Biochem. J. (1979) **178**, 133–137
Printed in Great Britain

Energy Coupling to the Transport of Inorganic Phosphate in *Escherichia coli* K12

By HARRY ROSENBERG, ROBERT G. GERDES* and FRANKLIN M. HAROLD†
Department of Biochemistry, John Curtin School of Medical Research, Australian National University, Canberra, A.C.T., Australia

(*Received* 25 *July* 1978)

The nature of the energy source for phosphate transport was studied in strains of *Escherichia coli* in which either one of the two major systems (PIT, PST) for phosphate transport was present. In the PIT system, phosphate transport is coupled to the proton-motive force. The energy source for the PST system appears to be phosphate-bond energy, as has been found in other systems involving binding proteins. High concentration gradients of phosphate (between 100 and 500) are established by both systems.

Two major transport systems (PIT and PST) for inorganic phosphate have been recognized in *Escherichia coli* (Willsky *et al.*, 1973). In a recent publication from this laboratory (Rosenberg *et al.*, 1977) the two systems were compared and were found to differ in a number of aspects, including their sensitivity to the uncoupler, carbonyl cyanide *m*-chlorophenylhydrazone, which inhibited aerobic glucose-dependent phosphate uptake in one system (PIT), but had no effect on the second, the PST system. This indicated that the PIT system may be coupled to the proton-motive force (Mitchell, 1966), whereas the PST system was possibly energized by phosphate-bond energy (Berger & Heppel, 1974). In the present work we examined this aspect in detail with the aid of mutants (*uncB*) defective in coupling oxidative phosphorylation to electron transport. The results confirmed the earlier conclusions.

Experimental

Materials

Chemicals were of the highest purity commercially available. Triethanolamine (puriss grade; Fluka AG, Roche–Sirius, Dee Why, N.S.W., Australia) was crystallized from ethanol as the hydrochloride. Carbonyl cyanide *m*-chlorophenylhydrazone was a product of Calbiochem (Australia), Carlingford, N.S.W., Australia. Carrier-free [^{32}P]P_i was obtained from the Australian Atomic Energy Commission, Lucas Heights, N.S.W., Australia. [U-^{14}C]Proline and [U-^{14}C]glutamine were from The Radiochemical Centre, Amersham, Bucks., U.K. N_2 used for purging the cell suspensions before and during anaerobic uptake assays was passed through a two-stage NILOX gas-purification apparatus (Jencons, Hemel Hempstead, Herts., U.K.), stated by the manufacturers to produce gas containing less than 1 p.p.m. of O_2.

Bacterial strains. The strains used in this work are listed in Table 1.

Methods

Growth of cells. The growth medium, P-free 'uptake medium' and essential supplements used were as described by Rosenberg *et al.* (1977). Cells were usually grown to stationary phase overnight at 37°C, aerobically with shaking or anaerobically in screw-capped culture flasks filled to capacity and stirred magnetically. Exponential-phase cells were obtained by growth on limiting carbon source followed by supplementation of carbon source as described by Simoni & Shallenberger (1972). Specific growth conditions are mentioned in the text. Strains containing the PST system for phosphate transport were starved for 2h in the P-free medium to achieve maximal uptake rates (Rosenberg *et al.*, 1977). All cells were washed three times with the uptake medium, resuspended in that medium and stored at 3°C until required for uptake assays.

Uptake assays. The techniques and media used for measuring the uptake of phosphate and amino acids under aerobic and anaerobic conditions have been described (Rosenberg *et al.*, 1975, 1977). All uptakes were performed at pH 6.6.

Abbreviations used: PIT, P_i transport; PST, phosphate-specific transport.

* Present address: Department of Biochemistry and Nutrition, University of New England, Armidale, N.S.W. 2351, Australia.

† Permanent address: Division of Molecular and Cellular Biology, National Jewish Hospital and Research Center, Denver, CO, 80206, U.S.A.

