Transport of α -Aminoisobutyrate into *Trypanosoma brucei brucei*

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The uptake of α -aminoisobutyrate (AIB) by washed cell suspensions of bloodstream forms of *Trypanosoma brucei brucei* has been shown to be an energy-dependent process. No metabolism of AIB was detected under conditions leading to a 100-fold accumulation of AIB within the organism. Kinetic studies revealed that AIB uptake involved two components; that operating at low substrate concentrations had an apparent K_m of 4.6 mM. Experiments with ionophores such as gramicidin and carbonylcyanide *m*-chlorophenylhydrazone were consistent with the AIB uptake system operating as a H^+ -symporter responding to the electrochemical gradient of H^+ , the major component of which was the membrane potential.

INTRODUCTION

The mechanisms of energy-coupling to active transport mechanisms in bloodstream forms of trypanosomes are currently unknown. Amino acid transport has been studied in some detail by Voorheis (1971, 1977, 1980), who has shown that at least four distinct systems operate for these compounds. It was proposed, following studies on threonine uptake, that a proton electrochemical gradient ($\Delta\bar{\mu}_{H^+}$) or an alkali-metal cation gradient did not play a significant role in amino acid accumulation but that a glycolytic intermediate played a key role (Voorheis, 1980).

Previous experiments, concerned with the transport of the non-metabolizable amino acid α -aminoisobutyrate (AIB) into the trypanosomatid *Crithidia fasciculata*, have produced results consistent with the operation of a proton-symport system for the active transport of this compound (Midgley, 1978). Further evidence for this was the demonstration of a significant, metabolically-generated membrane potential ($\Delta\psi$) in this organism (Stephenson & Midgley, 1980). Such a potential often constitutes the major component of an ion electrochemical gradient, e.g. $\Delta\bar{\mu}_{H^+}$ or $\Delta\bar{\mu}_{Na^+}$ (Eddy, 1978; Hamilton, 1975).

Recent work has provided evidence for the existence of an appreciable $\Delta\psi$ (-130 mV) across the cytoplasmic membrane of *Trypanosoma brucei brucei* (Midgley, 1983*a, b*). The maintenance of this $\Delta\psi$ was sensitive to ionophores such as gramicidin and tetrachlorosalicylanilide (TCS), suggesting that it arose as a result of electrogenic H^+ extrusion (Midgley, 1983*a*). Thus bloodstream forms of *T. b. brucei* probably maintain an appreciable $\Delta\bar{\mu}_{H^+}$ and this has prompted an examination of the role of this gradient in energy-coupling for AIB transport in this organism. Initial experiments were concerned with the general characteristics of AIB transport in order to justify its use in studies of energy-coupling.

METHODS

Growth and harvesting of the organism. The organism used was *Trypanosoma brucei brucei* 8/18 maintained, grown for experimental work in laboratory rats and harvested as previously described (Midgley, 1983*a*). Buffer solutions based on 4-(2-hydroxyethyl)-1-piperazine propane sulphate (HEPPS) were used for the resuspension of the organism for transport assays and related studies. The composition of the medium designated Na/HEPPS/glucose

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TCS, tetrachlorosalicylanilide; TPP, tetraphenylphosphonium ion; AIB, α -aminoisobutyrate.

has been specified (Midgley, 1983*b*); K/HEPPS/glucose had the same composition except that Na^+ was replaced by K^+ . A third version of this buffer was designated Tris/HEPPS/glucose and had the following composition: 128 mM-Tris base; 132 mM-HEPPS; 50 mM-glucose. The composition of the buffer designated PBSC has been described (Midgley, 1983*a*).

Measurement of AIB uptake. This was measured using a rapid filtration technique as previously described for $\Delta\psi$ measurements with this organism (Midgley, 1983*b*). The basic incubation system has been described (Midgley, 1983*b*). To vary the cation present, the organisms were harvested in Tris/HEPPS/glucose and diluted ~20 fold into the appropriate buffer.

Cells were preincubated for between 1 to 5 min prior to initiating transport by the addition of AIB. In experiments using ionophores or inhibitors, these were included in the preincubation period and were added as previously described (Midgley, 1983*a*). Initial rates were computed from duplicate incubations sampled over time intervals for which uptake was linear, generally during the first 20 min of uptake. Uptake was corrected for extracellular and filter binding using a control incubated on ice.

