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Critical parameters for functional reconstitution of glucose transport in *Trypanosoma brucei* membrane vesicles

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Abstract

The glucose transporter of *Trypanosoma brucei* was reconstituted by incorporating *Escherichia coli* phospholipid liposomes into detergent-solubilised trypanosome membranes. Proteoliposome vesicles were formed by detergent dilution and used in glucose-uptake assays. The minima for functional reconstitution of the glucose transporter were established and used to probe the mechanism of glucose transport. The uptake pattern of radiolabelled glucose showed a counterflow transient at about 3 s, after which the sugar equilibrated across the proteoliposomal membrane. This observation is consistent with a facilitated transporter. There was a six-fold increase in the initial rate of glucose uptake compared to non-reconstituted or native membranes. In addition, the transporter exhibited stereospecificity to D-glucose but poorly transported L-glucose. Directionality, stereoselectivity or substrate specificity and *cis*-inhibition by phloridzin were therefore the main criteria for validation of glucose transport. The observed counterflow transient also provided further evidence for a facilitated glucose transporter within the trypanosome plasma membrane, and was the single most important criterion for this assertion. A stoichiometry of 0.78 mol of glucose per mol of transporter was estimated.

Keywords: Glucose transporter; Reconstitution; Trypanosome; Proteoliposome; Counterflow

1. Introduction

The African trypanosome *Trypanosoma brucei* relies entirely on the blood glucose supplies of its mammalian host for energy; glucose transport has been considered to be the rate-limiting step in its energy metabolism. This is thought to occur by facilitated diffusion [1–5]; others however perceive this process as an active one [6]. This discrepancy may be the result of intrinsic differences in the assay procedures used, and also the criteria used to assign the mechanism of transport. This is further complicated by the fact that the trypanosome has a compartmentalised metabolic system that involves the seclusion of the glycolytic enzymes within the glycosome [7], which is surrounded by a selectively permeable glycosomal membrane. For glucose to reach this compartment therefore, it would have to traverse the plasma membrane and that of the glycosome. Teleologically, the two membranes should have glucose transporters. Because glucose transport has often been measured in whole live trypanosomes, there is an obvious difficulty in assigning any kinetic observations to either type of transporter or transporter isoform.

Because of the complexities of the cell and the

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several confounding physiological processes which may be directly or indirectly linked to transport, several attempts have been made to study membrane/carrier proteins in isolation by reconstitution [8,9]. This involves recreating a unit bilayered membrane in as near-to-native state as possible using membrane vesicles and phospholipid and then detecting the relevant functional activity. Several techniques of reconstitution have been described to study various membrane protein functions [10–13]. There are several variables to optimise and to control for. Here we report the isolation and reconstitution of glucose transport using plasma membranes of *T. brucei*, and show that the uptake of glucose in the reconstituted transporter proximates the physiological situation. Our study is largely in agreement with previous observations [14], but we went a step further to establish the minima for reconstitution of the trypanosome transporter, and the stoichiometry of glucose transport. We have therefore established here that, at least in the plasma membrane, the glucose transporter is a facilitated one. The single most important criterion for this assertion is a counterflow transient in transport kinetics.

2. Materials and methods

2.1. Parasite growth and purification

Previously cryopreserved stabilates of *T. brucei* clone MiTat 1.1 were resuspended in 1% glucose/3% sucrose in Krebs Ringer phosphate buffer pH 8.0 (KRP, 22 mM KH_2PO_4/98 mM NaCl/2 mM KCl/1 mM $MgSO_4$). Viable trypanosomes were counted in a Neubauer chamber, and then used to infect 32-week-old inbred male Wistar rats at about $1–2\times10^7$ cells per rat, by intraperitoneal injection. At peak parasitaemias (72 h), the rats were exsanguinated under diethyl ether anaesthesia. Blood was collected into 20 ml syringes containing 200 μg/ml heparin (Sigma), and centrifuged at 2800 rpm for 10 min at 4°C in a benchtop refrigerated centrifuge. The upper layer of plasma was aspirated, and the white band of trypanosomes was collected and resuspended in KRP/glucose/sucrose. Trypanosomes were then purified from contaminating red blood cells following the method of Lanham [15]. Briefly, the cells were run down a DEAE-cellulose column (5×1.5 cm), previously equilibrated with KRP, supplemented with 10 mM glucose and eluted with the same. Elution was monitored by observing a decrease in opalescence of the effluent. The cell harvest was calculated after counting in a Neubauer chamber. The yield per rat was routinely 10^9 cells/ml.

