-type CbbRR1 was very poorly phosphorylated in the presence of D696NCbbSR^{R96} (Fig. 5A) and virtually no phosphotransfer occurred to H171DCbbRR1 (Fig. 5B). Variations of the protein molar ratios over a wide range of ATP concentration and incubation times gave identical patterns of phosphorylation (data not shown). By contrast, the more truncated D696NCbbSR^{T189} protein exhibited activities consistent with the nonmutant CbbSR^{T189} construct, with rapid phosphorylation of CbbRR1 (Fig. 5C). Phosphotransfer to H171DCbbRR1 occurred both at a slower rate and to a lesser extent (Fig. 5D), with accentuated transient dephosphorylation of CbbSR and slightly decreased phosphotransfer efficiency, much like wild-type CbbSR^{R96} and H171DCbbRR1 (Fig. 4C and D). Incubation of the mutant kinase preparations, D696NCbbSR with D54NCbbRR1, prevented any phosphotransfer (data not shown).

Properties of the truncated sensor kinase proteins. The basic autophosphorylation reactions catalyzed by all four truncated constructs—CbbSR^{T189}, D696NCbbSR^{T189}, CbbSR^{R96}, and D696NCbbSR^{R96}—were characterized. The D696NCbbSR^{R96} construct had a much higher affinity for ATP than the other constructs (Fig. 6A), with this protein showing optimal phosphorylation at concentrations as low as 2.5 nM ATP (Fig. 6B, F, and G) compared to CbbSR^{T189}, D696NCbbSR^{T189}, and CbbSR^{R96} (Fig. 6A, C, D, and E), all of which required at least 20 μM ATP. In addition, there was no difference in the relative rate of autophosphorylation of CbbSR^{R96} and D696NCbbSR^{R96} at 20 μM and 25 nM [γ-^{32}P]ATP, respectively (data not shown). No significant differences in ATP affinity were noted for the CbbSR^{T189} wild-type and mutant constructs (11).

Previous studies on the chemical stability of phosphorylated CbbSR^{T189} constructs indicated that the wild-type sensor kinase protein was mainly acyl-phosphorylated on Asp-696, with rapid phosphotransfer from the autophosphorylated His-409 residue to Asp-696 within the receiver domain of the protein (11). This internal phosphotransfer occurred even in the presence of CbbRR1. However, after histidine autophosphorylation, the absence of a phosphoacceptor residue within the receiver domain of CbbSR and/or the absence of a response regulator protein would presumably cause dissociation of the phosphoryl group, which would then be released into solution. Thus, to further assess the properties of the four kinase constructs and their state of phosphorylation, pulse-chase experiments were performed in order to follow the time-dependent displacement of the label in the presence of a large excess of unlabeled ATP. Unlabeled ATP should compete with any labeled ATP remaining in solution as a substrate for the autophosphorylation reaction, ultimately allowing measurements of the rate of dephosphorylation. With the exception of D696NCbbSR^{R96}, which was phosphorylated with 25 nM [γ-^{32}P]ATP, all constructs were preincubated with 20 μM [γ-^{32}P]ATP to allow sufficient autophosphorylation prior to the addition of 100 mM unlabeled ATP. Both wild-type truncated constructs (CbbSR^{T189} and CbbSR^{R96}) showed virtually identical rates of dephosphorylation (Fig. 7A, B, and D), with gradual label decay throughout the whole experimental time frame. However, the two D696NCbbSR constructs exhibited completely different rates of γ-^{32}P label loss; the D696NCbbSR^{T189} protein rapidly lost more than 50% of its label within 1 min of the addition of unlabeled ATP and maintained only about 10% of the original level of phosphorylation, that is, prior to the addition of unlabeled ATP (Fig. 7A and C). By contrast, the phosphorylation level of D696NCbbSR^{R96} remained substantially unchanged after 2.5 h (Fig. 7A and E). The lack of dephosphorylation shown by D696NCbbSR^{R96} was interesting, in particular with respect to its high affinity for ATP (Fig. 6) and weak ability to catalyze phosphotransfer to CbbRR1 (Fig. 5). Since H409DCbbSR^{R96}, much like H409DCbbSR^{T189}, had no autophosphorylation activity (11) and contained no label (data not shown), the high affinity of D696NCbbSR^{R96} for ATP was strictly dependent on its kinase activity and not an indirect effect of covalent ATP binding to the sensor region of the protein, as seen, for instance, in the sporulation master kinase, KinA (12).

