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Nucleotidylation, Not Phosphorylation, Is the Major Source of the Phosphotyrosine Detected in Enteric Bacteria

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The majority of the phosphotyrosine recovered from partial acid hydrolysates of ^{32}P-labeled *Escherichia coli* is derived from a single prominent protein. We show here by biochemical, genetic, and immunological criteria that this protein is actually glutamine synthetase adenylylated (not phosphorylated) at tyrosine. Furthermore, all of the phosphotyrosine detectable in partial acid hydrolysates of ^{32}P-labeled *Salmonella typhimurium* was eliminated in a strain deficient in both glutamine synthetase and uridylyltransferase, an enzyme which uridylylates the regulatory protein P_{II} at a tyrosine residue. These results suggest that protein-tyrosine phosphorylation represents a rare modification in eubacterial cells.

In eucaryotic organisms, protein-tyrosine phosphorylation is thought to play an important role in the regulation of cell growth and the evolutionary origin of protein-tyrosine kinases is of considerable interest. A number of protein kinases have now been identified in enteric bacteria. These include isocitrate dehydrogenase kinase (14); the CheA protein, which phosphorylates the products of the *cheB* and *cheY* genes (15, 16, 30); and the NR_{II} (NtrB) protein, which phosphorylates the transcriptional regulator NR_I (NtrC) (19, 28, 30). However, those bacterial kinases whose specificity has been determined phosphorylate their protein substrates at serine or threonine residues (27, 39, 40). By contrast, it has not been possible to demonstrate directly protein-tyrosine kinase activity in *Escherichia coli* extracts (32). Furthermore, Wang et al. (41) were unable to detect phosphotyrosine in partial acid hydrolysates of *E. coli* labeled with $^{32}P_i$. These observations suggest that bacterial phosphoproteins do not contain phosphotyrosine. However, protein-tyrosine phosphorylation may be less abundant in procaryotes and, therefore, may have escaped attention.

Unlike Wang et al. (41), Manai and Cozzone were able to detect phosphotyrosine in partial acid hydrolysates of ^{32}P-labeled *E. coli* (23), and we have confirmed this finding. The same group subsequently reported that a major *E. coli* protein resolved by two-dimensional electrophoresis was the sole ^{32}P-labeled protein detectable after treatment of the gels with alkali prior to autoradiography (11, 12). Phosphoserine is alkali labile; hence, treatment with base has been used as a means to enrich for phosphotyrosine (9, 10). Partial acid hydrolysis of this species yielded phosphotyrosine. For these reasons, Cozzone and co-workers suggested that the modification carried by this *E. coli* protein is phosphotyrosine and, therefore, that protein-tyrosine phosphorylation occurs in bacterial cells.

As we have pointed out previously (32), phosphotyrosine can be generated by partial acid hydrolysis of proteins to which a nucleotide or nucleic acid is linked via a phosphodiester bond to the phenolic hydroxyl of a tyrosine residue (3, 31, 35). Hence, we reexamined the evidence for the existence of protein-tyrosine phosphorylation in enteric bacteria.

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MATERIALS AND METHODS

Strains and growth conditions. Bacterial stocks used were all provided by Sydney Kustu, University of California, Berkeley: *E. coli* YMC21 [Δ(*glnA-ntrC*) Δ*lacU169 endA hsdR thi*] (4) and NCM530 (a *glnA*$^+$ derivative of YMC21 prepared by P1 transduction); and *Salmonella typhimurium* TA831 (Δ*hisF645*) (5), SK35 [Δ(*glnA-ntrC*)*60* Δ*hisF645*], SK103 (*glnD79* Δ*hisF645*) (5), and SK2295 (Δ(*glnA-ntrC*)*60 glnD79* Δ*hisF645*].

To label cells during balanced growth, cultures (6 ml) were grown at 37°C to an A_{600} of 0.2 in minimal salts medium (23) containing 5 mM P_i. Cells were collected by filtration, washed, and suspended in an equal volume of the same medium containing 50 μM P_i. $^{32}P_i$ was added to a final concentration of 400 μCi/ml, and the cultures were incubated for three generations (about 3 h). The labeled cells were chilled on ice, collected by centrifugation, washed several times in chilled 1 M $NaPO_4$ (pH 7.0), and used immediately for analysis (or stored frozen at −70°C prior to use).

To label cells under adenylylating conditions, cultures (6 ml) were first grown at 37°C to late exponential phase in a minimal salts medium (5) containing 5 mM glutamate and 5 mM P_i; then they were collected, washed, and suspended in an equal volume of the same medium containing 5 mM glutamate and 50 μM P_i. $^{32}P_i$ was added to a final concentration of 400 μCi/ml. After 30 min, NH_4Cl was added to a final concentration of 10 mM to induce adenylylation (21). At 20 min after the addition of NH_4Cl, the cells were chilled on ice, collected, and washed as described above.

