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Phosphorylation Site of NtrC, a Protein Phosphatase Whose Covalent Intermediate Activates Transcription

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The NtrC transcription factor is a member of a family of homologous prokaryotic regulatory proteins that participate in the transduction of extracellular and nutritional signals. It has been demonstrated that the phosphate group from a histidine residue of the phosphorylated NtrB protein autokinase is transferred to the NtrC protein. Phosphorylation of the NtrC protein is transient and activates its transcriptional enhancement activity. We have investigated the site of phosphorylation of the *Salmonella typhimurium* NtrC protein and find that it is an aspartate residue (Asp-54) that is found within a sequence conserved in all of the members of the family of regulatory proteins. We propose that this phosphorylation is an NtrC protein histidine phosphatase catalytic intermediate. This conclusion suggests that the NtrC family should be viewed not as kinase substrates but as enzymes that can catalyze the hydrolysis of their activated forms in a concentration-independent fashion. They are similar in this sense to eukaryotic signal-transducing GTPases.

Biological signal transduction relies upon the enzymatic catalysis of reactions involving phosphate groups. Protein phosphorylation and dephosphorylation, the formation and destruction of cyclic nucleotides and inositol phosphates, and the hydrolysis of nucleotides such as GTP play pivotal roles in the propagation and attenuation of many biological signals. A complete understanding of cellular signal transduction therefore depends upon the elucidation of the mechanism and regulation of chemical reactions involving phosphate groups.

The prokaryotic response to a large variety of extracellular and nutritional conditions is paradigmatic of the central role of phosphate chemistry in signal transduction (3, 21, 24). An alteration in the bacterial chemical environment evokes a change in one or more of the rates of phosphotransfer reactions that are linked in a pathway. In the first reaction, a member of a family of homologous sensor proteins undergoes ATP-dependent autophosphorylation. The phosphate group is then transferred to a member of a second family of homologous regulatory proteins, whose phosphorylated forms directly evoke the cellular responses to environmental changes, normally through the activation of transcription of particular genes. The phosphate is finally removed from the second protein.

The response to nitrogen starvation by *Escherichia coli* and *Salmonella typhimurium* is one of the most extensively investigated prokaryotic signal transduction pathways. The two central components of this pathway, the NtrB and NtrC proteins, are members of the sensor and response regulator families, respectively (3, 24). The NtrB protein autophosphorylates on a histidine residue, and the phosphate group is subsequently transferred to the NtrC protein (16, 17, 27). The level of phosphorylation of the NtrC protein is determined by the activities of the PII and NtrB proteins, which in turn appear to be regulated by the cellular ratio of glutamine to α-ketoglutarate (24). Phosphorylation of the NtrC protein activates its transcriptional enhancement of a number of genes, including the gene encoding glutamine synthetase (17, 19, 24).

The NtrC protein has been shown to be phosphorylated transiently on one or more aspartate residues (14, 27). In order to increase our understanding of the mechanism of transfer of phosphate to the NtrC protein and of the structural consequences of phosphorylation, identification of the site of aspartyl phosphorylation within the protein was undertaken. The data presented here and those discussed below indicate that the NtrC protein possesses an amino-terminal protein-phosphatase domain whose catalytic intermediate allosterically enhances activation of transcription by the carboxy-terminal region of NtrC.

MATERIALS AND METHODS

Purification of tritium-labeled NtrC peptides. Phosphorylation of 1 nmol of the *S. typhimurium* NtrC protein in the presence of 500 pmol of the NtrB protein (gifts of the laboratory of Sidney Kustu) and reduction of the phosphorylated NtrC protein by NaB^3H_4 were carried out as previously described for the CheY protein, except that all reaction volumes were reduced 10-fold (21). The subsequent purification of the ^3H-labeled NtrC protein by reversed-phase high-performance liquid chromatography (HPLC) was performed exactly as described previously for the CheY protein (21). The ^3H-labeled NtrC protein was digested with trypsin at pH 7.8 in 100 mM NH_4HCO_3 and 1 mM $CaCl_2$ and subsequently with *Staphylococcus aureus* V8 protease at pH 4.0 in $NH_4CH_3CO_2$. The resulting peptides were separated by reversed-phase HPLC on a Vydac C_{18} column. After 5

