Comparison of Methods for Specific Depletion of ATP in *Salmonella typhimurium*

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Three methods of ATP depletion in *Salmonella typhimurium* were compared. ATP concentrations were lowest after arsenate treatment. Arsenate or α-methylglucoside-plus-azide treatment nonspecifically lowered all nucleotide triphosphate levels. Histidine starvation in a *hisF* mutant was relatively specific for ATP depletion and therefore has potential in distinguishing ATP-dependent processes from processes dependent on other nucleotides.

Three common ATP depletion methods have been used in *Escherichia coli* and *Salmonella typhimurium*, but no comparisons of these methods have been published. (i) Arsenate's structural similarity to phosphate causes ATP depletion by forming the labile arsenylated compound ADP~As (5, 13, 15). (ii) The nonmetabolizable glucose analog α-methylglucoside depletes ATP via phosphoenolpyruvate carboxykinase, which restores phosphoenolpyruvate exhausted during phosphotransferase transport; inclusion of sodium azide inhibits ATP repletion (14). (iii) We routinely use a novel method for ATP depletion, whereby the limitation for ATP synthesis is neither available phosphate nor oxidative phosphorylation but the adenine moiety itself (6, 11, 12, 22–24). Histidine tightly regulates its own biosynthesis through feedback inhibition of phosphoribosyltransferase (E.C. 2.4.2.17), which blocks the formation of phosphoribosyl-ATP, the first step in the pathway for histidine biosynthesis (17) (Fig. 1). Histidine starvation releases phosphoribosyl-transferase from feedback inhibition (2, 18, 19, 26), thereby increasing the consumption of ATP. However, *hisF* mutants do not regenerate the adenine moiety of ATP (2, 22, 23). ATP regeneration is limited to adenine synthesis through the de novo pathway (9, 10), which maintains ATP levels at approximately 100 μM in histidine-starved *S. typhimurium hisF* strains (6, 24). The addition of exogenous adenine rapidly restores ATP levels to normal.

S. typhimurium ST171 (*hisF thyA cheB*) (1) was grown to exponential phase (optical density at 600 nm [OD$_{600}$] of 0.4 to 0.6) at 30°C in Vogel-Bonner medium E (28) fortified with histidine (0.002%), thymine (0.001%), and glucose (0.7%). For histidine starvation, the pellets were washed twice in histidine-free growth medium and shaken at 30°C for 4 h. For arsenate treatments, the pellets were washed twice in an arsenate buffer [0.01 M piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) (PIPES) buffer (pH 7.0), 1 mM sodium arsenate, 0.7% glucose, 0.1 mM methionine, 0.1 mM EDTA, 1 mM magnesium sulfate, 1 mM ammonium sulfate] and starved for 1 h. For ATP depletion by α-methylglucoside and azide, cells were washed three times in growth medium without glucose and resuspended in the same medium with 10 mM α-methylglucoside and 20 mM sodium azide (14).

Cell extracts were prepared as described by Payne and Ames (21) with some modifications. A single 90-mm-diame-

ter glass fiber filter (Costar Corp.; no. 211625) on a coarse fritted-glass funnel was washed with 150 ml of deionized water to swell the membrane and thereby limit the loss of cells (less than 5%). A 100-ml aliquot of 4×10^8 cells ml^{-1} was filtered under vacuum (2 to 3 s), and the filter was placed upside down in 15 ml of ice-cold 1 M formic acid, all within 10 s. A second extraction was performed on each filter with another 10 ml of formic acid. Extracts were pooled, filtered to remove fibrous debris, lyophilized, dissolved in 400 μl of high-performance liquid chromatography (HPLC)-grade water, and filtered through a 0.45-μm-pore-size Teflon filter (Millipore; type EH). Nucleotide recovery was estimated from parallel luciferin/luciferase ATP assays (16) of prefiltered samples.

Ion-pair HPLC analyses of 20-μl samples utilized an Alltech Adsorbosphere nucleotide/nucleoside column. Solvent A contained 5 mM tetrabutylammonium phosphate, 1% acetonitrile, and 30 mM (Fig. 5) or 60 mM (Fig. 2) potassium phosphate, pH 6.0. Solvent B was 100% acetonitrile. Gradients were from 0 to 37.4% solvent B over 34 min (1.1%/min) at a flow rate of 1 ml/min. Absorbance was measured at 254 nm.

