A novel aspect of the inhibition by arsenicals of binding-protein-dependent galactose transport in Gram-negative bacteria

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The inhibitory effects of arsenate and arsenite on binding-protein-dependent transport systems are reconsidered. It is shown that arsenate inhibits binding-protein-dependent galactose transport in proteoliposomes energized either by dihydrolipoamide and NAD^+ or by a membrane potential (under conditions where ATP metabolism is not implicated); this result is in contradiction with the current interpretation of arsenate inhibition of binding-protein-dependent transport systems (which is based on ATP depletion) and can be explained by reference to the recently discovered ATP inhibition of the binding-protein-dependent galactose transport. In whole cells, the greater inhibition by arsenate of lipoamide-dependent transport than of protonmotive-force-dependent transport may be explained by a modification by arsenate of the pools of several compounds metabolized by 2-oxo-acid dehydrogenases (which have been implicated in binding-protein-dependent transport). The inhibition of binding-protein-dependent galactose transport by arsenite is probably linked to the inhibition by arsenite of the galactose-stimulated lipoamide dehydrogenase activity implicated in this transport and is reminiscent of the known arsenite inhibition of lipoamide dehydrogenases.

INTRODUCTION

The inhibition of the binding-protein-dependent transport systems by arsenate has been described by several groups and was interpreted as evidence for a direct involvement of ATP or a closely related molecule in the energization of these transport systems (Berger, 1973; Berger & Heppel, 1974; Curtis, 1974; Kobayashi *et al.*, 1974; Wilson, 1974; Hengge & Boos, 1983). In recent reports I have suggested that binding-protein-dependent transport systems were energized either by the oxido-reduction of dihydrolipoamide and NAD^+ or by the protonmotive force (Richarme, 1985a,b, 1986; Richarme & Heine, 1986; Richarme, 1987a,b), and I have recently shown that ATP inhibits binding-protein-dependent galactose transport in proteoliposomes (Richarme, 1987a). This new function of ATP as an inhibitor of transport demands a reconsideration of the arsenate inhibition of binding-protein-dependent transport systems. In the present study I show that arsenate inhibits binding-protein-dependent galactose transport in proteoliposomes (without any implication of ATP metabolism).

The arsenite inhibition of several binding-protein-dependent transport systems has been reported by me (Richarme, 1985a). Arsenite inhibits the lipoamide-dependent transport and does not significantly affect protonmotive-force-dependent transport. This has led me to suggest that the arsenite inhibition of transport is a consequence of the inhibition of a lipoamide dehydrogenase activity (Richarme, 1985a) involved in the energization of transport. The inhibition by arsenite of a galactose-stimulated lipoamide dehydrogenase activity implicated in galactose transport (Richarme & Heine, 1986) is described in the present study.

In whole cells (but not in proteoliposomes) arsenate inhibits more severely the lipoamide-dependent transport

than it does the protonmotive-force-dependent transport (Richarme, 1985a); this has led me to suggest that arsenate may act after its possible reduction to arsenite *in vivo*. The present results support another interpretation of this preferential inhibition of the lipoamide-dependent galactose transport in whole cells; this interpretation is based on a modification by arsenate of several pools of compounds, namely NAD^+ , NADH , CoA , acetyl- CoA , succinyl- CoA , pyruvate and 2-oxoglutarate, that are metabolites or cofactors of 2-oxo-acid dehydrogenases.

MATERIALS AND METHODS

Bacterial strains

Strain of *Escherichia coli* K12 AW546K⁻ (a galactokinase-less mutant obtained from Dr. S. Szmecman, Unité de programmation moléculaire, Institut Pasteur, Paris, France) was grown in minimal glycerol medium supplemented with threonine, leucine, histidine and methionine (50 $\mu\text{g}/\text{ml}$ each). Strains of *E. coli* K12, JRG 301 (F⁻, *lpd-1*, *trpA*, *trpE*) and T3A58 (F⁺, *trpA*, *trpE*) were kindly provided by Professor J. R. Guest (Department of Microbiology, University of Sheffield, Sheffield, U.K.) (Guest & Creaghan, 1973); they were grown in Luria-broth-rich medium (Miller, 1972), supplemented with 2 mM-fucose and 4 mM-acetate and -succinate for the preparation of the toluene-treated cells and in minimal glycerol medium supplemented with fucose, acetate, succinate and 50 μg of tryptophan/ml for the other experiments. Strain LA 5732 (F⁻, *lpd-1*, *trpA*, *trpE*, *mgl* 512 zee 700::Tn 10) transformed with plasmid pHG30 (containing the *mgl* operon of *Salmonella typhimurium*) (Müller *et al.*, 1985) was given by Dr. H. G. Heine (originally from Professor W. Boos, Fachbereich Biologie, Konstanz University, Konstanz, Germany) and was grown as described previously (Richarme & Heine, 1986).