2.2. Preparation and purification of plasma membranes

TES (*N*-tris[hydroxymethyl]methyl-2-aminoethane sulphonic acid), EDTA (disodium methylene diaminetetraacetic acid), PMSF (phenylmethylsulphonyl fluoride), leupeptin (synthetic hemisulphate), DNase 1 and 2-mercaptoethanol (2-ME) were obtained from Sigma. All analytical grade reagents were obtained from BDH. Buffers: TES/EDTA buffer, pH 7.5 (2 mM TES, 150 mM KCl, 1 mM EDTA, 1 mM 2-ME and 0.1 mM PMSF); 'swelling' buffer, pH 7.5 (1 mM TES, 1 mM 2-ME, 0.1 mM PMSF (in dimethyl sulfoxide), and 5 μg/ml leupeptin (dissolved in methanol); both protease inhibitors were added to prewarmed buffer with stirring; TES/$MgCl_2$ was essentially the same as TES/EDTA except that EDTA was replaced with 5 mM $MgCl_2$; assay buffer (100 mM sodium dihydrogen orthophosphate buffer, pH 7.4, containing 0.1 mM PMSF/0.1 mM EDTA). The procedure for the isolation and purification of membranes followed that of Voorheis et al. [16], based on the mechanical shearing of cells under osmotic swelling conditions. Trypanosomes purified above were washed with 10 ml TES/EDTA buffer by centrifuging for 10 min at 2800 rpm at 4°C. The cells were resuspended in swelling buffer and then transferred to a precooled Dounce homogeniser and then lysed with one to three strokes of the pestle. To the homogenate, 2 ml of 3 M KCl was added and then centrifuged for 10 s at $7500\times g$ at 4°C in an MSE 18 benchtop centrifuge. The resulting pellet was resuspended in 50 ml TES/leupeptin. After a repeat centrifugation as above, the pellet was resuspended in 10 ml TES/$MgCl_2$ containing 1 mg/ml DNase 1. After incubation for 5 min at room temperature, 40 ml of cold TES/EDTA was added and briefly spun as above. The resulting pellet was resuspended in 4–6 ml of 40% sucrose in TES/EDTA and transferred onto a chilled preformed 40–60% sucrose density

gradient in Beckman L65 ultracentrifuge tubes. This gradient was formed by gently layering various dilutions of 60% sucrose in TES/EDTA into the tubes using a pipette. The order of layering was as follows: 60%, 55%, 50%, 45% and 40% sucrose. This gradient was left on ice for at least 3 h before use. After layering the membrane preparation, the gradient was centrifuged for 3 h at 4°C in a swing-out SW28 rotor at $70\,000\times g$. The resulting membrane band was removed with a pipette and washed with 50 ml TES/EDTA by centrifugation at $7500\times g$ for 20 s at 4°C. The membrane pellet was resuspended in 2–3 ml assay buffer and used immediately or stored in 50 μl aliquots at −78°C.

2.3. Protein determination

This was performed by the Bio-Rad method. Membranes and bovine serum albumin standard were solubilised in 0.5% decanoylmethylglucamide, MEGA-10 (Sigma). Absorbances were measured at 595 nm. Membrane protein content was determined from a standard curve.

2.4. SDS–PAGE analysis

In order to monitor the integrity of the membrane preparation and to follow the enrichment of the transporter during the reconstitution procedure, whole membranes and reconstituted vesicles were resolved on a 10% polyacrylamide gel following standard techniques.

