UNCOUPLER AND ANAEROBIC RESISTANT TRANSPORT OF PHOSPHATE IN ESCHERICHIA COLI

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SUMMARY

Active transport of inorganic phosphate into whole cells of a strain (AB3311) derived from Escherichia coli K12 was found to be partially resistant to 50 μM carbonyl cyanide m-chlorophenyl hydrazone (CCCP), a powerful uncoupler of oxidative phosphorylation. The presence of 10 mM dithiothreitol (DTT) before the addition of CCCP completely prevented the inhibition of phosphate uptake caused by the uncoupler. The addition of DTT to the CCCP-inhibited system restored phosphate uptake to the control rate even when added 5 min after the phosphate transport assay was started. This uncoupler resistant transport is insensitive to anaerobiosis, or the addition of 10 mM KCN which reduces oxygen consumption to less than 1% that of aerobic controls. Additional studies of transport in a mutant (CBT302) deficient in membranebound Ca^{2+}-, Mg^{2+} -ATPase activity also demonstrated the retention of appreciable inorganic phosphate uptake under anaerobic conditions.

INTRODUCTION

Inorganic phosphate (Pi) transport in Escherichia coli has been defined as an active process since the substrate is concentrated many hundred fold within the cells under conditions requiring metabolic energy (1). However, detailed study of this transport system is made difficult by the inherent metabolic drag in which a significant percentage of the phosphate transported is rapidly modified chemically. Presumably this is one major reason why those studying transport have, in general, addressed themselves to the phenomenon of biological transport with other systems (e.g. amino acids, sugars, etc.). However, it was considered that, if a suitable means could be devised for observing the size of the Pi intracellular pool generated under a variety of metabolically inhibited conditions, this inherent problem would be largely overcome. To determine this pool size, we have refined a technique of whole cell high voltage electrophoresis combined with radioautography (1) and applied this procedure to two strains of Escherichia coli. It was observed

Abbreviations used: CCCP: Carbonylcyanide m-chlorophenylhydrazone; DTT: dithiothreitol.

that a significant transport of inorganic phosphate persisted in the presence of the uncoupler, CCCP which is unaffected by anaerobiosis or by KCN treatment. It was also surprising to note that an ATP'ase-negative mutant actively transported Pi under anaerobic conditions.

These data from both the mutant and uncoupler studies indicate that phosphate apparently is concentrated in the absence of respiratory activity and conditions traditionally accepted as necessary for generation of a transmembrane proton or electrochemical gradient (for discussion see Harold, 2). Thus, some alteration in existing models of energy coupling to bacterial transport would seem to be necessary to account for this, hitherto, anomalous active transport.

MATERIALS AND METHODS

Two strains of E. coli, derived from K12, were used in these studies. These were: AB3311 (Reeves met$^-$) kindly supplied by Dr. H. Rosenberg, John Curtin School of Medical Research, Australian National University (characteristics in ref. 3) and CBT 302 kindly supplied by Dr. B. D. Sanwal of this Department (described in ref. 4). Both strains were maintained on rich solid media (1% tryptone, 1% beef extract, 0.5% NaCl, 0.3% yeast extract and 2% agar). Cell cultures were grown overnight in 60 ml TSYG media (1) at 37°C in a gyrotory shaker. Cells in the stationary phase were harvested by centrifugation and then resuspended in phosphate-free TSG media (1) and incubated for 2 h with shaking at 37°C. The cells were then collected by centrifugation just prior to phosphate uptake or transport studies.

Phosphate uptakes were carried out essentially as described by Medveczky and Rosenberg (1). The final concentration of [^{32}P]- labelled Pi (2-5 x 10^2 counts per min per nanomole) was 100 µM. Normally 0.5 ml samples were taken and filtered on Gelman membranes (0.22 µ, Gelman Instrument Co., Ann Arbor, Mich.) at timed intervals. Each sample was washed twice with 2 ml of KH_2PO_4 (7 mM)-NaCl (140 mM) solution. Uptakes under anaerobic conditions were carried out using Warburg flasks containing the cell suspension in the main compartment and the ^{32}Pi-stock solution to be added in the side arm. The flasks were sealed with a serum cap through which three hypodermic needles were inserted. One was used to flush the media with N_2 (99.99%), one to provide a vent for pressure release and one for sampling. Preflushing of the cell suspension for 5 min produced a completely anaerobic system. A flow of nitrogen was also maintained over the filtration assembly during the uptake assay. In the uncoupler studies CCCP (Sigma Chemical Co., St. Louis, Mo.) was added to give a 50 µM concentration 5 min before the initial uptake. A