In summary, the results of the kinetic experiments indicated the following: (i) that the state of phosphorylation of the wild-
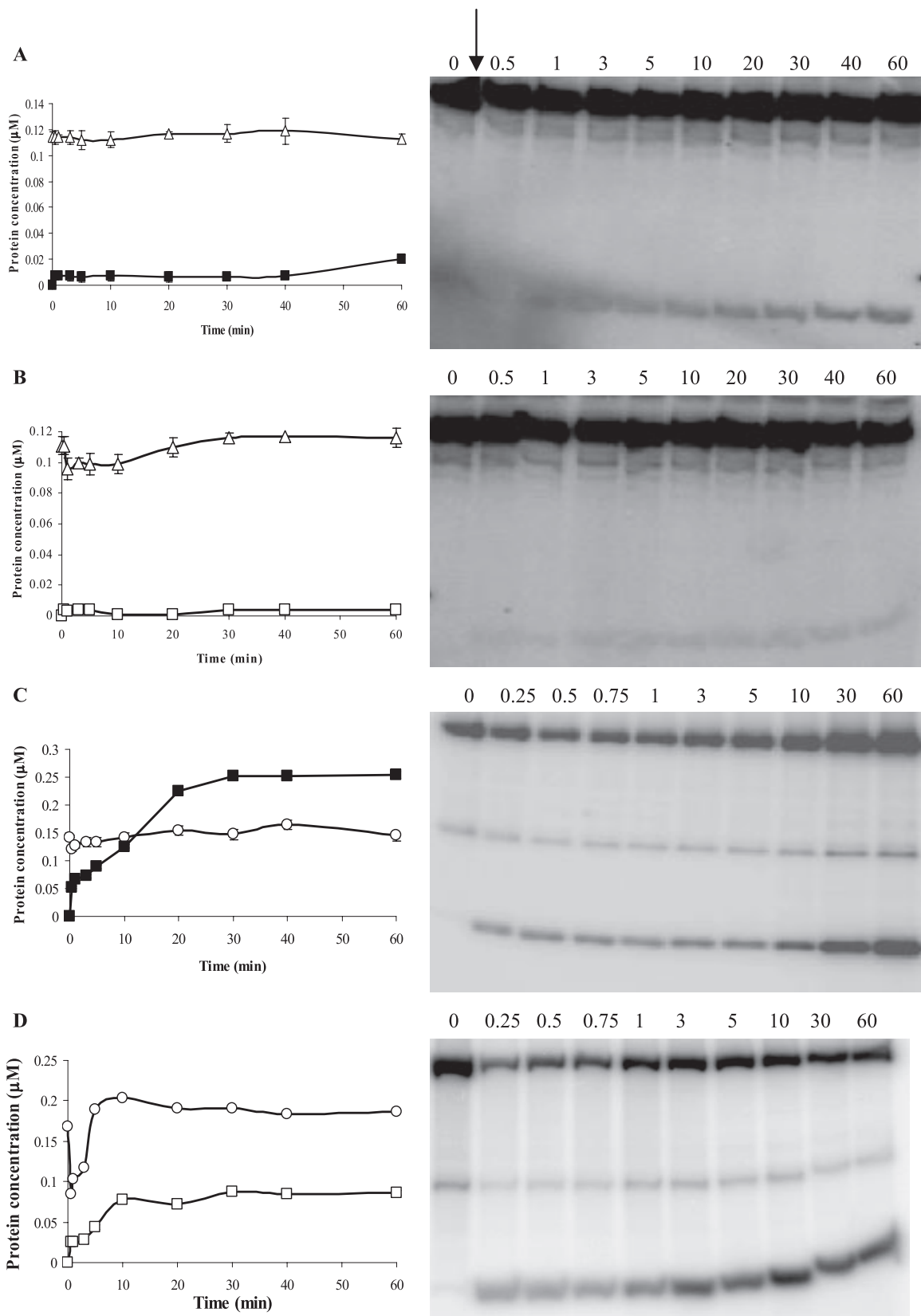
FIG. 5. Time course of phosphorylation of CbbRR1 (■) and H171DCbbRR1 (□) catalyzed by D696NCbbSR^{R96} (△) (A and B) and D696NCbbSR^{T189} (○) (C and D). Reaction conditions were as described in the legend of Fig. 4. Quantitation of phosphorylated products from time courses (left) was obtained from phosphotransfer reactions depicted in gels (right). The arrow indicates where CbbRR1 preparations were added to the reaction mixture.
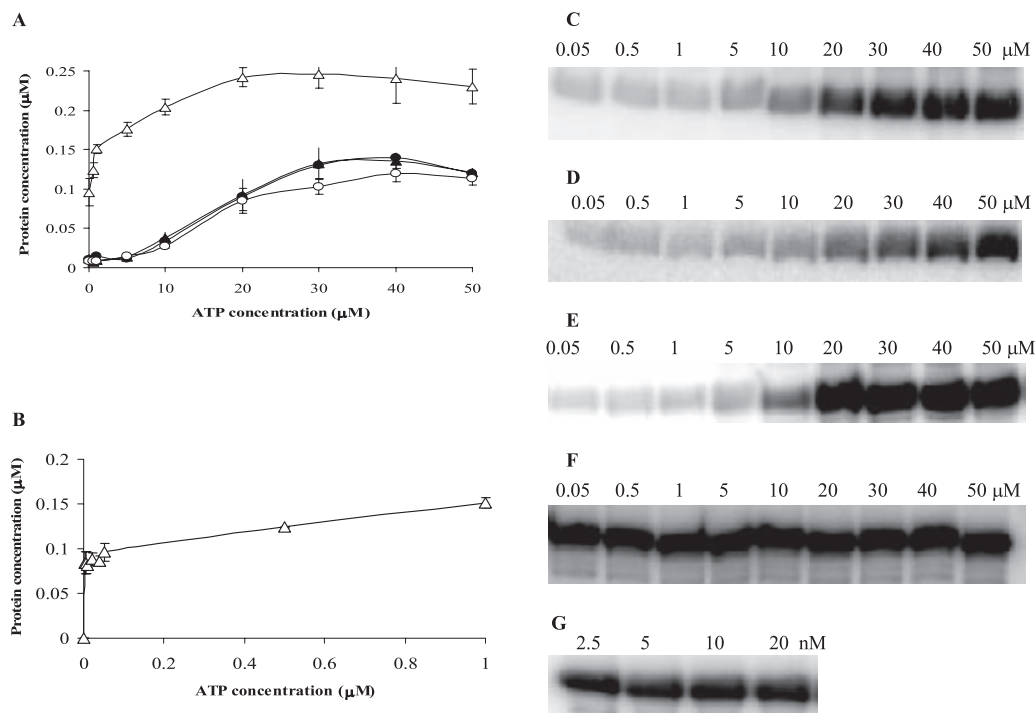
FIG. 6. Kinase autophosphorylation reactions at various concentrations of $[\gamma\text{-}^{32}P]ATP$. Each reaction was carried out with 380 pmol of protein at various concentration of $[\gamma\text{-}^{32}P]ATP$ for 20 min at room temperature in a final volume of 10 µl. The reactions were terminated by adding 5 µl of 3× sample buffer. (A) Levels of phosphorylated proteins obtained at increasing concentrations of $[\gamma\text{-}^{32}P]ATP$ for CbbSR^{T189} (●), D696NCbbSR^{T189} (○), CbbSR^{R96} (▲), and D696NCbbSR^{R96} (△). (B) Levels of phosphorylated products obtained at increasing concentrations of $[\gamma\text{-}^{32}P]ATP$ for D696NCbbSR^{R96}. Representative patterns of autophosphorylation at the various concentrations of $[\gamma\text{-}^{32}P]ATP$ of CbbSR^{T189} (C), D696NCbbSR^{T189} (D), CbbSR^{R96} (E), and D696NCbbSR^{R96} (F and G) at the indicated concentrations of ATP. The range of $[\gamma\text{-}^{32}P]ATP$ concentrations (µM or nM) used in the autophosphorylation assays is reported above each gel.