2.5. Reconstitution of the glucose transporter

The method of reconstitution by detergent dilution followed that of Kasahara and Hinkle [17] with some modifications. Trypanosome plasma membranes were solubilised in 5% MEGA-10 to give a final concentration of 1% detergent and 1 mg/ml of membrane protein. *E. coli* L-α-phosphatidylethanolamine, L-α-PE (Sigma), was dissolved at a concentration of 30 mg/ml in assay buffer. The phospholipid was sonicated to clarity under an atmosphere of nitrogen using a bath type Decon FS100 sonicator. The sample was centrifuged at 20 000 rpm for 30 min, at 4°C in an Ole Dich refrigerated microfuge. The solubilised membrane was similarly treated. Approximately 450 μl of the solubilised membranes was mixed with the phospholipid solution in a polypropylene tube and let stand on ice for 1 h to allow vesicles to form. The mixture was then diluted to 4 ml with assay buffer. Vesicles were used immediately or stored in 20 μl aliquots at −78°C until required.

2.6. Infinite-trans glucose transport assay

Frozen proteoliposomes were thawed slowly on ice and sonicated for 20 s. They were subsequently washed two to three times with 2.5 ml of assay buffer, by centrifuging at 20 000 rpm for 30 min at 4°C to remove detergent. The vesicles were resuspended in 500 μl of 200 mM D-glucose. After a final centrifugation, they were preloaded for 30 min in 50 μl of 100 mM D-glucose.

Transport assays were performed at room temperature, and were initiated by adding 5 μl (∼10 μg membrane protein) of preloaded vesicles to 500 μl of assay buffer containing either 4 μCi [^{14}C]D-glucose or [^{14}C]L-glucose (Amersham) and mixed rapidly. The final protein concentration in this assay mixture was 0.02 μg/μl. The mixing process produced an internal concentration of 100 mM inside the vesicles and 1 mM outside. Uptake of label was monitored over a time course of 1 min using a metronome, which gives two beats per second. All time points were run in duplicate. Each uptake was terminated with 1 ml of 0.1% phloridzin in assay buffer (hereafter referred to as stopping buffer). Background or zero time point values were obtained by adding vesicles to the stopping buffer before adding the label. After quenching, the mixture was immediately filtered (in under 5 s) through 0.22 μm Millipore filters (type GSWP) previously soaked in stopping buffer, and mounted on a filtration turret linked to a suction tap. The filters were washed two times with 10 ml of stopping buffer, and left to dry at room temperature on 3MM paper. They were then transferred to scintillation vials containing 5 ml of scintillation cocktail for counting.

2.7. Efflux assay

Reconstituted proteoliposomes (50 μl) were preloaded with [^{14}C]D-glucose (Amersham) for 10 min at room temperature. Aliquots (5 μl) were removed

and transferred into a tube containing 200 μl assay buffer supplemented with a defined concentration of unlabelled glucose (up to 100 mM). The mixture was incubated for 1 min at room temperature and the efflux of radiolabelled glucose from the vesicles was stopped by adding 1 ml of stopping buffer. The entire mixture was then rapidly filtered as above and washed two times with 10 ml of stopping buffer. The filters were air-dried and transferred to 5 ml scintillation cocktail for counting. To correct for background, 5 μl of unloaded vesicles (i.e. without radiolabelled glucose) was added directly to a vial containing radiolabelled glucose in stopping buffer. The vesicles were then filtered, washed and processed as above.

2.8. Data analysis

All data were analysed and graphical representations were performed using the GraphPad Prism version 2.0 software (GraphPad, San Diego, CA, USA).

3. Results

3.1. Characterisation of the reconstitution procedure

Protein recovery after solubilisation and reconstitution in liposome vesicles was estimated at 3–5% of total membrane protein, with a concentration of 2–3 μg/μl. However, the final concentration of membrane protein in reconstituted vesicles in the assay mixture approximately ranged from 0.02–0.06 μg/μl; this was routinely used and sufficient for all transport assays.

