Characterisation of glucose transport in the hyperthermophilic Archaeon *Sulfolobus solfataricus*

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Abstract ***Sulfolobus solfataricus*** **is a hyperthermophilic Archaeon growing at 80°C, pH 3. The glucose transport system of this organism has been characterised kinetically at this temperature and pH using 2-deoxy-D-glucose: the sugar analogue is transported into the cells with a $K_m = 1.8 \pm 0.3$ μM and a $V_{max} = 3.6 \pm 0.1$ nmol min^{-1} (mg protein)$^{-1}$, with an intracellular accumulation of up to 200-fold over the extracellular concentration. Transport was significantly reduced at pH 5. Inhibition of 2-deoxy-D-glucose transport was investigated using a variety of sugars and sugar analogues; D-glucose, D-galactose and D-mannose showed the highest affinity for the transporter, with D-glucose possessing a $K_i = 120 \pm 20$ nM.**

Key words: Archaea; Thermophile; Glucose transport; *Sulfolobus solfataricus*

1. Introduction

Sulfolobus solfataricus belongs to the phylogenetically distinct group of organisms known as the Archaea (reviewed in [1]). It is an extreme thermoacidophile typically found in geothermally heated acidic springs, with optimal growth rates at 75–80°C, pH 3–4 [2]. The organism is a facultative, sulphur-oxidising autotroph and is able to grow on simple media with a mono- or disaccharide as the sole carbon source. The pathways of glucose catabolism in two genera of the thermoacidophilic Archaea, *Sulfolobus* and *Thermoplasma*, have been well characterised (reviewed in [3,4]). In contrast, the transport of carbohydrates into these archaeal cells has hardly been investigated, although initial studies on the uptake of sugars in members of the hyperthermophilic and extremely halophilic Archaea have recently been reported [5–7].

The genes encoding sugar transporter proteins from a variety of non-archaeal organisms have been cloned and sequenced [8–10]. The alignment of the deduced primary structures has revealed a large family of related proteins from Eukarya and Bacteria, and they include transporters capable of both passive and active transport processes. In the present paper, we report the kinetic characterisation of a glucose transport system in *S. solfataricus* at 70°C, pH 3, and show that it has a remarkably high affinity for, and ability to accumulate, its substrate. We have also investigated its stereospecificity for various sugar analogues. This work forms the basis of future molecular biological studies on the archaeal transporter that, from the very nature of the organism's growth conditions, must operate at pH 3 at its external face and pH 6–7 intracellularly.

2. Materials and methods

2.1. Culture conditions

S. solfataricus (DSM 1616) was cultivated in 500 ml Erlenmeyer's flasks containing 200 ml of medium at 80°C with mechanical agitation (100 rpm). The standard culture medium comprised (per l): sucrose, 2 g; yeast extract, 1 g; $(NH_4)_2SO_4$, 1.3 g; KH_2PO_4, 0.28 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; $CaCl_2 \cdot 2H_2O$, 0.07 g; $FeSO_4 \cdot 7H_2O$, 0.028 g; $Na_2B_4O_7 \cdot 10H_2O$, 4.5 mg, $MnCl_2 \cdot 4H_2O$, 1.8 mg; $ZnSO_4 \cdot 7H_2O$, 0.22 mg, $CuCl_2 \cdot 2H_2O$, 0.05 mg; $Na_2MoO_4 \cdot 2H_2O$, 0.03 mg; $VOSO_4 \cdot 2H_2O$, 0.03 mg; $CoSO_4 \cdot 7H_2O$, 0.01 mg. The culture medium was adjusted to pH 3 with 98% H_2SO_4. Cell growth was followed turbidimetrically at 600 nm.

2.2. Preparation of cell suspension for transport assays

The cells were collected from mid-exponential phase cultures by centrifugation (800 × *g*, 15 min) and were washed twice with buffer A (mineral background of culture medium, pH 3). The cell pellet was resuspended in this buffer to a density of 1.0 mg protein/ml. The protein content of this suspension was determined, after cell solubilisation with 10 mM NaOH, by the Bradford assay [12] using bovine serum albumin as standard.