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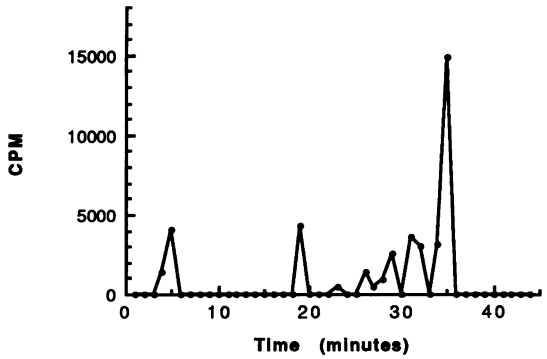

FIG. 1. Radioactivity profile of reversed-phase HPLC separation of peptides derived from sodium borohydride-treated phosphorylated NtrC protein. The incorporation of tritium into peptides in 1-min fractions eluted from the column was analyzed as described in Materials and Methods.

min of isocratic elution with 0.5 ml of 0.1% trifluoroacetic acid min^{-1}, elution of the peptides was achieved with a mobile-phase gradient of 0 to 64% aqueous acetonitrile containing 0.1% trifluoroacetic acid over 40 min at a flow rate of 0.5 ml min^{-1}. Fractions eluting from the column were collected and analyzed for tritium content by liquid scintillation counting.

Mass spectrometric analysis of tritium-labeled NtrC peptides. Liquid secondary-ion mass spectra were recorded with a Kratos MS 50-S double-focusing mass spectrometer as described earlier (21). Tandem mass spectrometry spectra were obtained on a Kratos Concept II HH four-sector instrument operating in the EBEB configuration with an electro-optical multichannel array detector as previously described (21). The molecular ions of interest were allowed to pass through the first mass spectrometer and were then collisionally activated. The resulting fragment ions were analyzed by mass to form the daughter ion (tandem mass spectrometry) spectra. Fragment ion nomenclature is based on the work of Roepstorff and Fohlman (20), as amended by Johnson et al. (13).

RESULTS

It had been previously demonstrated that a 12.5-kDa N-terminal tryptic fragment of the NtrC protein could be phosphorylated in an NtrB-dependent fashion and that the phosphorylated fragment possessed autophosphatase activity (14). These processes had the same kinetics as those measured with the intact NtrC protein (14). Therefore, we identified the sites of phosphorylation of both the 12.5-kDa fragment and the intact NtrC protein.

The identification of the site of phosphorylation of the *S. typhimurium* NtrC protein required the replacement of the highly labile aspartyl phosphate by a more stable modification. NaB^3H_4 reductively cleaves acyl phosphate bonds, thereby producing a ^3H-labeled alcohol from the anhydride while leaving free carboxyl groups unaltered (6). Identification of the site of acyl phosphorylation of the NtrC protein can therefore be achieved through the isolation of a tritium-labeled peptide from the NaB^3H_4-treated phosphorylated NtrC protein (21). Peptides obtained by cleaving the NaB^3H_4-treated phosphorylated NtrC protein with trypsin and *S. aureus* V8 protease were injected onto a C_{18} reversed-phase HPLC column. During the acetonitrile gradient elution of peptides from the column, a single predominant peak of radioactivity was obtained (Fig. 1). A single radioactive peak eluting at the same concentration of acetonitrile was obtained from a sample containing the trypsin- and NaB^3H_4-treated phosphorylated NtrC N-terminal fragment.

