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EFFECT OF THE ANTIBIOTIC MICROCIN 140 ON THE ATP LEVEL AND AMINO ACID TRANSPORT OF *Escherichia coli*

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SUMMARY

Microcin 140, a novel antibiotic of low molecular weight, rapidly depletes the ATP pool of sensitive *E. coli* strains. This effect seems to be caused by a stimulation of the ATPase since it is not observed either in a mutant strain that lacks activity of this enzyme or in the presence of the ATPase inhibitor dicyclohexylcarbodiimide. Under these conditions the microcin does not affect the incorporation of labelled glucose into macromolecules. Microcin 140 also produces a strong inhibition of the transport of proline and phenylalanine, which is known to be coupled to the proton gradient across the bacterial membrane. Therefore, the stimulation of the bacterial ATPase can be a consequence of collapsing the proton gradient maintained by this enzyme through a proton pumping activity.

These results point to a similar mechanism of action for both microcin 140 and colicin K, despite their great difference in molecular size (C.A. Plate et al., J. Biol. Chem, 249: 6138-6143, 1974).

INTRODUCTION

According to their mechanism of action two kinds of antimicrobial substances can be differentiated: inhibitors of vital enzymes and modifiers of membrane permeability. Most of the classical antibiotics belong to the first group and the field of membrane acting substances is much less developed. The finding of new substances of this later group may prove valuable not only because of their therapeutical possibilities but also as tools in the analysis of membrane functions (1).

The recent developments of the chemiosmotic theory have identified the energized state of bacterial membranes, which is coupled to oxidative phosphorylation and active transport, with the electrochemical gradient of protons (2). Therefore, this form of biological energy may

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be altered by antibiotics that increase ion permeability of the membranes (1,3). The immediate effects of an antibiotic which collapses the proton gradient across the bacterial membrane should include the inhibition of transport mechanisms coupled to this form of energy and the depletion of ATP level. This last effect is a consequence of oxidative phosphorylation being blocked and also because the proton gradient is in equilibrium with the ATP pool by the action of bacterial $F_o.F_1$ ATPase (2). The ion composition of the cytoplasm could also be altered, an effect which could be due to the permeability induced by the antibiotic or to the block of ion transport systems coupled to the proton gradient (3).

In line with this working hypothesis, we have studied the mechanism of action of microcin 140, a member of a new group of low molecular weight antibiotics produced by Enterobacteriaceae (4,5). The results indicate that this compound inhibits the active transport of amino acids and depletes the ATP pool by a mechanism which suggests the dissipation of the energized state of the bacterial membrane.

MATERIALS AND METHODS

Bacterial strains. Escherichia coli strain 140, isolated from human feces (4), was utilized for microcin production. For testing the effects of the antibiotic, E. coli strains 405 (Mc Leod strain), AN428 and N144 were employed. AN428 is a K 12 derivative (F^-, pro^-, lac_1^-, T_6^R, gal_2^-, ara^-, his^-, xyl^-, man^-, B_1^-, Str^R) and N144 is a mutant defective in $F_o.F_1$ ATPase derived from AN428 (6). These two strains were obtained from Dr. D.L. Gutnick (Tel Aviv University, Israel) by mediation of Dr. F. Fernández del Campo (CSIC, Madrid).

Media and growth of bacteria. The bacteria were grown in minimal medium 63 (7) supplemented with 0.2% glucose. In the case of strains AN428 and N144 the medium included histidine and proline (50 μg/ml each) and vitamin B_1 (1 μg/ml). Growth was at 37ºC with shaking until the absorbance at 660 nm reached 0.2-0.5.

Microcin preparation. Full details of the purification of the microcin 140 will be published elsewhere (A.F. Duro, in preparation). Briefly, the supernatant fluid from early stationary cultures of E. coli strain 140 were concentrated, precipitated with methanol and subjected to chromatography on Dowex and silica gel columns. The final preparation gave a single spot by thin layer chromatography in silica gel plates developed with butanol-acetic acid-water (12:3:5). Studies on the molecular structure of this microcin are in progress and preliminary results indicate

that it is an oligopeptide of molecular weight below 1000. Its biological activity is sensitive to brief exposure to strong acids.

Microcin concentration is expressed in arbitrary antibiotic units. One unit is defined as the minimal amount of microcin which gives a visible inhibitory effect in the antibiosis assay previously described (4).

Measurement of the levels of ATP and other metabolites. ATP was extracted and assayed with luciferin-luciferase as described elsewere (8). Phosphoenolpyruvate (10) ADP and AMP (11) were converted enzymatically to ATP as indicated in the references.

