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5-Methoxyindole-2-carboxylic acid a potent inhibitor of binding protein dependent transport in *Escherichia coli*

Gilbert Richarme

Institut Jacques Monod, Université Paris VII, 2, place Jussieu, 75251 Paris Cedex 05 (France)

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5-Methoxyindole-2-carboxylic acid a known inhibitor of α-ketoacid dehydrogenases in animal cells inhibits the binding protein dependent transport of ribose galactose and maltose while not affecting the lactose permease and the phospho*enol*pyruvate-glucose phosphotransferase; inhibition of transport occurs before a significant decrease in ATP concentration, showing that inhibition of transport does not result from ATP depletion. The binding protein-ligand interaction is not seriously affected by 5-methoxyindole-2-carboxylic acid indicating that another component of these transport systems is probably implicated in the inhibition.

Introduction

The energetics of binding protein-dependent transport systems has been the subject of controversial propositions during the past ten years. The arsenate sensitivity of these transports along with a requirement for a source of ATP be it oxidative or glycolytic, has been generally interpreted to indicate that binding protein-dependent transport is driven by ATP [1–5]. However, this interpretation has been questioned by results showing a lack of correlation between ATP levels and binding protein-dependent transport activity under certain conditions [6,7]. In addition several studies have shown that a membrane potential may be required [8,9]. In other studies acetylphosphate has been implicated in the energization of binding protein-dependent transport [10]. In the present study we show that 5-methoxyindole-2-carboxylic acid, which is known as an inhibitor of α-ketoacid dehydrogenases in animals cells [11,12], is a potent inhibitor of binding protein-dependent transport while not affecting the lactose permease [13–15] and the phospho*enol*pyruvate-glucose phosphotransferase system [16].

Materials and Methods

Bacterial strains. Escherichia coli K12 strains 3000 and AW 546K$^-$ (a galactokinase-deficient mutant obtained from S. Szmelcman) were grown aerobically at 37°C with 0.2% glucose as carbon source in minimal medium M63 [17] supplemented with thiamine (5 μg/ml) (and 50 μg/ml each of threonine, leucine, histidine and methionine for AW 546K$^-$) and the appropriate inducers, 5 mM ribose, 1 mM fucose, 5 mM maltose, 0.4 mM IPTG. Unless otherwise indicated, the experiments have been performed with strain 3000.

Transport measurements. The bacteria were washed twice at 0°C with minimal medium M63 containing 0.2% glycerol and 5 μg/ml thiamine; they were resuspended in the same medium and kept on ice. The washed bacteria were incubated for 10 min at 22°C before transport measurements. Ribose galactose and maltose transport were assayed at 22°C in a volume of 1 ml contain-

Abbreviation: IPTG, isopropyl β-D-thiogalactopyranoside.

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ing the equivalent of 50 μg of cell protein per ml. D-[^{14}C]Ribose (60 μCi/μmol), D-[^{14}C]galactose (250 μCi/μmol) or D-[^{14}C]maltose (36 μCi/μmol) was added to the assay mixture to a concentration of 2 μM. At this low concentration, galactose and ribose, for which several transport systems have been described, are transported almost exclusively through their respective binding protein-dependent transport systems [18,19]. A 200 μl sample of the assay mixture was removed from each flask at 15 s, 30 s, 60 s after the addition of the radioactive substrate, filtered on cellulose ester filters (millipore HAWP 0.45 μm pore size), washed with 3 × 1 ml of the transport medium and counted for radioactivity. The transport of methylthiogalactoside was assayed in an assay volume of 5 ml containing the equivalent of 70 μg of cell protein per ml [^{14}C]Methylthiogalactoside (2 μCi/μmol) was added to the assay mixture to a concentration of 1 mM. A 1 ml sample of the assay mixture was removed from the flask at 15 s, 1 min, 3 min after the addition of the radioactive substrate, filtered, washed and counted as described above. The transport of methyl-α-glucoside was assayed in a volume of 2 ml containing the equivalent of 70 μg of cell protein per ml. [^{14}C]Methyl-α-glucoside (1 μCi/μmol) was added to the assay mixture to a concentration of 0.3 mM. A 500 μl sample of the assay mixture was removed from the flask at 0.5 min, 2 min, 4 min after the addition of the radioactive substrate filtered, washed and counted as described already. In experiments with 5-methoxyindole-2-carboxylic acid, the inhibitor was added to the bacteria 10 min (at 20°C) before the addition of the labelled sugar.

