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Inducer Expulsion in *Streptococcus pyogenes*: Properties and Mechanism of the Efflux Reaction

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Expulsion of preaccumulated methyl-β-D-thiogalactoside-phosphate (TMG-P) from *Streptococcus pyogenes* is a two-step process comprising intracellular dephosphorylation of TMG-P followed by rapid efflux of the intracellularly formed free galactoside (J. Reizer, M. J. Novotny, C. Panos, and M. H. Saier, Jr., J. Bacteriol. 156:354–361, 1983). The present study identifies the mechanism and the order and characterizes the temperature dependency of the efflux step. Unidirectional efflux of the intracellularly formed [^{14}C]TMG was only slightly affected when measured in the presence of unlabeled TMG (25 to 400 mM) in the extracellular medium. In contrast, pronounced inhibition of net efflux was observed in the presence of relatively low concentrations (1 to 16 mM) of extracellular [^{14}C]TMG. Since net efflux was nearly arrested when the external concentration of [^{14}C]TMG approached the intracellular concentration of this sugar, we propose that a facilitated diffusion mechanism is responsible for efflux and equilibration of TMG between the intracellular and extracellular milieus. The exit reaction was markedly dependent upon temperature, exhibited a high energy of activation (23 kcal [ca. 96 kJ] per mol), and followed first-order kinetics, indicating that the permease mediating this efflux was not saturated under the conditions of expulsion employed.

Carbohydrate transport in gram-positive bacteria can be governed by at least seven regulatory mechanisms which respond to distinct intracellular or extracellular signals (7, 10; J. Reizer, J. Deutscher, F. Grenier, J. Thompson, W. Hengstenberg, and M. H. Saier, Jr., Crit. Rev. Microbiol., in press). Regulation of this pivotal step can serve to maintain a fine balance between energy-consuming biosynthetic pathways and energy-producing catabolic reactions and confer the means for effective adaptation of growing bacteria to rapidly changing environmental conditions. Whereas most of these mechanisms appear to regulate carbohydrate transport by exclusion of extracellular sugars, a unique vectorial device has been shown to modulate accumulation of sugars by expulsion of intracellular sugar phosphate (9, 14). Thus, the addition of a metabolizable hexose (such as glucose, mannose, or glucosamine) to streptococcal cultures preloaded with methyl-β-D-thiogalactoside-phosphate (TMG-P) or isopropyl-1-thio-β-D-galactopyranoside-phosphate results in rapid displacement of the sugar analog from the cells and the appearance of free β-galactoside in the medium (8, 9, 14). The expulsion process in *Streptococcus pyogenes* proceeds by a two-step mechanism consisting of intracellular dephosphorylation of the phosphate ester followed by efflux of the intracellularly formed free galactoside (8). Provision of both ATP and early glycolytic intermediates appears to be required for activation of the dephosphorylation-expulsion process, and enzyme II^{Lac} of the phosphotransferase system was implicated in the efflux reaction (11). A similar expulsion phenomenon was recently demonstrated in *Lactobacillus casei* preloaded with TMG-P or xylitol phosphate (1, 2, 4). Because the phosphotransferase system provides the only route for xylitol transport in this organism, it was concluded that the enzyme II^{Xtl} catalyzes efflux of the intracellularly formed free xylitol (2, 4).

In the present study, we demonstrate that in bacteria undergoing expulsion a facilitated diffusion mechanism rather than an active transport process is responsible for efflux and promotes equilibration of TMG between the external milieu and the cell cytoplasm. In addition, the temperature dependency and the order of the exit reaction were determined; because efflux was highly temperature dependent but obeyed first-order kinetics, we concluded that the permease responsible for efflux probably functions by a mechanism involving substantial conformational changes rather than by a simple channel-type mechanism but that it is not saturated under the expulsion conditions employed.

MATERIALS AND METHODS

Growth of *S. pyogenes* type 12, loading of these bacteria with [^{14}C]TMG, and expulsion studies with the preloaded bacteria were performed as previously described (8). Preloading of resting cells (8) resulted in the accumulation of TMG-P to 25 to 35 nmol/mg of dry weight, corresponding to an intracellular TMG-P concentration range of 13 to 19 mM. Specific conditions used for individual expulsion experiments are indicated below or in the figure legends. In some cases net flux (the net consequence of movement of radioactive substrate in both directions) was measured, whereas in other cases undirectional flux (the movement of a radioactive substrate across a membrane when it is initially present on only one side) was measured. Thus, the experimental conditions employed defined the event under study.