A number of variables that are relevant to achieving functionally reconstituted vesicles were investigated. Among these was the nature of the phospholipid. Fresh desiccated L-α-PE worked better than old lots. This was then sonicated in assay buffer

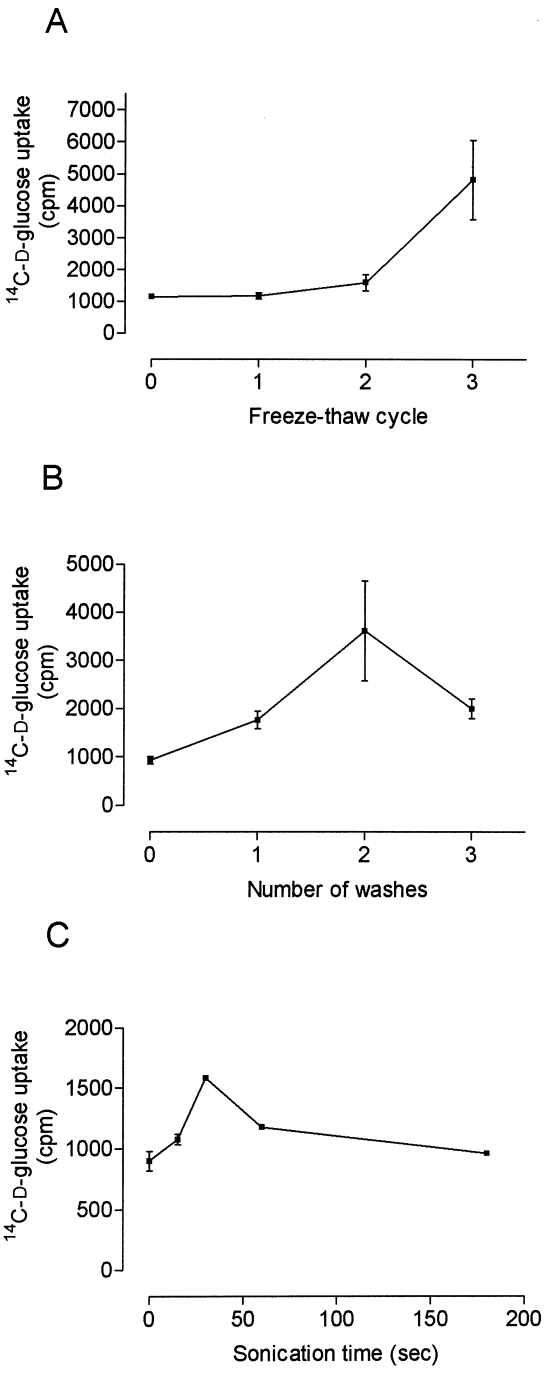

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Fig. 1. Critical parameters for reconstitution of transport activity. (A) Effect of freeze–thaw on reconstitution efficiency. Reconstituted proteoliposomes were subjected to cycles of freezing (at −78°C) and thawing in a water bath at room temperature. Following these steps, glucose uptake was measured as described above. The amount of D-glucose uptake (cpm) was expressed as a function of the freeze–thaw cycle. (B) Effect of proteoliposome washing/detergent dilution on glucose uptake. Proteoliposomes were washed one to three times with assay buffer by centrifugation as described above, and then used for transport assays. (C) Effect of sonication time on level of glucose transport. Proteoliposomes were sonicated for various times and used in transport assays. For each graph, the points represent the means ± S.E.M. of two replicates. Each figure is representative of at least three independent experiments.

under an atmosphere of nitrogen to prevent lipid peroxidation. This step was vital to a successful reconstitution. We also observed that fresh membranes were better suited for reconstitution than frozen ones.

The rate of transport was also observed to be a direct function of the number of freeze–thaw cycles that the membranes were taken through. It was found that three to four cycles were adequate (Fig. 1A). This observation probably results from the formation of larger proteoliposomes or the creation of a homogeneous vesicle population. There was also an observable increase in uptake when the vesicles were washed after detergent dilution. An optimum of two washes was permissible as transport diminished after this number (Fig. 1B). The length of sonication of the proteoliposomes was also critical (Fig. 1C). Transport was optimal between sonication times of 15 and 30 s, thereafter tailing off at 1 min and decreased sharply at 3 min. These observations are consistent with those of Kasahara and Hinkle [17] in reconstituting the human erythrocyte glucose transporter. Thus, the reconstitution procedure was performed with the above parameters optimised accordingly. In all cases in which the above parameters were being optimised, transport assays were performed over a period of 1 min.

