Energy Coupling in *Salmonella typhimurium* Nicotinic Acid Phosphoribosyltransferase: Identification of His-219 as Site of Phosphorylation†

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ABSTRACT: Energy coupling between ATP hydrolysis and other enzyme reactions requires the phosphorylation of substrate-derived intermediates, or the existence of enzyme-derived intermediates capable of storage and transfer of energy. *Salmonella typhimurium* nicotinic acid phosphoribosyltransferase (NAPRTase, EC 2.4.2.11) couples net ATP hydrolysis to formation of NAMN and PP_i from α-PRPP and nicotinic acid [Vinitsky, A., & Grubmeyer, C. (1993) *J. Biol. Chem. 268*, 26004−26010]. In the current work, we have determined that the enzyme reacts with ATP to produce a covalently phosphorylated form of the enzyme (E-P), which is common to both the ATPase and NAMN synthesis functions of NAPRTase. We have isolated E-P and verified its catalytic competence. E-P showed acid lability and base stability, diagnostic of a phosphoramidate linkage. Pyridine and hydroxylamine-catalyzed hydrolysis of E-P gave second-order rate constants consistent with published values for phosphohistidine. Two-dimensional thin-layer chromatography of alkaline-hydrolyzed E-^{32}P showed that the phosphorylated residue co-migrated with authentic 1-phosphohistidine. Chymotrypsin and trypsin proteolysis followed by HPLC and peptide sequencing localized the phosphopeptide to Ala-210 to Phe-222 of the 399-residue protein. This peptide contains a single histidine residue, His-219. NAPRTase phosphorylated at His-219 is an intermediate in the energy transduction mechanism of NAPRTase.

Nicotinic acid phosphoribosyltransferase (NAPRTase, EC 2.4.2.11)[1] couples the hydrolysis of ATP to the formation of nicotinate mononucleotide (NAMN) and pyrophosphate (PP_i) from α-D-5-phosphoribosyl 1-pyrophosphate (PRPP) and nicotinate (NA; Vinitsky & Grubmeyer, 1993). The cleavage of ATP distinguishes NAPRTase from other known PRTases (Musick, 1981). We have cloned and purified the 399-residue *Salmonella typhimurium* enzyme (Vinitsky et al., 1991) and shown that ATP hydrolysis, stoichiometric with NAMN formation, serves to drive the NAMN synthesis reaction toward products (Vinitsky & Grubmeyer, 1993). The use of ATP is optional for the *S. typhimurium* enzyme, with readily detectable "uncoupled" NAMN formation in the absence of ATP. However, the presence of ATP lowers K_m values for NA and PRPP by about 200-fold and increases V_{max} 10-fold. Vinitsky and Grubmeyer (1993) proposed that the binding and hydrolysis of ATP induce a series of conformational changes in NAPRTase, increasing both affinity for substrates and V_{max}.

Many enzymes couple ATP hydrolysis to a defined enzymatic process without the use of phosphorylated substrate-derived intermediates. The sarcoplasmic reticulum Ca^{2+}−ATPase, RecA, GroEL, and Lon protease are several examples of what Tanford (1983) describes as conformationally coupled enzymes, in which ATP hydrolysis drives a series of conformational changes in the enzyme which promote completion of the coupled process. The mechanisms of conformationally coupled ATPases are generally difficult to dissect. Typically the intermediates are difficult to distinguish chemically, and frequently the enzymes are membrane bound. Therefore the mechanism by which the energy of ATP hydrolysis is utilized has not been fully determined. NAPRTase provides an unusual opportunity to investigate conformational coupling. NAPRTase is a soluble protein which catalyzes a well-defined and easily assayed phosphoribosyltransferase reaction conformationally coupled to ATP hydrolysis. ATP sulfurylase, in which a G-protein-like subunit couples net GTP hydrolysis to the ATP sulfurylation reaction that proceeds on the other subunit (Liu et al., 1994), represents the only other known case in which conformational coupling is used to drive a metabolic reaction.

In defining the criteria for coupling ATP hydrolysis to other enzymatic processes, Jencks (1980, 1989) states that the two processes must be intertwined such that neither can proceed to completion without distinct stages of progress in the other reaction. This logic is readily applied to NAPRTase. We have proposed that an unliganded form of NAPRTase with poor affinity for NA and PRPP is phosphorylated by ATP, forming a covalent phosphoenzyme (E-P). Rajavel et al. (accompanying paper) have used partial proteolysis to detect an ATP-induced conformational change. E-P tightly binds substrate NA and PRPP which are converted to tightly bound products NAMN and PP_i. Product PP_i then promotes the hydrolysis of E-P, and products are released from the low affinity form of the enzyme (Vinitsky & Grubmeyer, 1993).

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[1] Abbreviations: NAPRTase, nicotinic acid phosphoribosyltransferase; NA, nicotinic acid; PRPP, α-D-5-phosphoribosyl 1-pyrophosphate; NAMN, nicotinate mononucleotide; PP_i, pyrophosphate; TFA, trifluoroacetic acid; PRTase, phosphoribosyltransferase; E-P, phosphoenzyme.

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Thus formation of E-P, which induces product formation, and E-P hydrolysis, required for product release, serve to link the ATPase and NAMN synthesis reactions. Although an E-P form of the enzyme has been proposed by several groups (Kosaka et al., 1971; Hanna et al., 1983; Vinitsky & Grubmeyer, 1993), it has not been isolated or characterized.