[^{14}C]TMG was obtained from New England Nuclear Corp., nonradioactive sugars were from Sigma Chemical Co., and all other chemicals were of analytical grade from standard sources.

RESULTS

Starved cultures of *S. pyogenes* were loaded with [^{14}C]TMG-P as previously described (8). The loaded cells were washed and suspended in 50 mM Tris-maleate buffer (pH 7.2) containing 5 mM $MgCl_2$–10 mM NaF–5 mM arginine and preincubated (5 min) at the indicated assay temperature (15, 20, 25, 30, 35, or 40°C) before glucose (20 mM) was added to elicit expulsion. At 30-s intervals samples of the

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cells were collected on membrane filters, and the intracellular concentrations of [^{14}C]TMG-P and [^{14}C]TMG were determined (8). The intracellular TMG and TMG-P levels were plotted as a function of time (Fig. 1), and the expulsion process was analyzed as a two-step reaction: $[TMG\text{-}P]_{in} \rightarrow [TMG]_{in} \rightarrow [TMG]_{out}$. As expected, both the rate of dephosphorylation of TMG-P and that of efflux of free TMG were directly related to the assay temperature (Fig. 1). A similar extent of accumulation of intracellularly formed free TMG was observed at all assay temperatures up to 30°C. However, the duration of this transient accumulation period increased with decreasing assay temperature. The temporary accumulation of free TMG under these conditions reinforced our previous conclusion that expulsion comprises two distinct reactions which can be manipulated and analyzed independently (8).

The rate of accumulation of free intracellular TMG at any time during expulsion is determined by the rate of its formation by dephosphorylation of TMG-P and the rate of its removal from the cells due to efflux

$$\frac{d[TMG]_{in}}{dt} = -\frac{d[TMG - P]_{in}}{dt} - \text{efflux rate} \quad (1)$$

$$\text{efflux rate} = -\frac{d[TMG - P]_{in}}{dt} - \frac{d[TMG]_{in}}{dt} \quad (2)$$

The values of the two derivatives in equation 2 at various time points were estimated from the slopes (tangents) of the plots shown in Fig. 1, and from these data the efflux rates were calculated according to equation 2. For all temperatures except 15°C, a linear dependency was obtained when the calculated values of the rate of efflux were plotted as a function of internal TMG concentration, consistent with a first-order reaction (Fig. 2). The first-order kinetics exhibited by the exit of free TMG suggest that the permease which catalyzes efflux was not saturated under the conditions of expulsion. This conclusion is consistent with our earlier findings that demonstrated first-order kinetics for the overall expulsion process (9). Table 1 presents the first-order rate constants and corresponding half-time values of the efflux reactions obtained from the data shown in Fig. 2. An Arrhenius plot was constructed with the data in Table 1, and the activation energy of the efflux reaction over the temperature range studied was calculated from its slope (Fig. 3). The calculated activation energy, 23 kcal (ca. 96 kJ) per mol, and the corresponding temperature coefficient [$Q_{10(25-35)}$] of 3.6 indicated a high degree of temperature dependency for the permease-mediated efflux reaction.

Two distinct mechanisms may account for efflux of intracellular TMG during the expulsion process: (i) a facilitated diffusion mechanism, which can promote net efflux of TMG only from a region of higher to one of lower electrochemical potential; or (ii) an active transport mechanism, which can catalyze efflux of TMG counter to its electrochemical potential gradient. To distinguish between these possibilities, we elicited expulsion in the presence of increasing concentrations of [^{14}C]TMG in the external medium. If a facilitated diffusion mechanism were responsible for efflux, a gradual