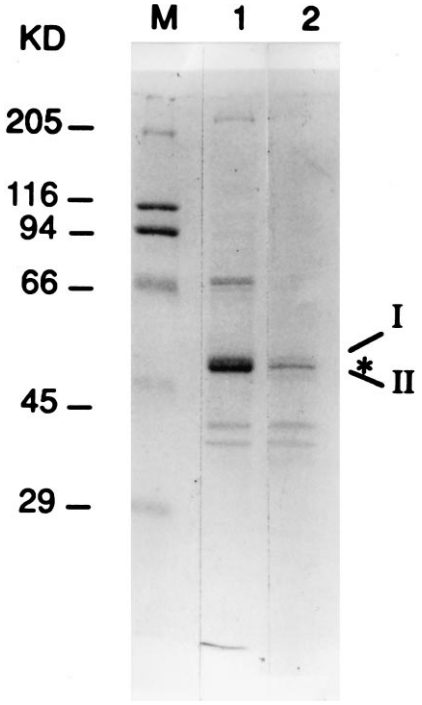

Fig. 2. SDS–PAGE analysis of total membrane proteins (1) and reconstituted vesicles (2). A putative enriched glucose transporter is indicated with an asterisk. The profile of molecular weight markers (M) is also shown.

3.2. SDS–PAGE analysis

Two groups of proteins were selectively enriched in the reconstituted vesicle fraction comprising a 35–38 kDa set and a second group running at about 50 kDa (Fig. 2, lane 2) compared with total membrane protein profile (lane 1). It is difficult to assign glucose transport function to any of these proteins in view of the potential for anomalous migration of proteins under SDS–PAGE. However, the 50 kDa protein (indicated by an asterisk) was apparently enriched. This appeared to migrate between the two prominent bands observed in the total membrane protein profile.

3.3. Transport assay and validation of transport

The uptake of glucose was measured under infinite-*trans* conditions. This involved preloading the vesicles with 100 mM glucose to serve as the 'driving' substrate. The addition of tracer/radioactive glucose to the *cis* side of the vesicles caused a very rapid uptake of this substrate, being driven by the 'cold' substrate. The 100-fold higher concentration of this substrate in the proteoliposomes induced a counterflow transient. At the point of equilibration, the exit of cold and hot substrates was equal. This equilibration point occurred within 3 s of uptake and corresponded to maximal glucose transport, and diminished over 1 min (Fig. 3A). From the time course profile, the half-time ($t_{1/2}$) of transport was approximately 1.4 s. This is in very good agreement with that obtained using live trypanosomes where a $t_{1/2} = 1.6$ s was obtained [1]. This rate of transport therefore appears to be much more rapid than for the human erythrocyte transporter which has a $t_{1/2}$ of 6 s [17]. Although unreconstituted or native membrane vesicles took up substrate in a similar fashion, that process was erratic and may be attributed to a large binding or diffusion component. In these membranes, there was a lag phase following the counterflow transient at 3 s, and then an exponential phase from 15 s upward. The absence of a measurable trap-