In all experiments involving YMC21, the medium was supplemented with 5 mM glutamine.

Preparation of cell extracts and two-dimensional gel electrophoresis. Cell pellets were suspended in 0.3 ml of 5 mM $MgCl_2$–10 mM Tris hydrochloride (pH 7.5)–50 μg of RNase A per ml and disrupted by sonication. DNase I was added to a final concentration of 50 μg/ml, and the lysates were incubated for 15 min at 4°C. Following addition of 0.12 volume of 3% sodium dodecyl sulfate–10% β-mercaptoethanol, the lysates were clarified by centrifugation at 30,000 × *g* for 25 min. The supernatant fraction (S30) was extracted with phenol, and protein was precipitated from the washed phenol phases with 15% trichloroacetic acid (17, 33). To remove phospholipids, the protein pellets were extracted

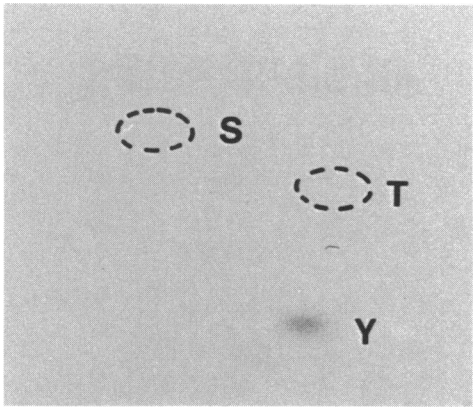

FIG. 1. Phosphoamino acid analysis of immunoprecipitated glutamine synthetase. Glutamine synthetase was immunoprecipitated from lysates prepared from *E. coli* NCM530 labeled with $^{32}P_i$ following a shift from glutamate- to ammonia-containing medium. A gel slice containing the immunoprecipitated protein was subjected to acid hydrolysis, and the phosphoamino acids were purified by ion-exchange chromatography and separated by two-dimensional electrophoresis on thin-layer plates, as described in Materials and Methods. The positions of each phosphoamino acid were revealed by autoradiography and by ninhydrin staining of authentic phosphoamino acids added to each sample as standards. S, Phosphoserine; T, phosphothreonine; and Y, phosphotyrosine.

with 0.3 volume of chloroform-methanol (2:1) and then washed successively three times with 5 ml of absolute ethanol and 5 ml of 75% acetone.

Proteins were separated by two-dimensional gel electrophoresis (29). Protein pellets prepared as described above were redissolved in a urea- and detergent-containing buffer appropriate for isoelectric focusing (29), with an Ampholine range of pH 3.5 to 10, and subjected to isoelectric focusing in a tube gel. The gel was extruded and placed on top of a slab gel of 10% acrylamide containing sodium dodecyl sulfate and subjected to electrophoresis in the second dimension (22). The slab gels were fixed, dried under vacuum, and analyzed by autoradiography, as described previously (25). For treatment of slab gels with alkali, dried gels were swollen in 1 M KOH at 55°C for 2 h (9), washed in 10% acetic acid–10% isopropanol, redried, and subjected to autoradiography again.

Immunoprecipitation. Pellets of labeled cells from 6-ml cultures were suspended in 0.3 ml of 2% sodium dodecyl sulfate and boiled for 3 min. The lysates were diluted with an equal volume of 300 mM NaCl–2% Triton X-100–2% sodium deoxycholate–0.2% sodium dodecyl sulfate–2% aprotinin–20 mM Tris hydrochloride (pH 7.5) and clarified by centrifugation at 13,000 × *g* for 3 min. Preclearing of the clarified lysates and subsequent immunoprecipitation of glutamine synthetase with rabbit polyclonal anti-glutamine synthetase antibodies (gift of S. Kustu) were carried out by methods described in detail previously (18), with the following mod-

