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Role of Phosphorylated Metabolic Intermediates in the Regulation of Glutamine Synthetase Synthesis in *Escherichia coli*

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Transcription of the Ntr regulon is controlled by the two-component system consisting of the response regulator NR_I (NtrC) and the kinase/phosphatase NR_{II} (NtrB), which both phosphorylates and dephosphorylates NR_I. Even though in vitro transcription from nitrogen-regulated promoters requires phosphorylated NR_I, NR_{II}-independent activation of NR_I also occurs in vivo. We show here that this activation likely involves acetyl phosphate; it is eliminated by mutations that reduce synthesis of acetyl phosphate and is elevated by a mutation expected to cause accumulation of acetyl phosphate. With purified components, we investigated the mechanism by which acetyl phosphate stimulates glutamine synthetase synthesis. Acetyl phosphate, carbamyl phosphate, and phosphoramidate but not ATP or phosphoenolpyruvate acted as substrates for the autophosphorylation of NR_I in vitro. Phosphorylated NR_I produced by this mechanism exhibited the properties associated with NR_I phosphorylated by NR_{II}, including the activated ATPase activity of the central domain of NR_I and the ability to activate transcription from the nitrogen-regulated glutamine synthetase *glnAp*$_2$ promoter.

The Ntr regulon of enteric bacteria is a collection of genes and operons that are regulated by the availability of ammonia and whose products facilitate survival under nitrogen-limiting growth conditions. The most important enzyme for the assimilation of ammonia under nitrogen-limiting conditions is glutamine synthetase (GS), the product of the *glnA* gene. Under nitrogen-excess conditions, a low intracellular concentration of this enzyme results from transcription initiated at a σ^{70}-dependent promoter known as *glnAp*$_1$. Under nitrogen-limiting conditions, a much higher intracellular concentration of this enzyme results from transcription from a nitrogen-regulated promoter known as *glnAp*$_2$. The activation of transcription from the *glnAp*$_2$ promoter of enteric bacteria and other nitrogen-regulated promoters in intact cells and by purified components has been studied in some detail (reviewed in references 12, 13, 19, and 32). Transcription from the *glnAp*$_2$ promoter requires RNA polymerase containing the alternative sigma factor σ^{54} (8, 9). This polymerase binds tightly to the *glnAp*$_2$ promoter sequence, but it lacks the capacity to melt the DNA in the region surrounding the transcription start site (26, 29). The formation of an open complex by σ^{54} RNA polymerase requires a transcriptional activator; for the *glnAp*$_2$ promoter, this activator is the phosphorylated form of NR_I (NtrC [21]). The efficient action of phosphorylated NR_I (NR_I-P) in bringing about the activation of transcription is facilitated by high-affinity binding sites on the template; these sites can be located far from the promoter and are functionally analogous to enhancer sequences (23, 28). The enhancers serve to increase the local concentration of NR_I-P near the promoter (38). NR_I-P, bound to its enhancer, interacts with σ^{54} RNA polymerase at the promoter by means of a DNA loop (33) and, by so doing, somehow brings about the formation of the open complex.

The ability of NR_I to activate transcription is regulated by protein phosphorylation (21). The dominant enzyme in this regulation is the protein kinase/phosphatase NR_{II} (NtrB). NR_{II} catalyzes the transfer of the gamma phosphoryl group of ATP to a histidine residue (His-139) within its kinase domain (20, 42). This phosphoryl group is then transferred from the His-139 residue of NR_{II}-P to an aspartyl residue (42) within the N-terminal domain of NR_I (11). The acylphosphate group in NR_I-P is unstable at neutral pH, with a half-life of hydrolysis of about 4 min (autophosphatase activity [11]), and the ability of NR_I to activate transcription is lost after hydrolysis of the phosphoryl group (39). The dephosphorylation of NR_I-P is accelerated by the concerted action of NR_{II} and another signal transduction protein known as P_{II} (11, 21). Thus, P_{II}, at least superficially, appears to convert NR_{II} from a kinase to a phosphatase. Nitrogen regulation of transcription in vivo is accomplished by the control of the availability of P_{II} for this reaction (1, 4).

Mutant forms of NR_I have been identified in which the requirement for phosphorylation has been reduced; that is, the unphosphorylated form of the mutant protein has some ability to activate transcription (26, 39). Studies with such mutant activators have indicated that NR_I has an intrinsic ATPase activity and that ATP is required for the formation of open transcription complexes in vitro, in addition to the requirement for ATP in the phosphorylation of NR_I by NR_{II} (26). Furthermore, the phosphorylation of NR_I by NR_{II} may stimulate the NR_I ATPase activity, which is required but not sufficient for the activation of transcription by NR_I (40). The mechanism by which NR_I-P ATPase activity facilitates the formation of the open transcription complex has not been elucidated.

Mutants lacking NR_{II} are able to activate transcription from *glnAp*$_2$ (27), and in previous work, it had been observed that the expression of the *glnA* product, GS, is affected by the availability of ammonia (4). Subsequent work showed

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that expression of GS on nitrogen-excess medium containing glucose seemed to be related to the growth phase of the culture; GS levels were low in lag-phase and early log-phase cultures (2), approximately normal in mid-log-phase cultures (4), but elevated in late-log-phase cultures (2, 6). In this report, we will show several other conditions under which GS was expressed at high levels in nitrogen-excess medium in the strain lacking NR_{II}.

NR_I and NR_{II} are related to a large number of bacterial regulatory proteins that control various adaptive responses, known collectively as the two-component systems (reviewed in references 19 and 32). Each of these systems contains a protein related to NR_{II} (histidine kinase) and one or more proteins related to NR_I (response regulator). The histidine kinase proteins have a conserved domain corresponding to the C-terminal 60% of NR_{II}, and the response regulators have a highly conserved domain corresponding to the N-terminal domain of NR_I. In a number of cases, histidine kinases and response regulators have been purified and characterized in vitro. In such cases, a phosphotransfer mechanism similar to the one for NR_{II} and NR_I has been observed. The purified histidine kinases generally exhibit some ability to donate phosphoryl groups to nonconsonant response regulators (10, 22). In at least one case, this cross-reactivity may be a physiologically relevant form of cross-regulation (reviewed in reference 34). One possible mechanism to explain the activation of *glnA* transcription in cells deleted for NR_{II} would be the transfer of phosphoryl groups from another histidine kinase to NR_I (reviewed in references 32 and 34). Under most conditions, the effect of such transfer would be observable only in cells lacking NR_{II}, since only these mutants lack the NR_{II}/P_{II} phosphatase activity that removes phosphoryl groups from NR_I-P under conditions of nitrogen excess.

Studies of adaptive responses controlled by other two-component systems indicate that, like NR_I, other response regulators can function in the absence of the cognate kinase (reviewed in reference 19). For example, the phosphate (*pho*) regulon of *Escherichia coli* is regulated by a two-component system consisting of PhoB and PhoR, which are analogous to NR_I and NR_{II}, respectively. Transcriptional activation requires phosphorylated PhoB (PhoB-P), whose phosphorylation, as determined from both in vivo and in vitro studies, is catalyzed by PhoR or, in mutants lacking PhoR, by the related CreC (PhoM) kinase. In mutants lacking both PhoR and CreC, the *pho* regulon is still expressed; in such mutants, PhoB-dependent transcription is activated by mutations that cause an accumulation of acetyl phosphate (37). Furthermore, experiments with the purified chemotaxis response regulator proteins CheY and CheB showed that small molecules containing high-energy phosphoryl groups, including acetyl phosphate, carbamyl phosphate, and phosphoramidate, can donate phosphoryl groups to CheY (14), while the CheB response regulator is phosphorylated and activated by phosphoramidate but not by acetyl phosphate or carbamyl phosphate (14).