Ligand binding activity of binding proteins. Ligand-binding activity of ribose-binding protein and galactose-binding protein were measured as described previously for the galactose-binding protein using a filter assay [20]. Ribose-binding activity was measured in a crude shock fluid prepared as described in Ref. 21. Galactose-binding activity was measured with purified galactose-binding protein prepared as described in [22].

Lipoamide dehydrogenase assay. Lipoamide dehydrogenase was measured using a toluenized cell system [23]. Bacteria were centrifuged (5000 × *g*, 10 min) at 0°C and suspended to 10 mg/ml, at 20°C, in 100 mM Tris-HCl (pH 8.0 at 20°C) which contained 5 mM EDTA; toluene was added to a final concentration of 0.1% (v/v); the bacteria were shaken (320 rev./min) for 15 min at 30°C, incubated for 1 h at 0°C, centrifuged for 15 min at 0°C and resuspended in the original Tris-EDTA buffer to 20 mg/ml. Lipoamide dehydrogenase was assayed by following the dihydrolipoate-dependent reduction of 3-acetyl-NAD at 366 nm [24]. To 900 μl of 100 mM Tris-HCl (pH 8) containing 1 mM 3-acetyl-NAD and toluenized bacteria (100 μg cell protein) 50 μl of 100 mM dihydrolipoate (dissolved in ethanol) was added; the rate of increase of absorbance at 366 nm as a function of time was taken as a measure of the activity of lipoamide dehydrogenase. No variation of absorbance was observed in the absence of dihydrolipoate or upon addition of 50 μl of ethanol.

ATP determination. The reaction mixtures were identical to those used for ribose transport except 120 μg of cell protein were used in a 1 ml volume. After 10 min incubation at 22°C, the reactions were terminated by the addition of 300 μl of ice-cold 10% perchloric acid. The acid samples were neutralized with 5 M KOH and centrifuged before use [25]. The extracts were assayed for ATP by a luciferin-luciferase assay. To a vial containing 100 μl of 100 mM Tris maleate buffer (pH 7.2), 40 μl of luciferin luciferase, 20 μl of the experimental perchloric extract was added and the chemiluminescence was measured in a LKB-Wallac luminometer 1250.

Materials. D-[^{14}C]Galactose and D-[^{14}C]methylthiogalactoside were from C.E.A., France; D-[^{14}C]maltose and D-[^{14}C]ribose were from ICN; D-[^{14}C]methyl-α-glucoside was from Amersham. 3-Acetyl-NAD, dihydrolipoate and 5-methoxyindole-2-carboxylic acid were from Sigma. The ATP monitoring reagent was obtained from LKB-Wallac. All other chemicals were reagent grade and were obtained from Sigma and Merck.

Results

5-Methoxyindole-2-carboxylic acid inhibition of ribose, galactose, maltose, methylthiogalactoside and methyl-α-glucoside transport

The effect of 20 mM 5-methoxyindole-2-carboxylic acid on the transport activity of strain

3000 is shown in Fig. 1; 5-methoxyindole-2-carboxylic acid produces a 100% inhibition of ribose transport, a 100% inhibition of galactose transport and a 30% inhibition of maltose transport while not affecting methylthiogalactoside and methyl-α-glucoside transport. The complete inhibition of two binding protein-dependent transport systems compared to the lack of inhibition of the lactose permease and the glucose phosphotransferase suggests that the inhibition is specific for binding protein-dependent transport systems. The 30% inhibition observed in the case of maltose transport compared to the complete inhibition of ribose and galactose transports reflects the lower sensitivity of maltose transport to several inhibitors which has been already observed by others [26]. The inhibition of ribose and galactose transport by 5-methoxyindole-2-carboxylic acid is stronger than the inhibition of these transport systems by other inhibitors including arsenate which is generally considered as the most potent inhibitor of binding protein-dependent transport activity [1–5].