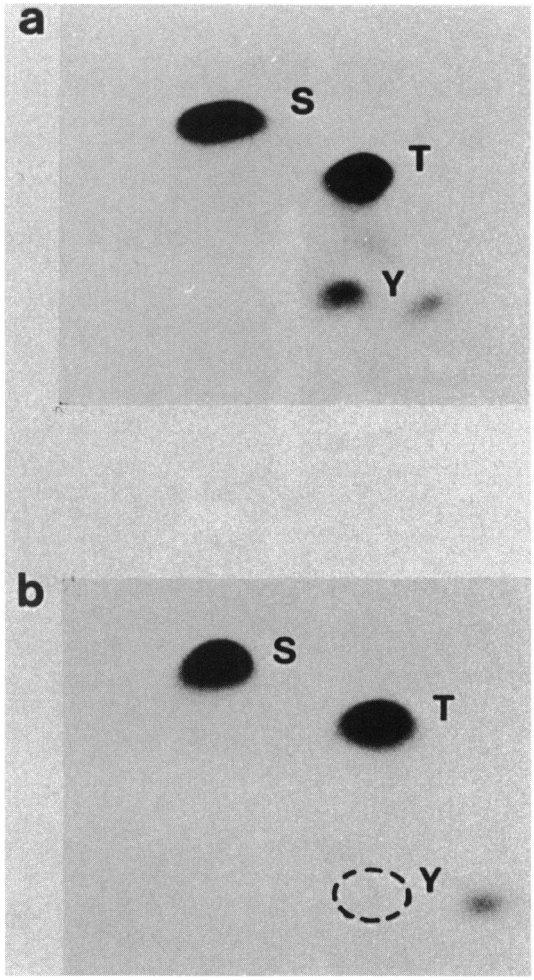

FIG. 2. Phosphoamino acid analysis of total *S. typhimurium* proteins from wild-type cells and from a *glnA glnD* double mutant. Proteins were extracted from *S. typhimurium* cells labeled with $^{32}P_i$ following a shift to ammonia-containing medium and subjected to acid hydrolysis, as described in Materials and Methods. The phosphoamino acids were separated as described in the legend to Fig. 1. (a) *glnA*$^+$ cells (TA831); (b) isogenic *glnA glnD* mutant (SK2295). Approximately equivalent amounts of radioactivity were loaded at the origin of each plate. S, Phosphoserine; T, phosphothreonine; Y, phosphotyrosine.

TABLE 1. Phosphoamino acid composition of *S. typhimurium* partial acid hydrolysates

Strain[a]	No. of independent determinations	% Radioactivity (SD) in[b]:		
		P-Ser	P-Thr	P-Tyr
TA831 *glnA*$^+$ *glnD*$^+$	4	55.0 (12.1)	36.2 (14.4)	8.8 (2.7)
SK35 Δ(*glnA-ntrC*)*60 glnD*$^+$	5	40.3 (7.9)	54.8 (7.2)	4.9 (1.1)
SK103 *glnA*$^+$ *glnD79*	4	46.9 (11.7)	50.5 (11.0)	2.1 (1.4)
SK2295 Δ(*glnA-ntrC*)*60 glnD79*	4	38.8 (7.4)	61.1 (7.4)	0.1 (0.1)

[a] Cells were grown in medium containing glutamine and labeled with $^{32}P_i$ in the presence of ammonium. Cellular protein was subjected to partial acid hydrolysis, and the phosphoamino acid composition was determined as described in Materials and Methods.

[b] Radiolabel in each phosphoamino acid as a percentage of the total recovered in all three phosphoamino acids. P-Ser, P-Thr, and P-Tyr are phosphoserine, phosphothreonine, and phosphotyrosine, respectively.