In the current work, we have isolated the E-P intermediate which is common to the ATPase and NAMN synthesis activities of NAPRTase and have identified the site of phosphorylation as N1 of His-219. The work provides important confirmation of the model for conformational coupling in NAPRTase and allows for its further study.

EXPERIMENTAL PROCEDURES

Materials. High specific activity [γ-^{32}P]ATP (3000 Ci/mmol) was from Dupont NEN, and intermediate specific activity [γ-^{32}P]ATP was obtained from Amersham or synthesized by Dr. Barbara Stitt, Temple University (Grubmeyer et al., 1982). Silica gel thin-layer chromatography plates (K6, 250 μm) were from Whatman, Sephadex G50 was from Pharmacia, trypsin was from Worthington, and CAPSO buffer was from Research Organics. α-Chymotrypsin and all other biochemicals were from Sigma. HPLC solvents and other chemicals were from Fisher.

Protein. S. typhimurium NAPRTase was purified as described by Rajavel et al. (accompanying paper). An extinction coefficient of E_{280} (1 mg/mL) of 1.27 and a molecular weight of 45 529 (Vinitsky et al., 1991) were used to calculate concentrations of the monomeric 399-residue protein (Vinitsky & Grubmeyer, 1993).

Phosphorylation. Phosphorylation reaction mixtures (200 μL) contained 200 μg of NAPRTase in 1 mM [γ-^{32}P]ATP (2000−20 000 cpm/nmol), 1 mM PRPP, 7 mM $MgCl_2$, 200 mM monopotassium glutamate (K-glu), 20 mM Tris-HCl, and 1 mM DTT (pH 8.3). The reaction mixture was incubated at 0 °C for 10 min and then passed over a column (1 × 10 cm) of Sephadex G50 equilibrated in buffer A (200 mM K-glu, 20 mM Tris-HCl, pH 8.3) containing 6 M urea and 1 mM NaP_i. In some cases as noted, urea was replaced by 6 M guanidine-HCl or 0.1% SDS.

For isolation of native E-^{32}P, the phosphorylation reaction was identical, but E-^{32}P was separated from other reaction components on a 1 mL centrifuge column (Penefsky, 1977) packed with Sephadex G50 and previously equilibrated with buffer A supplemented with 1 mM PRPP and either 5 mM $MgSO_4$ or 1 mM EDTA.

The rate of NAPRTase phosphorylation was determined in a 300 μL reaction containing 1 mg of NAPRTase/mL, 1 mM [γ-^{32}P]ATP (6500 cpm/nmol), 1 mM PRPP, and 7 mM $MgCl_2$ in buffer A supplemented with 1 mM DTT. The phosphorylation reaction was vigorously stirred and maintained at 30 °C in a thermostated bath. The reaction was initiated by the addition of NAPRTase and was quenched in 1 M NaOH. E-^{32}P was isolated by passage of reaction mixtures through centrifuge columns packed with Sephadex G50 and equilibrated in buffer A containing 6 M urea.

SDS−PAGE. SDS−PAGE followed methods of Laemmli (1970) using the modified loading buffer described by Wright et al. (1993).

Hydrolysis of E-P. The rate of hydrolysis of E-^{32}P was measured with phosphorylated NAPRTase made and denatured as described above, which was chromatographed on a column (1 × 10 cm) of Sephadex G50 equilibrated with a hydrolysis buffer (see below) adjusted to pH 10.0 and containing 6 M urea. After chromatography, the pH was readjusted to the desired value using 1 M HCl, and the solution was maintained at 37 °C. At specified times, aliquots of E-^{32}P were rechromatographed. For individual pH values the following buffers were employed: pH 2.0, 20 mM Na_2SO_4, 20 mM CAPSO, 1 mM NaP_i; pH 3.5 and 6.1, 20 mM citrate, 20 mM CAPSO, 1 mM NaP_i; pH 10, 50 mM CAPSO, 1 mM NaP_i. For determinations at pH 8.3 and 13.2, initial chromatography was performed in buffer A with 1 mM NaP_i, and the pH was then adjusted with 1 M NaOH. Radioactivity in column fractions was quantitated by Cerenkov radiation in a liquid scintillation counter. The total radioactivity associated with the protein and the free phosphate peaks was used to determine the percentage of residual E-^{32}P. Semilogarithmic plots of residual E-^{32}P vs time were linear to 5% remaining E-^{32}P, and a linear least-squares fit was used to calculate rate constants.

The behavior of the enzyme in the presence of catalysts of E-^{32}P hydrolysis was examined using E-^{32}P prepared as above. E-^{32}P was then applied to and eluted from a column (1 × 10 cm) of Sephadex G50 equilibrated in the buffers described below. To determine the rate of hydroxylamine-catalyzed E-^{32}P hydrolysis, E-^{32}P was eluted in buffer A containing 1 mM NaP_i and 6 M urea, pH 8.3, and fractions containing E-^{32}P were diluted with an equal volume of the identical buffer supplemented with 100 mM NH_2OH, pH 8.3. Pyridine-catalyzed E-^{32}P hydrolysis was investigated by eluting E-^{32}P in 20 mM CAPSO, 1 mM NaP_i, and 6 M urea, pH 9.9. Fractions containing E-^{32}P were diluted with an equal volume of 100 mM citrate, 100 mM pyridine, 1 mM NaP_i, and 6 M urea, pH 5.97, to a final pH of 6.04 (Spronk et al., 1976). To investigate I_2-catalyzed E-^{32}P hydrolysis, E-^{32}P was eluted in 150 mM NH_4HCO_3, 0.1% SDS, pH 8.5, and fractions containing E-^{32}P were diluted with an equal volume of the identical buffer supplemented with 0.8 mM I_2 (Wo et al., 1992). In each case, samples were chromatographed at various times, and the proportion of E-^{32}P remaining was plotted as above to generate rate constants. Controls were performed identically, except that the catalyst was omitted.