Histidine starvation depleted ATP to 7% (135 μM) of the control levels without severely lowering GTP, UTP, and CTP levels (Fig. 2B). Here, a peak shoulder contributed by dATP became evident. Subsequent addition of adenine to 10 μM resulted in full recovery of ATP within 2 min (Fig. 3). The ADP peak was obscured (Fig. 2B) by an unidentified peak present in extracts of histidine-starved cells (see Fig. 5). Separation of ADP from the unidentified peak was achieved by decreasing the phosphate in solvent A to 30 mM. Starvation levels of ADP increased from 26% (190 μM) to 70% (493 μM) of control values within 2 min (Fig. 3) after the addition of adenine.

Average GTP levels dropped by 28%, with a range of 20 to 50%, after histidine starvation but remained constant during the 2-min post-adenine addition ATP recovery phase (Fig. 3). Concentrations of CTP and UTP changed little during histidine starvation or after adenine supplementation (Fig. 3).

Treatment with α-methylglucoside and azide rapidly lowered ATP concentrations to 23% (415 μM) of control values within 10 min and to 6% (110 μM) within 1 h (data not shown). Glucose (1.5 mM) restored glycolysis and the ability to synthesize ATP by substrate level phosphorylation (Fig. 4). ATP levels rose rapidly over 2 min, followed by a slow
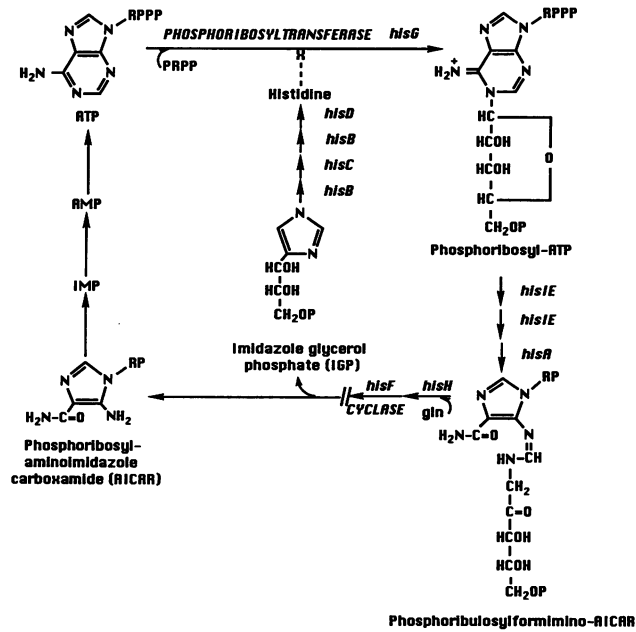
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FIG. 1. The purine nucleotide cycle in histidine biosynthesis. Mutations preceeding the cyclase (*hisF*) step prevent the synthesis of histidine and the resynthesis of the adenine moiety (modified from references 2 and 6 to include the bifunctional *hisIE* [4] gene product).

FIG. 3. Levels of various nucleotides in *S. typhimurium* ST171 prior to histidine starvation (unstarved), after histidine starvation for 4 h (starved), and after the addition of adenine to 10 μM for 20, 45, and 120 s. Intracellular concentrations were calculated using a volume of 0.68 μl per OD_{600} unit, or 3.1 μl mg^{-1} of total protein (conversion units: 0.2 OD_{600} unit = 1.5 × 10^8 cells ml^{-1} = 43.6 μg [dry weight] ml^{-1}; cell volume = 0.9 × 10^{-15} liter). Error bars represent standard deviations (n = 4 to 25).

rise over the next 10 min. The pre-glucose addition GTP level was 7% (37 μM) of control values, and there was a lag phase of more than 1 min before the level rose to 140% (730 μM) of control levels 5 min after the addition of glucose. The CTP level decreased to 6% (43 μM) and UTP decreased to

less than 5% (20 μM) of control values; recovery was protracted, and levels rose slowly over a 10-min interval.