2.3. Transport of 2-deoxy-D-[2,6-^{3}H]glucose

In a standard assay procedure, the cell suspension (prepared as above) was incubated in a water bath at 70°C for 40 min. Transport was then initiated when 160 μl of this cell suspension was transfered to 80 μl of a solution of 2-deoxy-D-[2,6-^{3}H]glucose in buffer A equilibrated to 70°C. The final concentration of 2-deoxy-D-glucose assayed ranged from 0.5 to 100 μM, with each assay containing 1 μCi of 2-deoxy-D-[2,6-^{3}H]glucose (spec. act. 52 Ci/mmol). Transport was arrested after 10 to 60 s (according to the concentration of 2-deoxy-D-glucose assayed) by dilution with 3 ml of ice-cold, non-radioactive 2 mM 2-deoxy-D-glucose. The cells were rapidly filtered through glass fibre filters (Whatman GF/F, 0.45 μm) and washed with 10 ml of buffer A. Radioactivity remaining on the filters was estimated by scintillation counting. Non-specific adsorption of 2-deoxy-D-[2,6-^{3}H]glucose to the filters was estimated in a similar manner but the cell suspension was replaced with buffer.

The rate of 2-deoxy-D-glucose uptake was expressed in nmol min^{-1} (mg protein)$^{-1}$, and K_m and V_{max} values for 2-deoxy-D-glucose transport were calculated from the data using the direct linear plot [11].

2.4. Stereospecificity of the transport system

Unlabelled sugars were tested as possible inhibitors of 2-deoxy-D-glucose uptake by their simultaneous addition with the 2-deoxy-D-glucose (final concentration 0.5 μM) to the transport assay described above. The following sugars were tested at a final concentration of 25 μM: D-glucose, D-galactose, D-mannose, D-fructose, D-xylose, 1-deoxy-D-glucose, α-methyl-D-glucoside, 3-*O*-methyl-D-glucose, 6-*O*-methyl-D-galactose, 4,6-*O*-ethylidene-D-glucose and maltose. The percentage inhibition was expressed with respect to the uptake rate of 2-deoxy-D-glucose in the absence of any inhibiting sugar.

2.5. Determination of K_i for glucose

For determinations of K_i, the rate of uptake of 0.5 μM 2-deoxy-D-glucose was measured in the presence of D-glucose at concentrations

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in the range of 0.1–1 μM. The inhibition constant, K_i, was determined from the relationship $v = v_0K_i/\{K_i+[I]\}$, where v is the observed rate of 2-deoxy-D-glucose uptake in the presence of glucose at concentration [I], v_0 is the rate of 2-deoxy-D-glucose uptake in the absence of glucose, and K_i is the dissociation constant for D-glucose.

2.6. Thin-layer chromatographic analysis of transported 2-deoxy-D-glucose

Transport assays were carried out as described above using 0.5 and 10 μM final concentrations of 2-deoxy-D-glucose, for 10 s and 1 min assays, respectively. Transport was arrested by the addition of 1 ml ice-cold 2 mM 2-deoxy-D-glucose and rapid centrifugation at 10000×*g* for 10 min. The pellet was washed with buffer and resuspended in 70% (v/v) ethanol (40 μl). After centrifugation at 10000×*g* for 10 min, 20 μl of the supernatant was loaded onto a silica-gel TLC plate alongside 2-deoxy-D-[2,6-^{3}H]glucose, non-radioactive 2-deoxy-D-glucose and 2-deoxy-D-glucose 6-phosphate in separate tracks. The plate was developed in butan-1-ol/ethanol/water (62:14:24), acidified with acetic acid, for approx. 1.5 h. It was then air-dried and non-radiolabelled compounds were visualised by dipping the plate in 5% (v/v) H_2SO_4 in methanol and heating. For radiolabelled compounds, the plate was divided horizontally into 5 mm sections, which were cut and placed into scintillation vials for counting of radioactivity.

2.7. Glucose dehydrogenase assays

The activity of glucose dehydrogenase with 2-deoxy-D-glucose as substrate was measured by monitoring the reduction of $NAD(P)^+$ at 340 nm and 70°C [13]. The assays were carried out in a 1 ml solution of 100 mM triethanolamine-HCl buffer, pH 9, containing 20 mM $MgCl_2$, 5 mM NAD^+ or 0.4 mM $NADP^+$, and 2–500 mM 2-deoxy-D-glucose.

3. Results and discussion

3.1. Transport of 2-deoxy-D-glucose

The data describing the uptake of 2-deoxy-D-glucose at 70°C were analysed by the direct linear plot [11] and are displayed in Fig. 1 as a Hanes-Woolf plot. The K_m was calculated to be 1.8 ± 0.3 μM and V_{max} to be 3.6 ± 0.1 nmol min^{-1} (mg protein)$^{-1}$. Assuming a similar value for the water content of *S. solfataricus* cells to that determined for *Escherichia coli* (1.1 μl water per mg dry weight of cells; P.J.F. Henderson, personal communication), it was calculated that the 2-deoxy-D-glucose was accumulated to an intracellular concentration of up to 200-fold of that in the external medium, indicating an active transport process.