The effect of 5-methoxyindole-2-carboxylic acid on galactose transport has also been measured in the galactokinase deficient strain AW 546 K^-; a complete inhibition of galactose transport was observed at a 20 mM concentration of 5-methoxyindole-2-carboxylic acid (not shown). This indicates that the inhibition of sugar transport by 5-methoxyindole-2-carboxylic acid is not a consequence of an inhibition of the metabolism of the transported sugar. A complete inhibition of galactose transport was also observed at a 20 mM concentration of 5-methoxyindole-2-carboxylic acid in glycerol-grown cells (not shown).

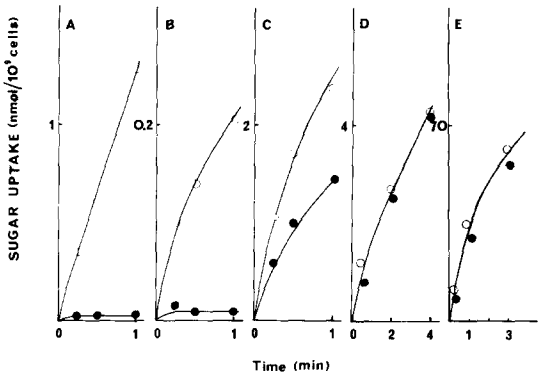

Fig. 1. Inhibition of bacterial transports by 5-methoxyindole-2-carboxylic acid. Transport of ribose, galactose, maltose, methyl-α-glucoside and methylthiogalactoside are shown respectively in panels A, B, C, D, E. The transport activity of whole cells in the absence (○) or in the presence (●) of 20 mM 5-methoxyindole-2-carboxylic acid was measured as described in Methods. 10^9 cells contain the equivalent of 350 μg of cell protein.

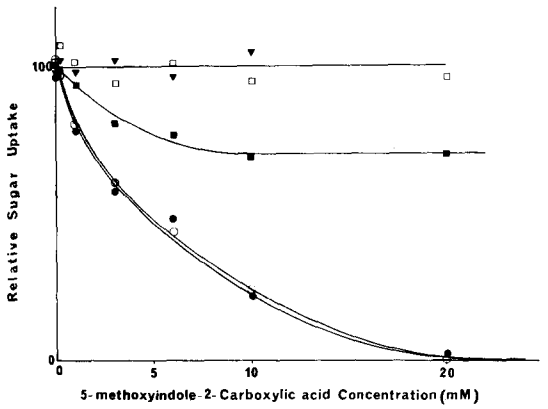

Fig. 2. Concentration dependence of 5-methoxyindole-2-carboxylic acid inhibition. Transport activity of whole cells for the substrates was measured as described in Fig. 1, and the sugar accumulated between 5 s and 1 min was taken as measurement of transport. Transport of ribose (●), galactose (○), maltose (■), methylthiogalactoside (□), methyl-α-glucoside (▼) are shown as a function of 5-methoxyindole-2-carboxylic acid concentration. The maximal rates of transport for ribose, galactose, maltose, methylthiogalactoside and methyl-α-glucoside were, respectively, 1.2, 0.21, 2.3, 62, and 4.1 nmol/min per 10^9 cells.

The dependence of transport inhibition on 5-methoxyindole-2-carboxylic acid concentration is shown in Fig. 2. For ribose, galactose and maltose transport half-maximal inhibition is observed around a 4 mM concentration of 5-methoxyindole-2-carboxylic acid. At all concentrations tested, this compound produces no inhibition of methylthiogalactoside and methyl-α-glucoside transport.

Lack of correlation between inhibition of transport by 5-methoxyindole-2-carboxylic acid and ATP concentration

Since 5-methoxyindole-2-carboxylic acid produces a decrease of the ATP pool in animal cells [27] and since a direct requirement for ATP has been suggested for binding protein-dependent

transport [1,2,4], we compared the characteristics of ribose transport inhibition with the intracellular pool of ATP. The experiments have been made with cells resuspended in minimal medium containing glycerol or lactate as carbon source. In minimal medium containing glycerol (Fig. 3A) ribose transport is 100% inhibited in less than 20 s (at this time the pool of ATP is 80% of the control value) while the pool of ATP is reduced to 15% after 2 min. In minimal medium containing lactate (Fig. 3B) ribose transport is 100% inhibited in less than 20 s whereas the ATP concentration falls to a level which is 50% of the control in 1.5 min. Both results show a lack of correlation bteween ATP levels and the inhibition of ribose transport by 5-methoxyindole-2-carboxylic acid.