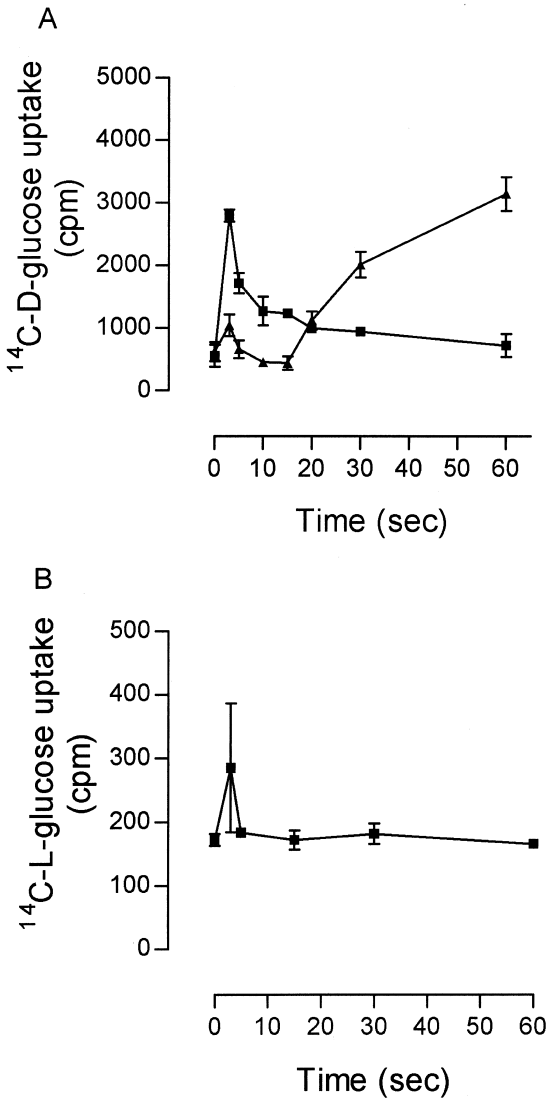

Fig. 3. (A) Infinite-*trans* glucose uptake in vesicles reconstituted with phospholipid (■), and non-reconstituted or native membrane vesicles (▲). Transport assays were performed as described above, and the amount of radiolabelled glucose trapped within the vesicles was determined as a function of time. (B) Uptake of L-glucose was determined for reconstituted vesicles as in A. Also, note that there is approximately a 10-fold difference between the amounts of D-glucose and L-glucose taken up by the vesicles. Each point represents the mean ± S.E.M. of two replicates. At least five independent reconstitution and transport assay experiments were performed. The above are representative of these experiments.

ping volume possibly accounted for this observation. It also resulted in a three to six-fold less uptake, i.e. reconstitution enhanced glucose uptake by up to six times at the counterflow transient. This value is consistent with that obtained from the reconstitution of the human erythrocyte glucose transporter [17–19]. It is significant however that the transporter recognised its substrate even in the native membrane vesicles, although the initial rate of transport was much slower than in reconstituted vesicles. For example, in native membrane vesicles, it took 1 min to trap an equivalent amount of tracer compared with 3 s for reconstituted vesicles.

The susceptibility of the transporter to specific inhibition by phloridzin was another justification for transport (data not shown). Addition of this inhibitor to vesicles before adding tracer abolished uptake of the latter at $t = 0$, which is in agreement with its inhibition of glucose uptake in live trypanosomes. The transporter was also incapable of binding and translocating L-glucose (Fig. 3B) with the same level of specificity or competence as it did D-glucose. As further evidence of specificity, an efflux assay was performed. The release of tracer by the vesicles appeared to be non-random, in response to a concentration differential between the internal and external milieu of the vesicles (Fig. 4). This shows that the rate of efflux of radioactive substrate is determined by the concentration of external cold glucose; the

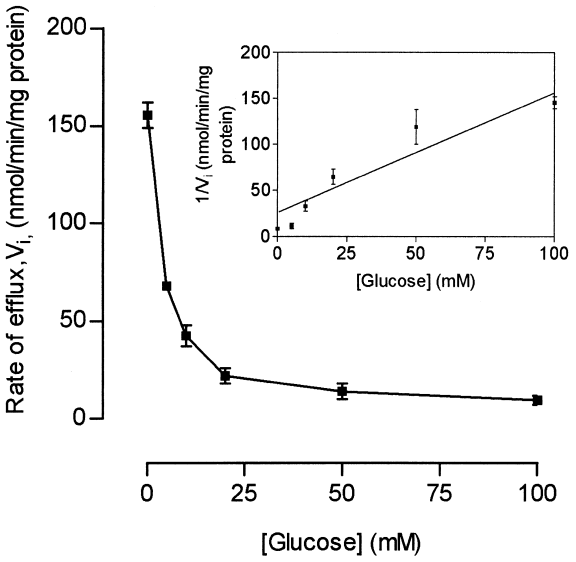

Fig. 4. Efflux of radiolabelled glucose from reconstituted vesicles as a function of external glucose concentration. The inset is a Dixon plot of the rate of exit of glucose from preloaded vesicles, with an estimated $K_m = 13.73 \pm 2$ mM. This is representative of at least three independent experiments. Each point represents the mean ± S.E.M. of two replicates.