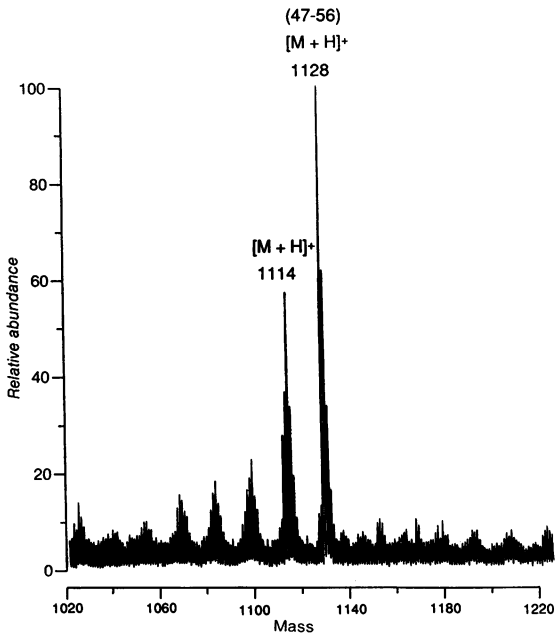

FIG. 2. Liquid secondary-ion mass spectrum of radioactive HPLC fractions from separation of peptides from sodium borohydride-treated phosphorylated NtrC protein. Two peptide molecular ions from the HPLC fractions containing the highest incorporation of tritium are shown at *m/z* values 1,114 and 1,128. The 1,128-Da peptide corresponds to the peptide TPDVLLSDIR (amino acids 47 to 56 in the NtrC sequence), which suggests that the 1,114-Da peptide results from the reduction of one of the aspartate residues in the 1,128-Da peptide. Nominal masses are reported.

Peptides in the radioactive fractions were identified through mass spectrometric analysis. Ions of approximately equal amounts corresponding to peptides of 1,128 and 1,114 Da were detected during analysis of the fractions which contained the highest level of radioactivity and which were obtained by the separation of proteolytic peptides from the complete NtrC protein (Fig. 2) and from the NtrC N-terminal fragment. The 1,128-Da ion corresponds uniquely to the peptide containing amino acids 47 to 56 of NtrC (*m/z* 1,128.6). There are no unmodified peptides that are predicted to be 1,114 Da in the NtrC protein; however, if one of the two aspartate residues within the 1,128-Da peptide was converted into a homoserine through reduction of a carboxyl phosphate by the sodium borohydride, then a 1,114-Da peptide would be obtained.

In order to confirm the identity of the peptides and identify which aspartate residue was modified, tandem mass spectrometric analysis of the peptides was performed. This analysis permits the reading of the sequences of the peptides and the direct identification of where a modification has occurred. Figure 3 displays sequences and mass spectra of the fragment ions observed from the tandem mass spectrometric analyses of the 1,128- and 1,114-Da peptides. A number of the low-mass ions are shared by the two peptides, indicating a similar amino acid composition and carboxy terminus. However, among the higher-mass ions, the predominant