Table 1. *Strains of E. coli K12 used*

Gene designations follow those of Bachman *et al.* (1976). The *pit* and *phoT* genes code for essential components in the PIT and PST phosphate-transport systems respectively.

Strain	Relevant genetic loci	Source and other information
K10	*pit*	Obtained from B. Bachman, C.G.S.C. no. 5023; Echols *et al.* (1961)
AN283	*uncB402,argH,entA*	Butlin *et al.* (1973); Gibson *et al.* (1977)
AN518	*pit,ilv*	Isolated after u.v. mutagenesis of strain K10 and penicillin selection
AN713	*pit,uncB402*	Isolated after transduction with strain AN283 as donor and strain AN518 as recipient
AN749	*pit,uncB*$^+$	Produced as described for AN713
AN248	*ilv,arg,entA*	Butlin *et al.* (1973)
C.101a	*pit,phoT101*	Obtained from A. Garen
AN710	*phoT101,arg,entA*	Isolated after transduction with strain C-101a as donor and AN248 as recipient
AN393	*hfr,ilv,his,pro*	Obtained from F. Gibson
AN746	*phoT101,ilv,entA*	Isolated from *arg*$^+$ conjugants prepared with strain AN393 as donor and AN710 as recipient
AN747	*phoT101,uncB402,entA*	Produced from AN746 as described for AN713

Table 2. *Aerobic phosphate uptake in Escherichia coli grown aerobically with* 20mM-*glucose as carbon source*

Washed cells (see under 'Methods'), supplemented with carbon source as shown, were warmed for 5 min by shaking on the water bath. The assay was started by the addition of [^{32}P]P$_i$ (70–150 kBq/μmol, 2–4μCi/μmol) to 50μM, and uptake was measured over the first minute. Where present, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was added to 40μM, 5 s before the [^{32}P]P$_i$. The pH was 6.6. The results, representing initial rates (nmol of P/min per mg dry wt.) are all corrected for endogenous uptake (measured with no added carbon source). Each value is a mean of five independent experiments and is shown with the standard error.

	Initial rate (nmol of P/min per mg dry wt.)			
Strain ...	AN747	AN710	AN713	AN749
Relevant genotype ...	*phoT,uncB*	*phoT,uncB*$^+$	*pit,uncB*	*pit,uncB*$^+$
P-system operating ...	PIT	PIT	PST	PST
Additions				
10mM-Sodium succinate	2.8 ±0.81	1.9 ±0.55	0.54±0.34	15.8±2.7
10mM-Sodium succinate+CCCP	0.20±0.14	0.3 ±0.14	0.66±0.28	3.2±0.7
10mM-DL-Potassium lactate	21.2 ±1.7	18.1 ±0.8	11.8 ±0.3	19.5±0.8
10mM-DL-Potassium lactate+CCCP	0.62±0.23	1.4 ±0.54	6.3 ±0.5	6.1±0.9
5mM-Glucose	39.1 ±2.2	33.1 ±1.3	42.4 ±1.7	37.6±1.0
5mM-Glucose+CCCP	1.4 ±0.5	0.72±0.31	40.8 ±2.4	37.2±2.0

Results

Phosphate uptake in aerobically grown cells

As reported earlier (Rosenberg *et al.*, 1977), aerobic glucose-energized phosphate uptake in cells grown aerobically with glucose as carbon source was practically abolished by carbonyl cyanide *m*-chlorophenylhydrazone in the PIT system, but not in the PST system. To verify further the nature of energy coupling in the two systems, we studied the effect of several carbon sources on phosphate uptake in a series of mutant strains carrying the *uncB402* allele and the corresponding isogenic *uncB*$^+$ strains.