We studied further NR_I-dependent transcription from the $glnAp_2$ promoter in mutants lacking NR_{II}. In many ways, the growth conditions that led to activation of *glnA* expression were similar to those that led to activation of the *pho* regulon in response to acetyl phosphate synthesis (2, 34, 37; this study). We therefore tested the effect of mutations in acetyl phosphate metabolism on the NR_{II}-independent activation of NR_I. We show here that mutations or growth conditions expected to decrease acetyl phosphate synthesis largely abolished NR_{II}-independent GS synthesis, while mutations or growth conditions expected to increase acetyl phosphate accumulation increased NR_{II}-independent GS synthesis. Therefore, acetyl phosphate may also be an inducing signal for the *ntr* regulon, as it appears to be for the *pho* regulon. We then examined whether small molecules containing high-energy phosphoryl groups are able to phosphorylate NR_I and by so doing bring about the stimulation of *glnA* transcription in vitro. Indeed, acetyl phosphate, carbamyl phosphate, and phosphoramidate were able to stimulate *glnA* transcription in an NR_I-dependent manner. We directly demonstrated that acetyl phosphate transfers phosphoryl groups to purified NR_I and that NR_I-P so formed had the characteristic autophosphatase activity and could be used to drive the NR_{II}-dependent phosphorylation reaction in reverse.

Our finding of a way to phosphorylate NR_I with small molecules allowed us to examine the requirement for ATP in open complex formation with wild-type NR_I protein in the absence of NR_{II}. We show that ATP is required for the formation of the transcription complex by wild-type NR_I and that phosphorylation of NR_I by small molecules greatly stimulates its ATPase activity. The ATPase activity of NR_I-P was found to be strongly influenced by the concentration of NR_I and by the concentration of the small phosphorylated molecules used as substrates for the autophosphorylation of NR_I. Our results are consistent with a model in which at least two of the dimeric NR_I-P molecules must associate to form the active ATPase required for open complex formation (40).

MATERIALS AND METHODS

Bacterial strains. All strains used are listed in Table 1. The Gln^+ Ntr^+ "wild-type" strain YMC10 has been described previously (3). The otherwise isogenic strain RB9132 (4) contains an internal *glnL* deletion which fuses codon 23 to codon 182. This deletion removes the site of autophosphorylation (His-139) and much of the non-conserved N-terminal domain (residues 1 to ~125) and conserved C-terminal kinase/phosphatase domain (residues ~125 to 349) of NR_{II}. Strain BW13711 (18) is an F^- λ^- *Escherichia coli* strain into which the *lacX74* deletion was introduced in a P1 cross. This strain has no other known mutations. BW13713 is similar but contains a *phoR* mutation. The *glnL* deletion was introduced into these strains in two steps. First, a *glnA*::Tn*5* or *glnA*::Tn*5-132* mutation was introduced by P1 transduction, with selection for kanamycin or tetracycline resistance, respectively. The *glnL2001* deletion was then introduced by transduction with P1 grown on a *glnL2001* mutant, with selection for glutamine prototrophy ($glnA^+$). We verified the presence of *glnL2001* in the glutamine prototrophs by polymerase chain reaction amplification of *glnL* sequences (2). The *pta*, *ackA*, and Δ(*pta ackA*) mutations were introduced by cotransduction with a linked Tn*10* and identified by testing transductants for growth on acetate, as described before (37) (Table 1). P1-mediated transduction was performed as described previously (30, 37).

GS assays. Cetyltridecylammonium bromide-treated cells were assayed for glutamyl transferase activity as described previously (25). Cells were grown overnight in the indicated medium and subcultured into 20 ml of warm fresh medium at an optical density at 600 nm (OD_{600}) of 0.02. Cultures were then grown at 30°C to the indicated OD_{600} and harvested. Defined W-salts and rich media were described previously (25). The GS microassay was performed as described previously (15).

Purified proteins. NR_I, NR_{II}, σ^{54}, core RNA polymerase, and CheB were purified as described previously (9, 23, 31).

TABLE 1. *E. coli* strains

Strain	Relevant genotype	Construction or reference
YMC10	*hutC*$_{Klebs}$(*hut*$^+$) Δ*lac-169 hsr hsm*	3
RB9132	As YMC10, *glnL2001*	4
RB9040	As YMC10, *glnD99*::Tn*10*	4
RB9060	As YMC10, *glnB*	4
DD13	As RB9132, *glnD99*::Tn*10*	RB9132 × RB9040 (P1)
YMC15	As YMC10, *glnL2302*	3
TH16	As YMC10, *glnA*::Tn*5*	28
BW3627	*lac-169 phoR68*(Oc)	35
BW9795	*lac-169 phoR68*(Oc) *glnA*::Tn*5*	BW3627 × TH16 (P1)
BW10015	As BW3627, *glnL2001*	BW9795 × RB9132 (P1)
BW12863	As BW9795, *glnA*::Tn*5-132*	
BW13711	*lacX74*	18
BW13713	As BW13711, *phoR68*(Oc)	36
BW16167	Δ(*his gnd*)*461 dhuA1 pta-200 zej-223*::Tn*10*	37
BW16168	Δ(*his gnd*)*461 dhuA1 ackA200 zej-223*::Tn*10*	37
BW16169	Δ(*his gnd*)*461 dhuA1* Δ(*pta ackA hisQ hisP*) *zej-223*::Tn*10*	37
WS6000	As YMC10, *pta-200 zej-223*::Tn*10*	YMC10 × BW16167 (P1)
WS6001	As YMC10, *ackA200 zej-223*::Tn*10*	YMC10 × BW16168 (P1)
WS6002	As YMC10, Δ(*pta ackA hisQ hisP*) *zej-223*::Tn*10*	YMC10 × BW16169 (P1)
WS6003	As RB9132, *pta-200 zej-223*::Tn*10*	RB9132 × BW16167 (P1)
WS6004	As RB9132, *ackA200 zej-223*::Tn*10*	RB9132 × BW16168 (P1)
WS6004	As RB9132, Δ(*pta ackA hisQ hisP*) *zej-223*::Tn*10*	RB9132 × BW16169 (P1)
BW16462	As BW13711, *pta-200 zej-223*::Tn*10*	BW13711 × BW16167 (P1)
BW16545	As BW13711, *ackA200 zej-223*::Tn*10*	BW13711 × BW16168 (P1)
BW16463	As BW13711, Δ(*pta ackA hisQ hisP*) *zej-223*::Tn*10*	BW13711 × BW16169 (P1)
BW18042	As BW13711, *glnA*::Tn*5*	BW13711 × TH16 (P1)
BW18302	As BW13711, *glnL2001*	BW18042 × RB9132 (P1)
BW18499	As BW18302, *ackA200 zej-223*::Tn*10*	BW18302 × BW16168 (P1)
BW18500	As BW18302, Δ(*pta ackA hisQ hisP*) *zej-223*::Tn*10*	BW18302 × BW16169 (P1)
BW18501	As BW18302, *zej-223*::Tn*10*	BW18302 × BW16169 (P1)
BW18793	As BW18302, *pta-200 zej-223*::Tn*10*	BW18302 × BW16167 (P1)
BW16465	As BW13713, *pta-200 zej-223*::Tn*10*	BW13713 × BW16167 (P1)
BW16466	As BW13713, *ackA200 zej-223*::Tn*10*	BW13713 × BW16168 (P1)
BW16467	As BW13713, Δ(*pta ackA hisQ hisP*) *zej-223*::Tn*10*	BW13713 × BW16169 (P1)
BW17931	*lac-169 phoR68*(Oc) *glnL2001 zej-223*::Tn*10*	BW10015 × BW16167 (P1)
BW17932	As BW17931, *pta200*	BW10015 × BW16167 (P1)
BW17933	As BW17931, Δ(*pta ackA hisQ hisP*)	BW10015 × BW16169 (P1)
BW17940	As BW17931, *ackA200*	BW10015 × BW16168 (P1)