Toluene/EDTA treatment

The toluene/EDTA treatment [100 mM-Tris(pH 8.0)/5 mM-EDTA/0.1% toluene] was similar to the treatment described by De Smet *et al.* (1978) and was described previously (Richarme & Heine, 1986).

Transport measurements

Transport measurements in whole cells and in toluene-treated cells were described previously (Richarme & Heine, 1986). A low concentration of galactose was chosen for transport measurements (0.2 μ M), since at this concentration galactose is transported mainly through the binding-protein-dependent transport system and not through the galactose permease ['GalP' transport system (Boos, 1969; Macpherson *et al.*, 1983)]. Unless otherwise stated, transport was measured during the first 20 s after galactose addition, and transport activities were calculated from the linear uptake observed during that time.

Lipoamide dehydrogenase assay

Lipoamide dehydrogenase was assayed as described previously (Richarme & Heine, 1986) by monitoring (in toluene-treated cells) the dihydrolipoate-dependent reduction of 3-acetyl-NAD⁺ at 366 nm.

2-oxo-acid dehydrogenases assay

The activities of the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes were measured with bacterial extracts from strain T3A58 as described by Guest & Creaghan (1973).

Determination of ATP

ATP was determined by the luciferin-luciferase assay as described previously (Richarme, 1985a).

Measurements of ATPase activity

ATPase activity was measured in 10 μ l of 50 mM-Tris (pH 7.5)/1.5 mM-[³H]ATP (0.4 μ Ci)/3 mM-MgCl₂. ATP and ADP were separated by chromatography on polyethyleneimine-cellulose (developed with 1 M-formic acid/0.5 M-LiCl) as described by Shlomai & Kornberg (1980).

Measurements of intracellular pools

For each determination a bacterial HClO₄ extract was prepared as follows. Bacteria (strain T3A58) were grown at 37 °C in minimal glycerol medium supplemented with 50 μ g of tryptophan/ml, collected by centrifugation in the exponential phase of growth, washed twice with 50 mM-Tris, pH 7.4, and resuspended at $A_{600} = 1$ in the same buffer. The cells were incubated 10 min at 20 °C in the presence of 3 mM-fructose (or 3 mM-fructose and 5 mM-arsenate and rapidly filtered (Millipore HAWP filter; 0.45 μ m pore size); filtration lasted less than 10 s, and the filters were put in a tube containing 1 ml of 0.3 M-HClO₄. The HClO₄ extract was centrifuged (10000 g, 10 min), adjusted to pH 7 with 2.5 M-K₂CO₃ and centrifuged again. A 200 μ l portion of the supernatant was used for the determination of the metabolite pool.

Pyruvate and 2-oxoglutarate were determined by a spectrofluorimetric assay of NADH, using lactate dehydrogenase and 2-oxoglutarate dehydrogenase respectively (Von Korf, 1969).

Determinations of CoA, acetyl-CoA and succinyl-CoA were made by spectrofluorimetric assays of NADH [in the presence of phosphotransacetylase and arsenate for acetyl-CoA and succinyl thiokinase and arsenate for succinyl-CoA (Tubbs & Garland, 1969)].

Determinations of NAD⁺ and NADH were made by spectrofluorimetric assay of NADH using alcohol dehydrogenase and lactate dehydrogenase respectively (Williamson & Corkey, 1969).

Purification of the galactose-binding protein

This was described previously (Richarme & Kepes, 1983). Arsenate or arsenite have no effect on the binding of galactose to the galactose-binding protein [measured as described previously Richarme & Kepes (1983)].