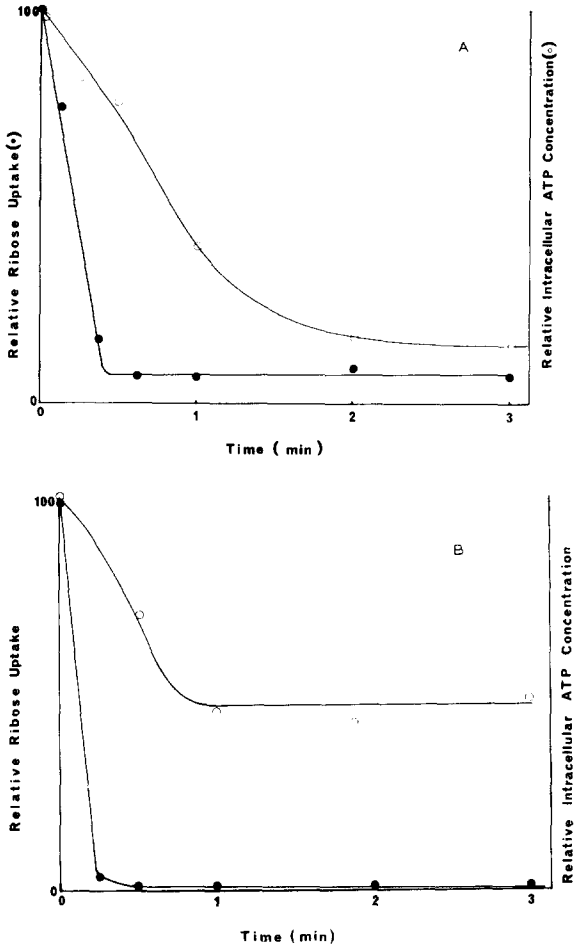

Fig. 3. Effect of 5-methoxyindole-2-carboxylic acid on ribose transport and intracellular ATP. At the times indicated after the addition of 10 mM 5-methoxyindole-2-carboxylic acid to the bacterial cells, ATP concentrations and ribose transport were measured. ATP determination commenced with the addition of perchloric acid to the bacteria as described in Methods. Ribose transport was initiated by adding radioactive ribose, and the bacteria were filtered 5 s and 10 s after the addition of ribose; experiments were made with cells suspended in minimal medium containing 0.2% glycerol (A) or 20 mM D,L-lactate (B).

Effect of 5-methoxyindole-2-carboxylic acid on the binding protein-ligand interaction

Since the binding protein-ligand interaction is the only well characterized process in binding protein-dependent transport [28] we have checked the effects of 5-methoxyindole-2-carboxylic acid on this interaction; Fig. 4 shows that, even at a concentration of 40 mM 5-methoxyindole-2-carboxylic acid, there is only a slight inhibition of ribose-binding and galactose-binding to the ribose-binding protein and galactose-binding protein, respectively; this inhibition is not likely to