type sensor kinase protein is most probably a combination of its autophosphorylation activity, its internal phosphotransfer activity, and the inherent stability of the phosphorylated residue; and (ii) that the N-terminal region of the sensor kinase between residues Arg-96 and Thr-189, which contains a PAS motif, must play a pivotal role in sensing the signal that affects the phosphorylation state of the protein (i.e., the in vivo signaling mode of the protein). This region apparently dictates downstream phosphotransfer reactions.

Phosphotransfer between D696NCbbSR^{R96} and CbbRR2. Although D696NCbbSR^{R96} inefficiently catalyzed phosphorylation of CbbRR1 (Fig. 5), this mutant protein, but not wild-type CbbSR^{R96} (Fig. 3), was able to catalyze a slow rate and moderate level of phosphotransfer to CbbRR2 (Fig. 8A). In particular, if Asp-195, one of the conserved and potential aspartate phosphoacceptor residues from the two receiver domains of CbbRR2 was mutated, i.e., by using construct D195NCbbRR2, the apparent rate of phosphotransfer from D696NCbbSR^{R96} to the mutated CbbRR2 proteins was markedly enhanced (Fig. 8C, D, and E). As for the reaction in the presence of wild-type CbbSR^{R96} (Fig. 3), concomitant mutation of both aspartate residues completely prevented phosphorylation of CbbRR2 (data not shown). Moreover, throughout the 4 h that the phosphorylation experiments were carried out in the presence of wild-type CbbRR2, further accumulation of phosphorylated D696NCbbSR^{R96} was observed (Fig. 8A), suggesting that possible competition for the phosphate group be-