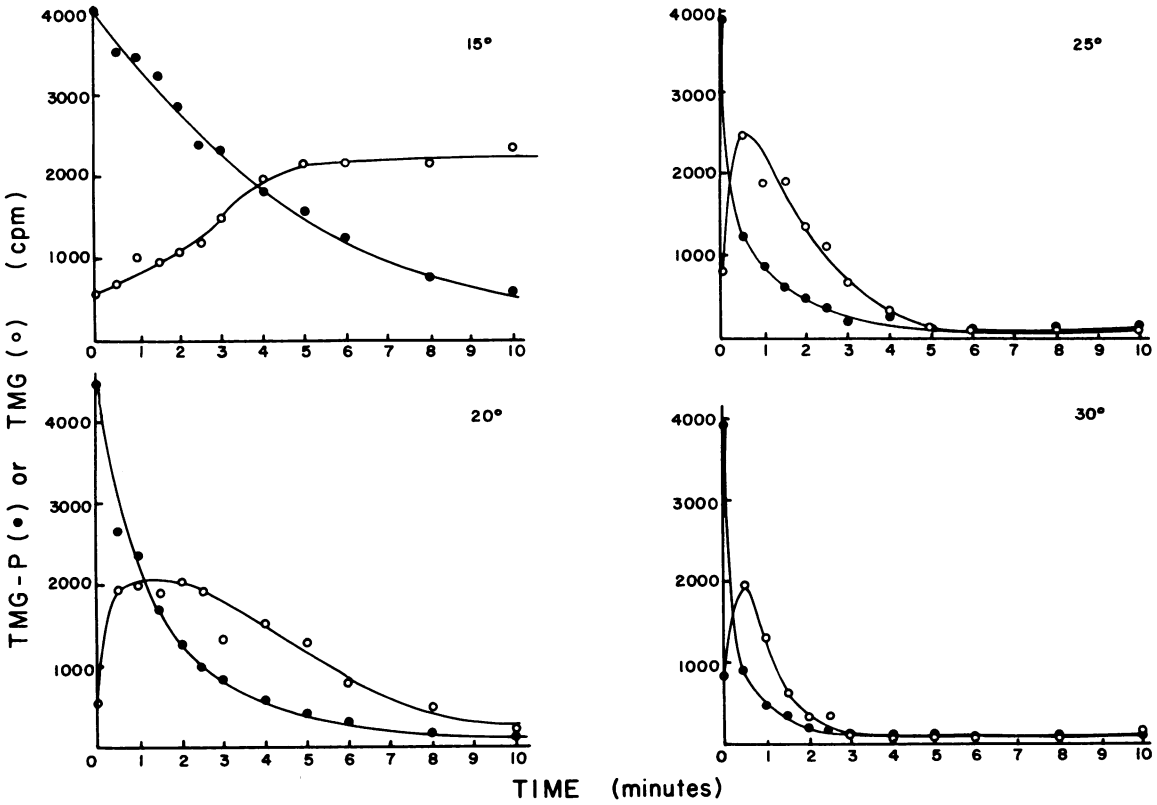

FIG. 1. Temperature dependence of expulsion. Starved cells of *S. pyogenes* preloaded with [^{14}C]TMG-P were washed and suspended in TMG-free buffer containing NaF and arginine. After equilibration (5 min) at the indicated temperatures, glucose (20 mM) was added to elicit expulsion. At t_0 (before the addition of glucose) and at 30-s intervals thereafter, samples were removed, and the intracellular concentrations of TMG-P (●) and free TMG (○) were determined as described in the text.

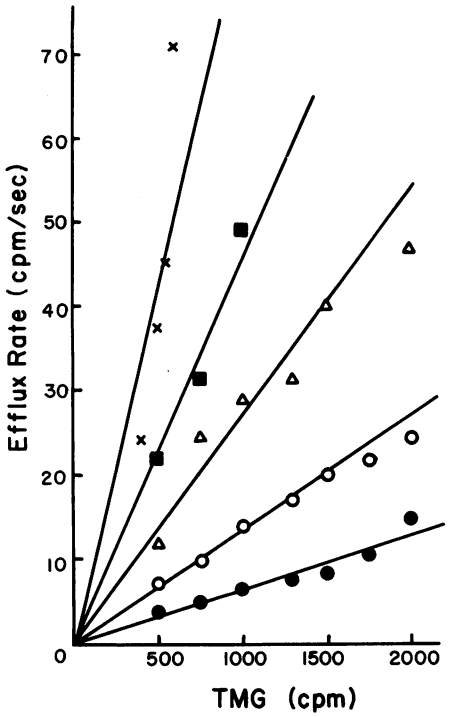

FIG. 2. Dependence of the rate of efflux on the intracellular concentration of TMG. Efflux rates at 20°C (●), 25°C (○), 30°C (△), 35°C (■), and 40°C (×) were calculated as described in the text.