Each protein appeared to be at least 90% pure, as judged from Coomassie-stained gels.

Transcription assays. In all experiments, the template for transcription assays was supercoiled pTH8 (9). This plasmid contains a strong rho-independent terminator from bacteriophage T7 positioned 308 bp downstream from the wild-type $glnAp_2$ promoter. The initiated-complex assay (see Fig. 2) was done as described previously (23). The buffer contained 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 0.1 mM EDTA. Reaction mixtures (44 μl) contained 10 nM template, the nucleotides ATP, CTP, and GTP (0.4 mM each), RNA polymerase (100 nM), and σ^{54} (100 nM). Where indicated, NR_{II} was used at 200 nM and NR_I was used at 80 nM. Acetyl phosphate, carbamyl phosphate, phosphoramidate, phosphoenolpyruvate, or ATP was added at the indicated concentrations. Reaction mixtures were incubated at 37°C for 25 min to allow formation of the initiated complexes. A solution (6 μl) containing labeled UTP (0.1 mM final concentration) and heparin (200 μg/ml final concentration) was then added, and incubation at 37°C was continued for an additional 25 min. Reactions were stopped by addition of an equal volume of a solution containing 0.01 M Tris (pH 8), 100 μg of tRNA per ml, 50 mM EDTA, and 500 mM NaCl. RNA was ethanol precipitated, subjected to electrophoresis in 4% acrylamide–7 M urea gels, and detected by autoradiography as described before (9). The open-complex assay was similar, except that transcription complexes were formed in the presence of ATP (0.4 mM) as the sole nucleotide. In this case, after incubation to permit the formation of transcription complexes, a labeling mixture containing heparin and all four nucleotides (unlabeled ATP, CTP, and GTP at 0.4 mM and labeled UTP at 0.1 mM) was added.

For the experiments shown in Fig. 3, lanes 3, 7, and 10, the reaction components were incubated as described above except that no nucleotide was present. A mixture containing heparin, unlabeled ATP, CTP, and GTP, and labeled UTP was then added as in the experiments involving uninitiated complexes.

Phosphorylation of NR_I by acetyl-^{32}P. Acetyl-^{32}P was prepared as described previously (14). For the experiment shown in Fig. 2, proteins (50 to 75 pmol) were incubated in a 20-μl reaction mix containing 50 mM Tris-Cl (pH 7.0), 5 mM $MgCl_2$, 1 mM dithiotreitol, and 5 mM acetyl-^{32}P. Reactions were stopped after 5 min by addition of 10 μl of a solution containing 100 mM Tris-Cl (pH 6.8), 1% (wt/vol) sodium dodecyl sulfate (SDS), 10 mM EDTA, 10 mM 2-mercaptoethanol, 10% (vol/vol) glycerol, and 0.025% (wt/vol) bromophenol blue. The proteins were subjected to electrophoresis on an SDS–15% polyacrylamide gel, which was autoradiographed and then stained with Coomassie brilliant blue.

To measure the stability of NR_I-P formed by autophosphorylation from acetyl-^{32}P, NR_I (2.4 μM) was incubated with acetyl phosphate (10 mM) for 45 min at 37°C in a reaction buffer consisting of 50 mM Tris-Cl (pH 7.5), 10 mM $MgCl_2$, and 50 mM KCl. A sample was taken and mixed with an equal volume of SDS sample buffer as above. The remaining portion of the reaction mixture was then split into aliquots, to which were added (i) reaction buffer, (ii) reaction buffer plus 40 or 80 mM phosphoramidate, (iii) reaction buffer plus 1.25 mM ADP, (iv) reaction buffer plus 1.25 mM ATP, (v) reaction buffer plus 1.25 μM NR_{II}, (vi) reaction buffer plus 1.25 mM ADP plus 125 nM NR_{II}, or (vii) reaction buffer plus 1.25 mM ATP plus 125 nM NR_{II}. The reaction mixes were then incubated at 37°C, and aliquots were removed at various times into SDS sample buffer. Samples were then subjected to electrophoresis and autoradiography, and the relative amount of NR_I-P was estimated by visual inspection of the autoradiographs and by densitometry.

ATPase assay. The procedure for assaying ATPase was based on that of Norby (24), with the following modifications. The reaction volume for all the experiments was 700 μl. In the standard assay, reaction mixtures contained transcription buffer, phosphoenolpyruvate (1 mM), lactate dehydrogenase (46 U; Sigma), and pyruvate kinase (14 U; Boehringer Mannheim). NR_I, NR_{II}, acetyl phosphate, carbamyl phosphate, and phosphoramidate were added as indicated. Some of the reaction mixtures were incubated for 5 min at 37°C before transfer into a cuvette and then placed in a Beckman DU65 spectrophotometer equipped with a temperature control device (VWR circulating water bath). The A_{340} was calibrated to 0.00, and NADH was then added to 0.15 mM, which gave an A_{340} of 1.0 to 0.9. ATP (1 mM) was then added to start the reaction. Adsorbance readings were taken every 30 s under the control of Beckman kinetics Soft-pac software. At the conclusion of each experiment, ADP (10 mM) was added to each reaction mix; this addition caused the A_{340} to drop to 0.05 within 1 min (not shown in all cases).

In Fig. 5A, the data come from a series of experiments in which NR_I was incubated with phosphoramidate prior to the addition of ATP, as described above. The rates were calculated for each indicated concentration point (phosphoramidate, Fig. 5A; NR_I, Fig. 5B) as follows. The slopes of the change in OD_{340} versus time plots were determined by using the statistical functions of the Sigmaplot 3.1 program and converted to rates as described previously (24). The slope obtained in control experiments for unstimulated NR_I was used to determine the background rate, which was then subtracted from all values. The concentration of phosphoramide was plotted against rate by using the Enzfitter program (Fig. 5A), which also provided the Hill coefficients. In Fig. 5B, the data come from a series of experiments in which NR_I at the indicated concentrations was incubated with 40 mM phosphoramidate for 5 min prior to the addition of ATP. Rates were calculated as described above; in this case, a control was used for each NR_I concentration, in which the unstimulated rate was determined under identical conditions.