tween residues Asp-54 and Asp-195 of CbbRR2 indirectly favored the kinase autophosphorylation reaction. Indeed, the final absolute amount of phosphorylated D696NCbbSR^{R96} in the presence of wild-type CbbRR2 was significantly greater than that obtained in the presence of the mutant CbbRR2 proteins (Fig. 8D). Finally, D195NCbbRR2 showed greater than double the level of phosphorylation compared to wild-type or mutant D54NCbbRR2, strongly suggesting that Asp-54 from CbbRR2 possessed the highest affinity for the phosphoryl group derived from D696NCbbSR^{R96} (Fig. 8E).

Independent activity of the kinase receiver domain and phosphorylation of the CbbRR1 HPt domain. Because the receiver domain of the sensor kinase appeared to play a critical role for the reactions thus far described, its function was further addressed by assaying the phosphoacceptor activity of inactivated kinase constructs, that is, H409DCbbSR, since residue His-409 is essential for kinase activity (11). In a reaction containing 20 µM $[\gamma\text{-}^{32}P]ATP$ and equimolar concentrations of the D696NCbbSR^{T189} and H409DCbbSR^{R96} proteins (Fig. 9A and B), there was rapid and extensive phosphotransfer from His-409 of the D696NCbbSR^{T189} protein to the receiver domain of the inactive H409DCbbSR^{R96} kinase protein. A similar pattern of phosphorylation was observed with D696NCbbSR^{R96} and H409DCbbSR^{T189} (data not shown). This result clearly proves that rapid phosphotransfer occurred within the kinase protein; therefore, the kinase receiver domain functions as a discrete functional unit.
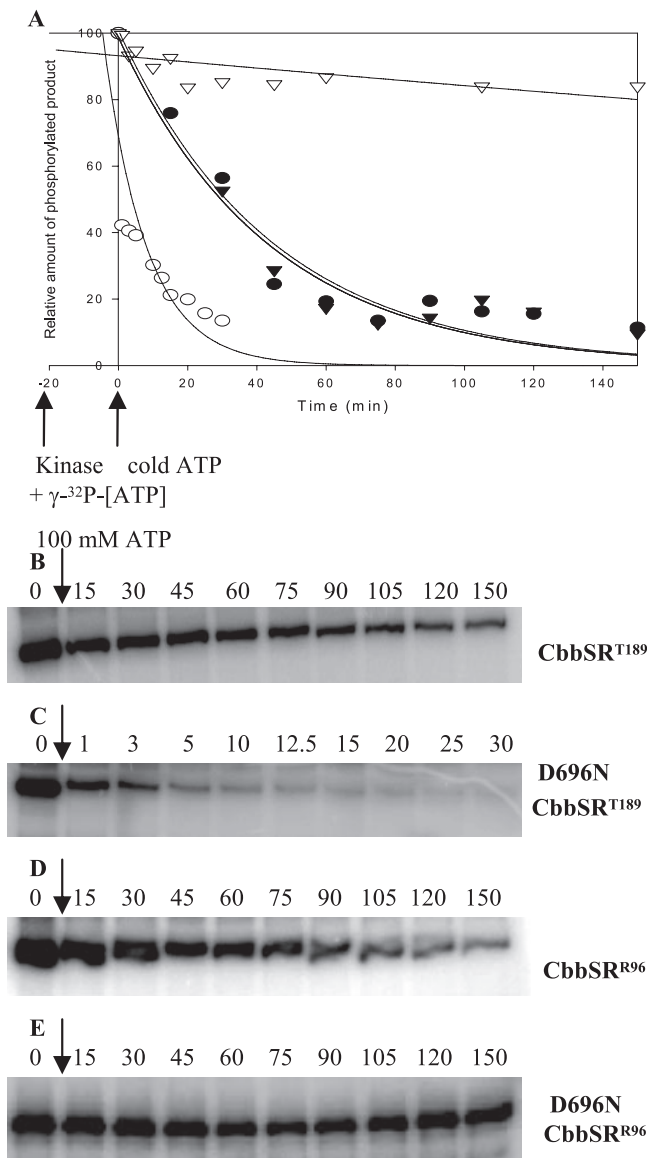
FIG. 7. Pulse-chase analysis of the CbbSR^{R96} and CbbSR^{T189} wild-type truncated histidine kinase and their D696N site-directed mutants. The phosphorylation levels of the four constructs were normalized to the maximum intensity of signal (100%) of each construct prior to the addition of an excess of unlabeled (100 mM) ATP and plotted against time. Label disappearance (γ-^{32}P) is expressed as the percentage of the phosphorylated products obtained at any given time after the addition of unlabeled ATP. The kinase proteins were preincubated with 20 μM [γ-^{32}P]ATP for 20 min, with the exception of D696N CbbSR^{R96}, which was preincubated with 25 nM [γ-^{32}P]ATP. The reaction was carried out in a 100-μl final volume. At time zero, 10 μl of the phosphorylation reaction mixture was removed, and 10 μl of unlabeled ATP (100 mM final concentration) was added. Aliquots were removed at the time points indicated. CbbSR^{T189}, ($\bullet$); D696NCbbSR^{T189}, ($\circ$); CbbSR^{R96}, ($\blacktriangledown$); D696NCbbSR^{R96}, ($\triangledown$). Raw data were analyzed, normalized, and plotted with SigmaPlot 8.0. Representative pulse-chase patterns of phosphorylation are shown for CbbSR^{T189} (B), D696NCbbSR^{T189} (C), CbbSR^{R96} (D), and D696NCbbSR^{R96} (E).