For the determination of the rate of hydrolysis of native E-^{32}P, aliquots of the E-^{32}P solution were passed through 1 mL centrifuge columns (Penefsky, 1977) equilibrated with buffer A supplemented with 1 mM PRPP and either 5 mM $MgSO_4$ or 1 mM EDTA.

The rate of native E-^{32}P hydrolysis was determined in the presence of substrates for the coupled forward reaction. NAPRTase was first phosphorylated in a 250 μL reaction containing 1.2 mg/mL NAPRTase, 0.12 mM [γ-^{32}P]ATP (32 000 cpm/nmol), 1.2 mM PRPP, and 10.5 mM $MgCl_2$ in buffer A supplemented with 1 mM DTT. The phosphorylation reaction was vigorously stirred and maintained at 30 °C in a thermostated bath. A solution containing NA and nonradioactive ATP was added directly to the phosphorylation reaction to induce the coupled reaction, yielding final concentrations of 1 mg of NAPRTase/mL, 1 mM NA, 10.1 mM [γ-^{32}P]ATP (300 cpm/nmol), 1 mM PRPP, and 17 mM $MgCl_2$ in buffer A supplemented with 1 mM DTT. The reaction was quenched with 1 M NaOH, and E-^{32}P was isolated using centrifuge columns packed with Sephadex G50 and equilibrated in buffer A containing 6 M urea.

Identification of Phosphohistidine. NAPRTase was phosphorylated with [γ-^{32}P]ATP as described above and a 100 μL portion was passed through a centrifuge column which had been equilibrated with a 10 mg/mL solution of synthetic phosphohistidine (Wei & Matthews, 1991). The eluate was collected directly in 100 μL of 6 N KOH in a 2 mL Sarstedt polypropylene screw-top tube and incubated at 105 °C in a thermostated block heater for 5.5 h. The hydrolyzed sample was brought to pH 10 with perchloric acid, and the precipitate of $KClO_4$ was removed by centrifugation at 14 000*g* for 3 min. A portion of the supernatant (2 μL) was applied to a thin-layer chromatography plate of silica gel. Development in the first dimension used chloroform:methanol:17% aqueous ammonia (2:2:1, v/v). After drying, chromatography in the second dimension was carried out in 90% aqueous phenol:water (4:1, v/v) (Hess et al., 1988). To detect amino acids, plates were sprayed with 0.1% ninhydrin in water-saturated *n*-butanol and heated at 105 °C for 5 min. The chromatography plate was then exposed to a Fuji phosphorimager screen for 37 h, and the screen was developed in a Fuji BAS 2000 phosphorimager.

Phosphohistidine was synthesized from phosphoramidate (Wei & Matthews, 1991). The synthetic product is reported to contain 1- and 3-phosphohistidine (Hess et al., 1988).

Proteolysis. For trypsin digestion, E-^{32}P was isolated by gel filtration in buffer A containing 6 M urea. The solution was brought to 10 mM $CaCl_2$, and trypsin was added at 1:20 (w:w) with respect to NAPRTase. After incubation for 30 min at 37 °C, a second portion of trypsin was added at 1:20 (w:w) and the digestion continued for 30 min. The digested protein was injected at a flow rate of 1 mL/min onto a Vydac C18 column (0.46 × 25 cm, 218TP54) equilibrated in 0.1% TFA in water (solvent A), using a Waters 600E controller and pump system. The peptide separation was performed by applying a 100 mL linear gradient from 0% to 100% solvent B (0.1% TFA in acetonitrile). The peptides were detected at 280 nm (not shown) and 215 nm employing a Waters 490E detector. Where noted, a preliminary purification employed the same column and gradient, but with 0.25 M urea in 50% isopropanol substituted for solvent B. Fractions containing radioactivity from the preliminary purification were concentrated under vacuum and rechromatographed using the system and conditions originally described.

For chymotrypsin proteolysis, E-^{32}P was isolated in buffer A containing 3.5 M urea. The solution was brought to 10 mM $CaCl_2$, and chymotrypsin was added to NAPRTase at 1:20 (w:w). After a 30 min digestion at 37 °C, a second aliquot of chymotrypsin (1:20, w:w) was added and digestion continued for 30 min. Peptides were isolated by HPLC using the system and conditions described above.

The purified peptides were applied to a Porton 2090 sequencer interfaced with a Hewlett Packard 1090A HPLC for PTH−amino acid identification. The results were analyzed manually or by Porton chromatography software.

RESULTS

Covalent Phosphorylation of NAPRTase. When NAPRTase was incubated with [γ-^{32}P]ATP and the mixture was subjected to gel filtration chromatography in the presence of 6 M urea, radioactivity was found associated with the protein. The presence of 6 M guanidine-HCl or 0.1% SDS during chromatography gave similar results. To further demonstrate the covalent nature of the E-^{32}P bond, phosphorylated NAPRTase was subjected to SDS−PAGE. The autoradiogram and the Coomassie Blue staining (Figure 1) indicated that the radioactivity co-migrated with NAPRTase protein.