preincubation of 5 min was also used for KCN (1 or 10 mM). DTT (Sigma Chemical Co., St. Louis, Mo.), 10 mM was added at the times indicated.

^{32}P-labelled inorganic phosphate and water-soluble ester phosphate intermediates (glycolytic, nucleotides, etc.) were separated by high-voltage electrophoresis of cells that were collected on Gelman membranes and directly applied to 3 MM Whatman filter paper as follows: The membranes after filtration and washing were applied right side up onto flat cakes of dry ice. Before application onto Whatman 3 MM paper (57 x 46 cm) the filters were dried by gentle suction. These were then folded and glued. Each folded membrane was glued (book binder glue) to the origin marked on the 3 MM paper. Two or three drops of buffer, pH 2.0 (43.5 ml glacial acetic acid and 12.4 ml 90% formic acid per 1) were applied during this procedure. The 3 MM paper was wetted with buffer (final wetting around filters was allowed to occur by surface action). High voltage electrophoresis was carried out at 3000 volts and a current of 200-250 mamps for 45 min (High Voltage Electrophorator, Model D, Gilson Medical Electronics, Middleton, Wisc.). After drying of the electropherogram, radioactive areas were located by exposure to Kodak X-ray film. Quantitation was achieved by cutting out each labelled area and counting the radioactivity present using a gas-flow counter (Model 1043 Automatic Low Background Counting System, Nuclear Chicago, Des Plaines, Ill.).

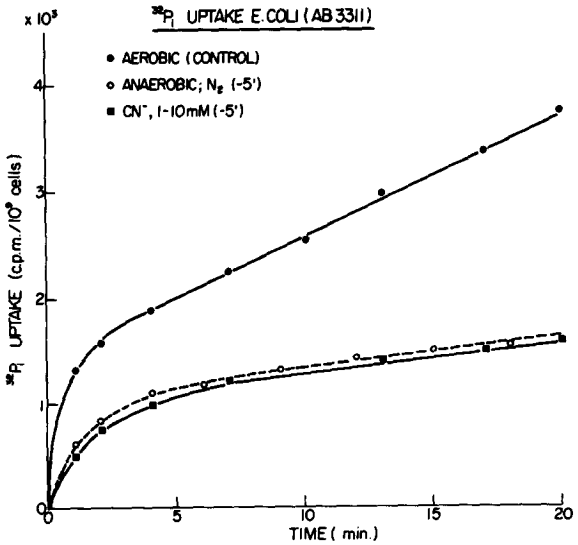

Fig. 1 Uptake of Pi by E. coli strain AB3311 under aerobic and anaerobic conditions and in the prescence of KCN. Preflushing with N_2 or the addition of KCN was carried out 5 min before the uptake commenced. Uptakes were done as described in the methods.

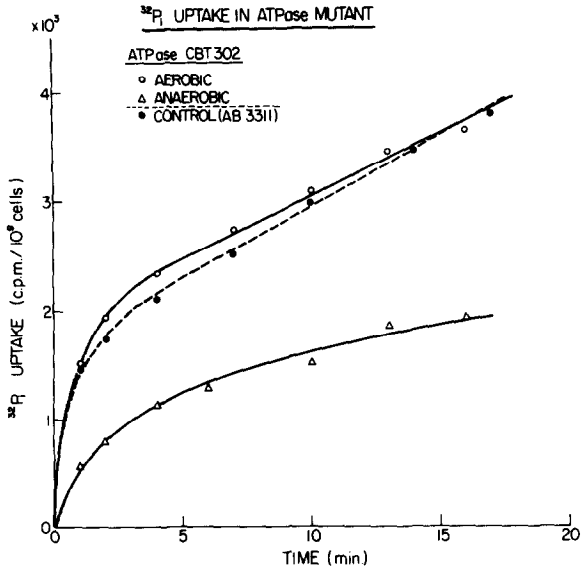

Fig. 2 Uptake of Pi by E. coli strains, AB3311 and CBT302 (ATP'ase negative mutant) under aerobic and anaerobic conditions.