Transport assays. Amino acid transport was measured after addition of chloramphenicol (80 μg/ml) to the cultures. Transport was initiated by the addition of either 10 μM L-(U-^{14}C) proline, 10 μM L-(U-^{14}C) glutamine, 10 μM L-(U-^{14}C) leucine or 25 μM L-(U-^{14}C) phenylalanine with a specific radioactivity of 20 Ci/mol. Initial rates of transport were obtained by removing aliquots of 0.050 ml after 30 seconds at 37ºC, and filtering on nitrocellulose filters as described (8).

Incorporation of glucose into cellular macromolecules. Cells were washed by centrifugation in growth medium without glucose and resuspended in this medium with addition of 200 mM KCl. Incorporation was initiated with (U-^{14}C) glucose to a final concentration of 5 mM. The specific radioactivity was 0.2 Ci/mol. After 5 min of incubation at 37ºC samples of 1 ml were mixed with 2 ml of 7.5% trichloroacetic acid and filtered as in the transport assays.

Chemicals. Firefly extract (FLE-50) pyruvate kinase (type II) and myokinase (grade III) were obtained from Sigma. Dicyclohexylcarbodiimide and ATP were from Merck. (U-^{14}C) glucose, L-(U-^{14}C) proline, L-(U-^{14}C) leucine, L-(U-^{14}C) glutamine and L-(U-^{14}C) phenylalanine were from The Radiochemical Center, Amersham.

RESULTS AND DISCUSSION

Preliminary studies on the action of microcin 140 on E. coli 405 indicated that it produces an inmediate and simultaneous arrest of DNA, RNA and protein synthesis. Cell lysis did not occur under these circumstances because neither protein nor nucleotides were released into the medium (data not shown). A plausible mechanism for this general inhibition of macromolecular biosynthesis would be that the antibiotic interferes with the energy transducing processes. In order to test this possibility we have measured ATP level of the sensitive E. coli strain 405 after the addition of the microcin.

The results of figure 1 indicate that the microcin produces a rapid and dramatic drop of ATP concentration to values of about 15% of those

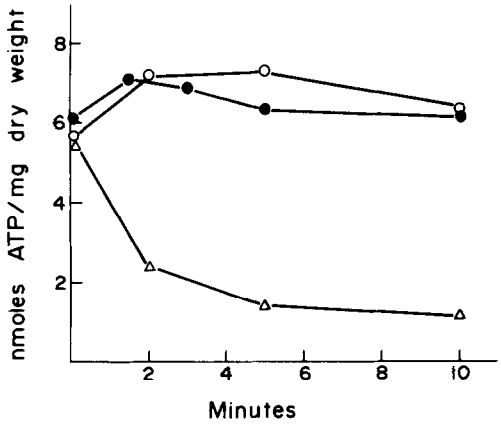

Figure 1. Effect of microcin 140 on the ATP level of E. coli strain 405. Cells were treated with Tris-EDTA as described (12) in order to increase the accesibility of dicyclohexylcarbodiimide and were resuspended in fresh growth medium in the absence (o , Δ) or presence (•) of 0.2 mM dicyclohexylcarbodiimide. After 15 minutes of incubation microcin was added at zero time in the experiments symbolized by (Δ) and (•) but not to (o). At the times indicated, samples were taken for ATP determination as described in Methods.

of untreated cells. The levels of ADP (0.5-0.7 nmol/mg) and phosphoenolpyruvate (2-2.5 nmol/mg) were not significantly altered by the microcin. Concerning AMP, the method of determination (11) was not accurate in our hands, with variations between duplicates up to 100%. Nevertheless, a consistent increase in the microcin treated samples was observed, with mean values of 0.05 and 2 nmol/mg before and after microcin addition. The observed decrease of the "energy charge" (11) of the cells may explain the inhibition of macromolecular biosynthesis previously described. On the other hand, the lack of effect on phosphoenolpyruvate levels indicates that glycolysis is not significantly affected by the microcin.

The decrease of ATP levels produced by the microcin could be a consequence of blocking its synthesis, stimulating its degradation or both. ATP splitting occurs during biosynthesis but it is also hydrolysed specifically by the bacterial $F_o.F_1$ ATPase sensitive to dicyclohexylcarbodiimide (2). It can be observed in figure 1 that the fall of ATP levels produced by the microcin is prevented by this ATPase inhibitor, suggesting that it was caused by a stimulation of the ATPase. In the absence of the mi-

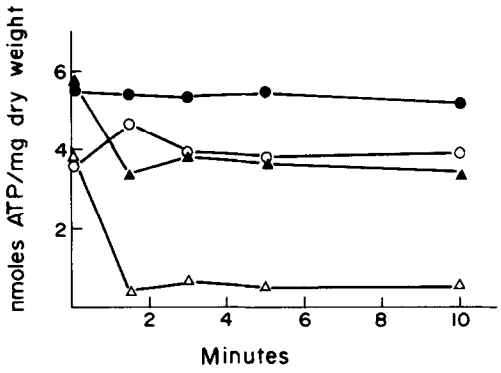

Figure 2. Effect of microcin 140 on the ATP level of E. coli strains AN428 and N144. The experiment was carried out as in figure 1 except that the Tris-EDTA treatment was ommited. Open symbols refer to the AN428 parental strain and closed symbols to its ATPase negative derivative N144. Microcin was added at zero time in the curves symbolized with triangles.

crocin, addition of carbodiimide did not affect ATP levels (data not shown).