Treatment of *S. typhimurium* with 1 mM arsenate for 1 h in phosphate-free buffer depleted ATP to 1.6% (30 μM) of control levels (data not shown). GTP, UTP, and CTP were depleted to levels evident only as ripples on the chromatogram and could not be quantitated. Nucleoside diphosphates were also affected, but to a lesser extent.
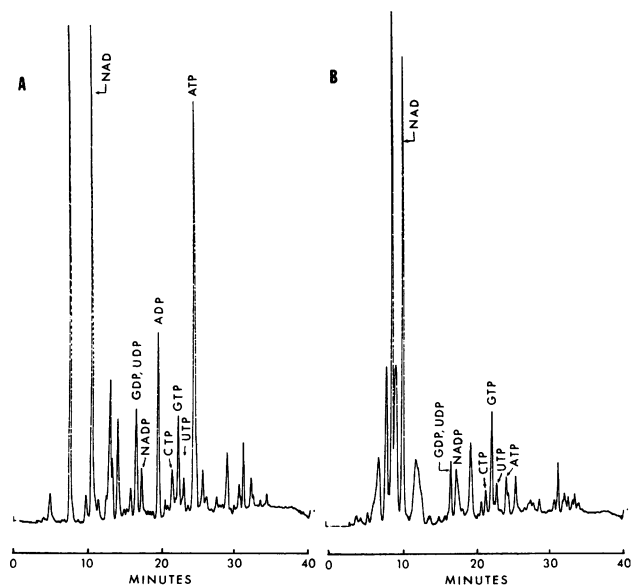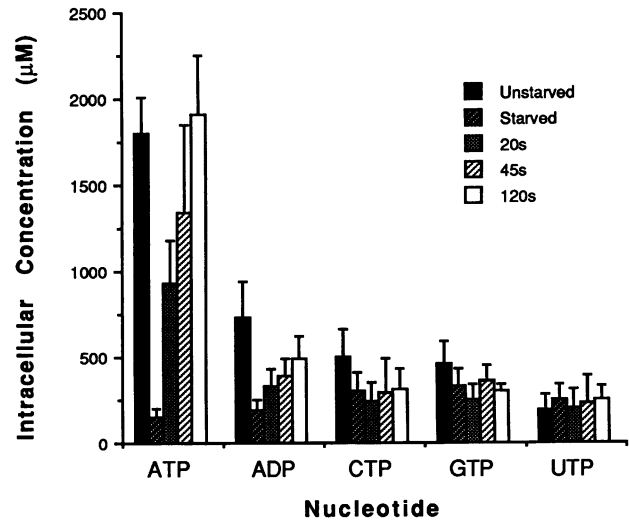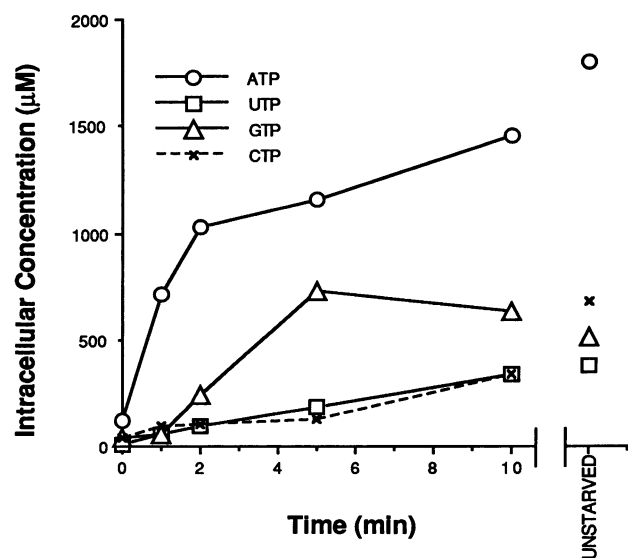

FIG. 2. Specific depletion of ATP in *S. typhimurium* ST171 after histidine starvation. (A) Separation of nucleotide pools prior to histidine depletion; (B) nucleotide pools 4 h after withdrawal of histidine. See text for HPLC conditions.