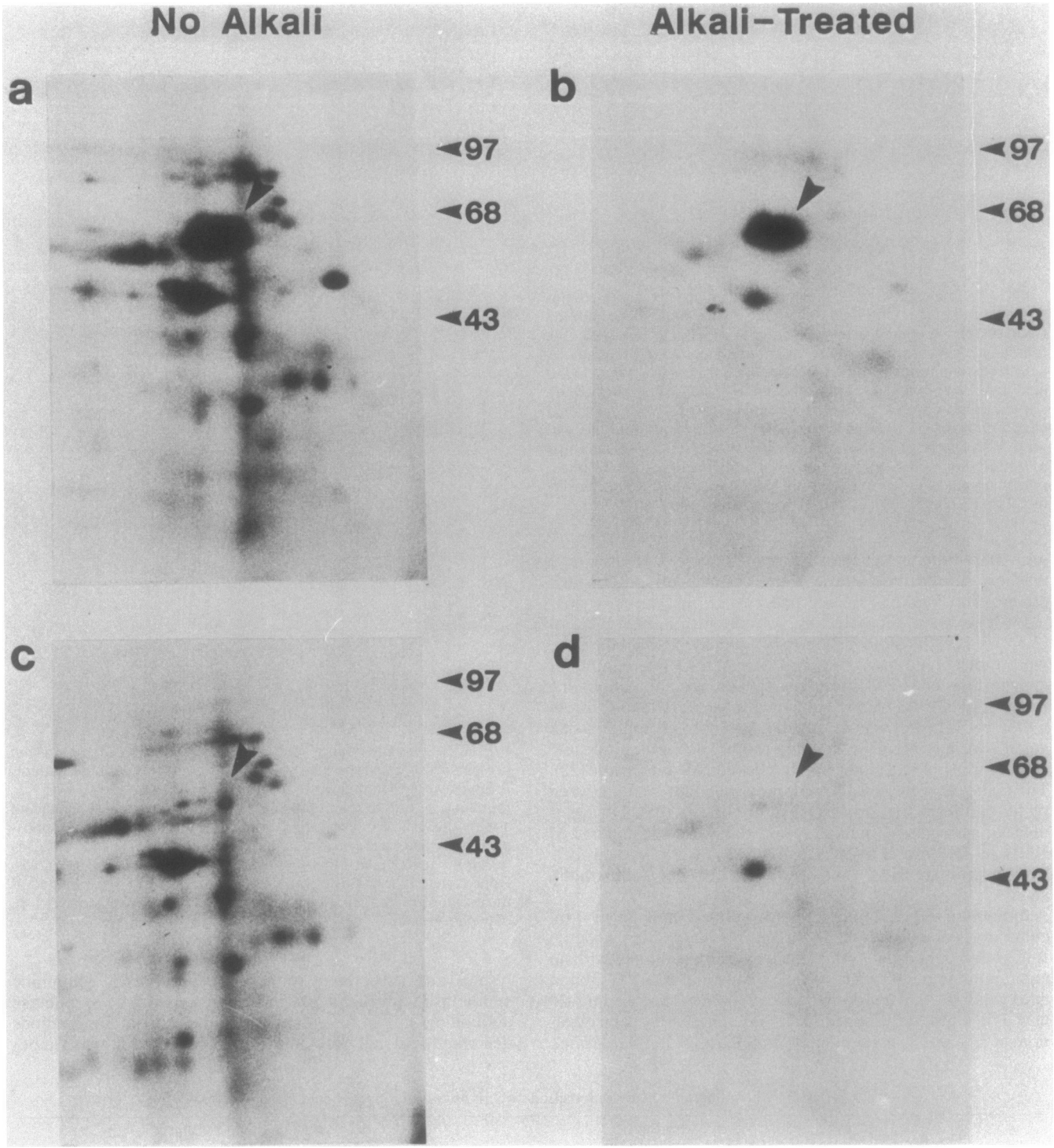

FIG. 3. Two-dimensional polyacrylamide gel electrophoresis of ^{32}P-labeled *E. coli* proteins. S30 fractions were prepared from *E. coli* cells labeled with $^{32}P_i$ during a shift from glutamate- to ammonia-containing medium. Proteins were extracted, precipitated, resolubilized, and subjected first to isoelectric focusing (for all two-dimensional gels, the basic region is at the left) and then to electrophoresis under denaturing conditions, by procedures described in Materials and Methods. The gels were fixed, washed, and subjected to autoradiography before and after alkali treatment of the gel. The migration position of marker proteins of known molecular weight (10^3) are indicated. The position of glutamine synthetase is indicated by the arrowhead. Equal amounts of protein were loaded initially onto each gel. (a) *glnA*$^+$ cells (NCM530) before alkali treatment; (b) same gel after alkali; (c) isogenic Δ*glnA* mutant (YMC21) before alkali treatment; (d) same gel after alkali treatment.

ification. Samples were incubated with anti-glutamine synthetase serum overnight at 4°C and an excess of *Staphylococcus aureus* Cowan I cells (10% suspension in 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 1% aprotinin, 10 mM Tris hydrochloride, pH 7.5) was used to recover the antigen-antibody complexes.

After the final wash, the immunoprecipitated material was released from the staphylococcal cells by suspension in isoelectric focusing buffer and incubation at room temperature for 20 min. The *Staphylococcus aureus* cells were removed by centrifugation at 13,000 × *g* for 15 min, and the supernatant solution was subjected to analysis by two-dimensional gel electrophoresis as described above.