Further evidence in favour of this mechanism was obtained by testing the effect of the microcin in an ATPase negative mutant and its corresponding parental strain (figure 2). Microcin addition produced a 90% decrease of ATP in the parental strain and only a 30% decrease in the mutant.

Our next question was related to the mechanisms by which the microcin could stimulate ATPase activity. This enzyme constitutes a reversible proton pump driven by ATP (2) and therefore, in addition to a direct effect of the microcin on the enzyme there is the possibility that the antibiotic collapses the proton gradient across the bacterial membrane and, consequently the ATPase would be stimulated to restore this gradient.

The transport of some amino acids such as proline and phenylalanine is known to be coupled to the electrochemical proton gradient (9), therefore we tested indirectly the effect of the microcin on this gradient by measuring amino acid transport. The uptake of amino acids in the presence of microcin stopped completely after 30 seconds, while in the controls uptake increased for several minutes. The results of Table 1 indicate that the microcin strongly inhibits the initial rate of transport of proline and phenylalanine in several strains tested. The trans-

Table 1. Effect of microcin 140 on amino acid transport

Amino acid	E. coli strain	Initial transport rate (nmoles/mg dry weight x 30 s)	
		control	with microcin
Proline	405	0.8	0.3
	AN428	1.7	0.4
	N144	2.0	0.1
Phenylalanine	405	1.9	0.1
	AN428	1.5	0.4
	N144	1.2	0.4
Glutamine	405	0.8	0.1
	AN428	2.0	0.1
	N144	1.4	0.3
Leucine	405	4.3	0.1
	AN428	1.1	0.1
	N144	5.8	0.1

Transport was measured as described in Methods. When indicated microcin was added simultaneously with the labelled amino acid to a final concentration of 16 units/ml.

port of some other amino acids such as glutamine and leucine does not depend on this gradient, rather it is coupled to phosphate bond energy, probably ATP (8). It can be observed in Table 1 that these transport systems are also inhibited by the microcin. The inhibition produced in the strains 405 and AN428 can be easily understood because the microcin depletes the ATP content of the cells. What seems more difficult to understand is that a similar inhibition is observed under conditions where the fall of ATP is mostly prevented, as in the ATPase negative strain N144 (Table 1) and in the 405 strain treated with dicyclohexylcarbodiimide (not shown).

A clarification of this unexpected result was attempted by measuring the effect of microcin 140 on another cellular function dependent of ATP. For this we studied the incorporation of (U-^{14}C) glucose into macromol-

Table 2. Effect of microcin 140 on the incorporation of glucose into cellular macromolecules.

Strain	Glucose incorporation (nmoles/mg dry weight x 5 min)	
	control	with microcin
AN428	21.1	6.3
N144	11.9	13.1

Glucose incorporation was measured as described in Methods. Microcin was added simultaneously with the labelled glucose to a final concentration of 16 units/ml

ecules. The transport of this sugar is not dependent on the proton gradient (10) but the incorporation of its radioactivity into macromolecules involves biosynthetic ATP-requiring processes. As can be observed in Table 2 the microcin inhibited this incorporation in the ATPase positive strain AN428, where ATP is depleted, but not in the ATPase negative mutant where the microcin had only minor effects on the ATP content of the cells. When longer incubation times were employed glucose incorporation was also inhibited in the mutant despite normal ATP levels and this may reflect secondary effects of the antibiotic (data not shown).

These results indicate that when ATP levels are preserved by blocking bacterial ATPase, some ATP-requiring functions are not immediately affected by the antibiotic whereas the transport systems coupled to phosphate bond energy are inhibited.

One important aspect of these results is the similarity of effects between the microcin 140 and colicin K despite their clear structural differences. Colicin K also depletes ATP levels by stimulating bacterial ATPase and inhibits proline and glutamine transport even when ATP concentration is preserved in an ATPase negative mutant (12). A possible explanation for the inhibition of glutamine transport in the presence of normal ATP levels would be a change in the membrane permeability in such a way that the amino acid can not be retained by the cells. In this regard it has recently been reported that colicin K forms voltage-

dependent channels in artificial membranes which have low selectivity (13). The movement of ions across the channels would explain the reduction of membrane potential, which has been in fact observed in bacteria treated with the colicin (14,15). The nature of the permeability changes caused by the microcin in bacterial and artificial membranes is currently under investigation in this laboratory.

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