The function of the CbbRR1 HPt domain was tested for its ability to receive the phosphoryl group from Asp-696 of the sensor kinase. In general, proteins containing an HPt domain have been shown to participate in multistep phosphorelay

processes whereby the active histidine residue, although incapable of being autophosphorylated, participates in a phosphorylation cascade by providing a "phosphorylation" bridge between two distinct Asp residues (2, 3, 6, 14) either as a mono-domain protein or as a functional module of a tripartite hybrid kinase. To assess the interaction between CbbSR and CbbRR1, phosphotransfer was initiated by first preincubating D696NCbbSR^{T189} with 20 μM [γ-^{32}P]ATP for 20 min prior to the simultaneous addition of both the H409DCbbSR^{R96} and D54NCbbRR1 proteins. If there was substantial phosphorylation of the CbbRR1 mutant protein, this would presumably be due to phosphotransfer from Asp-696 of the sensor kinase to His-171 of the CbbRR1 protein. Previously, it was established that Asp-54 on CbbRR1 directly received the phosphoryl group from His-409 of the sensor kinase (Fig. 4A); in addition, Asp-696 of the sensor kinase is not required for this reaction (11). The pattern of phosphorylation (Fig. 9C, lanes 1 and 2) substantiated the above reasoning. Similar results were obtained by using D696NCbbSR^{R96} and H409D CbbSR^{T189} (data not shown). A control experiment showed no CbbRR1 phosphorylation with a D54NH 171DCbbRR1 double mutant (Fig. 9C, lanes 3 and 4). These studies indicated that a multistep phosphorylation reaction occurred whereby the active kinase (mutant D696NCbbSR) catalyzed phosphotransfer to the inactive kinase (mutant H409DCbbSR containing the phosphate acceptor residue Asp-696) and eventually to His-171 of the D54NCbbRR1 construct. Thus, phosphotransfer occurred from His-409 to Asp-696 on the sensor kinase to His-171 on CbbRR1. Since radiolabeled phosphate on the kinase constructs was considerably diminished by the end of the reaction (Fig. 9C, lane 2), phosphotransfer appeared to be unidirectional, and His-171 of CbbRR1 did not apparently allow reverse phosphotransfer to Asp-696 of the sensor kinase (4). Moreover, D696NCbbSR^{R96} reacted poorly with CbbRR1 constructs (Fig. 5). This is consistent with the idea that the kinase receiver domain and the HPt domain interact as independent functional domains. Finally, experiments with combinations of all three CbbRRS proteins (i.e., sensor kinase CbbSR and both response regulators CbbRR1 and CbbRR2, in their wild-type as well as mutant forms) indicated that phosphorylated CbbRR1, in particular, its phosphorylated His-171 residue, was incapable of passing the phosphoryl group to CbbRR2 (data not shown).