higher this concentration the smaller the rate of efflux from the proteoliposome vesicles, and may suggest that external substrate inhibits the efflux of the trapped tracer glucose. A Dixon plot showed that the K_m for efflux was about 13.73 ± 2 mM (Fig. 4, inset), and differs from the K_m for influx under both zero-*trans* and infinite-*trans* conditions, where this value is considerably lower [1,5,14]. This means simply that inward flux of glucose mediated by the parasite transporter is more favoured than outward transport, a fact that may be teleological.

3.4. Estimation of stoichiometry of transport

To estimate the stoichiometry of glucose uptake, the rates of transport were calculated at various concentrations of glucose. The logarithmic values of rates of transport were then plotted against the log-[glucose], and the stoichiometry was estimated from the correlation coefficient to be 0.789 ± 0.038 mol of glucose per mol of transporter (Fig. 5). This is similar to that observed for the phosphotransferase system and the arabinose transporter of *E. coli* [20,21]. It also correlates well with the stoichiometry of 0.5 mol cytochalasin B (cyt B) per mol of the human erythrocyte glucose transporter [19]. This last comparison is made on the basis of the fact that cyt B is a competitive inhibitor of glucose in GLUT1 transport.

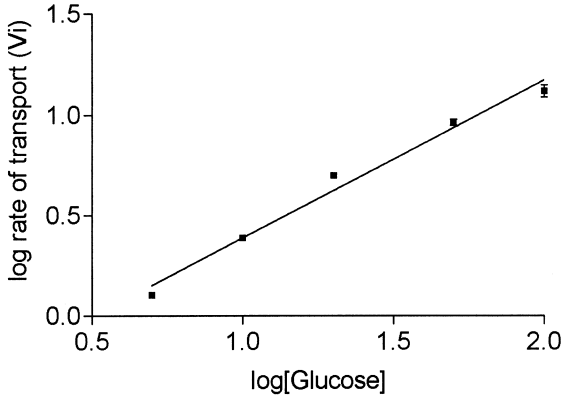

Fig. 5. Estimation of stoichiometry of glucose binding and transport. Log values of rates of glucose transport were plotted against log of glucose concentration. The stoichiometry was estimated from the correlation coefficient based on the equation $y = mx + c$. Three independent experiments were performed, of which the above is representative. Each point represents the mean ± S.E.M. of two replicates.

4. Discussion

The literature on the reconstitution of intrinsic membrane proteins is replete with methods of isolation, solubilisation, and incorporation into liposomes, but there is no consensus on an ideal and generally applicable approach [22]. Nonetheless, the approach presents a means for assaying a particular functional attribute of a cell separated from other cellular functions or structures. This is a more preferred method than the 'black box' approach. However, any functional observations must correlate directly with the whole cell regarding specificity. The objective for any reconstitution procedure would therefore involve the determination of the components or minima required to reconstitute function. This is in view not only of the plethora of variables mentioned above, but also assuring right-side-out, unidirectionally oriented intact vesicles, and a homogeneous vesicle size or population, optimal protein to lipid ratios, etc. While some of these variables were not confounding in our study, others were less easy to control for such as the size of the vesicles. It is not only difficult to obtain a homogeneous vesicle population, but also to ensure a measurable trapping volume. For instance, a vesicle of 25-nm diameter has an internal volume of 2×10^{-8} ml, and has the capacity for only one molecule of solute at a solute concentration of 1 mM [23]. Equally limiting is the integrity of the vesicles. Leaky vesicles are of no use in transport assays, for example.