Respiration studies. These were carried out as previously described using the appropriate buffer system (Midgley, 1983*a*).

Extraction and identification of the intracellular product of AIB accumulation. This was based on the previously described method (Midgley, 1978) in which cells that had accumulated [^{14}C]AIB were treated, after washing on a filter, with ice-cold 60% (v/v) ethanol. This procedure released >96% of the radioactivity present within the cells. The extract was concentrated by rotary evaporation and applied to the chromatographic and electrophoretic systems previously described (Midgley, 1978). Standards and radioactivity were detected as previously described (Midgley, 1978).

Protein determination. The method of Lowry was employed using cells digested in 1 M-NaOH for 10 min at 100 °C.

Chemicals. These were obtained from sources previously specified (Midgley, 1983*a*). Radiochemicals were obtained from Amersham.

RESULTS

General characteristics of AIB transport

Preliminary experiments using the Na/HEPPS/glucose system, established that when washed cell suspensions of *T. b. brucei* 8/18 were incubated at 25 or 37 °C in a simple buffered system of adequate osmolality, glucose-dependent uptake of [^{14}C]AIB could be observed (Figs 1 and 3). As shown, uptake at 25 °C was linear for the time period studied whereas at 37 °C the amount accumulated reached an apparent plateau and subsequently declined. This phase of decline occurred to a varying extent with different batches of the organism and probably reflected the acknowledged lability of the bloodstream forms at 37 °C once removed from the host, rather than a characteristic of the transport system. Glucose-dependent respiration, which was used as an index of cell stability, decreased rapidly at 37 °C. The respiration of two batches of organisms [respiring initially at 160 and 130 $\mu\text{mol O}_2 \text{ min}^{-1}(\text{g protein})^{-1}$ respectively] declined to 50% of the initial values in 60 min and 35 min. Greater stability was observed at 25 °C. However, since observations at 37 °C were considered more relevant to bloodstream forms, the majority of experiments were conducted at this temperature.

Disruption of the cellular permeability barrier by the inclusion of nystatin (10 $\mu\text{g ml}^{-1}$) or Triton X-100 (10 $\mu\text{g ml}^{-1}$) in the incubation medium abolished AIB uptake. The intracellular material accumulated after incubation with AIB under conditions as specified in Fig. 1 was isolated as described in Methods. This material had the same mobility as authentic AIB when tested in the systems described in Methods. Thus AIB was not metabolized significantly by the organisms under the conditions employed. Using a value for the cell water content of ~2 ml (g protein) $^{-1}$ derived from published data (Voorheis, 1971), the peak accumulation value observed at 37 °C in Fig. 1 corresponded to an intracellular concentration of 10.3 mM and an accumulation ratio of ~100. The specific uptake rate was independent of protein concentration over the range 0.2 to 2 mg protein ml^{-1} .

Kinetic studies of AIB transport

Studies on the initial rate of AIB transport demonstrated the contribution of at least two components (Fig. 2). The apparent K_m for the saturable component was $4.6 \text{ mM} \pm 1.1 \text{ mM}$ ($n =$

Fig. 1

Fig. 2

Fig. 1. The uptake of α -aminoisobutyrate by *T. b. brucei* 8/18. Transport was measured in duplicate in the Na/HEPPS/glucose system using an AIB concentration of $100 \mu\text{M}$ ($4 \mu\text{Ci } \mu\text{mol}^{-1}$) and a cell density of $0.36 \text{ mg protein ml}^{-1}$; $\blacktriangle$, 37°C ; $\bullet$, 25°C .

Fig. 2. Double reciprocal plot for AIB transport by *T. b. brucei* 8/18. The initial rate of AIB transport was measured over a concentration range of 0.5 mM to 40 mM in the Na/HEPPS/glucose system as described in Methods.

5) while the apparent V_{max} ($\pm\text{SD}$) observed for this system with different batches of the organisms was $8.6 \pm 3 \mu\text{mol min}^{-1} (\text{g protein})^{-1}$ ($n = 5$). The behaviour of the system detected at high AIB ($> 15 \text{ mM}$) was compatible with either entry by simple diffusion or the operation of a mediated system with a high K_m relative to the concentration range employed, since the Lineweaver-Burk plot for this range extrapolated through the origin. Thus for this system AIB transport was directly proportional to AIB with a proportionality constant ($\pm\text{SD}$) of $0.46 \pm 0.23 \mu\text{mol min}^{-1} (\text{g protein})^{-1} \text{ mM}^{-1}$ ($n = 5$).