Phosphoamino acid analysis. To determine the phosphoamino acid content of total cellular proteins, cells were labeled under the adenylylating conditions described above with the following modifications. All strains were grown in media supplemented with 5 mM glutamine instead of 5 mM glutamate, and labeling was carried out in the presence of 500 μM P_i. Although glutamine auxotrophs (*glnA* and *glnD* mutants) will grow on medium supplemented with glutamine, transport of this amino acid is inefficient and growth is glutamine limited. $^{32}P_i$ was added to the cultures (2 ml) at a final concentration of 1.5 mCi/ml. To prepare samples for acid hydrolysis, cell pellets were suspended in 50 μl of 2% sodium dodecyl sulfate and boiled for 3 min. The lysates were adjusted to 0.3% sodium dodecyl sulfate–1% β-mercaptoethanol and clarified by centrifugation at 13,000 × *g* for 3 min. The supernatants were treated with 0.1 volume of 500 mM Tris hydrochloride (pH 7.0)–50 mM $MgCl_2$–0.5 mg of RNase A per ml–1 mg of DNase I per ml for 15 min at 4°C and extracted with phenol. The extracted protein was collected from the phenol phase by precipitation with 15% trichloroacetic acid. This material was washed with organic solvents, as described above for the S30 fractions analyzed by two-dimensional gel electrophoresis. The final protein pellet was suspended in 6 N HCl and hydrolyzed at 110°C for 2 h in a sealed tube. The hydrolysate was lyophilized, redissolved in water, and subjected to two-dimensional electrophoresis on cellulose thin-layer plates, as described previously (9). Following autoradiography, the relative amount of each phosphoamino acid was determined by scraping the material that comigrated with ninhydrin-stained authentic phosphoamino acid standards and quantifying the radioactivity present by scintillation counting.

Acid hydrolysis of immunoprecipitated glutamine synthetase. NCM530 was labeled under adenylylating conditions with $^{32}P_i$ at a final concentration of 5 mCi/ml. Lysates were prepared and glutamine synthetase was immunoprecipitated as described above. The immunoprecipitated material in the final washed pellet was released from the staphylococcal cells by suspension in electrophoresis sample buffer (10% [wt/vol] glycerol, 5% [vol/vol] β-mercaptoethanol, 2.3% [wt/vol] sodium dodecyl sulfate, 62.5 mM Tris hydrochloride, pH 6.8) and boiling for 3 min. The *Staphylococcus aureus* cells were removed by centrifugation, and the supernatant solution was subjected to one-dimensional electrophoresis in a 10% polyacrylamide gel containing sodium dodecyl sulfate. After autoradiography of the wet unfixed gel, a gel slice containing glutamine synthetase was incubated with 6 N HCl at 110°C for 2 h in a sealed tube. The hydrolysate was lyophilized and redissolved in water. This solution was subjected to anion-exchange chromatography on Dowex I (Ag 1-X8, Bio-Rad Laboratories) to separate the phosphoamino acids from contaminating material (9). The phosphoamino acid fraction was then lyophilized and redissolved in water. Separation of the phosphoamino acids was carried out as described above.

RESULTS AND DISCUSSION

A prominent protein resolved by two-dimensional gel electrophoresis was identified by Cozzone and co-workers as the major alkali-resistant, phosphotyrosine-generating, ^{32}P-labeled species in extracts of *E. coli* and was designated pp40 (11, 12). We noted that this protein has an apparent molecular weight and pI close to the values reported for purified glutamine synthetase (42). In addition, glutamine synthetase is an abundant intracellular protein in enteric bacteria (37) and is known to be modified by adenylylation at a specific tyrosine residue present in each of its 12 subunits (2, 8, 34–36). Moreover, the adenylyl moiety linked to the phenolic hydroxyl of tyrosine is converted to phosphotyrosine and adenosine upon partial acid hydrolysis (T. Martensen, personal communication). We have confirmed this observation in the following experiment. *E. coli* NCM530 was labeled with $^{32}P_i$ under conditions which promote extensive adenylylation of glutamine synthetase (21). The enzyme was immunoprecipitated and subjected to partial acid hydrolysis (Fig. 1). Clearly, phosphotyrosine is generated from adenylylated glutamine synthetase during partial acid hydrolysis. These considerations suggested that adenylylated glutamine synthetase might be a major source of the phosphotyrosine detected in partial acid hydrolysates of ^{32}P-labeled enteric bacteria and might represent the species reported by Cozzone and his collaborators. The reversible adenylylation of glutamine synthetase is catalyzed by the enzyme adenylyltransferase (20, 26), whose activity is modulated by the regulatory protein P_{II} (7). P_{II} itself is modified by uridylylation at tyrosine through the activity of a uridylyltransferase (product of the *glnD* gene) (1, 5, 13, 24). We reasoned that uridylylated tyrosine would also yield phosphotyrosine upon acid hydrolysis and thus contribute to the phosphotyrosine recovered in partial acid hydrolysates.