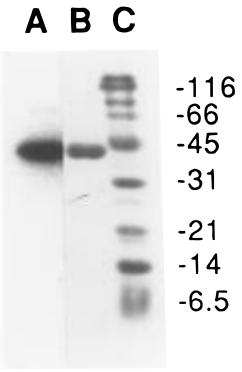

FIGURE 1: SDS−PAGE of phosphorylated NAPRTase. NAPRTase was phosphorylated with [γ-^{32}P]ATP, and the phosphoenzyme was electrophoresed on SDS−PAGE as described in Experimental Procedures. A. Phosphorimage of the radioactivity. B. Coomassie Blue staining of the gel. C. M_r markers.

The stoichiometry of phosphorylation was typically 0.6 nmol of ^{32}P/nmol of NAPRTase. Stoichiometry values remained unchanged for ATP concentrations ranging from 100 μM to 1 mM. Stoichiometry values were determined with four batches of NAPRTase and did not vary significantly. PRPP is known to inhibit the low intrinsic ATPase activity of NAPRTase (Vinitsky & Grubmeyer, 1993), and omission of PRPP from phosphorylation mixtures decreased stoichiometry values to 0.2 nmol of ^{32}P/nmol of NAPRTase. The apparent stoichiometry of phosphorylation was sensitive to the age of high specific radioactivity (3000 Ci/mmol) [γ-^{32}P]ATP purchased from NEN DuPont. Experiments using fresh NEN [γ-^{32}P]ATP yielded stoichiometries of 0.6 nmol of [^{32}P]/nmol of NAPRTase. However, in subsequent experiments using the same product, the stoichiometry declined continuously. This decline, similar to that noted by others (Wolodko et al., 1994), was not observed when we used lower specific activity [γ-^{32}P]ATP.

To address the possibility of enzyme dimerization and half-site reactivity, we performed size exclusion chromatography (Superdex-75, 1.6 × 60 cm) on NARPRTase both in the presence and absense of Mg−ATP. The elution volume of NAPRTase in conditions known to result in phosphorylation of the enzyme was unchanged and remained consistent with a monomeric aggregation state.

Rate of Phosphorylation. Reported k_{cat} values for NAPRTase range from 2 to 3 s^{-1} (Vinitsky & Grubmeyer, 1993) and require rapid phosphorylation of the enzyme. The rate of phosphorylation of NAPRTase was analyzed by mixing purified enzyme with [γ-^{32}P]ATP and quenching the reaction at various times with 1 M NaOH (Figure 2). Phosphorylation was 95% complete in 2 s, the shortest time obtainable.

E-P Stability under Native Conditions. The rate of native E-^{32}P hydrolysis was investigated at 37 °C in buffer A supplemented with 1 mM PRPP. The hydrolysis followed first-order kinetics with a rate constant (k_{hyd}) of 0.068 min^{-1} in buffer containing 1 mM EDTA. Replacement of EDTA with 5 mM $MgSO_4$ increased k_{hyd} to 0.18 min^{-1} (data not shown).

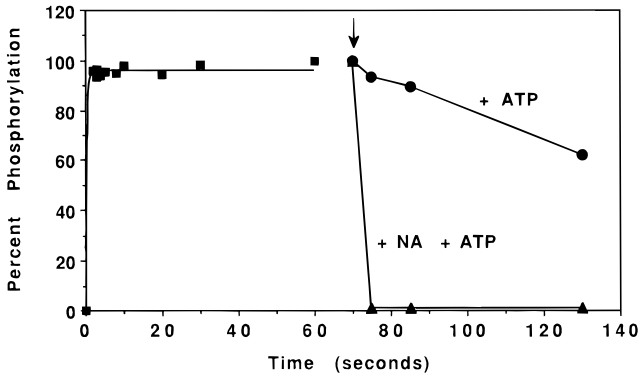

FIGURE 2: Rates of phosphorylation and E-^{32}P hydrolysis of NAPRTase. The rate of phosphorylation of NAPRTase (solid squares) was investigated by adding NAPRTase to the phosphorylation reaction described in Experimental Procedures and then quenching the reaction in 1 M NaOH. Native E-^{32}P hydrolysis was monitored by phosphorylating NAPRTase for 70 s, as above, and then, at the arrow, the reactions were supplemented with nonradioactive ATP (solid circles) or NA plus nonradioactive ATP (solid triangles) and finally quenching the reaction in 1 M NaOH.

When NAPRTase was phosphorylated with [γ-^{32}P]ATP in the presence of PRPP and Mg^{2+}, and the phosphorylation reaction mixed with NA and a 100-fold excess of nonradioactive ATP, conditions favoring the forward coupled reaction, only 1% of the ^{32}P initially incorporated remained associated with NAPRTase after 3 s, the earliest time point obtained (Figure 2). This loss of 99% of the covalently associated ^{32}P within 3 s yields a minimum rate of hydrolysis of 1.5 s^{-1}, close to the k_{cat} of 2−3 s^{-1}. The 1% of the original radioactivity which remained associated with the enzyme after addition of NA and nonradioactive ATP represents NAPRTase which was re-phosphorylated with [γ-^{32}P]ATP that had been isotopically diluted 100-fold. When NA was omitted from the cold chase, and only the 100-fold excess of nonradioactive ATP was added, E-^{32}P hydrolysis was at least 200-fold slower at a rate of 0.46 min^{-1}, a value slightly greater than k_{hyd} of native E-^{32}P. The ability of NAPRTase substrates to stimulate rapid and complete hydrolysis of E-^{32}P demonstrates that E-^{32}P is an enzymatic intermediate and not an artifact of nonspecific phosphorylation.