RESULTS

Phenotype of a mutant with an internal deletion in *glnL* (*ntrB*), inactivating NR_{II}. In order to understand the physiological basis of the NR_{II}-independent activation of GS synthesis, we examined the synthesis of GS in a strain containing the internal *glnL2001* deletion under a range of conditions not investigated previously. Since it is known that this activation of GS synthesis requires the transcriptional activator NR_I (4) and thus probably results from the activation of transcription from $glnAp_2$, it is important that the *glnL* null mutation used in these studies not display polarity on expression of the downstream *glnG* (*ntrC*) gene encoding NR_I. The *glnL2001* mutation is an internal deletion fusing codon 23 of *glnL* to codon 182; presumably, this is a null mutation. Since the internal deletion is in frame, we expect that it does not display polarity with regard to the expression of the downstream *glnG* (*ntrC*) gene, and this assumption is supported by the results of previous studies with this strain (4) and the experiments reported in this article.

TABLE 2. Regulation of GS synthesis in rich medium by various metabolites[a]

OD_{600}	Strain	GS sp act (nmol/min/mg of cell protein) in medium with addition:						
		None	Glc	Pyr	Ace	Glyc	Suc	Glc + NH_3
1.00	YMC10 (wild type)	83	30	67	78	90	87	45
	WS6002 (*pta ackA*)	104	100	100	81	123	135	63
	RB9132 (*glnL*)	129	257	181	810	209	157	442
	WS6005 (*glnL pta ackA*)	51	21	55	59	62	64	26
2.00	YMC10 (wild type)	82	119	78	98	68	100	107
	WS6002 (*pta ackA*)	56	89	89	88	128	91	69
	RB9132 (*glnL*)	132	571	647	523	546	260	500
	WS6005 (*glnL pta ackA*)	116	39	155	98	61	81	33

[a] The medium was LB plus glutamine (0.2%, wt/vol) and contained, in addition, the carbon source indicated (at 0.4%, wt/vol): Glc, glucose; Pyr, pyruvate; Ace, acetate; Glyc, glycerol; Suc, succinate; Glc + NH_3, glucose plus ammonium sulfate (0.2%, wt/vol).

To rapidly examine the synthesis of GS on many different growth media, we initially used a microassay technique to qualitatively assess the relative amount of GS in cells scraped from the surface of various solid media. On the very nitrogen rich complex LB plus glutamine medium, the synthesis of GS was low in strains containing the *glnL2001* mutation, but addition of pyruvate, glycerol, or glucose to the medium resulted in a clear stimulation of GS synthesis (not shown). Acetate was observed to provide a weak stimulation, while succinate provided no discernible stimulation (not shown). The effects of these metabolites were investigated further by the quantitative GS assay with cultures grown in liquid LB-glutamine medium (Table 2). As shown, GS activity was low in the wild-type YMC10 strain and was affected little by the presence of glucose, pyruvate, acetate, glycerol, succinate, or the combination of glucose and ammonia. In strain RB9132, containing the *glnL2001* mutation, GS expression was stimulated by acetate in cultures grown to an OD_{600} of 1.00 (mid-log phase) and was stimulated by glucose, acetate, pyruvate, glycerol, and the combination of glucose and ammonia in cultures grown to an OD_{600} of 2.00 (late log phase; Table 2). These studies thus revealed a metabolic regulation of GS synthesis that occurred only in the absence of NR_{II}.

We investigated the possibility that the metabolic regulation of GS synthesis observed in the *glnL2001* background was due to differences in the intracellular concentration of acetyl phosphate. The *pta* gene encodes the enzyme phosphotransacetylase, responsible for the reversible interconversion of acetyl coenzyme A and acetyl phosphate (reviewed in reference 34). The *ackA* gene encodes acetate kinase, responsible for the reversible interconversion of acetate and acetyl phosphate (reviewed in reference 34). We examined the effect of deletion of these genes on the synthesis

TABLE 3. Effect of *pta-200* and Δ(*pta ackA*) mutations on GS synthesis in cells grown in defined media[a]

Strain	Relevant genotype	GS sp act (nmol/min/mg of cell protein)			
		Ggln	GNgln	PNgln	ANgln
YMC10	Wild type	1,783	275	211	196
WS6000	*pta*	1,646	134	194	70
WS6002	Δ(*pta ackA*)	1,557	173	212	96
RB9132	*glnL*	2,077	499	2,257	590
WS6003	*glnL pta*	52	46	122	1,902
WS6005	*glnL* Δ(*pta ackA*)	59	50	341	99
BW13711	Wild type	2,101	336	274	214
BW16462	*pta*	1,982	161	230	105
BW16463	Δ(*pta ackA*)	1,971	169	210	110
BW18302	*glnL*	2,582	1,198	3,182	821
BW18501	*glnL*	2,881	1,011	2,812	959
BW18793	*glnL pta*	122	68	178	2,636
BW18500	*glnL* Δ(*pta ackA*)	118	93	173	81
BW13713	*phoR*	1,582	287	217	137
BW16465	*phoR pta*	1,738	117	218	152
BW16467	*phoR* Δ(*pta ackA*)	1,834	140	224	117
BW17931	*glnL phoR*	2,607	1,021	2,661	724
BW17932	*glnL phoR pta*	144	70	181	2,537
BW17933	*glnL phoR* Δ(*pta ackA*)	146	76	192	107

[a] Ggln medium contains glucose at 0.4% and glutamine at 0.2% and is nitrogen limiting. GNgln medium contains, in addition to glucose and glutamine, ammonium sulfate at 0.2% and is nitrogen-excess. PNgln contains pyruvate at 0.4%, ammonium sulfate at 0.2%, and glutamine at 0.2%. ANgln contains potassium acetate at 0.4%, ammonium sulfate at 0.2%, and glutamine at 0.2%. All cultures were assayed at an OD_{600} of 0.5.

of GS in cells grown on the nitrogen-excess LB-glutamine medium to which various carbon sources were added. Introduction of a deletion of *pta* and *ackA* into the wild-type background had little effect on the synthesis of GS under these conditions (Table 2). In contrast, in the RB9132 (*glnL2001*) background, induction in every case was decreased by introduction of the deletion removing *pta* and *ackA* (Table 2).

We also examined the synthesis of GS in liquid defined medium cultures containing various carbon and nitrogen sources (Table 3). Normal nitrogen regulation by ammonia was observed in the wild-type YMC10 strain, and this regulation was little affected by the presence of the deletion removing *pta* and *ackA* or by a point mutation in *pta* (Table 3). In contrast, the otherwise isogenic *glnL2001* strain (RB9132) displayed elevated GS activity on the nitrogen-rich glucose-ammonia-glutamine medium, as observed previously (2, 6). When pyruvate was used as the carbon source in medium containing ammonia and glutamine as nitrogen sources, the expression of GS in the *glnL2001* mutant was very high (Table 3). Acetyl phosphate is strongly implicated as the internal signal stimulating GS synthesis, as revealed by the effect of the deletion removing *pta* and *ackA*, which greatly reduced the expression of GS under all conditions in the RB9132 strain (Table 3). A point mutation in *pta* greatly reduced the synthesis of GS in the RB9132 strain lacking NR_{II} when the growth medium contained glucose or pyruvate as the carbon source, but led to an elevation of GS synthesis when acetate was the carbon source (Table 3). Since a *pta* mutant is expected to accumulate acetyl phosphate when grown on acetate, this result indicates that acetyl phosphate or a compound derived from acetyl phosphate is a positive regulator of GS synthesis.