increase of [^{14}C]TMG concentration in the external medium should progressively inhibit net [^{14}C]TMG efflux and arrest this reaction at the point where there are equimolar concentrations of the labeled galactoside in both intracellular and extracellular compartments. On the other hand, if an active transport mechanism were responsible for efflux, little or no inhibition by the extracellular labeled sugar would be expected even when present at a concentration equimolar to that present in the cell cytoplasm. The data presented in Fig. 4A are consistent with the facilitated diffusion mechanism, which involves equilibration of TMG across the membrane. Thus, [^{14}C]TMG at relatively low external concentrations (0.25 to 16 mM) reduced the rate of net efflux and increased the amount of intracellular sugar retained by the cells. Both these effects were directly proportional to the external concentration of [^{14}C]TMG; only a slow rate and a low extent of loss of the intracellular sugar were observed in the presence of 16 mM [^{14}C]TMG in the extracellular medium, whereas lower concentrations of external [^{14}C]TMG limited both of these parameters to a proportional degree. The results obtained for unidirectional efflux of intracellular [^{14}C]TMG are also shown for comparative purposes (Fig. 4B). The presence of unlabeled TMG (25 to 200 mM) in the

TABLE 1. First-order rate constants for efflux of TMG

Temp (°C)	k (per s)	$t_{1/2}$ (s)
15	—[a]	—[a]
20	0.0065	107
25	0.013	52
30	0.027	26
35	0.045	15
40	0.083	8

[a] —A plot of the efflux rate versus [TMG] was not linear.

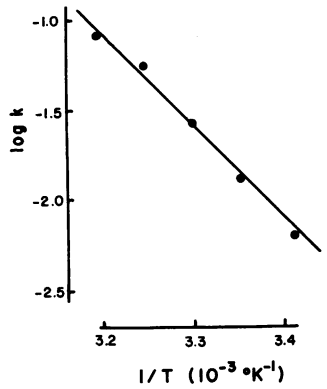

FIG. 3. Arrhenius plot for efflux of free TMG. The plot was constructed using the data in Table 1.

external medium did not accelerate efflux as would have been expected if a carrier-mediated accelerative exchange mechanism were operative. Instead, a slight inhibition (33%) of the rate of efflux was observed in the presence of 200 mM external TMG. This inhibition could be due to uptake of unlabeled TMG and subsequent competitive inhibition of the exodase. Increasing the concentration of external, unlabeled TMG to 400 mM did not result in a further inhibitory effect. Even in the presence of very high concentrations of unlabeled extracellular TMG, nearly all (more than 96%) of the [^{14}C]TMG was removed from the cells within 5 min.

DISCUSSION

Previous reports have demonstrated that expulsion of cytoplasmic sugar phosphate from gram-positive bacteria is a two-step process consisting of intracellular dephosphorylation of the sugar phosphate ester followed by efflux of the intracellularly generated free sugar (Reizer et al., in press). Efflux of TMG from *S. pyogenes* was 5 to 10 times faster than phosphorylative TMG uptake, but both processes appeared to be mediated by the lactose enzyme II of the