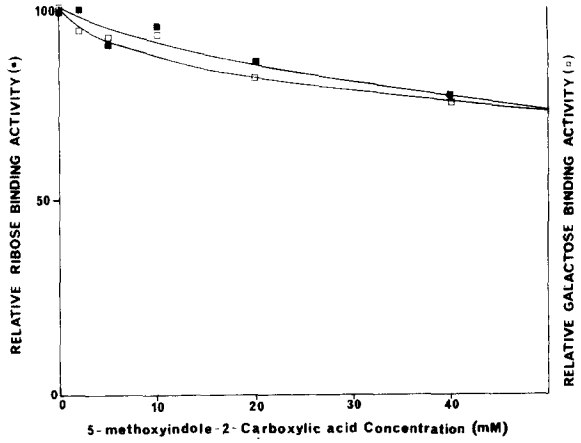

Fig. 4. Ligand binding activity of the binding proteins as a function of 5-methoxyindole-2-carboxylic acid concentration. The binding activity of the binding proteins was measured as previously described [34]. To 1 vol. of a mixture containing 100 mM potassium phosphate (pH 7.2), binding protein, radioactive ligand and 5-methoxyindole-2-carboxylic acid, 10 vol. of saturated ammonium sulfate were added at 0°C; the whole was filtered on cellulose esters filters, rinsed thrice with 1 ml of saturated ammonium sulfate and counted for radioactivity in a dioxane-based scintillation mixture. Galactose-binding activity was measured with 3 μg purified galactose binding protein and radioactive galactose at 1 μM in 500 μl. Ribose-binding activity was measured with 60 μg crude shock fluid and radioactive ribose at 1 μM in 500 μl; 50 pmol galactose and 220 pmol ribose were bound in the absence of inhibition.

account for the complete inhibition of transport. Another component of the binding protein-dependent transport systems should be the target of the inhibition by 5-methoxyindole-2-carboxylic acid, possibly one of the membrane components involved in such transport systems [29] or possibly another component involved in the coupling of these transport systems to metabolism [30].

Inhibition of bacterial lipoamide dehydrogenase by 5-methoxyindole-2-carboxylic acid

Since 5-methoxyindole-2-carboxylic acid is a known inhibitor of pyruvate metabolism in animal cells [11,12,31] where it acts as an inhibitor [12] of the lipoamide dehydrogenase component of the pyruvate dehydrogenase complex, it seemed necessary to test the sensitivity of the bacterial lipoamide dehydrogenase to 5-methoxyindole-2-carboxylic acid; as shown in Fig. 5 the bacterial lipoamide dehydrogenase activity is almost completely inhibited by 5-methoxyindole-2-carboxylic acid with a half-maximal inhibition at a concentration of 6 mM (half maximal inhibition of lipoamide dehydrogenase purified from rat liver occurs at a 3 mM concentration of 5-methoxyindole-2-carboxylic acid [12]). We have found no inhibitory effect of 5-methoxyindole-2-carboxylic acid at a 10 mM concentration on the NADH dehydrogenase activity in everted membrane vesicles of *Escherichia coli* and on the lactate dehydrogenase activity in whole cells of *E. coli* (not shown), thus indicating that lipoamide dehydrogenase may be a primary target for 5-methoxyindole-2-carboxylic acid.

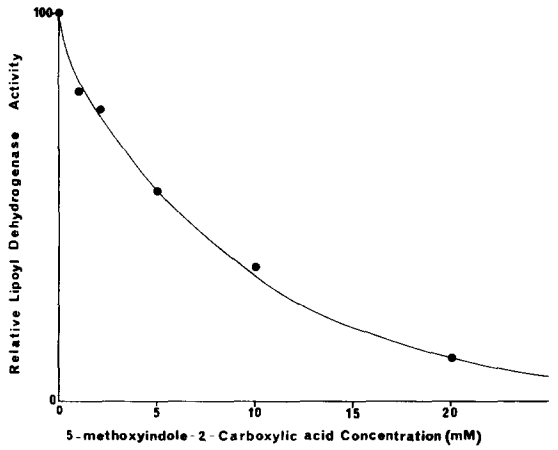

Fig. 5. Inhibition of bacterial lipoamide dehydrogenase activity by 5-methoxyindole-2-carboxylic acid. Lipoamide dehydrogenase activity was measured as described in Methods and the rate of increase of absorbance at 366 nm as a function of time was taken as a measure of the activity of lipoamide dehydrogenase. Maximal lipoamide dehydrogenase activity was 100 nmol of acetyl NADH per min per mg of cell protein.