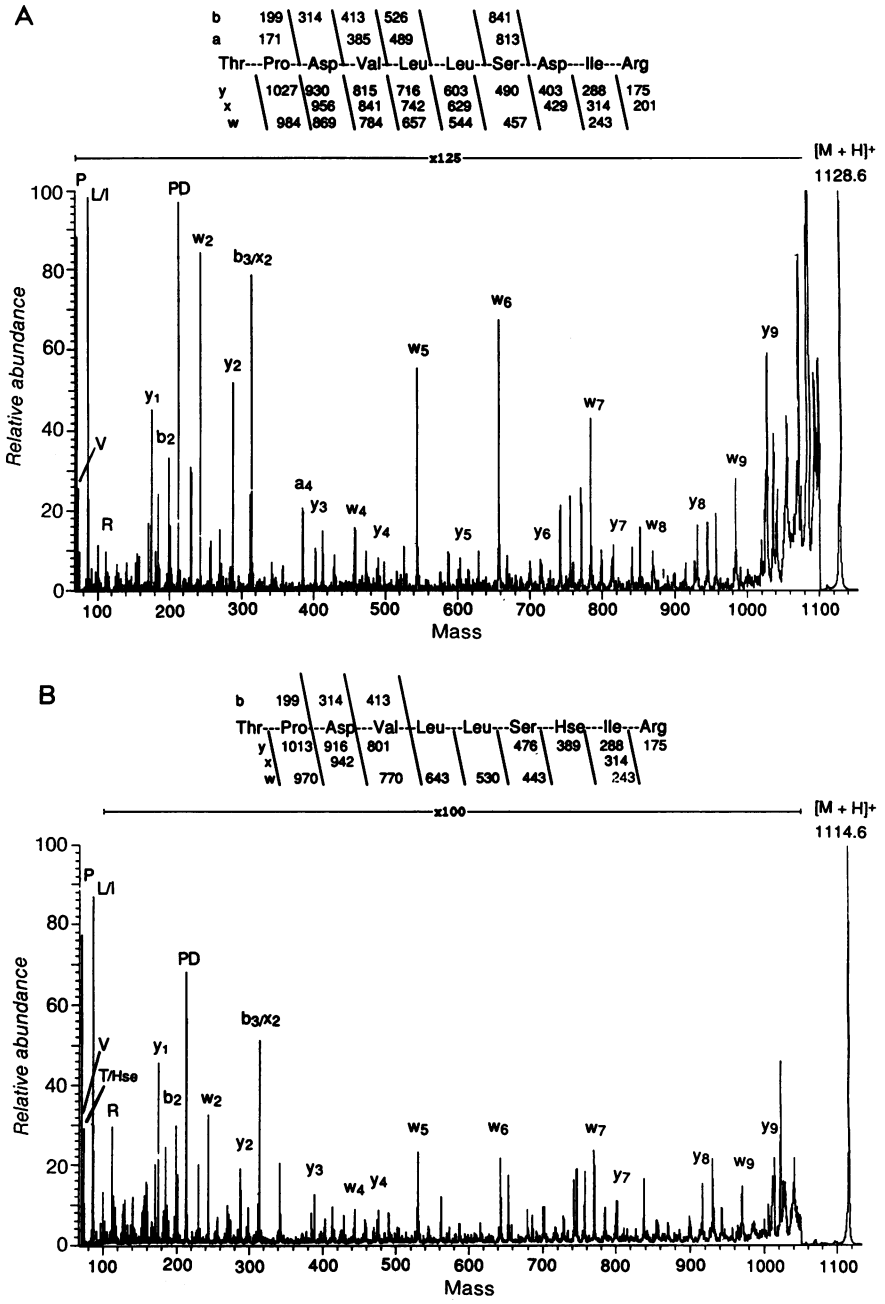

FIG. 3. Collision-induced dissociation mass spectra of the 1,114- and 1,128-Da peptides. (A) Sequence and amino acid ions obtained from the 1,128-Da peptide. The y sequence ions with *m/z* values of 1,027, 930, 815, 716, 603, 490, 403, 288, and 175 are the result of the sequential loss from the amino terminus of the peptide of threonine, proline, aspartate, valine, isoleucine or leucine, isoleucine or leucine, serine, aspartate, and isoleucine or leucine. The ion at *m/z* 213 is diagnostic of the presence of a proline-aspartate dipeptide. Other fragment ions involving side chain bonds (e.g., the w_2, w_5, and w_6 ions) are used to identify the isoleucine and leucine residues (13). This confirms the identification of the sequence as TPDVLLSDIR (amino acids 47 to 56 of the NtrC protein). The low-mass immonium ions confirm the identity of the amino acids present in the peptide. (B) Sequence and amino acid ions obtained from the 1,114-Da peptide. The immonium ions obtained are identical to those found in the spectrum in panel A (a homoserine residue gives an immonium ion of the same mass as that of a threonine residue), as is the ion diagnostic of the presence of a proline-aspartate dipeptide, indicating that the 1,114- and 1,128-Da peptides have similar compositions and furthermore that aspartate 49 has not been reduced in the 1,114-Da peptide. The y and w sequence ions at *m/z* values 175, 243, and 288 indicate that the peptides share a common isoleucine-arginine carboxy terminus, while the y sequence ion at *m/z* 389 present in the spectrum obtained from the 1,114-Da peptide, but not in that from the 1,128-Da peptide, indicates that aspartate 54 has been reduced to a homoserine residue. The other sequence ions obtained are consistent with the identification of the peptide as TPDVLLSHseIR (where Hse is homoserine).