To examine these possibilities, we analyzed the phosphoamino acid composition of hydrolysates prepared from both wild-type and mutant *S. typhimurium* strains. The mutant strains examined included a glutamine synthetase-deficient [Δ(*glnA-ntrC*)*60*] strain (SK35), a uridylyltransferase-deficient (*glnD79*) strain (SK103), and a doubly deficient [Δ(*glnA-ntrC*)*60 glnD79*] strain (SK2295). Since in wild-type cells the level of adenylylation varies considerably, even during exponential growth, cells were labeled with $^{32}P_i$ following a shift to ammonia-containing medium, a regimen that markedly stimulated adenylylation of glutamine synthetase (21).

Wild-type *S. typhimurium* was found to yield significant proportions of phosphotyrosine upon acid hydrolysis. Phosphotyrosine constituted 8.8% of the total recovered phosphoamino acids. Similar results were obtained with wild-type *E. coli* (not shown). Elimination of glutamine synthetase (*glnA* product) or deficiency of uridylyltransferase (*glnD* product) caused a significant reduction in the level of phosphotyrosine recovered in partial acid hydrolysates of ^{32}P-labeled *S. typhimurium* (Table 1). Neither mutation alone completely eliminated phosphotyrosine from the partial acid hydrolysates. (It should be noted, however, that as a result of physiological compensatory mechanisms, mutations in *glnA* result in an increase in the level of uridylylation of P_{II}, whereas in *glnD* mutants the degree of adenylylation of glutamine synthetase is increased [5]; therefore, the

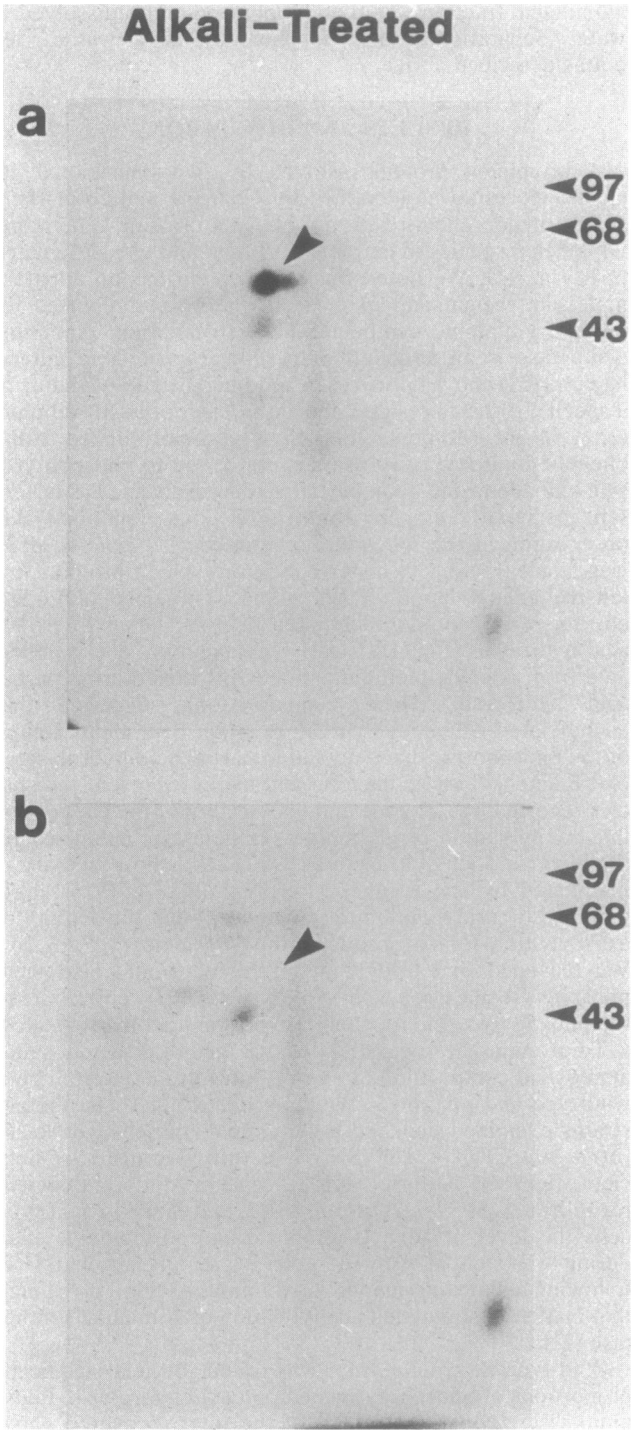

FIG. 4. Identification of major alkali-resistant ^{32}P-labeled protein in exponentially growing *E. coli*. Conditions were essentially identical to those in the experiment described in the legend to Fig. 3, except that the *E. coli* cells were labeled with $^{32}P_i$ under balanced growth conditions. Only autoradiograms of the alkali-treated gels are shown. (a) *glnA*$^+$ cells (NCM530); (b) isogenic Δ*glnA* mutant (YMC21).

resulting decrease in phosphotyrosine recovered in each instance does not strictly reflect the loss of either gene product.) The *glnA glnD* double mutant yielded no detectable phosphotyrosine in four independent trials (Table 1 and Fig. 2). These results indicate that the phosphotyrosine present in whole-cell partial acid hydrolysates of enteric bacteria is derived primarily, if not exclusively, from adenylylated and uridylylated tyrosine. Hence, phosphotyrosine generated by the authentic phosphorylation of cellular proteins is either absent altogether or occurs at levels too low (<0.1%) to be detected by the available analytical methods.