DISCUSSION

Previous results indicated that the CbbRRS system plays a significant role in the control of form I ribulose 1,5-bisphosphate carboxylase/oxygenase biosynthesis in *R. palustris*, with the proper balancing of sensor kinase and response regulator playing an important part in the regulatory mechanism under photoautotrophic (11) and photoheterotrophic (this laboratory, unpublished data) growth conditions. In the current study, phosphotransfer reactions catalyzed by the CbbRRS system were analyzed in vitro since a number of events were conceivable once autophosphorylation of the sensor kinase protein occurred. Since these phosphorylation events likely serve a profound function in vivo, it was particularly important to discern whether the single kinase, CbbSR, could potentially
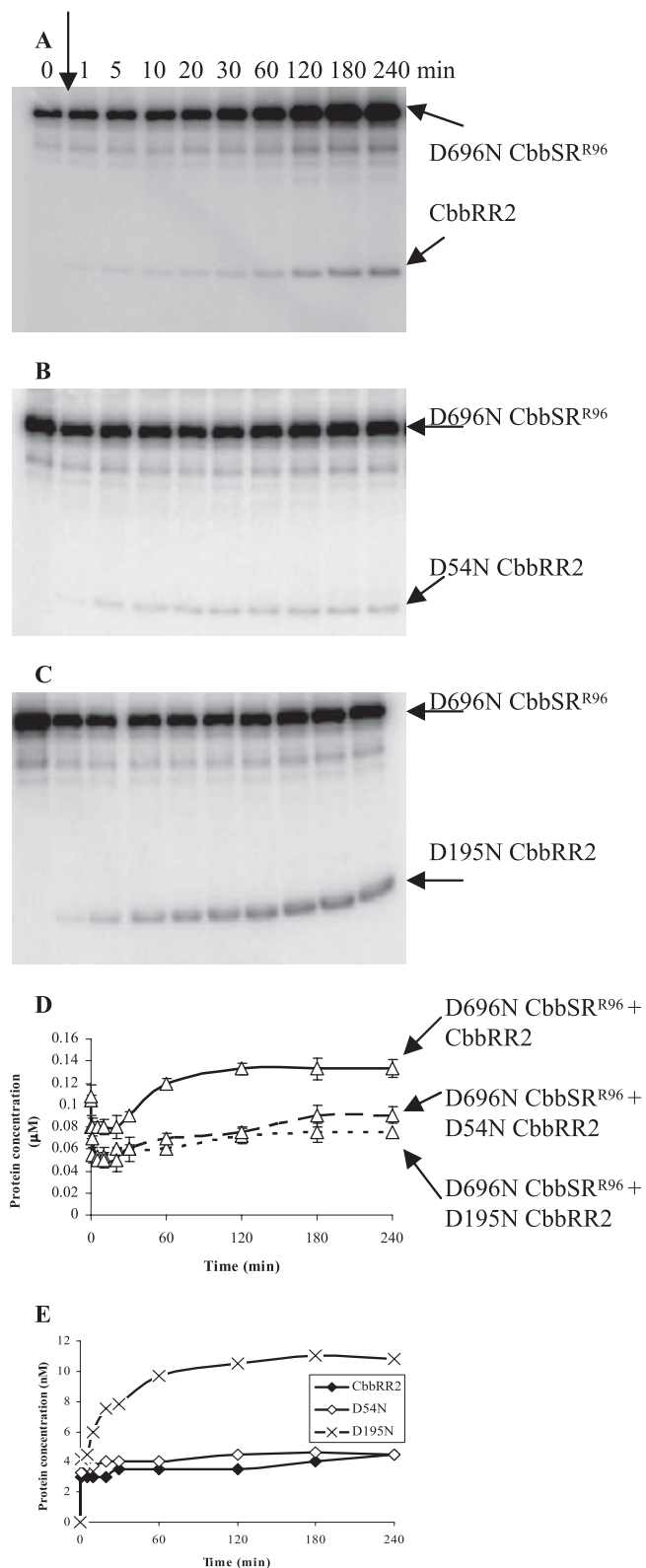
FIG. 8. Time course of CbbRR2 phosphorylation. To better resolve the phosphorylation products, the phosphotransfer reaction was performed under the following conditions: 1.2 μM D696NCbbSR[R96] was preincubated with 25 nM [γ-^{32}P]ATP for 20 min (time zero), prior to the addition of 24 μM CbbRR2 (A) and its site-directed mutants, D54NCbbRR2 (B) and D195NCbbRR2 (C). Ten-microliter aliquots

interact with four distinct receiver domains that show very limited sequence homology (Fig. 2). Thus, each of the four putative independent phosphotransfer reactions was considered here. Two truncated constructs of different lengths of CbbSR, in combination with various active-site residue mutations of CbbSR, CbbRR1, and CbbRR2, provided insight into different signaling states of the kinase protein. Based on the results of this investigation, hypothetical pathways for the CbbRRS phosphorelay were determined (Fig. 10), and several events were established upon autophosphorylation of residue His-409 of CbbSR: (i) the phosphate group may be transferred to Asp-696 within the kinase receiver domain and then to the His-171 residue of the CbbRR1 HPt domain; (ii) the phosphate group may also be transferred from His-409 of CbbSR to residue Asp-54 of the receiver domain of CbbRR1; (iii) the phosphate may be transferred from His-409 of CbbSR to either Asp-54 or Asp-195 of the two receiver domains of CbbRR2, with a preference for Asp-54. The first of these reactions occurred independently of the integrity of the N-terminal region of the sensor kinase protein; however, the second and third reactions were controlled by the presence of residues Arg-96 to Thr-189 at the N terminus of CbbSR. Furthermore, it is apparent from the in vitro data (Fig. 7) that the extent and degree of phosphorylation of the sensor kinase are influenced by the integrity of the N-terminal region, perhaps defining different conformational states of the sensor kinase. This observation raises the possibility that the phosphorylation state of the receiver domain of CbbSR, concomitant with the N-terminal region, functions as a major determinant to control the specificity of interactions of CbbSR with either response regulator CbbRR1 or CbbRR2. In the latter instance, two distinct regions of CbbSR clearly play a cooperative role in determining the signaling state of the kinase and ultimately the phosphotransfer to either CbbRR1 or CbbRR2.