Proteoliposomes

The partial purification of the components of the binding-protein-dependent galactose transport was, as described previously (Richarme, 1987a), by osmotic lysis of bacteria (LA 5732 pHG30), extraction with Triton X-100, DEAE-cellulose chromatography and lipoyl-Sepharose affinity chromatography. The protein fraction used for the proteoliposomes had a lipoamide dehydrogenase activity of 500 nmol/min per mg of protein (ATPase activity was measured and found to be lower than 2 nmol/min per mg of protein). Proteoliposomes were made with soya-bean asolectin by the cholate-dialysis method (Kagawa & Racker, 1971) as described previously (Richarme, 1987a). Galactose transport was measured in Tris buffer [10 mM-Tris (pH 7.1)/2 mM-EDTA/5 mM-MgCl₂/0.2 mM-dithiothreitol] by filtration of the proteoliposomes (0.2 mg of protein/ml) (Millipore GSWP filter; 0.22 μ m pore size) in the presence of 1 mg of purified galactose-binding protein/ml and 10 μ M-galactose (500 mCi/mmol) at 22 °C as described previously (Richarme, 1987a). Transport was entirely dependent on the presence of galactose-binding protein and varied linearly with the amount of proteoliposomes. The amount of galactose-binding protein bound to the proteoliposomes (energized by a K⁺ diffusion potential) was measured with ³⁵S-labelled galactose-binding protein prepared as described previously (Richarme, 1982); a low value of 0.4 nmol/mg of protein was found (50-fold lower than the maximal amount of galactose accumulated by the proteoliposomes); this suggests that transport of galactose does not represent galactose bound to the galactose-binding protein (bound to the proteoliposomes). NAD⁺ (1 mM), ATP and arsenate were introduced in proteoliposomes (before transport experiments) by incubation for 10 min at 50 °C and subsequent freeze-thawing; dihydrolipoamide (1 mM) was added at the end of the 50 °C incubation and immediately before freeze-thawing and transport experiments. Membrane potential was created by a valinomycin (20 μ M)-induced K⁺ efflux from proteoliposomes loaded with 50 mM-KCl as described previously (Richarme, 1987a). The ATPase activity of the proteoliposomes was measured and found to be below the detection limit of the method.

Materials

[³H]Galactose was obtained from Amersham International and was used at 500 mCi/mmol. Asolectin (mixed soya-bean phospholipids) was obtained from Associated Concentrates (Woodside, Long Island, NY

11377, U.S.A.). The ATP-monitoring reagent was from LKB-Wallac. All other products were of reagent grade and were obtained from Sigma.

RESULTS

Arsenate inhibition of transport in intact cells is not a consequence of ATP depletion

The known inhibitory effect of arsenate on binding-protein-dependent transport has generally been interpreted (Berger, 1973; Berger & Heppel, 1974; Curtis, 1974; Wilson, 1974; Hengge & Boos, 1983) as being the result of ATP depletion (Klein & Boyer, 1972). The kinetics of galactose-transport inhibition and ATP-pool depletion after the addition of 5 mM-arsenate to bacteria are shown in Fig. 1. Arsenate produces a strong inhibition of galactose transport and a depletion of ATP levels, as reported by others (Berger, 1973; Wilson, 1974). Maximal inhibition of galactose transport occurs in less than 20 s, whereas the intracellular ATP concentration is only diminished by 15%; the latter decreases in 90 s to its final low level. Similar results were obtained with a galactokinase-less mutant (AW546K⁻); this suggests that galactose metabolism is not implicated in the inhibition of galactose transport by arsenate. The rapid inhibition of galactose transport compared with the slower decrease in intracellular ATP does not accord with the proposal that inhibition of galactose transport *in vivo* is a consequence of ATP depletion. Besides which, the recently discovered ATP inhibition of binding-protein-dependent galactose transport in proteoliposomes (Richarme, 1987a) does not support a role for ATP in the energization of transport.