E-P Hydrolysis under Denaturing Conditions. E-^{32}P hydrolysis was investigated at pH 8.3 in buffer A containing 6 M urea. The hydrolysis followed first-order kinetics with a rate constant (k_{hyd}) of 0.0042 min^{-1}, approximately 16-fold slower than the rate for the native form of E-P. The denaturing conditions allowed sufficient time to investigate the chemical stability of the E-^{32}P bond.

Chemical Stability of E-P. Phosphoenzymes containing phosphoester, phosphoramidate, thiol phosphate, or anhydride linkages can be differentiatied by their chemical stabilities (Wo et al., 1992). Denatured E-^{32}P was incubated at pH values of 2, 3.5, 6.1, 8.3, 10.0, and 13.2. The k_{hyd} vs pH plot (Figure 3) was complex and showed that E-^{32}P was rapidly hydrolyzed at pH 2. At pH 3.5 and 6.1 the rate of hydrolysis was relatively constant, and above pH 8.3, E-^{32}P was stable. The base stability and acid lability of E-^{32}P definitively exclude phosphoester chemistry at serine, threonine, or tyrosine (Wo et al., 1992). Analysis of these hydrolysis data using programs of Cleland (1979) suggested a pK_a of 7.75, similar to the pK value of 7 reported for imidazole phosphate by Jencks and Gilchrist (1965) and identical to the pK value of 7.8, determined by NMR, for

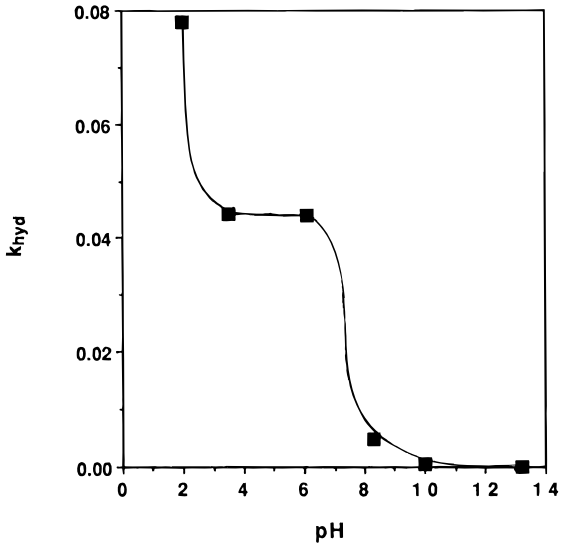

FIGURE 3: pH effects on E-^{32}P hydrolysis. The rate of E-^{32}P hydrolysis (k_{hyd}) was determined under denaturing conditions at the indicated pH values, as described in Experimental Procedures.

Table 1: Chemically Catalyzed E-^{32}P Hydrolysis

	k_2 for reaction (M^{-1} min^{-1})		
	hydroxylamine	pyridine	molecular iodine
NAPRTase	0.54	0.45	0
thiol phosphate[a]			170
acyl phosphate[b]	42	0.046	
phosphohistidine[c]	0.50	0.25	
phosphoramidate[d]	0.8	0.7	

[a] Wo et al. (1992). [b] Di Sabato and Jencks (1961). [c] Spronk et al. (1976). [d] Jencks and Gilchrist (1965).

the 1(π)-phosphohistidine in the histidine-containing phosphocarrier protein (Rajagopal et al., 1994). The relationship of the E-^{32}P hydrolysis rate with pH is nearly identical to that reported for 1-phosphohistidine, both in profile and rate, and is distinct from that of 3(τ)-phosphohistidine (Hultquist, 1968).

The behavior of E-^{32}P in the presence of chemical catalysts was used to discriminate phosphoramidate, acyl phosphate, and thiol phosphate chemistries for E-^{32}P. For these reactions, k_2, a second-order rate constant for the catalyzed hydrolysis,

$$\text{E-P} \xrightarrow{k_2[\text{catalyst}]} \text{E} + \text{Pi}$$

was defined and measured as described by Spronk et al. (1976).

The k_2 values for the hydrolysis of phosphorylated NAPRTase in hydroxylamine, pyridine, and molecular iodine (Table 1) were quite distinct from those of acyl phosphates (Di Sabato & Jencks, 1961) and thiol phosphates [see Table 2 in Wo et al. (1992)] and served to exclude these types of chemistry for the linkage in E-^{32}P.

Alkaline Hydrolysis of NAPRTase. E-^{32}P was treated with 3 N KOH for 5.5 h at 105 °C, the partially neutralized hydrolysate was analyzed by two-dimensional thin-layer chromatography as described (see Experimental Procedures), and the results were compared with chromatograms of synthetic phosphohistidine (Figure 4). The latter product contains three ninhydrin-positive compounds, one of which