To determine whether glutamine synthetase is the major alkali-resistant ^{32}P-labeled protein in lysates of *E. coli* cells, S30 fractions from isogenic *glnA*$^+$ and Δ*glnA* mutant strains were analyzed by two-dimensional electrophoresis, and autoradiography was performed before and after alkali treatment of the gels. To promote greater incorporation of label into glutamine synthetase and thereby assist in its identification, ^{32}P-labeling was carried out during a shift from glutamate-containing to ammonia-containing medium to enhance adenylylation. The most intensely radioactive species detected in alkali-treated gels of proteins from wild-type cells (Fig. 3b, arrow) was glutamine synthetase, based on the following criteria. First, the intensely labeled spot was completely absent in the isogenic Δ*glnA* mutant (Fig. 3d, arrow). Second, transfer of proteins from such gels to a nylon membrane and immunodetection with anti-glutamine synthetase antibodies (Western blotting [immunoblotting]) demonstrated that the intensely labeled species comigrates with the glutamine synthetase polypeptide (data not shown; see below also).

A similar pattern is observed in cells labeled with $^{32}P_i$ during balanced growth. A major alkali-resistant species was clearly observed in *glnA*$^+$ cells (Fig. 4a, arrow) that was completely undetectable in the isogenic Δ*glnA* mutant (Fig. 4b). Thus, a shift to adenylylating conditions enhances the yield of the alkali-resistant species, but the same species is also detectable in the absence of the shift.

To compare the efficiency of labeling of glutamine synthetase during exponential growth and during the shift from glutamate- to ammonia-containing medium and to demonstrate directly that the label incorporated into glutamine synthetase is alkali resistant, the enzyme was immunoprecipitated from extracts of cells labeled with $^{32}P_i$ under the two different growth conditions and subjected to two-dimensional gel electrophoresis. As anticipated, only a single radioactive component was immunoprecipitated with the anti-glutamine synthetase antibody (Fig. 5), although this spot appears to be a composite of several tightly clustered species (possibly due to carbamylation by cyanate contaminants in the 9 M urea used for isoelectric focusing). The labeling of these species is clearly greater under the nutritional shift conditions known to promote adenylylation (Fig. 5c and d) than it is in cells labeled during balanced growth (Fig. 5a and b). Again, it should be noted that some adenylylated glutamine synthetase is detected even under balanced growth conditions in the absence of a nutritional shift. Furthermore, this material has a mobility indistinguishable from that of the most intensely labeled species present in the total *E. coli* extract (cf. Fig. 3 and 5). Most important, regardless of the labeling conditions, the radioactivity present in the immunoprecipitated material is almost completely resistant to removal by alkali treatment.

In summary, we conclude that the pp40 protein previously identified by other workers (11, 12, 23) as the major alkali-resistant, phosphotyrosine-containing protein in *E. coli* extracts is glutamine synthetase. Using genetic, immunological, and biochemical approaches, we have demonstrated unequivocally that adenylylated glutamine synthetase yields