The HPt domain of CbbRR1 was also intriguing. First of all, CbbRR1 represented the first investigated instance where an HPt domain was found to be part of a response regulator protein. HPt domains appear usually as either independent mono-domain proteins or as subdomains of tripartite hybrid kinases (2, 3, 7, 9, 14). The results reported here indicate that the HPt domain of CbbRR1 seems to function as a type of release device, allowing the kinase and therefore the whole CbbRRS system to reset its signaling state by removing the phosphate group from residue Asp-696 of the kinase and transferring it to His-171 of CbbRR1. This hypothesis was supported by observations with the mutant H171DCbbRR1 protein, which seemed to react with the wild-type kinase as well as CbbRR1 (Fig. 4); however, no more sequential transfer of the phosphate group occurred, either back to the kinase (Fig. 9C) or to CbbRR2 (data not shown). After transfer of the phos-

were removed at the time points indicated. The reaction products were resolved by 12% SDS-PAGE. The phosphorylated proteins are indicated by arrows. (D) Levels of D696NCbbSR[R96] over the time course of phosphotransfer for D696NCbbSR[R96]+CbbRR2 (solid line), D696NCbbSR[R96]+D54NCbbRR2 (dashed line), and D696NCbbSR[R96]+D195NCbbRR2 (dotted line). (E) Levels of phosphorylation for wild-type CbbRR2 (◆), D54NCbbRR2 (◇), and D195NCbbRR2 (×).

A

0 0.25 0.5 0.75 1 3 5 10 30 60
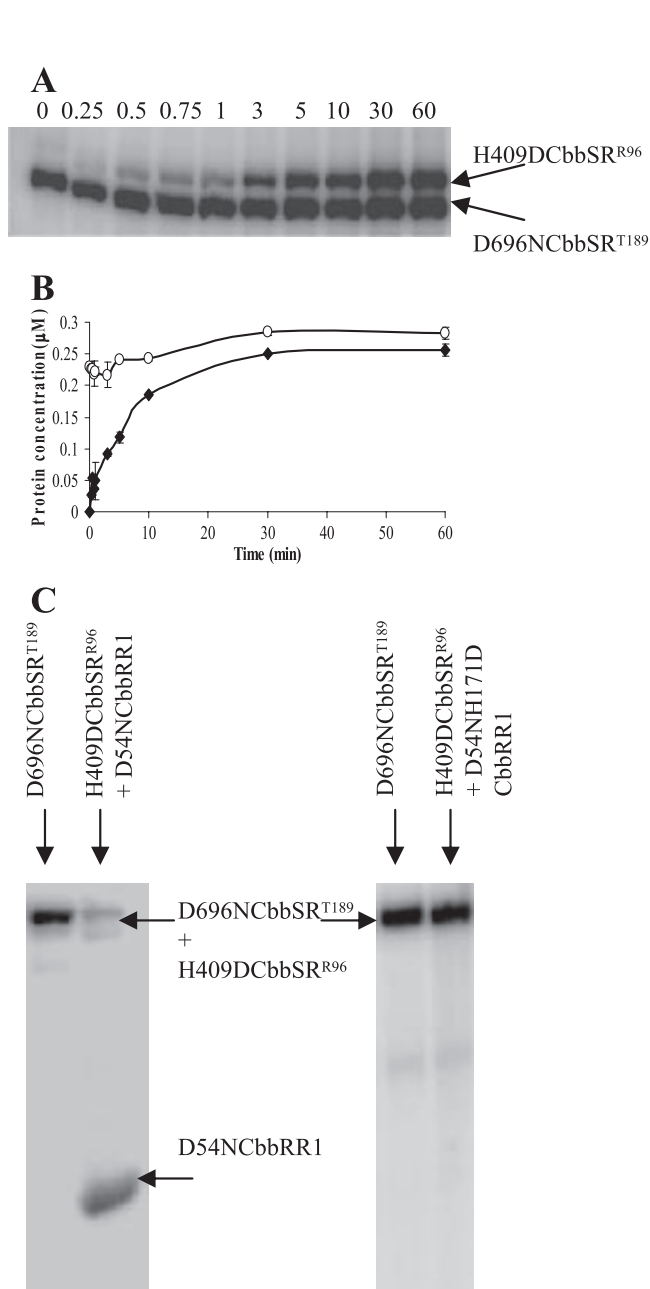
H409DCbbSR^{R96}