Membrane proteins have a uniform vectorial orientation in vivo, and reconstitution frequently results in randomisation largely due to inappropriate lipid:protein ratios. Although lipid:protein ratios for several membranes are known, it has not been possible to achieve such ratios in reconstituted vesicles. Solubilisation with detergent also sets limits to these ratios. Even though the amphiphilic nature of integral membrane proteins favours micelle formation, it is very difficult to ascertain the critical micelle concentration required for vesicle formation, and to assure their stability once formed. This inevitably depends on the nature of the detergent used, as this would ultimately determine the lipid:protein ratio to achieve functionally reconstituted vesicles. Hence, solubilisation of both lipid and protein based on appropriate detergent:lipid ratios is an essential feature

of any solubilisation/reconstitution strategy. Unfortunately, the choice of detergent is largely empirical and therefore leads to variations in reproducibility. Our choice of detergent was based on two main criteria: (a) it should have a high critical micelle concentration that would facilitate its removal by dialysis or dilution, and (b) it should be non-ionic and non-denaturing, thereby causing minimal disruption to the membrane protein structure. These two criteria are fully satisfied in MEGA-10 [24,25]. This is a mild detergent that is easily removed by dilution and a few steps of washing, and does not interfere with absorbance measurements of protein concentration. The essence of the detergent dilution reconstitution procedure described here is the reduction of detergent concentration below levels at which it can interfere with solute uptake by the vesicles, i.e. below its critical micelle concentration.

In this study we have provided further evidence for a trypanosome glucose transporter that satisfies all the criteria for a facilitator of glucose uptake. This transporter showed rapid kinetics similar to those observed in live trypanosomes; it also exhibited stereoselectivity or specificity with respect to D-glucose, and *cis*-inhibition by phloridzin (data not shown). Crucially, the countertransport or counterflow transient observed in response to a *trans*-stimulation by glucose, was the principal basis for the validation of transport. This observation is in agreement with theoretical or computer simulations for the simple carrier exhibiting facilitated diffusion [26]. Because this phenomenon is transitory, only initial rates can be measured in vitro (see Section 3.3). Although this study asserts that the trypanosome plasma membrane glucose transporter is facilitative (based on counterflow), other reports suggest that glucose transport in the related kinetoplastid *Leishmania donovani* may be an active one [27,28]; a similar conclusion was derived for *Plasmodium yoelii* [29].

Reconstitution of the trypanosome transporter allowed us to simultaneously observe the associated processes of glucose recognition and binding, translocation and accumulation within the proteoliposomes. The rapid initial phase of uptake may comprise the binding and translocation phase, while the plateau phase probably indicates the release of glucose and subsequent restitution of the transporter. By this deduction, it is assumed that the transporter is mobile and that the kinetics of transport are determined by only the vectorial components, viz., translocation of glucose and restitution of the transporter. At the saturation transient, these components are at a steady state in which the transporter is rapidly recycled *cis-trans*. This assay also enabled a proximate determination of the stoichiometric relationship between the glucose substrate and its transporter, which is close to equimolar (0.789:1). Since some of the transporter molecules may have been partially inactivated as a result of the nature of the reconstitution process or due to its partially impure form or for reasons of sidedness, etc., this value may be taken as the lower limit of the stoichiometry.

An important derivative of this study relates to the interdependence between bloodstream trypanosome energy metabolism and glucose uptake. The rapid kinetics of glucose flux are consistent with the high rate of metabolism of this substrate. In an earlier study (unpublished data), it was observed that the rate of glucose oxidation, as measured using an oxygen electrode, was about 20–50-fold higher than in mammalian systems, and was in agreement with previous findings [30,31]. Thus, there appears to be a tight link between the transport and metabolism of glucose in the African trypanosome. Rapid oxidation of glucose means that its intracellular concentration is permanently low, thus ensuring a sustained concentration gradient for facilitated glucose uptake to occur. This may be the basis for energy flux within the trypanosome.

Acknowledgements

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