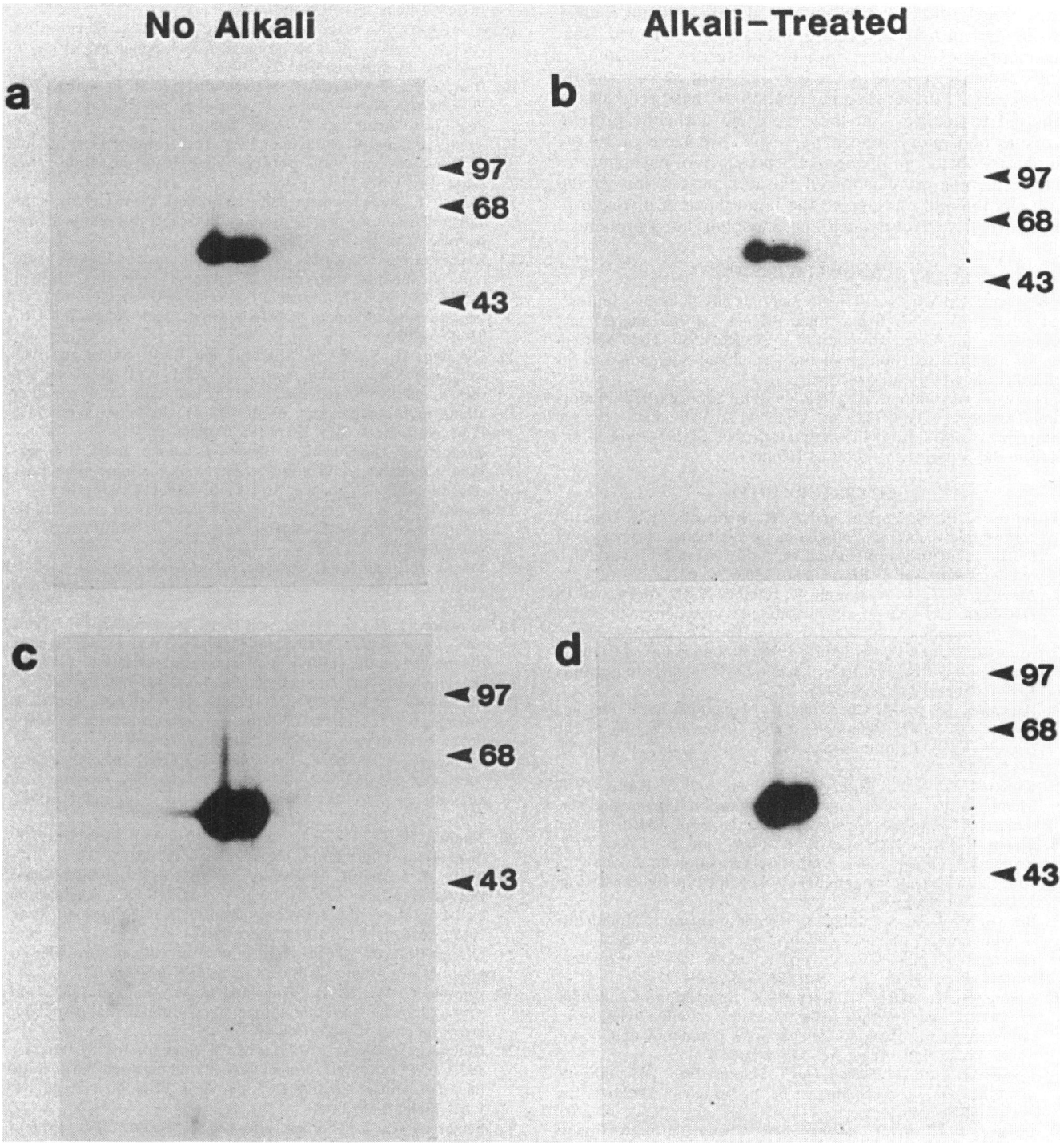

FIG. 5. Effect of a nutritional shift on alkali-resistant radioactivity in immunoprecipitated glutamine synthetase. Glutamine synthetase was immunoprecipitated with specific anti-glutamine synthetase antibodies from extracts prepared from *E. coli* NCM530 labeled with $^{32}P_i$ either under balanced growth conditions or during a shift from glutamate- to ammonia-containing medium. The immunoprecipitated protein was subjected to two-dimensional electrophoresis and analyzed by autoradiography both before and after alkali treatment, as described in Materials and Methods. (a) Balanced growth; (b) after alkali treatment of the same gel; (c) nutritional shift; (d) after alkali treatment of the same gel.

phosphotyrosine under the conditions of acid hydrolysis and that glutamine synthetase is the most prominent alkali-resistant ^{32}P-labeled protein in *E. coli* lysates. Moreover, most, if not all, of the phosphotyrosine present in *S. typhimurium* partial acid hydrolysates is derived from two nucleotidylated proteins, adenylylated glutamine synthetase and uridylylated P_{II}, and therefore does not arise from the action of protein-tyrosine kinases in vivo.

These results raise the possibility that authentic phosphorylation at tyrosine may be restricted to eucaryotic organ-

isms. In extracts of the purple nonsulfur bacterium *Rhodospirillum rubrum*, a kinase has been detected that phosphorylates certain synthetic substrates containing a single tyrosine residue as the only apparent phosphoacceptor site (38). Further characterization of these reactions is required to confirm that they represent authentic protein-tyrosine phosphorylation. The results we have presented emphasize that even the apparent presence of phosphotyrosine-containing proteins in cell extracts must be interpreted with caution and underscore the importance of distinguishing between nucleotidylated and phosphorylated proteins.

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