D696NCbbSR^{T189}

B

C

FIG. 9. The receiver domain on the sensor kinase functions as an independent catalytic unit. (A) Phosphotransfer between D696NCbbSR^{T189} and H409DCbbSR^{R96} (380 pmol each) in the presence of 20 μM [γ-^{32}P]ATP, resolved on an 8% SDS-PAGE gel. Analogous results were obtained with D696NCbbSR^{R96} and H409DCbbSR^{T189} in the presence of 25 nM [γ-^{32}P]ATP (data not shown). (B) Time-dependent accumulation of phosphorylated products when H409DCbbSR^{T189} (●) was incubated with D696NCbbSR^{R96} (○). (C) Residue Asp-696 is a productive phosphodonor to residue His-171 on the CbbRR1 HPt domain. D696NCbbSR^{T189} was preincubated with 20 μM [γ-^{32}P]ATP for 20 min (lane 1) prior to the simultaneous addition of both H409DCbbSR^{R96} (380 pmol) and D54NCbbRR1 (1 nmol). The complete reaction mixture was then incubated for 1 h (lane 2). As additional controls, D696NCbbSR^{T189} was incubated with 20 μM [γ-^{32}P]ATP (lane 3) prior to adding H409DCbbSR^{R96} and D54NH171DCbbRR1 (lane 4). The double mutant D54NH171DCbbRR1 was not phosphorylated.
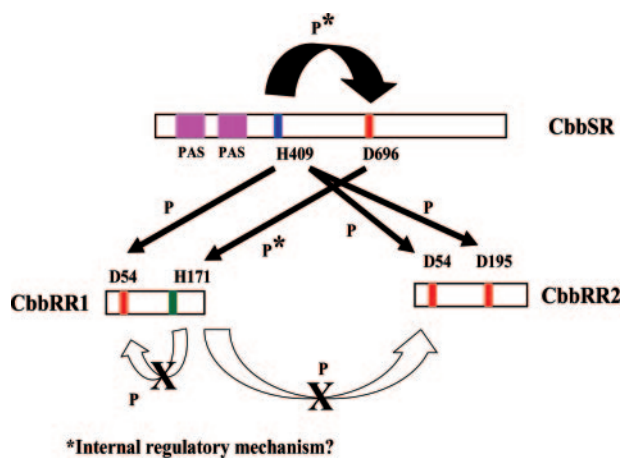
FIG. 10. Summary of the phosphorylation reactions catalyzed by the CbbRRS system. The conserved residues and motifs on the sensor kinase, CbbSR, and the two response regulators, CbbRR1 and CbbRR2, are indicated.

phate to His-171 of CbbRR1, the phosphorylated HPt domain likely decays, either by eventual spontaneous hydrolysis of the phosphate group or by means of some as yet unknown phosphatase.

In conclusion, the CbbRRS system was found to catalyze multiple phosphotransfer reactions with at least two functional checkpoints. The integrity of the N-terminal region of the sensor kinase clearly seems to influence the phosphorylation state of the kinase and specifies active complexes with either one of the two response regulators. On the other hand, the multistep phosphotransfer reaction to residue His-171 of response regulator CbbRR1 appeared to allow a turnover of the CbbRRS system, so that a new set of phosphorylation reactions might occur. Furthermore, based upon our findings, we propose that the sensor kinase protein assumes at least three separate signaling states: (i) an inactive mode, whereby phosphorylation of the kinase is ultimately terminated by phosphotransfer from His-409 to first its own receiver domain and then to the HPt domain of CbbRR1; (ii) an active mode whereby CbbSR can catalyze phosphorylation to CbbRR1; and (iii) a second active mode in which CbbSR can catalyze phosphorylation of CbbRR2. Transitions between these modes appeared to be determined by the signaling state of the N-terminal sensor region, possibly in response to some threshold level of intensity of the activating stimulus. The consequence would be the creation of an internal device to control downstream phosphotransfer reactions. Further studies will seek to identify the signal(s) that determine(s) each single step of the complex phosphorelay identified here and the physiological consequences of each of the phosphorylation steps.

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