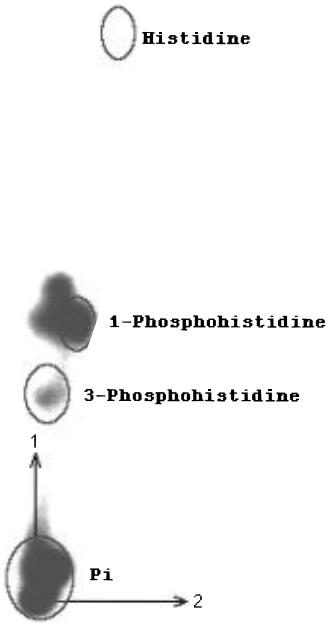

FIGURE 4: Two-dimensional TLC of alkaline-hydrolyzed E-^{32}P. Alkaline-hydrolyzed E-^{32}P was chromatographed on silica plates as described in Experimental Procedures. An autoradiogram is shown with dotted lines demarcating ninhydrin-positive areas. Dimensions 1 and 2 are identified.

co-chromatographs with histidine, and the other two of which are ascribed to 1- and 3-phosphohistidine (Hess et al., 1988; Jin et al., 1990). This identification was also validated by the kinetics of the appearance of the two products during our synthetic reaction. 1-Phosphohistidine is transiently formed during the first 2 h of the reaction, while 3-phosphohistidine predominates at later reaction times (Wei & Matthews, 1991), corresponding to the observed appearance of the two spots. In chromatograms of base-hydrolyzed ^{32}P-NAPRTase two major radioactive products were observed, one of which migrated with an R_f similar to that of $^{32}P_i$, and a second with an R_f identical to that of synthetic 1-phosphohistidine (Figure 4). Phosphoarginine does not survive hydrolysis in hot alkali (Fujitaki & Smith, 1984), ruling out this linkage as a potential intermediate in the reaction.

The spot assigned to 1-phosphohistidine consisted of three lobes. That in the lower right contained 58% of the radioactivity in this area of the plate, was ninhydrin-positive, and represents 1-phosphohistidine. The smear to the left contained 19% of the radioactivity in this area, was ninhydrin-negative, migrated at the R_f for P_i, and represents P_i from hydrolysis of 1-phosphohistidine during chromatography in the second dimension. (A ninhydrin-positive smear to the right of the 1-phosphohistidine spot represents free histidine released in this hydrolysis). The lobe above that for 1-phosphohistidine was ninhydrin-negative and contained 23% of the ^{32}P in this area. It may represent α-*N*-[^{32}P]-phosphohistidine originating from an intramolecular transfer during alkaline hydrolysis or chromatography. Transfer from the 1-isomer of phosphohistidine to the 3-isomer and the ϵ-amino group of lysine is facile under these conditions (Wålinder, 1968). In conclusion, pH profiles, chemical stability, and chromatographic identification all demonstrate that the phosphorylated residue in NAPRTase is a 1-phosphohistidine.

Localization of the Phosphorylated Residue. The phosphorylated residue of NAPRTase was localized in the

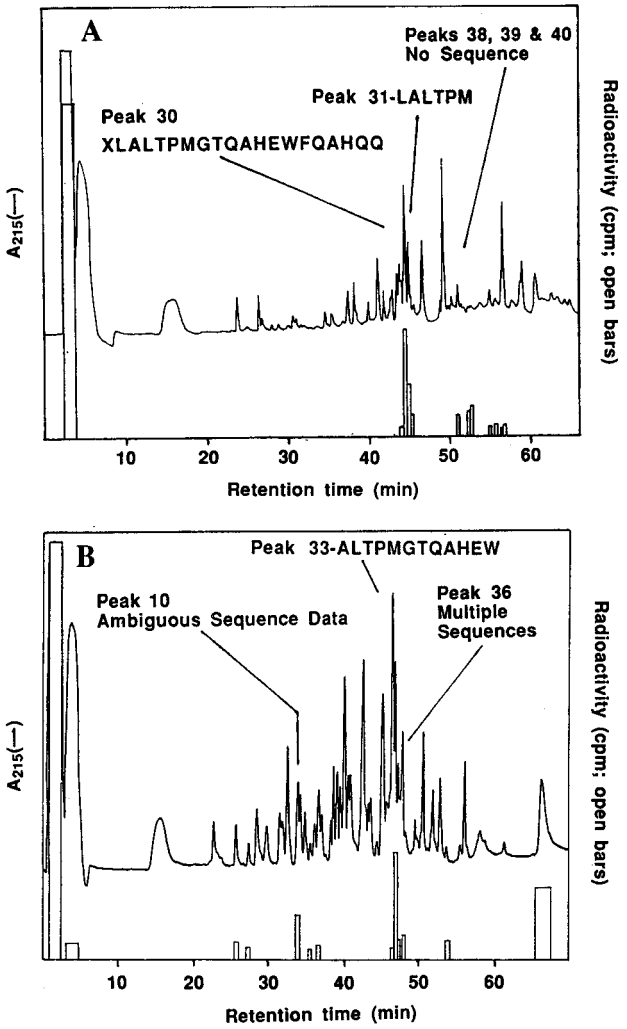

FIGURE 5: Proteolytic digests of E-^{32}P. E-^{32}P was digested with trypsin (A) or chymotrypsin (B) as described in Experimental Procedures. Peptides were separated by reverse phase HPLC, monitoring the absorbance at 215 nm. Radioactive peptides were subjected to automated peptide sequencing.

primary structure by degrading E-^{32}P with proteases, separating the peptides by reverse phase HPLC, and sequencing those peptides which contained radioactivity. The elution profile of trypsin-proteolyzed E-^{32}P is shown in Figure 5A, with the radioactivity in each peak shown under a trace of A_{215nm}. Peaks 30 and 31 contained the highest levels of radioactivity and appeared well resolved by absorbance at 215 and 280 nm. Peaks 38−40 were the only other regions of the chromatogram found to contain significant radioactivity; however, none of these peaks demonstrated significant absorbance at 215 or 280 nm.