The results (Table 2) show that the PIT system displays activation patterns typical of a protonmotive force-energized transport: under aerobic conditions the responses of the *unc*$^+$ and *unc*$^-$ strains are the same, and in all cases transport is severely inhibited by carbonyl cyanide *m*-chlorophenylhydrazone. The PST system is activated by a non-glycolytic substrate (succinate) only in the *unc*$^+$ strain, and then the activity is carbonyl cyanide *m*-chlorophenylhydrazone-sensitive. The PST system in both *unc*$^+$ and *unc*$^-$ strains is equally well activated by glucose, and this is carbonyl cyanide *m*-chlorophenylhydrazone-insensitive. The case of DL-lactate is intermediate since it can also provide substrate-level phosphorylation. Yet even here activation of the PST system is considerably greater in the *unc*$^+$ compared with the *unc*$^-$ strain.

Phosphate uptake in anaerobically grown cells

The same strains grown anaerobically in the presence of glucose and nitrate were tested for phosphate uptake under nitrogen (Table 3). With glucose

Table 3. *Rates of anaerobic phosphate uptake in anaerobically grown Escherichia coli*

The cells were grown on the standard medium (see under 'Methods') with 20mM-glucose as carbon source and were supplemented with 50mM-KNO_3 and 1 μM each of Se and Mo in the form of sodium selenite and ammonium molybdate respectively. Washed cells were purged with O_2-free N_2 for 10min before assay commenced. Other conditions, and data presentation are as in Table 2. Results represent means for four experiments. The daily cultures of the *uncB* strains (AN747 and AN713) used were tested for revertants to *uncB*$^+$ (aerobic growth on succinate as sole carbon source). When such growth occurred overnight, the day's results were discarded.

	Initial rate (nmol of P/min per mg dry wt.)			
Strain ...	AN747	AN710	AN713	AN749
Relevant genotype ...	*phoT,uncB*	*phoT,uncB*$^+$	*pit,uncB*	*pit,uncB*$^+$
System operating ...	PIT	PIT	PST	PST
Additions				
5mM-Glucose	9.0 ±1.0	25.0 ±1.1	13.6 ±2.7	20.7 ±2.5
5mM-Glucose+CCCP	0.5 ±0.1	1.2 ±0.2	5.1 ±0.19	4.9 ±0.7
5mM-Glucose+20mM-KNO_3	25.7 ±0.14	28.7 ±1.6	25.8 ±1.4	35.2 ±2.0
5mM-Glucose+20mM-KNO_3+CCCP	0.6 ±0.1	2.5 ±0.9	15.4 ±1.3	24.7 ±2.3
10mM-Potassium formate	0.15±0.12	0.13±0.09	0.09±0.03	0
10mM-Potassium formate+20mM-KNO_3	2.8 ±1.1	7.4 ±1.4	0.79±0.17	6.2 ±0.14
10mM-Potassium formate+20mM-KNO_3+CCCP	0	0.15±0.1	0.32±0.16	0.45±0.3

as a substrate, phosphate uptake in the *uncB* mutant strain carrying the PIT system was stimulated severalfold by nitrate. With glucose alone, uptake in the *uncB*$^+$ strain was almost 3 times that in the *uncB* mutant strain. All uptakes were severely inhibited by carbonyl cyanide *m*-chlorophenylhydrazone in the PIT system. In the PST system, glucose alone stimulated phosphate uptake in both the *uncB* mutant and *uncB*$^+$ strains, and the difference in response between these was less pronounced than in the PIT system. Nitrate increased the stimulation in the PST system in both *uncB* mutant and *uncB*$^+$ strains, and the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone inhibited transport in this system by from 30 to 74%.

With formate as substrate, none of the strains took up any phosphate under anaerobic conditions. The combination of formate and nitrate stimulated both the *uncB* and *uncB*$^+$ strains in the PIT system, but in the PST system there was significant stimulation only in the *uncB*$^+$ strain.

Uptakes of proline and glutamine

To ascertain that the observed differences in energy coupling was not due to some differences between the strains used, we studied the uptake of proline and glutamine in the same set of strains. The results (Table 4) show uptake patterns which reflected only the nature of the amino acid tested and the presence or absence of an *uncB* mutation. The presence of one or the other phosphate uptake system in any strain was without effect on the uptake of either amino acid. Thus all four strains took up both amino acids normally under aerobic conditions. Under anaerobic conditions with glucose, all strains took up glutamine normally, but only the two *uncB*$^+$ strains took up proline.