The specificity of the system operating at low AIB concentration was investigated in competition experiments with other amino acids using an AIB concentration of 0.5 mM (Table 1). At this concentration the flux via the saturable system constituted 60 to 84% of the total flux measured. The results were consistent with the saturable component being one of the neutral amino acid transport systems described by Voorheis (1971). The demonstration that valine acted as a competitive inhibitor with an apparent K_i of 1.5 mM (data not shown) was consistent with this interpretation.

Energy requirement for AIB transport

Bloodstream forms of *T. b. brucei* are dependent upon a supply of external substrate, such as glucose, for viability (Lumsden *et al.*, 1973) and this was included in all buffers used in harvesting since otherwise, suspensions showing little metabolic activity were produced. The role of energy generation in the uptake of AIB was assessed by preparing cell suspensions in the usual manner and immediately before use preparing a further suspension in the absence of glucose, compensating for the osmolarity of the medium by use of an increased buffer concentration. AIB transport was glucose-dependent (Fig. 3). Also shown is the inhibitory effect of 2-deoxyglucose. The initial rate of AIB transport was inhibited 50 to 70% by 1 mM -salicylhydroxamate, a concentration that abolished respiration and diverted glucose metabolism to an alternative mode generating less ATP (e.g. Voorheis, 1980). If inhibitory conditions that were too severe were employed, e.g. a combination of salicylhydroxamate plus glycerol (Brohn & Clarkson, 1978) or 2-deoxyglucose in the absence of glucose, then the organism lysed rapidly. In the experiments presented here, lysis was assessed by measuring the turbidity of the cell suspensions and did not account for the observed inhibition of AIB transport, since lysis occurred only to the extent of 10 to 20% in a 30 min incubation period in the absence of glucose.

Table 1. *Specificity of α -aminoisobutyrate transport by *T. b. brucei* 8/18*

Transport was measured in the Na/HEPPS/glucose system. Presumed competitors were added 10 to 15 s prior to AIB (0.5 mM, 1 μ Ci μ mol). Initial rates were calculated from four samples removed in the 10 min period after AIB addition. The values presented are the means of duplicate experiments with two independent batches of the organism. All the amino acids were of the L configuration. The absolute values of the control rates were 0.44 and 0.65 μ mol min⁻¹ (mg protein)⁻¹.

Amino acid	Concn (mM)	Relative rate of AIB transport
None	—	100
Serine	5	42
	25	16
Threonine	5	44
	25	17
Valine	5	31
	25	13
Lysine	5	103
	25	82
Glutamate	5	69
	25	30
Phenylalanine	5	30
	25	27
Alanine	5	32
	25	12

Mechanistic studies of AIB transport

The mechanism of energy-coupling to AIB transport was investigated by examining the effects of changing the composition of the medium used in the transport assays. Thus the buffer system used contained as cation either Na⁺, K⁺ or trishydroxymethylammonium (Tris⁺). The organism was harvested and resuspended in Tris/HEPPS/glucose and then diluted so that the major cation was Na⁺, K⁺ or Tris⁺. In a series of experiments the Na⁺- and K⁺-systems showed equivalent rates of AIB uptake, while the Tris⁺-system showed rates that were somewhat lower (Table 2). When initial rates of AIB transport were compared in the Na/HEPPS system and the PBSG system, a traditional medium for handling trypanosomes (Lumsden *et al.*, 1973), the rates obtained in the PBSG system were 60 to 76% of the rates in the Na/HEPPS system. Thus no stringent cation requirement for AIB transport emerged from these studies.