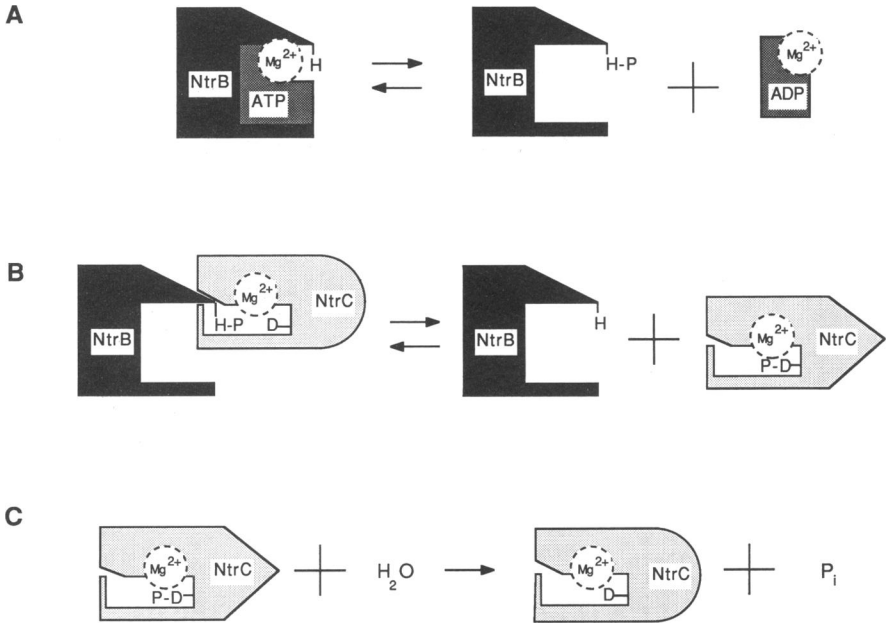

FIG. 4. Model for the activity of the NtrC protein. (A) The NtrB protein autophosphorylates on a histidine residue. (B) The NtrC protein then acts as a protein histidine phosphatase catalyzing the transfer of the phosphate moiety from the substrate, the phosphorylated NtrB protein, to an aspartate residue at the phosphatase active site. The magnesium ion that catalyzes phosphate transfer and other catalytic groups are present on the NtrC protein. When the NtrC protein is phosphorylated as part of its catalytic mechanism, its conformation changes so that it can activate transcription initiation. (C) The NtrC protein completes the catalytic cycle through autodephosphorylation and returns to a conformation that is inactive in the promotion of transcription initiation. Note that the net reaction is an ATPase cycle and that if each of the transfers of phosphate (from ATP to NtrB, from NtrB to NtrC, and from NtrC to water) proceeds with stereochemical inversion of configuration around the phosphorus atom, then net inversion would be observed just as has been found during catalysis of hydrolysis of GTP by the Ras protein (7).

fragments differ by 14 Da. Analysis and comparison of the fragment sizes obtained from the two peptides indicate that aspartate 54 has been reduced to a homoserine residue in the 1,114-Da peptide. In the other radioactive HPLC fractions that were analyzed by mass spectrometry, no peptides indicating reduction of any other acyl bond were observed (data not shown). It can therefore be concluded that aspartate 54 is the site of phosphorylation of the NtrC protein.