Discussion

The experiments presented above confirm our previous finding about the different nature of the energy coupling to phosphate transport in the two distinct systems operating in *Escherichia coli.*

A comparison of the data in Tables 2, 3 and 4 shows that phosphate uptake in the PIT system resembles that of proline generally. This is evident from the difference in response to glucose, under anaerobic conditions, between the *uncB* and *uncB*$^+$ strains, and the lack of significant difference between them in response to any energy source under either aerobic conditions, or under anaerobic conditions in the presence of nitrate. Finally, under all conditions phosphate uptake in the PIT system was fully sensitive to uncoupler. This system thus appears to be energized by the protonmotive force (Mitchell, 1966).

Phosphate transport in the PST system resembles that of glutamine, and therefore the PST system is identified in this respect with the other systems which involve periplasmic binding proteins (Berger & Heppel, 1974) and utilize phosphate-bond energy from an unidentified metabolite. Thus, under aerobic conditions, succinate supports uncoupler-sensitive transport in the control strain (AN749) only, but not in the *uncB* strain (AN713), whereas glucose supports uncoupler-insensitive transport in both strains. Under anaerobic conditions glucose alone supports transport in both strains, whereas formate-plus-nitrate does so only in the control strain. The effect of glucose is only partly inhibited by uncoupler, but that of formate plus nitrate is fully uncoupler-sensitive.

Certain inconsistencies in the data should be pointed out. In the aerobic transport of phosphate in glucose-grown cells, succinate is much more effective

Table 4. *Initial rates of uptake of proline and glutamine in the four strains of Escherichia coli used*

The results represent the means of three determinations and are shown with the standard deviations of the mean. Exponentially growing cells (see under 'Methods') were washed thrice and depleted of carbon source by aerating for 2h in glucose-free medium. Proline (2.5kBq/nmol, 70nCi/nmol) or glutamine (1.2kBq/nmol, 35nCi/nmol) were added to 10μM. Chloramphenicol was present in all suspensions at 50μg/ml. Other conditions are as shown in Tables 2 and 3.

Strain ...	AN747	AN710	AN713	AN749
	Aerobic uptakes			
(*a*) Proline				
With 10mM-succinate	0.0	0.02±0.02	6.2 ±0.13	2.6 ±0.31
With 10mM-succinate+CCCP	0.0	0.02±0.02	0.0	0.0
With 5mM-glucose	7.6 ±0.2	3.9 ±0.58	4.1 ±0.37	5.1 ±0.65
With 5mM-glucose+CCCP	0.1 ±0.1	0.02±0.02	0.0	0.0
(*b*) Glutamine				
With 10mM-succinate	0.06±0.06	0.0	0.21±0.05	3.6 ±0.15
With 10mM-succinate+CCCP	0.0	0.0	0.0	0.12±0.01
With 5mM-glucose	5.8 ±0.40	4.1 ±1.6	5.3 ±0.63	6.2 ±0.52
With 5mM-glucose+CCCP	3.9 ±0.81	1.8 ±0.25	4.3 ±0.41	2.4 ±0.09
	Anaerobic uptakes			
(*a*) Proline				
With 5mM-glucose	0.12±0.08	2.2 ±0.22	0.0	2.5 ±0.20
With 5mM-glucose+CCCP	0.0	0.0	0.0	0.0
(*b*) Glutamine				
With 5mM-glucose	1.1 ±0.15	2.3 ±0.55	1.6 ±0.12	2.7 ±0.09
With 5mM-glucose+CCCP	0.33±0.02	0.28±0.02	0.53±0.15	0.06±0.03

in strain AN749 than in either AN747 or AN710 (Table 2). These differences are observed even more sharply in the case of proline transport (Table 4). This phenomenon is not understood, and is seen in cells after growth on glucose, which represses succinate utilization in cells of the *pit*$^+$ genotype.