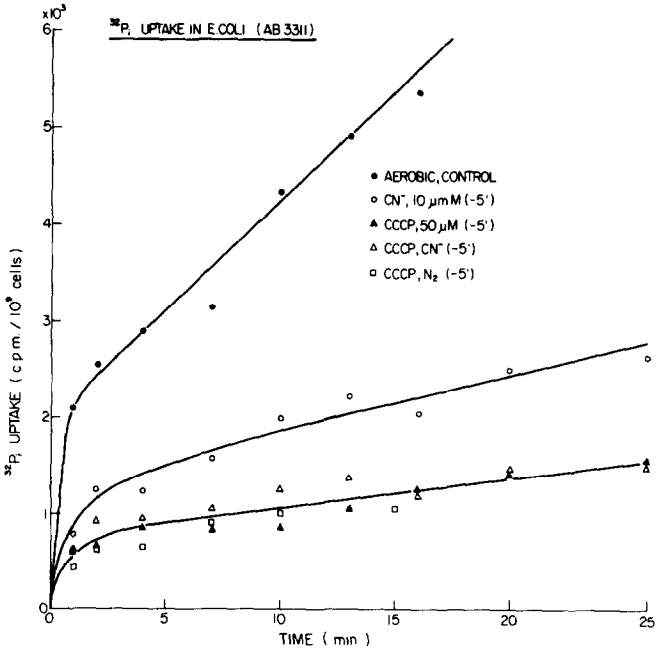

Fig. 3 Uptake of Pi by E. coli strain AB3311 in the presence of (a) KCN, 10 mM, (b) CCCP, 50 μM, (c) CCCP, 50 μM plus KCN, 10 mM, and (d) CCCP, 50 μM and N_2. Preflushing with N_2 or addition of inhibitors was carried out 5 min before the uptake commenced.

RESULTS AND DISCUSSION

Phosphate uptakes were performed on cells grown to the stationary phase and starved for 2 h in phosphate-free TSG media. These yielded a biphasic curve (fig. 1) under aerobic conditions similar to that reported by Medveczky and Rosenberg (1). Fig. 1 depicts also that the anaerobic technique used is valid in that the uptake curves for both anaerobiosis and for 10 mM KCN (a concentration that completely abolishes respiration) were practically superimposable. Analysis of the electropherogram (not shown) from a similar experiment showed the pool of intracellular phosphate in the aerobic to be approximately twice that of the KCN-treated cells.

Fig. 2 shows the same uptake (aerobically for both the wild-type strain (AB3311) and the ATPase-negative mutant (CBT302). Anaerobiosis lowered the uptake of the mutant by about one-half as observed for AB3311 (Fig. 1). Thus, it appears that a functional ATPase is not obligatory for phosphate uptake under both aerobic and anaerobic conditions. The latter finding was somewhat surprising in view of the many studies which implicate an ATPase in anaerobic or glycolytic supported transport (5-9).

Since a number of workers (5, 7, 10) have found that the presence of uncouplers (e.g. DNP or CCCP) completely inhibits the transport of β-galactosides (lactose or thiomethyl-β-D-galactoside) or amino acids (proline, leucine), it was decided to test the effect of uncouplers on the phosphate transport system that we had under study. Results from this study appear in Fig. 3-5 for the uncoupler, CCCP. Two observations of interest appear in Fig. 3. First, there is still a significant phosphate uptake (which as discussed later is mainly active transport) in the presence of CCCP (50 μM). Second, this residual uptake apparently is independent of any respiratory activity since it is identical to the uptake obtained in the presence of 10 mM KCN or under anaerobiosis. This uncoupler-resistant transport would appear to occur independently of any "energized-state" or"proton motive" force (both dissipated by uncouplers) generated (see 2 for ref.) in the membrane by either respiration or glycolysis.