Discussion

5-Methoxyindole-2-carboxylic acid is a strong inhibitor of binding protein-dependent transport: (i) It is the most potent inhibitor of binding protein-dependent transport activity including arsenate [1–3] and arsenite [32] which do not produce a 100% inhibition of transport as 5-methoxyindole-2-carboxylic acid does; the concentrations of 5-methoxyindole-2-carboxylic acid necessary for a complete inhibition of transport are in a millimolar range as the concentrations necessary for inhibition of purified rat liver lipoamide dehydrogenase [12] and bacterial lipoamide dehydrogenase in toluenized cells. However this similitude is difficult to discuss since the permeability of bacteria to 5-methoxyindole-2-carboxylic acid has not been studied. (ii) 5-Methoxyindole-2-carboxylic acid is a specific inhibitor of binding protein-dependent transport activity since it produces a 100% inhibition of ribose and galactose transport while not affecting the lactose permease and the phospho*enol*pyruvate-glucose phosphotransferase system. In contrast arsenate at millimolar concentrations inhibits binding protein-dependent transport by 20% to 90% [1–4,5,26] and lactose permease by 40% [33]; carbonyl cyanide *m*-chlorophenylhydrazone at concentrations close to 20 μM inhibits the lactose accumulation by 90% [34] and the binding protein-dependent transport by 20% to 70% [2,5,26]. A partial inhibition of maltose transport was observed contrasting with the complete inhibition of ribose and galactose transport. This may be due to alternative modes of energization for maltose transport. It is interesting to recall that maltose transport is much less sensitive to arsenate inhibition than ribose and galactose transport [26]. (iii) Though 5-methoxyindole-2-carboxylic acid produces a decrease of the ATP pool (probably as a consequence of inhibition of

the pyruvate dehydrogenase) there is no correlation between ATP depletion and inhibition of transport since inhibition of transport occurs before a significant decrease of ATP levels; this observation reinforces previous results showing a lack of correlation between ATP levels and binding protein-dependent transport activity [6,7]; (however, the results concerning ATP can not be extended to other phosphorylated compounds, such as acetylphosphate which have been implicated in the binding protein-dependent transport [10]). (iv) The binding protein-ligand interaction is only slightly affected by 5-methoxyindole-2-carboxylic acid, thus making it likely that another component of transport should be the target for 5-methoxyindole-2-carboxylic acid inhibition; such components are not sufficiently well characterized to permit a study of their interaction with 5-methoxyindole-2-carboxylic acid.

The known action of 5-methoxyindole-2-carboxylic acid as an inhibitor of lipoamide dehydrogenase in animal cells [12] has also been found in bacteria. Though it is not possible to say whether transport inhibition is related to this inhibition, it would be interesting to look for a possible link between binding protein-dependent transport and α-ketoacid dehydrogenases; it is of interest to note that a possible implication of (ketoacid) dehydrogenases in binding protein-dependent transport would fit with several results reported in the literature: (i) The sequence of gene *malK* which encodes a cytoplasmic membrane protein involved in maltose transport exhibits homology to the sequence of *ndh* the structural gene for the respiratory NADH dehydrogenase; this raises the possibility that oxido-reduction mechanisms might be implicated in the energization of binding protein-dependent transport [35]. (ii) A partial purification of the product of gene *malK* has shown that this protein was enriched along with four other polypeptides with apparent molecular weights of 69 000, 58 000, 32 000 and 26 000 [30]. We have checked these molecular weights as being very similar to several subunits of the pyruvate dehydrogenase complex; dihydrolipoyl transacetylase, 70 000 [36], and dihydrolipoyl transhydrogenase, 56 000 [36]. Former studies of dihydrolipoyl transacetylase have revealed molecular weights of 35 000 [37] and 26 000 [38] which could represent degraded or dissociated forms of the 70 000 component [38]. (iii) The reconstitution of the binding protein-dependent transport of glutamine in isolated membrane vesicle of *E. coli* requires the addition of pyruvate and NAD suggesting a role for some metabolite of pyruvate in transport [39,40]. (iv) Arsenite which is a powerful inhibitor of pyridine nucleotide disulfide oxidoreductases (including the lipoamide dehydrogenase component of α-keto-acid dehydrogenases) has recently been shown by us to inhibit strongly ribose transport [32]. (v) Some characteristics of the arsenate inhibition of ribose transport suggest the possibility that arsenate inhibition may be due to interaction of arsenate with a thiol group possibly after in vivo reduction of arsenate to arsenite [32]. Experiments are actually in progress (manuscript in preparation) showing a possible involvement of lipoic acid, a cofactor of α-ketoacid dehydrogenases in binding protein dependent transport. Oxidoreduction processes have been recently shown to play a radical function in several biological processes [41–43] and seem to control the affinity of the lactose permease [44], the proline permease [44] and the phospho*enol*pyruvate-glucose phosphotransferase [45].

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