Also, in the PST system, phosphate transport is insensitive to uncoupler under aerobic conditions with glucose as the energy source (Table 2, strains AN713 and AN749), but the same unexpectedly does not hold under anaerobic conditions (Table 3). Again, a similar effect is observed in the case of glutamine (Table 4). These observations indicate that carbonyl cyanide *m*-chlorophenylhydrazone may have effects other than collapsing the proton gradient. It is possible that, under anaerobic conditions where the protonmotive force can only be maintained at the expense of ATP, the sudden collapse of the pH gradient may trigger rapid ATP hydrolysis. It is for this reason that in all our experiments carbonyl cyanide *m*-chlorophenylhydrazone was added 5s before the start of the assay, but even this short period may have been sufficient to deplete some of the phosphate-bond energy required for the PST system and for glutamine uptake. The breakdown of intracellular energy reserves through some side effects of proton conductors has attracted comment (cf. Harold, 1977).

It can be calculated, from the data presented in Tables 2 and 3, that both uptake systems establish a high concentration gradient of phosphate within the cell. In short-term uptakes either of the two systems established a gradient of at least 100 within 20s, when esterification of phosphate was still negligible (Medveczky & Rosenberg, 1971; Rosenberg *et al.*, 1977). Ultimately the values were in excess of 500. These values refer to glucose-grown cells, where phosphate exchange does not occur (Rosenberg *et al.*, 1977). Both systems therefore carry out active transport of phosphate.

The present finding of different modes of energy coupling to phosphate transport in the two systems provides an explanation for the discrepancies in published results regarding this function. Thus Rae & Strickland (1976) found phosphate transport to be energized by phosphate-bond energy, in a manner similar to that found for phosphate transport in *Streptococcus faecalis* by Harold & Spitz (1975), while previous results from this laboratory (Rosenberg *et al.*, 1975) indicated dependence on the protonmotive force. The predominance of one or the other uptake system, depending on strain, growth conditions and degree of P-deprivation, may well have been at the source of the differences observed.

The visit of F. M. H. was made possible by a grant from the United States National Science Foundation under the United States–Australia Cooperative Science Program, and by an Australian National University Visiting Fellowship. We thank Ms. Bridget F. Beaver for carrying out most of the transport assay work and Ms. Jane Jones for skilled technical assistance.

References

Bachman, B. J., Low, K. B. & Taylor, A. L. (1976) *Bacteriol. Rev.* **40**, 116–167

Berger, E. A. & Heppel, L. A. (1974) *J. Biol. Chem.* **249**, 7747–7755

Butlin, J. D., Cox, G. B. & Gibson, F. (1973) *Biochim. Biophys. Acta* **292**, 366–375

Echols, H., Garen, A., Garen, S. & Torriani, A. (1961) *J. Mol. Biol.* **3**, 425–438

Gibson, F., Cox, G. B., Downie, J. A. & Radik, J. (1977) *Biochem. J.* **162**, 665–670

Harold, F. M. (1977) *Annu. Rev. Microbiol.* **31**, 181–203

Harold, F. M. & Spitz, E. (1975) *J. Bacteriol.* **122**, 266–277

Medveczky, N. & Rosenberg, H. (1971) *Biochim. Biophys. Acta* **241**, 494–506

Mitchell, P. (1966) *Biol. Rev.* **41**, 445–502

Rae, A. S. & Strickland, K. P. (1976) *Biochim. Biophys. Acta* **433**, 564–582

Rosenberg, H., Cox, G. B., Butlin, J. D. & Gutowski, S. J. (1975) *Biochem. J.* **146**, 417–423

Rosenberg, H., Gerdes, R. G. & Chegwidden, K. (1977) *J. Bacteriol.* **131**, 505–511

Simoni, R. D. & Shallenberger, M. K. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 2663–2667

Willsky, G. R., Bennett, R. L. & Malamy, M. H. (1973) *J. Bacteriol.* **113**, 529–539