Kaback et al (11) have been concerned that certain of the uncouplers might simply be acting as sulfhydryl reagents. They have demonstrated not only protection against but also release of inhibition by CCCP with the addition of the thiol agent, DTT, to membrane vesicles carrying out amino acid transport. A similar effect has been observed here in respect to phosphate uptake (Fig. 4). DTT (10 mM) added before the CCCP (not shown) prevented any inhibition by the uncoupler. DTT, added 5 min after the transport assay started, returned the uptake rate back to that of the control.

The intracellular Pi pools included in Fig. 4 come from radioactive

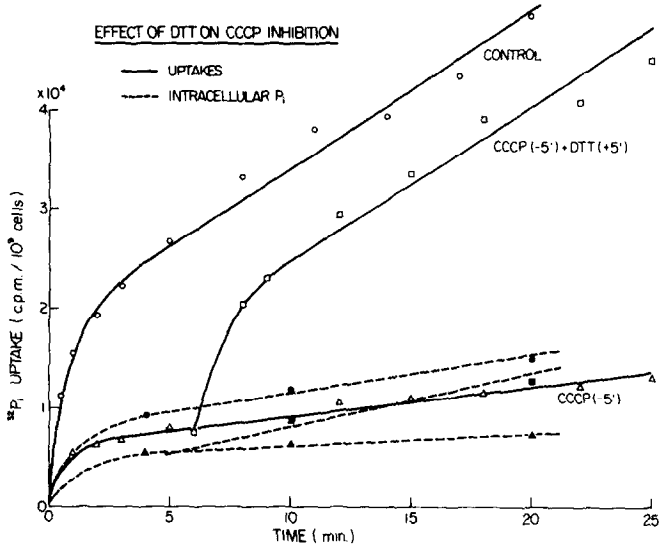

Fig. 4 The effect of DTT on CCCP inhibition of Pi uptake and intracellular Pi of E. coli strain AB3311. CCCP (50 μM added 5 min before and DTT (10 mM) added 5 min after uptake commenced. Intracellular Pi measured by high voltage electrophoresis technique described in the methods. One nanomole of Pi corresponds to 1.8×10^3 c.p.m.

analysis of the areas depicted in the autoradiograph shown in Fig. 5. Cells used in this electrophoresis run were treated as in Fig. 4. The three columns on the left show the radioactive metabolite profile for control cells incubated with ^{32}P-labelled Pi for 4, 10 and 20 min. Three regions of interest may be noted: (1) the dark circular spot two thirds of the way up the column is Pi, (2) the radioactive areas above this area correspond to glycolytic intermediates and (3) the activity at the origin is the result of incorporation primarily into phospholipids and nucleic acids (1, also unpublished data). This latter region is observed to increase as a direct function of time. The central three columns arise from cells treated with 50 μM CCCP. Under these conditions the intracellular pool of Pi (see Fig. 4) is approximately one-half that of the control. This uptake is definitely active transport as calculations performed on the control at 20 min give a concentrating effect of 400 times and CCCP has only reduced this effect by one-half. Interestingly the glycolytic intermediates are greatly reduced. This would seem to indicate the formation of little labelled ATP for incorporation of ^{32}P label into glycolytic intermediates. This deduction is further supported by the almost total loss of incorporation into the phospholipids and nucleic acids as judged by the activity at the origin. The three columns to the right demonstrate the relief by DTT of the inhibition by CCCP. In the presence of DTT, increased activity occurred in all