Arsenate inhibits binding-protein-dependent galactose transport in proteoliposomes

I have recently reconstituted binding-protein-depen-

Fig. 1. Kinetics of arsenate inhibition in intact cells

The bacteria (strain T3A58) were washed twice at 0 °C with 50 mM-Tris, pH 7.2, containing 0.2% glycerol and resuspended at 20 °C in the same medium at a concentration of 200 µg of protein/ml. At the time indicated after the addition of 5 mM-arsenate, ATP concentrations (●) and galactose transport (○) were measured. ATP determination commenced with the addition of HClO₄ to the bacteria as described in the Materials and methods section. Galactose transport was initiated by adding radioactive galactose, and bacteria were filtered 5 s and 10 s after the addition of galactose. Transport activity was calculated from the linear uptake of galactose observed during the first 10 s.

Fig. 2. Inhibition of galactose transport by arsenate in proteoliposomes

Galactose transport in proteoliposomes energized either by dihydrolipoamide and NAD⁺ (○) or by a membrane potential (●) was measured in the presence of several concentrations of arsenate as described in the Materials and methods section. Maximal transport activities were 12 nmol/min per mg of proteoliposomal protein (energization by dihydrolipoamide and NAD⁺) and 38 nmol/min per mg of proteoliposomal protein (energization by membrane potential).

dent galactose transport of *Salmonella typhimurium* in proteoliposomes. Galactose transport is energized either by dihydrolipoamide and NAD⁺ or by a membrane potential and is inhibited by ATP (Richarme, 1987a). As shown in Fig. 2, arsenate inhibits galactose transport in proteoliposomes with a K_i of 10 mM under both conditions of energization. The inhibition of galactose transport by arsenate in the proteoliposomal system does not involve any effect of arsenate on ATP formation and suggests a direct interaction of arsenate with a component of transport. Since ATP itself inhibits transport (with a K_i of 100 µM) (G. Richarme, unpublished work), and taking into account the similarity between arsenate and ATP, we suggest that the inhibition by arsenate may be due to an interaction of arsenate with the ATP-binding site. The latter suggestion is supported by a displacement of the apparent K_i for arsenate inhibition (towards higher concentrations) in the presence of concentrations of ATP producing partial inhibition of transport (G. Richarme, unpublished work).

The arsenate inhibition observed in proteoliposomes may explain (at least partially) the inhibition observed *in vivo*. The lower concentrations of arsenate required for transport inhibition in whole cells (less than 1 mM in some experiments; Wilson, 1974) may be explained by the concentrative uptake of arsenate by phosphate-transport systems *in vivo* (Willsky & Mallamy, 1980).

Modification by arsenate of the pools of metabolites of 2-oxo-acid dehydrogenases

Whereas in proteoliposomes arsenate inhibits galactose transport to a similar extent whether energization is by dihydrolipoamide and NAD⁺ or by a membrane potential, in whole cells arsenate produces a stronger inhibition of lipoamide-dependent transport than of protonmotive-

Table 1. Modification by arsenate of the pools of metabolites related to the 2-oxo-acid dehydrogenases

The pools of metabolites were measured as described in the Materials and methods section, and calculations were done by assuming 130 μg of cell protein/ml of bacteria at an A_{600} of 1, and 5 μl of cell volume/mg of protein (Winkler & Wilson, 1966). The calculated intracellular concentration represents the total (bound + free) concentration.

Parameter	Metabolite ...	Pyruvate	2-Oxoglutarate	CoA	Acetyl-CoA	Succinyl-CoA	NAD ⁺	NADH
Metabolite extracted from untreated cells								
Pool size (nmol/mg of cell protein)		2.1	0.4	1.3	1.0	0.14	3.4	< 0.03
Intracellular concn. (μM)		400	80	260	200	28	670	< 6
Metabolite extracted from arsenate-treated cells								
Pool size (nmol/mg of cell protein)		1.2	0.14	0.14	2.5	0.57	2.7	0.14
Intracellular concn. (μM)		240	28	28	500	110	540	28