FIG. 4. Hierarchy of nucleotide recovery in α-methylglucoside-plus-azide-treated *S. typhimurium* ST171 cells after the addition of glucose to 1.5 mM. Cells were incubated with 10 mM α-methylglucoside and 20 mM azide prior to the addition of glucose. Nucleotide concentrations were determined after HPLC as described for Fig. 3.
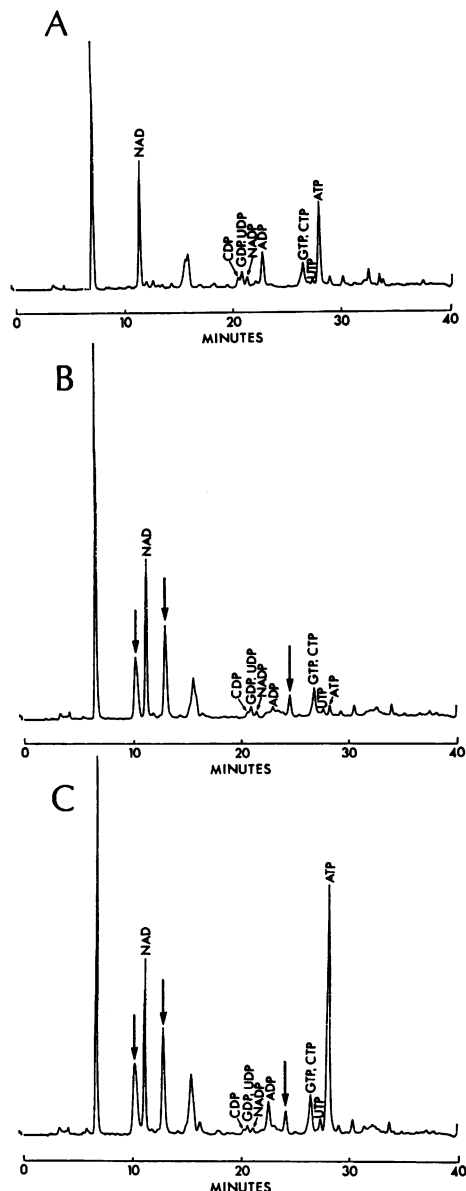
FIG. 5. Appearance of three new peaks (see arrows) after starvation for histidine in *S. typhimurium* ST171. (A) Unstarved cells; (B) 4 h after histidine withdrawal; (C) 2 min after the addition of adenine (10 μM) to starved cells. Chromatography conditions were as for Fig. 2 but with 30 mM phosphate (K⁺) in solvent A (see the text).

After the addition of guanine (10 μM) to histidine-starved cells, GTP rose to control levels within 1 min, whereas ATP increased to only 10% of control levels over the same period (data not shown).

Three additional unidentified peaks, separable by adjusting phosphate concentrations, were evident after histidine starvation (Fig. 5B), but their concentrations remained constant after the addition of adenine (Fig. 5C).

Histidine starvation can be used for ATP depletion in other organisms. We have observed ATP depletion after histidine starvation in histidine-requiring *Bacillus subtilis* OI1085 (provided by G. W. Ordal, University of Illinois) (27)

and in several *E. coli* UTH653-derived *hisF* strains (provided by P. E. Hartman, Johns Hopkins University) (7, 8, 25), although other nucleotide levels were not monitored.

These data suggest that cellular processes dependent on any triphosphonucleotide will be inhibited by arsenate or α-methylglucoside-plus-azide treatment. Histidine starvation, however, was relatively specific for the depletion of adenine nucleotides (Fig. 2). Moreover, recovery after the addition of adenine affected only adenine nucleotides (Fig. 3). The slow (>2 min) interconversion rates between guanine and adenine nucleotides are rather surprising considering that they are readily interconvertable (20). The α-methylglucoside-plus-azide method exhibited a temporal hierarchy of nucleotide recovery after the addition of glucose that in theory might be used to correlate levels with some cellular function (Fig. 4).

The reversibility of ATP depletion and repletion after histidine starvation can clearly be separated from the stringent response (3) because the cells remain starved for the limiting amino acid after the addition of adenine. However, histidine starvation may not be suitable for all studies of the role of ATP in *S. typhimurium* because the concentration of ATP remains in the 100 μM range, above the K_m for many ATP-requiring enzymes.

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