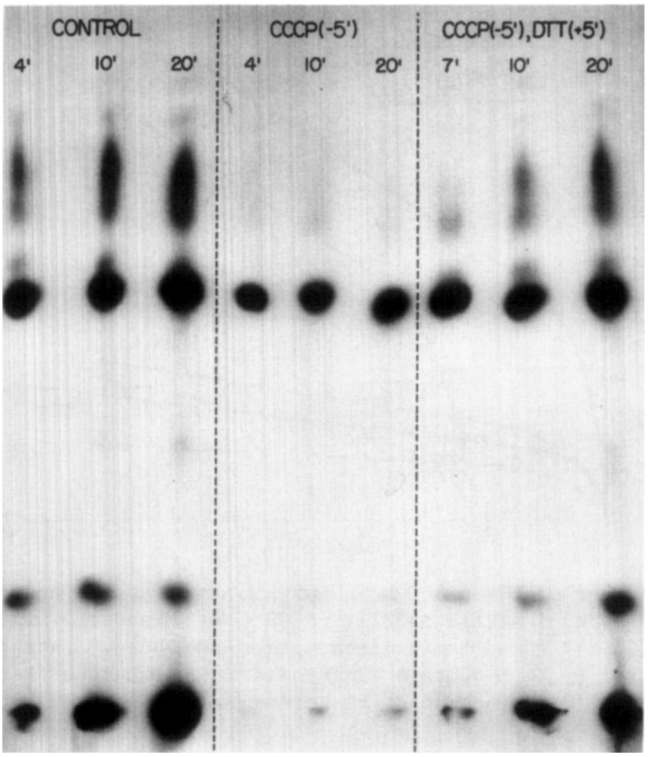

Fig. 5 Radioautograph of electropherogram obtained followed high voltage electrophoresis of cells as described in the methods. Conditions as in Fig. 4

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the areas labelled, that is the glycolytic intermediates, the Pi and the phosphate compounds at the origin.

Independent studies (7, 12) have shown that 50 μm CCCP does not affect respiration while this and much lower concentrations of CCCP cause complete uncoupling, the inhibition of many transport systems and the dissipation of proton or electrochemical gradients (see 1, 7, 10, 12). Our findings with CCCP suggest that part of the active transport of Pi in *E. coli* is accomplished independent of the generation of either an "energized state" or proton or electromotive gradient in the membrane either by respiration or glycolysis. The results obtained with the ATPase negative mutant, CBT302 suggest a non-obligatory role for ATP'ase either aerobically or anaerobically in respect to phosphate transport. Thus, some of phosphate transport would seem to occur via direct coupling to a step of glycolysis or to ATP utilization via a mechanism other than that involving ATP'ase. These findings most closely parallel those observed by Berger (13) for glutamine transport in *E. coli* where he concludes that "glutamine uptake is apparently driven directly by phosphate-bond energy formed by way of oxidative or sub-

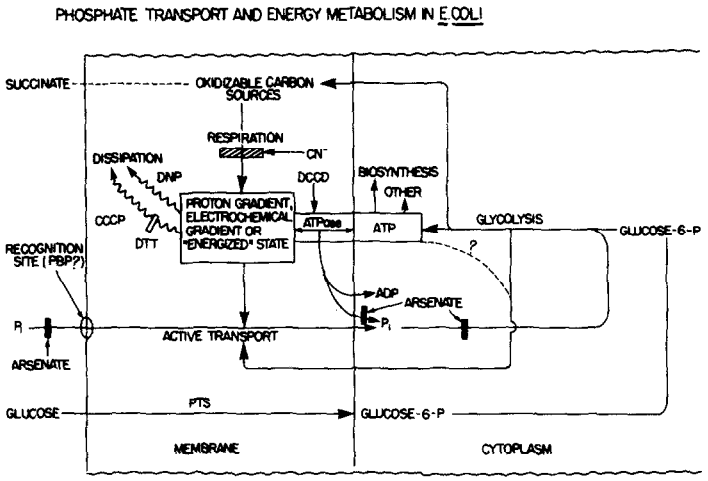

Fig. 6 Scheme to account for possible energy coupling mechanisms that drive the active transport of inorganic phosphate in E. coli.

strate level phosphorylations". It is interesting to note that in respect to both phosphate and glutamine transport binding proteins have been isolated and implicated (14, 15, 16). Boos (16) has commented on the possibility that substances whose transport is mediated by a periplasmic binding protein may exhibit further common features in respect to transport.

The above and additional data to be reported lead us to propose the scheme shown in Fig. 6 to account for the energy coupling mechanisms that drive the active transport of phosphate in E. coli.

ACKNOWLEDGEMENTS

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