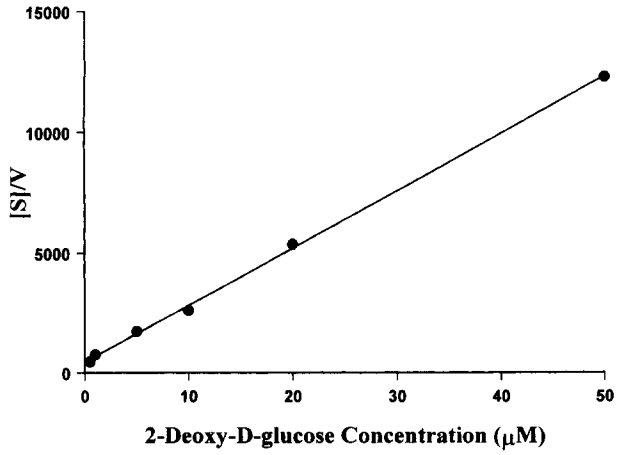

Fig. 1. Determination of the kinetic parameters for 2-deoxy-D-glucose uptake by *S. solfataricus*. Transport of 2-deoxy-D-[2,6-^{3}H]glucose was measured at 70°C and pH 3 as described in section 2. The data were analysed by the direct linear plot [11] and are displayed as a Hanes-Woolf plot. Values of substrate concentration [S] are expressed in μM, and values of uptake rate (V) are in μmol min^{-1} (mg protein)$^{-1}$.

Table 1
Inhibition by sugar analogues of 2-deoxy-D-glucose transport by *S. solfataricus* at 70°C

Sugar	% inhibition of 2-deoxy-D-glucose transort
D-Glucose	98
D-Galactose	97
D-Mannose	98
D-Fructose	7
D-Xylose	48
Maltose	0
1-Deoxy-D-glucose	55
α-Methyl-D-glucoside	0
3-*O*-Methyl-D-glucose	0
6-*O*-Methyl-D-galactose	0
4,6-*O*-Ethylidene-D-glucose	90

Uptake of 0.5 μM 2-deoxy-D-glucose was followed over a 10-s period in the presence of each sugar at 25 μM concentration.

It was necessary to determine whether the 2-deoxy-D-glucose was metabolised on entering the cells at 70°C during the assay period, since this would affect the interpretation of the kinetic data. The technique of thin-layer chromatography was applied to the radiolabelled sugar extracted from the cells after transport. The positions of the bands on the TLC plate for 2-deoxy-D-[2,6-^{3}H]glucose before and after transport showed that the majority of the 2-deoxy-D-glucose remained unaltered ($R_f = 0.67$) but that about 10% co-migrated with 2-deoxy-D-glucose 6-phosphate ($R_f = 0.08$).

Glucose dehydrogenase is the first enzyme to metabolise glucose in the non-phosphorylated version of the Entner-Doudoroff pathway in *S. solfataricus* [3,12]. Although 2-deoxy-D-glucose was found to be a substrate for the enzyme, the K_m was found to be at least 7500 times larger than the K_m observed for 2-deoxy-D-glucose transport at the same temperature. K_m values for 2-deoxy-D-glucose were determined to be 170 mM (with NAD^+ as cofactor) and 15 mM (with $NADP^+$). Therefore, in agreement with the TLC analysis, it seems unlikely that the glucose dehydrogenase will catalyse the dehydrogenation of 2-deoxy-D-glucose during the time course of the assay.

The transport was insensitive to the antibiotics forskolin and cytochalasin B. At 0.5 μM 2-deoxy-D-glucose (0.25 K_m), 10 μM forskolin did not inhibit sugar uptake, and 10 μM cytochalasin B produced < 25% inhibition. However, the proton gradient did influence transport, and at pH 5 the rate of 2-deoxy-D-glucose uptake was reduced to 37 ± 5% of that observed at pH 3.

3.2. Specificty of sugar transport

To obtain information on the stereospecificity of this transport system, several sugars and sugar analogues were examined for their ability to inhibit the uptake of 2-deoxy-D-glucose at 70°C. The results of these experiments are shown in Table 1 and indicate that the 2-deoxy-D-glucose transporter protein recognises D-glucose, D-galactose, D-mannose, D-xylose, 1-deoxy-D-glucose and 4,6-*O*-ethylidene-D-glucose as inhibitors. D-Glucose, D-galactose and D-mannose all show a similarly high affinity for the transporter and one feature common to these three sugars is the configuration of the hydroxyl group at the C-3 position on the pyranose ring. The methyl group on the C-3 hydroxyl of 3-*O*-methyl-D-glucose appears