DISCUSSION

The phosphorylated aspartate residue of the NtrC protein is homologous to those of the CheY and VirG proteins (4, 12, 21). The identity of the sites of phosphorylation suggests that the phosphate group of the NtrB family of histidine autokinases can be transferred to only one of the conserved aspartate residues on the surface of the NtrC-like proteins. This finding strengthens the concept that all of the members of the NtrB and NtrC families utilize a common mechanism for signal transduction and have common structures (3, 18, 21, 23, 24). It further implies that the conformational consequences of phosphorylation of individual members of the NtrC family of proteins are similar. Finally, it lends credibility to the hypothesis that the members of the NtrC protein family are not merely substrates for phosphorylation by the NtrB family of proteins (21). Protein modification enzymes generally do not possess the high degree of substrate specificity demanded by these results.

An alternative interpretation that has been previously discussed (21), that the members of the NtrC protein family are protein histidine phosphatases that possess a common covalent catalytic intermediate, affords a straightforward explanation for the conservation of the site of phosphorylation and also for a number of other experimental observations. The identification of the NtrC family as enzymes was originally suggested by the autophosphatase activity that they possessed (10, 14, 21, 27) and by the evidence that the magnesium ion that participates in the transfer of phosphate from the CheA histidine autokinase to the CheY protein is bound to the latter (15, 21). Further support for the hypothesis was derived from the fact that the domain of the CheA protein that contains the histidine that is phosphorylated is outside the region that is homologous to the NtrB protein family (9, 22). The phosphate group from a phosphorylated fragment of the CheA protein that lacks all of the conserved sequences, which are required for autophosphorylation, can still be transferred to the CheY protein (9). This demonstrates that the purpose of the conserved domains of the histidine autokinases is to transfer the γ-phosphate group of ATP to a histidine residue on the protein (21). Figure 4 summarizes the proposed mechanism for signal transduction by the histidine autokinases and protein histidine phosphatases.

Recently, evidence has been presented that the phosphorylated Spo0F histidine phosphatase can transfer its phosphate group from the conserved aspartate residue to a nitrogen atom (probably that of a histidine residue) of the Spo0B protein (5). This strengthens the identification of the phosphatases as enzymes and suggests that other members of the phosphatase family may transfer the phosphate from themselves to protein acceptors rather than to water.

If the NtrC protein is indeed a protein histidine phosphatase, then a novel form of regulation of protein activity has been discovered, in which the catalytic intermediate of an enzyme regulates allosterically a second function of the same polypeptide (21), in this case, its role in the activation of transcription (17, 19). A protein with this property possesses the advantage of determining autonomously the duration of its ability to perform the second function. For example, once the stimulus has been received through the NtrB protein, the NtrC protein can activate transcription initiation for an appropriate predetermined period without further stimulation. Thus, the protein histidine phosphatases are similar to eukaryotic GTPases that, once stimulated, activate an effector for a period determined by their rates of hydrolysis of GTP (2). Moreover, the hydrolytic activity of the phosphatases can be altered through interactions with other proteins (10, 11, 14, 17), just as the intrinsic GTPase activity of the Ras protein can be greatly enhanced by interaction with the GAP protein (8, 25).

An even closer analogy to GTPases is found in those proteins in which the sequences homologous to the histidine autokinases and the protein histidine phosphatases can be found within the same polypeptide (24). These proteins function as ATPases whose catalytic intermediates allosterically activate a second function. This idea can be extended conceptually to any histidine autokinase-phosphatase pair (Fig. 4). Indeed, the analogy to signal-transducing GTPases is strengthened by experiments that suggest that the affinity of the CheA histidine autokinase for adenine nucleotides is altered by chemotaxis receptors (1), just as transmembrane receptors regulate the affinity of the α-subunits of heterotrimeric G proteins for guanine nucleotides (2).

Viewing NtrB and NtrC together as an ATPase leads to the conception of their regulation of transcription as a cascade of ATPases. Phosphorylation of the NtrC protein has been shown to activate an ATPase activity that participates in the closed-open promoter complex isomerization catalyzed by the protein (19, 26). Thus, ATP would first be hydrolyzed at one site on the protein complex in order to activate the hydrolysis of ATP at a second site. Future experiments will examine the validity of these ideas and the prevalence of these forms of regulation.

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