In the course of our studies, we observed that the strain YMC10, originally derived from MM294, and its derivatives such as strain RB9132 are defective in their ability to grow on pyruvate or acetate as a sole carbon source compared with other *E. coli* strains (2). In order to determine whether the effects of internal *glnL* deletion, the *pta* mutation, and *pta-ackA* deletion are in part due to the presence of one or more uncharacterized mutations in the YMC10 strain background, we constructed single and double mutants in the BW13711 background (18), which is more nearly wild type and grows well on pyruvate and acetate. We observed that, as expected, the strains constructed in the BW13711 background grew much better than their analogs constructed in the YMC10 background when pyruvate or acetate was the carbon source (not shown). As shown in Table 3, in the BW13711 background, the internal deletion in *glnL*, the *pta* mutation, and the *pta-ackA* deletion had effects similar to those observed in the YMC10 background. One slight difference that was noted was that, in the BW13711 background, the *glnL2001* mutation resulted in a higher level of expression of GS on nitrogen-rich medium than was observed in the YMC10 background (Table 3); a similar effect was observed previously when *glnL2001* was placed in the W3110 strain background (6).

We also examined the effects of the *glnL* internal deletion, *pta* mutation, and *pta-ackA* deletion in strains that also contain a *phoR* mutation, inactivating the main kinase/phosphatase of the *pho* regulon (Table 3). In this strain background as well, the activation of GS expression in the absence of NR_{II} seemed to reflect the capacity to synthesize acetyl phosphate in a fashion identical to that observed in $phoR^+$ cells (Table 3).

We attempted to ascertain the effects of an *ackA* mutation on the expression of GS in cells containing the *glnL2001* mutation or wild-type $glnL^+$. Since the *ackA*-encoded acetate kinase enzyme is primarily responsible for the catabolism of acetyl phosphate in cells grown on carbon sources leading to acetyl coenzyme A, loss of this enzyme should result in very high levels of GS in cells lacking NR_{II} when grown on glucose or pyruvate. Unfortunately, cells containing both *ackA* and *glnL2001* mutations grew very poorly on all media tested and rapidly accumulated pseudorevertants that grew better. Thus, we were unable to grow pure liquid cultures of these strains. Nevertheless, we performed the quantitative GS assay on a number of the decidedly impure cultures of the *ackA glnL2001* mutant strains constructed in the YMC10 and BW13711 backgrounds and observed that GS expression was indeed elevated in glucose-grown cultures (not shown).

Acetyl phosphate, carbamyl phosphate, and phosphoramidate stimulate NR_I-dependent activation of transcription from the nitrogen-regulated $glnAp_2$ promoter by purified *E. coli* components. We used the $glnAp_2$ transcription assay to examine the ability of various potential phospho donors to stimulate transcription. Previous results have indicated that wild-type NR_I lacks the capacity to activate transcription in this assay unless it is phosphorylated (21). We screened for small molecules that could stimulate transcriptional activation by NR_I in the absence of any kinase. In order to detect with great sensitivity any activation of transcription, we used the initiated-complex assay described previously (23, 26). In this assay, the template and polymerase are incubated with NR_I and three of the four nucleotides (ATP, CTP, and GTP at 0.4 mM each) in the presence or absence of putative phosphorylating agents. Phosphorylation of NR_I results in the activation of transcription, and initiated polymerase-template-transcript ternary complexes stalled at positions requiring the absent nucleotide (UTP) are formed. Addition of heparin and UTP then results in a single cycle of tran-

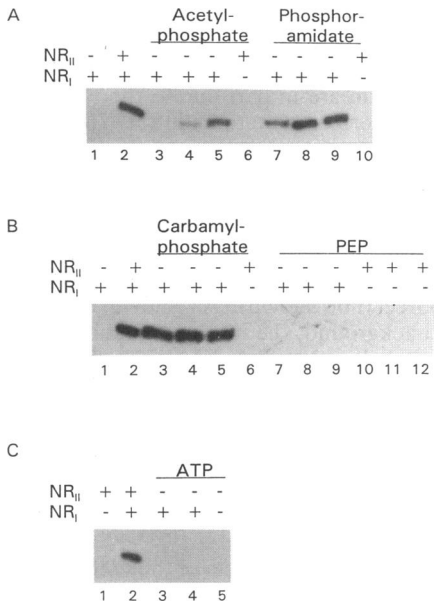

FIG. 1. Acetyl phosphate, carbamyl phosphate, and phosphoramidate stimulate *glnAp*$_2$ transcription in the absence of NR_{II}. NR_I, when present, was used at 80 nM, and NR_{II}, when present, was used at 200 nM. (A) Acetyl phosphate was present at 1 mM (lane 3), 5 mM (lane 4), or 10 mM (lanes 5 and 6). Phosphoramidate was present at 1 mM (lane 7), 5 mM (lane 8), or 10 mM (lanes 9 and 10). (B) Carbamyl phosphate was present at 1 mM (lane 3), 5 mM (lane 4), or 10 mM (lanes 5 and 6). Phosphoenolpyruvate (PEP) was present at 1 mM (lanes 7 and 10), 5 mM (lanes 8 and 11), or 10 mM (lanes 9 and 12). (C) ATP was present at 5 mM (lane 4) or 10 mM (lanes 3 and 5). Comparisons can only be made within each panel; differences in autoradiographic exposure do not permit direct comparison between panels.

scription, and the transcripts can be quantified on gels. We used this assay for screening purposes because the initiated ternary complexes are essentially irreversible and resistant to heparin. It was also possible to form uninitiated transcription complexes in a similar assay lacking all nucleotides except ATP, but fewer transcription complexes were detected, and the uninitiated transcription complexes that were obtained had a significant instability in heparin challenge experiments.

Acetyl phosphate, carbamyl phosphate, and phosphoramidate brought about NR_I-dependent activation of transcription at *glnAp*$_2$ in the absence of the NR_{II} kinase (Fig. 1). Addition of acetyl phosphate to 1 mM resulted in a barely detectable level of *glnAp*$_2$ transcription (Fig. 1A, lane 3), and addition of acetyl phosphate to 5 and 10 mM resulted in a considerable stimulation of *glnAp*$_2$ transcription (Fig. 1A, lanes 4 and 5). This stimulation of transcription was entirely dependent on the presence of NR_I (Fig. 1A, lane 6). Phosphoramidate and carbamyl phosphate at similar concentrations provided an even greater NR_I-dependent stimulation of *glnAp*$_2$ transcription (Fig. 1A, lanes 7 to 9; Fig. 1B, lanes 3 to 5). Again, the stimulation by these compounds required NR_I (Fig. 1A, lane 10; Fig. 1B, lane 6). Phosphoenolpyruvate and ATP at similar concentrations were entirely unable to substitute for a kinase and stimulate *glnAp*$_2$ transcription (Fig. 1B, lanes 7 to 12; Fig. 1C, lanes 3 to 5). The experiments shown in Fig. 1 were repeated several times, and various side-by-side comparisons were made under identical conditions (using single gels, identical specific activity of

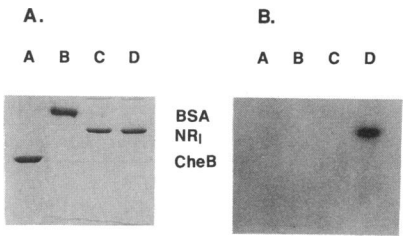

FIG. 2. Phosphorylation of NR_I by acetyl-^{32}P. CheB (lane A), bovine serum albumin (BSA, lane B), and NR_I (lanes C and D) were incubated with acetyl-^{32}P at 23°C for 5 min in the presence of either 5 mM $MgCl_2$ (lanes A, B, and D) or 1 mM EDTA (lane C). The proteins were separated on an SDS–15% polyacrylamide gel. (A) Coomassie-stained gel; (B) autoradiogram of the gel.

labeled UTP, exposure time of the X-ray film, etc.). Those experiments in the aggregate indicate that, of the three compounds that stimulated transcription in vitro, phosphoramidate was clearly the most efficient, carbamyl phosphate was the next most efficient, and acetyl phosphate was the least efficient.