The peptides in both peaks 30 and 31 were sequenced by automated Edman degradation. The peptide in peak 30 was sequenced for 20 cycles and gave the sequence XLALTPMGTQAHEWFQAHQQ, in which X represents an unidentified amino acid. The peptide in peak 31 was sequenced for 6 cycles and gave the sequence LALTPM. The sequences from the peptides found in peaks 30 and 31 thus differ only at the amino terminus and correspond to the following region of *S. typhimurium* NAPRTase (with the sequenced region in bold face type):

207-R**RLALTPMGTQAHEWFQAHQQ**ISPDLATSQR-237

Slow digestion of the Arg-208 to Leu-209 bond following

Table 2: Amino Acid Sequences Surrounding Some Known Phosphohistidines

NAPRTase (*S. typhimurium*)	A-L-T-P-M-G-T-Q-A-**H**-E-W-F-Q-A-H-Q-Q-I-S
VirA (*Agrobacterium tumefaciens*)[a]	V-G-T-L-A-G-G-I-A-**H**-E-F-N-N-I-L-G-A-I-L
NtrB (*E. coli*)[b]	A-R-D-L-V-R-G-L-A-**H**-E-I-K-N-P-L-G-G-L-R
EnvZ (*E. coli*)[c]	R-T-L-L-M-A-G-V-S-**H**-D-L-R-T-P-L-T-R-I-R
HPr (*S. typhimurium*)[d]	V-T-I-T-A-P-N-G-L-**H**-T-R-P-A-A-Q-F-V-K-E
III^{LAC} (*Staphylococcus aureus*)[e]	S-V-T-M-M-H-G-Q-D-**H**-L-M-T-T-I-L-L-K
EII^{mtl} (*E. coli*)[f]	Y-L-G-E-S-I-A-V-P-**H**-G-T-V-E-A-K
CheA (*E. coli*)[g]	Q-L-N-A-I-F-R-A-A-**H**-S-I-K-G-G-A-G-T-F-G
Histone H4 (calf thymus)[h]	I-R-D-A-V-T-Y-T-E-**H**-A-K-R-K-T-V-T-A-M-D
Succinyl-CoA synthetase (*E. coli*)[i]	T-A-P-K-G-K-R-M-G-**H**-A-G-A-I-I-A-G-G-K-G
Nucleoside diphosphate kinase (human RBC)[j]	C-I-Q-V-G-R-N-I-I-**H**-G-S-D-S-V-E-S-A-E-K
Bisphosphoglycerate mutase (human RBC)[k]	S-K-Y-K-L-I-M-L-R-**H**-G-E-G-A-W-N-K-E-N-R
Phosphoglycerate mutase (yeast)[l]	P-K-L-V-L-V-R-**H**-G-Q-S-E-W-N-E-K-N-L
Pyruvate, phosphate dikinase (*Bacteroides symbiosus*)[m]	G-G-M-T-S-**H**-A-A-V-V-A-R

[a] Jin et al. (1990). [b] Ninfa and Bennett (1991). [c] Roberts et al. (1994). [d] Weigel et al. (1982). [e] Deutscher et al. (1982). [f] Pas and Robillard (1988). [g] Hess et al. (1988). [h] Heubner and Matthews (1985). [i] Wang et al. (1972). [j] Gilles et al. (1991). [k] Hass et al. (1980). [l] Han and Rose (1979). [m] Goss et al. (1980).

cleavage at Arg-207 (Juhl & Soderling, 1983) is the likely reason for the incomplete proteolysis at Arg-208. The only candidate phosphoramidates present in the tryptic peptide Leu-209 to Arg-237 are the two histidine residues, His-219 and His-225, although it was not clear which was the site of NAPRTase phosphorylation.

Peaks 38−40 always appeared as minor peptide components based on A_{215nm}, but their ^{32}P content was highly variable, in several runs containing more ^{32}P than peaks 30 and 31. Amino acid sequencing of the material in these peaks demonstrated low levels of several amino acids at each cycle, and attempts to repurify this material resulted in all radioactivity behaving as $^{32}P_i$. We reasoned that these peaks might represent products of ^{32}P migration during chromatography, and modified the purification protocol. When tryptic peptides from E-^{32}P were prepurified on a urea-containing gradient (Experimental Procedures), only a single ^{32}P-containing peak was isolated, and subsequent purification by the original method gave a single peak of radioactivity. Edman degradation for 15 cycles gave the single sequence RLALTPMGTQAHXWF, in agreement with the tryptic peptide Arg-208 to Arg-237 which was isolated previously.