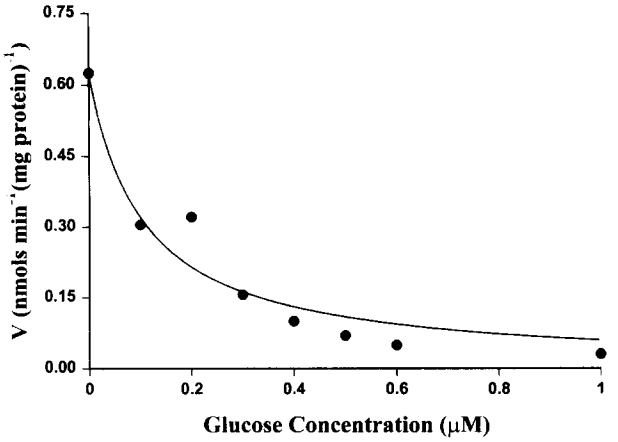

Fig. 2. Inhibition of 2-deoxy-D-glucose transport in *S. solfataricus* by D-glucose. The rate of uptake (V) of 0.5 μM 2-deoxy-D-glucose was measured in the presence of D-glucose at concentrations in the range of 0.1–1 μM, as described in section 2.

to have prevented any binding of the sugar to the transporter, suggesting that steric hindrance at this position has prevented a close approach of the hydroxyl group to the binding site.

The C-5 hydroxymethyl group is also a conserved feature in D-glucose, D-galactose and D-mannose. This group is missing in D-fructose and D-xylose in the pyranose form and is sterically hindered in 6-*O*-methyl-D-galactose. No inhibition of 2-deoxy-D-glucose transport was observed with D-fructose or 6-*O*-methyl-D-galactose. However, some inhibition of 2-deoxy-D-glucose transport was observed with D-xylose. This sugar is identical to D-glucose but for the missing C-5 hydroxymethyl group. One less interaction at this site may explain the reduced affinity of the transporter for D-xylose with respect to D-glucose. Hence, we may conclude that an interaction between this hydroxyl and the transporter may be important in the binding process.

The reduced affinity of 1-deoxy-D-glucose with respect to D-glucose may indicate an interaction between the C-1 hydroxyl and the binding site. This is also implied by the apparent failure of α-methyl-D-glucoside to bind, which again suggests that the methyl group is preventing a close approach to the transporter. However, 1-deoxy-D-glucose, which is a stable pyranose ring, does show some affinity for the transporter, suggesting that D-glucose may be transported in the pyranose form.

Finally, 4,6-*O*-ethylidene-D-glucose was observed to inhibit transport strongly despite its bulky C-4 to C-6 linkage. The C-6 oxygen may be locked into a position where it can interact with the transporter and the bulky ethylidene group may be forced away from the sugar-transporter interface.

A K_i for D-glucose inhibition of 2-deoxy-D-glucose uptake at 70°C was obtained from the data shown in Fig. 2 and was calculated to be 120 ± 20 nM. The K_m calculated for 2-deoxy-D-glucose uptake and the K_i for glucose inhibition of 2-deoxy-D-glucose uptake in *S. solfataricus* are extremely low, indicating a very high affinity of the transporter for the substrate.

4. Concluding remarks

The data reported in this paper represent the first kinetic characterisation of the glucose transport system in *S. solfataricus*. Of particular note is the very high affinity of the transporter for glucose and a number of other sugars, a property that may enable the organism to compete effectively for the carbon nutrients in hot acid environments. *Sulfolobus* species contain a protein S-layer outside the cytoplasmic membrane (reviewed in [13]); the interspace between the two might resemble the periplasmic space of Gram-negative Bacteria, although it is likely that the S-layer meshwork is too loose to retain nutrient-binding proteins [13].

The transport appears to be dependent on the proton gradient across the cell membrane, although further and more sophisticated experiments are required to determine if this is an obligatory proton-symport system or whether the transport is indirectly energised via the proton gradient [14]. Alternatively, the pH effect observed could reflect the pH dependence of the transporter protein rather than the existence of a proton-linked glucose transport system. Forskolin and cytochalasin B are powerful inhibitors (K_D values 0.1–10 μM) of some bacterial proton-linked sugar transport systems (e.g. [15]) and of some mammalian glucose transporters [16]. However, these antibiotics (at concentrations up to 10 μM) produced very weak or no inhibition of the *Sulfolobus* glucose transporter, although at this stage it is not known if the S-layer can prevent their access to the cell's membrane. Clearly, the molecular mechanism of sugar transport in this hyperthermophilic Archaeon awaits a structural analysis of the transport protein(s).

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