Transfer of phosphoryl groups from acetyl phosphate to NR_I. We directly examined whether acetyl phosphate could serve as a substrate for the autophosphorylation of NR_I. The transfer of labeled phosphoryl groups from acetyl-^{32}P to NR_I was observed at a concentration of acetyl-P similar to that used in the transcription assay (5 mM) (Fig. 2). Under these conditions, neither bovine serum albumin nor CheB was phosphorylated. The autophosphorylation of NR_I at the expense of acetyl phosphate was strongly inhibited by EDTA. In additional experiments, we observed that NR_{II} was not phosphorylated by acetyl-P under similar conditions, but that the combination of NR_{II} and ADP resulted in the rapid loss of label from [^{32}P]NR_I-P produced by autophosphorylation from acetyl-^{32}P (not shown). Since the phosphorylation of NR_I by ATP and NR_{II} is reversible (40), this latter result suggests that the vast majority of the phosphoryl groups in NR_I-P produced by autophosphorylation from acetyl phosphate are present at the same site phosphorylated by NR_{II} and ATP. We also examined the stability of the phosphoryl group in NR_I-P produced by autophosphorylation with acetyl-^{32}P. Previous results have indicated that NR_I-P produced by phosphotransfer from NR_{II}-P has a half-life of about 4 min at neutral pH (11). We observed that when chased with unlabeled phosphoramidate or the combination of NR_{II} and unlabeled ATP, as described in Materials and Methods, label was lost from [^{32}P]NR_I-P at a rate consistent with the previously determined half-life (not shown).

Transcription complex formation requires ATP. Having a method to phosphorylate NR_I in the absence of ATP allowed us to examine the dependence of transcription complex formation on ATP with wild-type NR_I. As shown in Fig. 3, transcription complexes were formed when the NR_{II} kinase was present in the mixture of ATP, CTP, and GTP (initiated complexes) or when only ATP was present (open complexes), but fewer transcription complexes were recovered after heparin challenge when initiation was prevented (Fig. 3, compare lanes 1 and 2). No transcription complexes were obtained when NR_I was present but either ATP or NR_{II} was omitted (Fig. 3, lanes 3 and 4). The results of these control experiments are similar to those obtained previously (26). Virtually the same results were obtained when the NR_{II} kinase was replaced by 10 mM carbamyl phosphate or 10 mM acetyl phosphate, except that fewer transcription com-

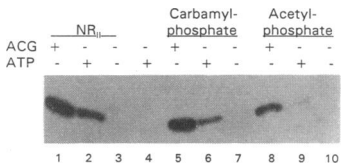

FIG. 3. Transcription complex formation requires ATP. Transcription complexes were formed in the presence of ATP alone or a mixture of ATP, CTP, and GTP (ACG) or in the absence of nucleotides. Carbamyl phosphate and acetyl phosphate, when present, were at used 10 mM, and NR_{II}, when present, was used at 200 nM. NR_I was present in all reaction mixes at 80 nM.

plexes were obtained. Thus, ATP was required for the formation of heparin-resistant transcription complexes stimulated by carbamyl phosphate and acetyl phosphate.

Phosphorylation of NR_I activates the ATPase activity of NR_I. Previous results have suggested that phosphorylation of NR_I by NR_{II} resulted in stimulation of an NR_I-P ATPase activity (40). Quantitation of the ATPase activity in those experiments was complicated by the fact that phosphorylation of NR_I by NR_{II} at the expense of ATP and the hydrolysis of NR_I-P result in the net hydrolysis of ATP. Since small phosphorylated molecules can bring about the activation of transcription and can phosphorylate NR_I directly, we were able to determine whether phosphorylation of NR_I stimulated the ATPase activity of NR_I in the absence of NR_{II}. Instead of measuring phosphate released, which can come from an ATPase activity or from the hydrolysis of the acyl-phosphate moiety of NR_I-P, we measured ADP produced from ATP in a common coupled assay system with pyruvate kinase and lactate dehydrogenase (24). This permitted us to measure the ATPase activity and study its control by phosphorylation with the wild-type protein.

We found that our purified preparation of NR_I contained a weak ATPase activity in the absence of any phosphorylating agent (Fig. 4). This activity may be due to a basal level of activity in the absence of phosphorylation or to the slight contamination of our NR_I preparation with another ATPase. In a series of control experiments, we measured the rate of ATP hydrolysis in reaction mixtures containing NR_I at 1 μM and a concentration of NR_{II} ranging from 25 to 200 nM (Fig. 4A) as described in Materials and Methods. We observed that at the lowest concentration of NR_{II}, there was a significant lag prior to the appearance of the ATPase activity and that this lag was greatly reduced at higher NR_{II} concentrations (Fig. 4A). Although further experiments will be required to fully understand this effect, in light of the experiments to be presented below, it seems likely that this lag reflects the need to assemble the active ATPase, which is dependent on the concentration of NR_I-P (see Fig. 5). The ATPase activity measured in these reactions (NR_I plus NR_{II}, Fig. 4A) is due to both the phosphorylation-dependent ATPase activity of NR_I and the conversion of ATP to ADP and P_i by the concerted action of NR_I and NR_{II} (due to the instability of NR_I-P), as noted previously (40).

The ability of acetyl phosphate, carbamyl phosphate, and phosphoramidate to stimulate the hydrolysis of ATP by NR_I was examined (Fig. 4B, C, and D). The combination of NR_I and any of these phosphorylating agents at a concentration of 25 mM resulted in an increase in measured ATPase activity, and this stimulation of the ATPase activity was enhanced by incubation of NR_I with the phosphorylating agent in the reaction mixture prior to the addition of ATP (Fig. 4). As in the transcription assay, phosphoramidate was the most effective compound, carbamyl phosphate was the next most effective, and acetyl phosphate was the least effective compound.

We determined the rate of the ATPase reaction in reaction mixtures containing 0.5 and 1.0 μM NR_I, 1.0 mM ATP, and various concentrations of phosphoramidate, as described in Materials and Methods. As shown in Fig. 5A, the response to increasing concentrations of phosphoramidate was clearly sigmoidal at both concentrations of NR_I; Hill coefficients were 2.44 (0.5 μM NR_I) and 2.89 (1 μM NR_I). We also measured the ATPase activity in reaction mixtures containing 1.0 mM ATP, 40 mM phosphoramidate (excess), and various concentrations of NR_I, as described in Materials and Methods. The ATPase activity of NR_I-P was strongly dependent on the enzyme concentration, and the response was clearly sigmoidal (Fig. 5B).