force-dependent transport. This has led me to suggest that arsenate might act after reduction to arsenite *in vivo* (Richarme, 1985a). This explanation is unlikely, since we have not found any inhibition of the galactose-stimulated lipoamide dehydrogenase by arsenate in toluene-treated cells (see below). However, the strong inhibition of the lipoamide-dependent transport *in vivo* by arsenate may be explained by a modification by arsenate of the pools of several metabolites related to the 2-oxo-acid dehydrogenases. As shown in Table 1, arsenate in whole cells (wild-type strain T3A58) produces an increase in the NADH/NAD⁺, acetyl-CoA/CoA, and succinyl-CoA/CoA ratios; these effects (especially the increase in the NADH/NAD⁺ ratio) are known to decrease activity of the 2-oxo-acid dehydrogenase complexes (Hansen & Henning, 1966; Schwartz *et al.*, 1968; Garland, 1964). The reduction of the pyruvate and 2-oxoglutarate concentrations may be important as well, since these concentrations are close to the K_m of these enzymes for pyruvate and 2-oxoglutarate (Schwartz *et al.*, 1968; Garland, 1964). The effects of the perturbation by arsenate of these metabolic pools on the activity of the 2-oxo acid dehydrogenases was ascertained by measuring the activity of these dehydrogenases *in vitro* at the metabolite concentrations found in untreated cells and in arsenate-treated cells [arsenate does not inhibit by itself the activities of 2-oxo-acid dehydrogenases complexes (results not shown)]; the metabolite concentrations were calculated as described in the legend to Table 1. For metabolite concentrations found in arsenate-treated cells, these activities were 18 and 20% (for pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase respectively) of the activities measured at metabolites concentrations found in untreated cells (results not shown). These results suggest that the strong inhibition of the lipoamide-dependent transport by arsenate *in vivo* could be exerted by an indirect inhibition of the 2-oxo acid dehydrogenases (implicated in lipoamide-dependent transport) caused by a perturbation of the pools of several metabolites of these dehydrogenases.

Effect of arsenite on binding-protein-dependent galactose transport and on the galactose-stimulated lipoamide dehydrogenase activity

The inhibitory effects of arsenite on binding-protein-dependent transport seem to be related to the involvement

of lipoamide dehydrogenase activities in these transport systems. In whole cells and in proteoliposomes, arsenite inhibits the lipoamide-dependent transport and has little effect on the protonmotive-force-dependent transport (Richarme, 1985a, 1987a). As shown in Fig. 3, 0.5 mM-arsenite inhibits the galactose-stimulated lipoamide dehydrogenase activity in toluene-treated cells and inhibits galactose transport (results not shown) [under the same conditions 5 mM-arsenate does not inhibit lipoamide dehydrogenase activities and moderately (20%) inhibits galactose transport (results not shown)].

These results suggest that the arsenite inhibition of binding-protein-dependent transport systems is related to the inhibition by arsenite of lipoamide dehydrogenase activities involved in transport. They are in agreement with the known inhibitory effect of arsenite on lipoamide dehydrogenases (Knowles & Benson, 1983; Webb, 1966).

Fig. 3. Inhibition by arsenite of galactose-stimulated lipoamide dehydrogenase activity

The lipoamide dehydrogenase activity of strain JRG 301 was measured in toluene-treated cells as described in the Materials and methods section, without any supplement (○), in the presence of 10 μM -galactose (●), in the presence of 0.5 mM-arsenite (□) and in the presence of 0.5 mM-arsenite and 10 μM -galactose (■).

DISCUSSION

Arsenate produces an inhibition of the binding-protein-dependent galactose transport in proteoliposomes energized either by dihydrolipoamide and NAD^+ or by a membrane potential. This inhibition does not involve any effect of arsenate on ATP formation and suggests a direct interaction of arsenate with a component of galactose transport. Since ATP produces a strong inhibition in the same system (Richarme, 1987a) and has a K_i 100-fold lower than arsenate (G. Richarme, unpublished work), we suggest that the arsenate inhibition mimics ATP inhibition and may reflect an interaction of a phosphate group of ATP with a component of galactose transport. This interpretation (in which arsenate mimics ATP inhibition) is different from the current interpretation, namely that arsenate, by its resemblance to phosphate, inhibits the synthesis of ATP (from ADP and phosphate) and that ATP is supposed to be the fuel for transport (Berger & Heppel, 1974); however, the implication of ATP and of the resemblance of arsenate to phosphate are common to both interpretations.

In whole cells arsenate inhibits transport before it depletes ATP; this suggests that, in whole cells, inhibition of transport is not a consequence of ATP depletion. The fast inhibition of transport by arsenate is consistent with a direct interaction of arsenate with a component of galactose transport [the limiting step would be arsenate transport, which has been shown to occur with rapid kinetics (Rosenberg *et al.*, 1977; Willsky & Mallamy, 1980)].