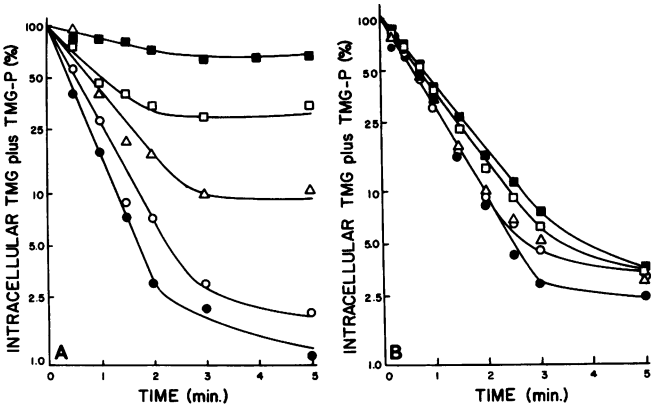

FIG. 4. Comparison of the rate of unidirectional versus net efflux of [^{14}C]TMG from *S. pyogenes*. (A) Net efflux from cells preloaded with [^{14}C]TMG-P was elicited by simultaneous addition of glucose (20 mM) and 0.25 mM (●), 1 mM (○), 4 mM (△), 8 mM (□), or 16 mM (■) [^{14}C]TMG. (B) Unidirectional efflux from cultures preloaded with [^{14}C]TMG-P was elicited by simultaneous addition of glucose and 0 mM (●), 25 mM (○), 50 mM (△), 100 mM (□), 150 or 200 mM (■) unlabeled TMG. Both experiments were performed at 37°C. The calculated initial intracellular concentration of TMG-P was 19 mM (8, 11).

phosphotransferase system (8, 11). Although the earlier reports served to define the process in general terms, the molecular mechanism was not elucidated.

In this report we present the results of initial studies aimed specifically at clarifying the mechanism of the efflux step. Efflux of intracellular TMG appears to be an energy-independent process which functions by facilitated diffusion. Thus, an equilibration mechanism rather than an active expulsion process appears to be operative. This conclusion is in agreement with previously reported results showing that uncouplers and inhibitors of the proton-translocating ATPase do not inhibit efflux (8). The rapidity of the process might lead to the suggestion that the mechanism merely involves the opening of a channel, allowing the sugar to passively diffuse out of the cell. The temperature dependency of the process argues against this possibility. The activation energy (23 kcal/mol) and corresponding Q_{10} value (3.6) for efflux are characteristic of an enzymatic process involving substantial molecular rearrangements. Since the substrate is not modified during efflux, the results lead to the proposal that substantial conformational changes in the responsible permease accompany sugar efflux. This proposal is inconsistent with a simple channel-type mechanism. The possibility that a highly temperature-dependent activation step (such as protein phosphorylation) or a change in membrane fluidity is involved has not been excluded.

Although further direct evidence for the involvement of the enzymes II in sugar efflux is needed, it appears that the enzymes II catalyze at least two rapid transport processes: group translocative sugar uptake, involving sugar phosphorylation, and facilitated sugar efflux, which does not involve sugar modification (11). This raises a number of interesting questions, the resolution of which will require further experimentation.

Our previous results (11) suggesting that enzyme II^{Lac} mediates efflux of free TMG and isopropyl-1-thio-β-D-galactopyranoside and the subsequent report of Hausman et al. (2) suggesting that enzyme II^{Xtl} mediates the analogous xylitol efflux were unexpected because it had previously been established that the enzymes II do not, in general, catalyze rapid facilitated uptake of free sugar (Reizer et al., in press). Slow uptake of free sugar via the enzymes II and acceleration of the process by mutations in the enzyme II structural genes have been demonstrated (3, 5). Thus, although most facilitated diffusion processes are equilibrating and rapid in both directions, efflux is rapid, whereas uptake of free sugar via the phosphotransferase system is normally very slow even in the presence of external sugar concentrations which are high relative to the internal TMG concentrations present during our efflux studies (6). One possible explanation for this apparent discrepancy is that the enzymes II can exist in two quite different states or conformations, each capable of catalyzing a distinct transport process. Conditions promoting one of these two processes may exclude the other. In fact, the two transport processes catalyzed by a single enzyme II have never been demonstrated under identical conditions. Development of special conditions may be required for further investigation of this phenomenon.

The following questions also remain to be answered. (i) Can all enzymes II catalyze facilitated diffusion under appropriate conditions, i.e., is the ability to catalyze sugar efflux a general characteristic of the enzymes II or is it a special characteristic of some of these enzymes? (ii) Does sugar efflux via other enzymes II (such as II^{Xtl}) also exhibit a high activation energy? (iii) Can the two transport processes be altered in parallel (i.e., by mutation or chemical modification), or do they represent two relatively independent processes involving distinct moieties and catalytic functions of the enzymes II? (iv) How does the activation energy for sugar uptake via group translocation compare with that for sugar efflux? With respect to this question, it should be noted that high activation energies for glucose or methyl-α-glucoside uptake via the phosphotransferase system have been reported in both *Escherichia coli* (19 to 25 kcal [ca. 80 to 105 kJ] per mol) (13) and mycoplasmas (36.5 kcal [ca. 153 kJ] per mol) (12).

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