DISCUSSION

The synthesis of GS in response to nitrogen limitation is regulated by the reversible phosphorylation of the enhancer-binding transcription factor NR_I by the kinase/phosphatase NR_{II} (21). In the absence of NR_{II}, another positive regulatory factor activates GS synthesis. We present evidence that acetyl phosphate is an important positive regulatory factor in the absence of NR_{II} and that it can act by serving directly as a substrate for NR_I autophosphorylation. The most important physiological evidence that we provide is that elimination of the capacity to synthesize acetyl phosphate by a deletion of the *pta* and *ackA* genes has little effect on the synthesis of GS in otherwise wild-type cells but eliminates this synthesis in a mutant lacking functional NR_{II}. Furthermore, strains lacking NR_{II} and the *pta*-encoded phosphotransacetylase enzyme had reduced GS synthesis when grown on glucose or pyruvate as the carbon source but had elevated GS synthesis when grown on acetate as the sole carbon source. Growth of this strain on glucose or pyruvate should result in a low intracellular concentration of acetyl phosphate, while growth on acetate in the absence of the *pta*-encoded enzyme should lead to an accumulation of acetyl phosphate (37). Thus, there seems to be a direct correlation between the ability to accumulate acetyl phosphate and the ability to produce GS in cells lacking NR_{II}. Our experiments do not exclude the possibility that, in intact cells, an unidentified enzyme mediates the phosphorylation of response regulator proteins at the expense of phosphorylated metabolic intermediates, nor do our experiments exclude the possibility that a compound derived from acetyl phosphate is the immediate donor of phosphoryl groups to NR_I.

We have shown that, like CheY and CheB, NR_I can catalyze its own phosphorylation. The best substrate for NR_I is the autophosphorylated His-139 phosphate of NR_{II}. We showed that phosphoramidate, acetyl phosphate, and carbamyl phosphate are also effective phosphoryl group donors, but ATP and phosphoenolpyruvate are not. The absence of any NR_I autophosphorylation at the expense of phosphoenolpyruvate strongly suggests that the energy of the phosphoryl group in the small molecule is less important than compatibility with the active site of NR_I. Previous results indicate that the substrate specificity for CheY autophosphorylation (using small molecules) was similar to that of NR_I but that CheB was phosphorylated only by phosphoramidate and not by acetyl phosphate and carbamyl phosphate (14). Owing to its small size and similarity to phosphohistidine, phosphoramidate may prove to be generally useful for the phosphorylation of the response regulator proteins in vitro.

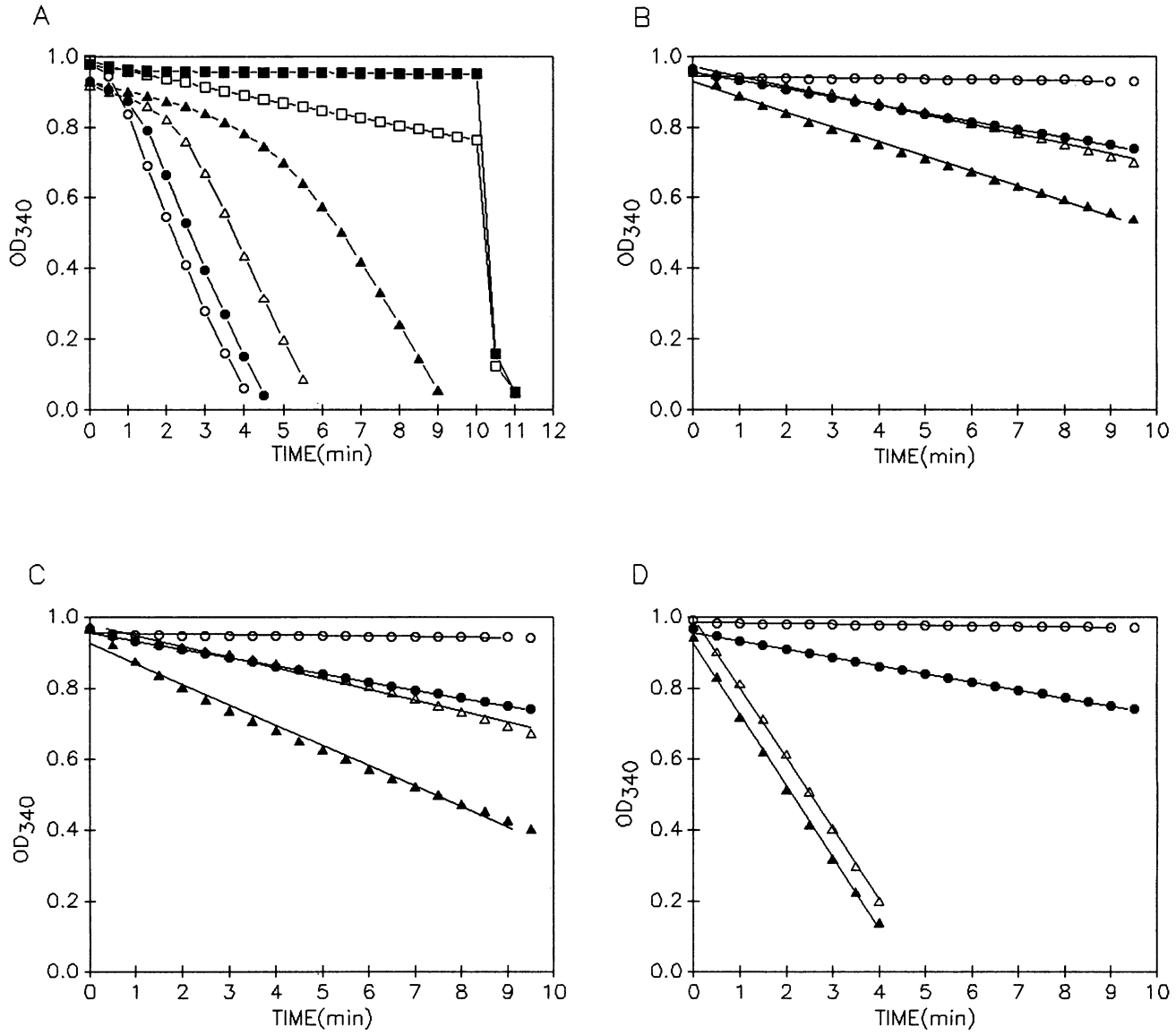

FIG. 4. Stimulation of the NR_I ATPase activity by NR_{II}, acetyl phosphate, carbamyl phosphate, and phosphoramidate. ATPase activity was measured as described in Materials and Methods. (A) Stimulation by NR_{II} present at 25 nM (▲), 50 nM (△), 100 nM (●), or 200 nM (○). Reaction mixtures were preincubated in the absence of ATP as described in the text. ■, control reaction containing NR_{II} alone at 200 nM. In all other cases, NR_I was used at 1 μM and ATP was used at 1 mM. □, control containing NR_I alone. (B, C, and D) Stimulation by acetyl phosphate, carbamyl phosphate, and phosphoramidate, respectively. ○, small-molecule phospho donor at 25 mM, in the absence of NR_I. In all other cases, NR_I was present at 1 μM. ●, control containing NR_I alone; △, small-molecule phospho donor at 25 mM, without preincubation; ▲, small-molecule phospho donor at 25 mM, with preincubation. In panels B, C, and D, the points shown are the actual data points, but the lines were drawn by regression analysis of the data.