The inhibition of galactose transport by arsenate *in vivo* may be related to the inhibition observed in the proteoliposomal system; the low affinity of arsenate observed *in vitro* may be overcome by the concentrative uptake of arsenate in whole cells (Willsky & Mallamy, 1980); this may explain why low concentrations of arsenate (less than 1 mM) inhibit transport *in vivo* (Wilson, 1974), but not in toluene-treated cells and in proteoliposomes (the present study).

However, the above interpretation does not explain the feeble inhibition by arsenate of the protonmotive-force-dependent transport compared with its strong inhibition of lipoamide-dependent transport (Richarme, 1985a) [unless arsenate transport itself is diminished under the conditions (lipoic acid deprivation) in which protonmotive-force-dependent transport was measured (leading to lower intracellular arsenate concentrations)]. The stronger inhibition of lipoamide-dependent transport may be related to the modification by arsenate of several pools of metabolites of 2-oxo-acid dehydrogenases [which are implicated in lipoamide-dependent transport (Richarme, 1987b)]. All the effects of arsenate on these metabolic pools in whole cells (NAD^+ , NADH , CoA, acetyl-CoA, succinyl-CoA, pyruvate and 2-oxoglutarate) tend to decrease the activities of the 2-oxo-acid dehydrogenases.

Arsenite in whole cells (Richarme, 1985a) and in proteoliposomes (Richarme, 1987a) inhibits lipoamide-dependent galactose transport and does not affect significantly protonmotive-force-dependent transport. This inhibition seems to be related to the known inhibitory effect of arsenite on lipoamide dehydrogenases (Webb, 1966; Knowles & Benson, 1983), including the galactose-stimulated lipoamide dehydrogenase activity

(the present study). In contrast, arsenate does not seem to inhibit in the toluene-treated-cell system the galactose-stimulated lipoamide dehydrogenase, and my formerly held hypothesis (Richarme, 1985a), namely that arsenate could act *in vivo* after a possible reduction to arsenite, seems unlikely.

The present study and my previous results support the following model. The binding-protein-dependent transport systems would be energized either by the protonmotive force (the target of the inhibition by carbonyl cyanide *m*-chlorophenylhydrazone) or by a lipoamide-dependent reaction (the target of the arsenite inhibition); the protonmotive-force- or lipoamide-dependent reaction would affect a common reaction centre (probably the target of the inhibition by 5-methoxyindole-2-carboxylic acid) (this would explain the complete inhibition of galactose transport and ribose transport by the latter compound, compared with their partial and additive inhibitions by arsenite and carbonyl cyanide *m*-chlorophenylhydrazone) (Richarme, 1985a; G. Richarme, unpublished work); ATP would be an allosteric inhibitor of transport designed to link transport activity to the metabolic state of the cells (in a way similar to that by which it controls catabolic pathways such as glycolysis). Arsenate would be a secondary inhibitor, acting partially by analogy to ATP (a phosphate group of ATP) and partially by diminishing (through modifications of metabolic pools) the activity of the 2-oxo-acid dehydrogenases implicated in transport.

The implication of ATP as an inhibitor of binding-protein-dependent transport may be related to the existence of nucleotide-binding sites in membrane components of several binding-protein-dependent transport systems (Hobson *et al.*, 1984; Higgins *et al.*, 1985). The implication of protonmotive force in binding-protein-dependent transport systems has been suggested by several investigators (Plate *et al.*, 1974; Plate, 1979), and the implication of the 2-oxo-acid dehydrogenase is in accordance with results showing a role for some metabolite of pyruvate in glutamine transport (Hunt & Hong, 1981, 1983). The importance of oxido-reduction phenomena in transport (Barnes & Kaback, 1970; Robillard & Konings, 1981, 1982; Konings & Robillard, 1983) and in other membrane processes (Holmgren, 1979, 1981) has already been reported.

I thank Professor J. R. Guest, Dr. H. G. Heine, Dr. N. Müller, Dr. S. Szmelema and Professor W. Boos for kindly supplying the bacterial strains used in this work, and Dr. M. Kohiyama and Dr. B. Alpert for helpful discussions.

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Received 5 August 1987/2 February 1988; accepted 31 March 1988