E-^{32}P digested with chymotrypsin and purified by reverse phase HPLC (Figure 5B) produced four A_{215nm} peaks which contained radioactivity: 10, 33, 36, and 54. Of the total radioactivity applied to the column, 68% eluted as [^{32}P]P_i, 19% was found in portions of the gradient that did not contain A_{215nm} peaks, with the remaining 13% distributed among A_{215nm} peaks. Peaks 10, 33, and 36 were analyzed by automated Edman degradation. Peak 33 contained the most radioactivity (3% of that applied) and was sequenced for 13 cycles, failing to give sequence after cycle 12. Two peptides could be identified in the Edman degradation: ALTPMGTQAHEW and NIVIXLV. The first peptide represents residues 210 to 221 of NAPRTase. This peptide includes His-219 but not His-225, clearly stopping at Trp-221, an excellent chymotryptic site. The yield of PTH−alanine in cycle one, 196 pmol, represented 20% of the theoretical maximum. The second peptide, which was less abundant, represents residues 352 to 358 of the NAPRTase sequence. Although quantitation was difficult, 28 pmol of PTH−valine were observed in cycle 3. Peaks 10 and 36 were sequenced for 6 and 11 cycles, respectively, and each peak yielded a mixture of amino acids at each cycle. The sequence VFSDNLDLPKA, observed in peak 36, corresponds to residues 310−320 of NAPRTase and was the only sequence from either peak which could be ascribed to any region of the protein. Peak 54 eluted late in the gradient and was poorly resolved, containing undigested and partially digested protein. No radioactivity was associated with the PTH amino acids during any of the cycles of Edman degradation. This was not unexpected as the E-P bond is acid labile and would be rapidly hydrolyzed in trifluoroacetic acid. Hydrolysis of E-P during sequencing would then allow identification of unmodified histidine residues.

DISCUSSION

The work reported here unambiguously identifies His-219 of *S. typhimurium* NAPRTase as the site of covalent phosphorylation by ATP during enzymatic turnover. First, the covalent nature of the linkage was established with chromatography and electrophoresis under denaturing conditions. The pH and chemical stability of the linkage were those of a phosphoramidate and clearly excluded phosphoesters, acyl phosphates, or thiol phosphates. Use of two-dimensional thin-layer chromatography allowed identification of histidine as the phosphorylated residue. Finally, on sequencing of phosphorylated tryptic or chymotryptic pep-

tides, His-219 was the only histidine residue that was present on all sequenced peptides. A covalently phosphorylated form of NAPRTase was proposed earlier. Kosaka et al. (1976) observed that radioactivity from [γ-^{32}P]ATP remained associated with the enzyme during gel filtration in nondenaturing buffer, and several workers (Kosaka et al., 1971; Hanna et al., 1983) observed ATP/ADP exchanges, most readily explained by a covalent E-P. Vinitsky and Grubmeyer (1993) reported that radioactivity from [γ-^{32}P]ATP remained associated with NAPRTase after gel filtration chromatography in SDS, but they did not further characterize the postulated E-P.

The reproducible stoichiometry of phosphorylation of NAPRTase, 0.6 nmol of [^{32}P]/nmol of NAPRTase, was lower than the unit value expected from the monomeric enzyme. Several factors might contribute to this non-unit stoichiometry. First, it may be that the enzyme as isolated is not fully active. However, although the NAMN synthesis activity of NAPRTase does decrease on storage, the ATP/ADP exchange activities and stoichiometry of phosphorylation remain relatively constant (unpublished). A second explanation comes from the hydrolytic instability of the native E-^{32}P. The rate of E-^{32}P hydrolysis was rapid for native enzyme compared to the denatured enzyme, suggesting that E-^{32}P is destabilized compared to phosphohistidine itself. It may be that the non-unit stoichiometry reflects breakdown occurring during the denaturation process. However, no variation in denaturation treatment that was tested yielded higher stoichiometries of phosphorylation.

The behavior of phosphorylated NAPRTase was most compatible with a 1-phosphohistidine. The 1-isomer of phosphohistidine is less common in enzyme phosphorylations than the 3-isomer, with only nucleoside diphosphate kinase (Edlund et al., 1969) and the histidine-containing phosphocarrier protein (Anderson et al., 1971) being unambiguously identified as containing 1-phosphohistidine.

The peptide sequence around His-219 shows little similarity to that near phosphohistidines in other enzymes (Table 2). The NAPRTase phosphopeptide does fit the consensus five-residue motif HyAHEHy (Hy designates a hydrophobic amino acid) which contains the phosphohistidine in the histidine kinase molecules of the bacterial two-component regulatory systems (Roberts et al., 1994), represented in Table 2 by NtrB, EnvZ, and CheA. The two-component histidine kinases also contain two other invariant sequences, DXDXG and GXG, separated by 20−50 residues (Stock et al., 1989; X indicates any residue), which could not be located in NAPRTase, reducing the likelihood of a common structural motif. NAPRTase does not show sequence similarity to other PRTases, which normally contain a PRPP binding sequence motif (Hove-Jensen et al., 1986). NAPRTase contains weak similarity to the consensus sequence for an ATP binding site at Gly372-Lys374 (Vinitsky et al., 1991), distant in the primary structure from the site of phosphorylation at His-219.

The use of ATP by NAPRTase remains enigmatic. It has been known since the work of Imsande and Handler (1961) on the mammalian enzyme that ATP promotes NAMN formation. It has also been reported by many workers that, when present, ATP is hydrolyzed in 1:1 stoichiometry with NAMN formation (Kosaka et al., 1971; Vinitsky & Grubmeyer, 1993). Vinitsky and Grubmeyer (1993) have shown that ATP provides a thermodynamic drive to the NAMN synthesis reaction catalyzed by the *S. typhimurium* enzyme, perturbing the steady state ratio of products to substrates by 1000-fold. The present work establishes that ATP use provides an intermediate in the reaction pathway and, together with studies of the partial proteolysis of NAPRTase (Rajavel et al., accompanying paper), the structural data linking ATP hydrolysis to NAMN synthesis, showing that ATP binding and hydrolysis drive a series of chemical and conformational events that result in energy capture. The molecular details of ATP use by NAPRTase are likely to provide insight into the basic process of enzymatic energy coupling.

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