The *glnAp*$_2$ transcription assay is particularly useful for the screening of small molecules that serve as substrates for the autophosphorylation of NR_I. The phosphorylation of wild-type NR_I results in great stimulation of transcription from *glnAp*$_2$. This assay eliminates the need to synthesize the various small molecules containing labeled phosphate and provides the added assurance that NR_I is phosphorylated at the correct site, since the activity of NR_I-P is measured. We demonstrated for one of the small molecules, acetyl phosphate, that labeled phosphoryl groups are indeed transferred to NR_I in the autophosphorylation reaction. This substrate was chosen because, although it is the least efficient substrate in vitro, it is the easiest to synthesize in labeled form. Also, our experiments indicate that it is likely to be a physiologically relevant substrate. In preliminary work, we have observed that *E. coli* grown under conditions in which GS synthesis is induced in the *glnL* mutant contains acetyl phosphate in approximately millimolar concentrations (17). Future work will be directed to determination of the regulation of the intracellular concentration of acetyl phosphate in cells grown under various conditions.

Having a method to phosphorylate NR_I in the absence of ATP allowed us to examine the ATP dependence of transcriptional activation and the ATPase activity of NR_I-P by

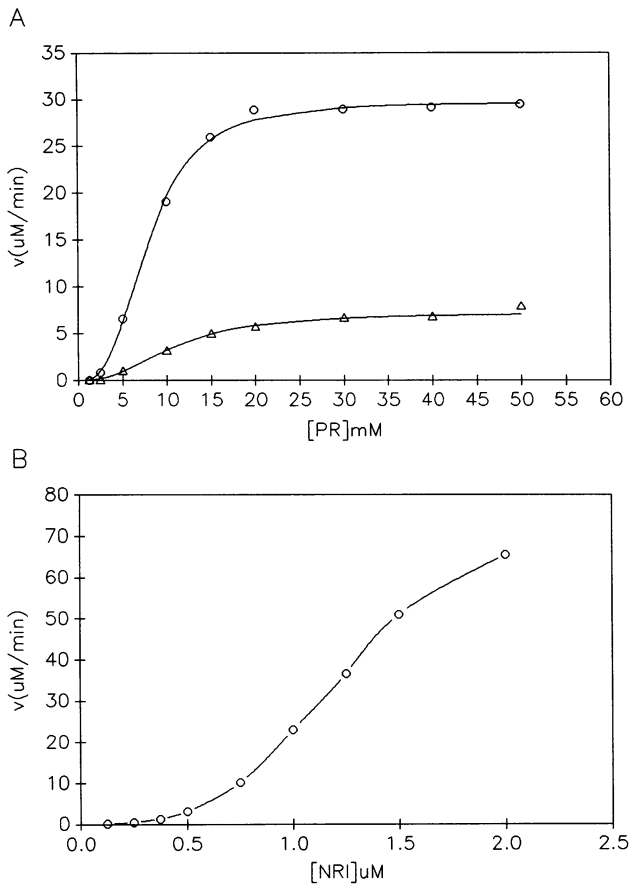

FIG. 5. ATPase activity is strongly dependent on the concentrations of phosphoramidate and NR_I. (A) Dependence of ATPase activity on phosphoramidate (PR) concentration. △, 0.5 μM NR_I; ○, 1.0 μM NR_I. (B) Dependence on NR_I concentration. The concentration of phosphoramidate was 40 mM.

using wild-type NR_I. Our results indicate that ATP is required for the activation of transcription by NR_I-P and that NR_I-P has a phosphorylation-regulated ATPase activity, as deduced previously (26, 40). We were able to study the ATPase activity in a coupled assay system and observed that there is a strong dependence of ATPase activity on the concentration of phosphoramidate used to phosphorylate NR_I, with a Hill coefficient of more than 2. We also found a sigmoidal dependence of the ATPase activity on NR_I concentration. Both of these experiments suggest that the ATPase results from the assembly of two (or more) molecules of the dimeric NR_I-P. Similar results were noted previously (40). Since transcriptional activation requires ATP, presumably due to the requirement for the ATPase reaction, we would predict that two NR_I-P dimers are required for the activation of transcription. Data from transcription experiments support this idea, as follows. The wild-type $glnAp_2$ promoter contains two adjacent high-affinity NR_I binding sites, centered approximately 110 and 140 bp upstream from the transcriptional start site. In the presence of these two strong binding sites, transcription from the promoter is very sensitive to NR_I, and footprinting studies showed that both sites fill simultaneously at low NR_I-P concentrations (23, 41). Interestingly, phosphorylation of NR_I greatly increases the cooperativity of its binding to this pair of sites (41). In the absence of any high-affinity sites, transcriptional activation has a clearly sigmoidal response to variations in the concentration of NR_I-P (5, 23, 41a). Furthermore, in recent experiments, we have observed a clearly sigmoidal response of transcriptional activation to variations in the NR_I-P concentration with a template containing only one high-affinity NR_I binding site. Thus, in both the transcription assay and the ATPase assay, the active species seems to be the tetramer of NR_I-P dimers.

The easily observable reactivity of NR_I with acetyl phosphate and carbamyl phosphate, together with our physiology results indicating that acetyl phosphate is a positive regulatory signal for the activation of *glnA* expression, suggests that the mechanism of signal transduction in an NR_{II} mutant could involve the direct phosphorylation of NR_I by acetyl phosphate. Results indicating that acetyl phosphate is a positive regulatory signal for expression of the *pho* regulon are probably the result of an analogous mechanism (34, 37). If so, then it is reasonable to ask what role this mechanism might play in wild-type cells. The synthesis of acetyl phosphate is formally a pathway in both carbon and phosphate metabolism, for it leads directly to the incorporation of phosphate into ATP. Therefore, acetyl phosphate may be an important signal in cross-regulation of carbon and phosphate metabolism, as described elsewhere (34). The role of acetyl phosphate in the control of the *ntr* regulon may be different.

One possible role for the activation of NR_I by acetyl phosphate in wild-type cells may be to facilitate the shift from nitrogen-rich conditions, in which the cells have few molecules of NR_{II}, to nitrogen-poor conditions. (The NR_{II} protein is present in very low concentrations in cells, and owing to the location of *glnL* within the *glnALG* operon, the level of NR_{II} is itself nitrogen regulated [2].) Since, under nitrogen-limiting growth conditions, the flow of 2-ketoglutarate out of the tricarboxylic acid cycle is retarded (owing to decreased glutamate synthase and glutamate dehydrogenase activity), it is possible that acetyl phosphate concentration becomes elevated under these conditions. Phosphorylation of NR_I by acetyl phosphate would then stimulate transcription from the $glnAp_2$ promoter. Transcription from this promoter leads to an increase in the cellular concentration of NR_{II} and NR_I. Thus, the alternative activation mechanism involving acetyl phosphate might reflect a requirement to prime the system under conditions in which the regulators are normally present at very low concentrations.

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The first two authors contributed equally to this work.

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ADDENDUM

We have observed that growth on glucose minimal medium containing arginine as the sole source of nitrogen, typically used as a measure of the capacity of the Ntr regulon to be expressed, is greatly decreased in mutants defective in either *glnL* (BW18302) or *pta* (BW16462).

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