A second approach was to use the ionophores gramicidin and valinomycin and the protonophores CCCP and TCS to eliminate specific electrochemical gradients of ions across the cytoplasmic membrane of the organism (Fig. 4). The uptake of AIB, measured at 105 μ M-AIB, was severely inhibited by 30 μ M-CCCP or 5 μ M-TCS, independently of whether the cation present was K⁺ or Tris⁺ (Fig. 4*c, d*) or Na⁺ (data not shown). When K⁺ was the cation present AIB transport was severely inhibited by the addition of 1 μ g gramicidin ml⁻¹ or 1 μ g valinomycin ml⁻¹ (Fig. 4*a*); when Na⁺ was the cation present transport of AIB was inhibited by gramicidin but insensitive to valinomycin (Fig. 4*b*). Such behaviour would be expected for a transport system driven by the $\Delta\psi$. When the sensitivity of the initial rate of AIB transport to gramicidin or valinomycin was tested in the Tris/HEPPS system the following rates, expressed as μ mol AIB min⁻¹ (g protein)⁻¹ $\pm$ SD ($n = 4$), were measured: control rate, 0.18 ± 0.013 ; plus 1 μ g valinomycin ml⁻¹, 0.17 ± 0.03 ; plus 1 μ g gramicidin ml⁻¹, 0.14 ± 0.023 . In these experiments the organism was incubated with valinomycin or gramicidin for 5 min prior to AIB addition. Increasing the preincubation time to 10 min resulted in no increase in inhibition. Thus under conditions where the addition of ionophores allowed either Na⁺ or K⁺ to reach passive flux equilibrium (i.e. $\Delta\mu_{K^+}$ or $\Delta\mu_{Na^+} = 0$), but where extensive membrane depolarization was limited by the low concentrations of Na⁺ or K⁺ present, then AIB transport was largely unaffected.

The initial rate of uptake of AIB measured using 52.5 μ M-AIB was shown to be inhibited by

Fig. 3

Fig. 4

Fig. 3. Energy dependence of α -aminoisobutyrate uptake by *T. b. brucei* 8/18. Transport was measured at 37 °C in the Na/HEPPS/glucose system (●), the Na/HEPPS/glucose system plus 2-deoxyglucose (▲) (the concentrations of glucose and 2-deoxyglucose were 20 and 30 mM respectively) and in Na/HEPPS alone (■).

Fig. 4. Effect of ionophores on the transport of AIB by *T. b. brucei* 8/18. AIB transport, measured at 105 μ M-AIB, was performed as described in Methods. The results presented were obtained with independent batches of cells. (a) K/HEPPS/glucose: ●, control; Δ , plus 1 μ g valinomycin ml^{-1} ; $\square$, plus 1 μ g gramicidin ml^{-1} . (b) Na/HEPPS/glucose: ●, control; Δ , plus 1 μ g valinomycin ml^{-1} ; $\square$, plus 1 μ g gramicidin ml^{-1} . (c) K/HEPPS/glucose: ●, control; Δ , plus 30 μ M-CCCP; $\square$, plus 5 μ M-TCS. (d) Tris/HEPPS/glucose: ●, control; Δ , plus 30 μ M-CCCP; $\square$, plus 5 μ M-TCS.

Table 2. Effect of cations on the transport of AIB by *T. b. brucei* 8/18

Initial rates of AIB transport were measured using 105 μ M-AIB as specified in Methods. The values quoted are $\pm$ SD.

Buffer system	Rate of AIB transport ($\mu\text{mol (min)}^{-1} \text{ (g protein)}^{-1}$)
Na/HEPPS/glucose	0.22 $\pm$ 0.07 ($n = 5$)
K/HEPPS/glucose	0.21 $\pm$ 0.08 ($n = 4$)
Tris/HEPPS/glucose	0.13 $\pm$ 0.06 ($n = 4$)

tetraphenylphosphonium chloride; when measured in the Na/HEPPS system 50 μ M-TPP produced 35 or 54% inhibition with two independent batches of the organism. Previous experiments have shown that the maintenance of a $\Delta\psi$ in this organism is sensitive to similar concentrations of TPP (Midgley, 1983b).

Interaction of inhibitors of AIB transport with respiration

In bloodstream forms of *T. b. brucei*, aerobic glycolysis produces pyruvate quantitatively as an end product and NADH is oxidized by a salicylhydroxamate-sensitive α -glycerophosphate oxidase system (e.g. see Gutteridge & Coombs, 1977). ATP is produced only by substrate level

Table 3. *Interaction of transport inhibitors with respiration in T. b. brucei 8/18*

Rates of respiration were measured as previously described (Midgley, 1983a) using an O₂-electrode. The values quoted are the means of two or three observations made with independent batches of the organism. The rates quoted in the presence of inhibitor were measured over the 2 min period immediately after the addition of inhibitor. The symbol * indicates that there was a time-dependent increase in inhibition following this period. The absolute value of the control rate ($\pm$ SD), corresponding to 100%, was compiled from results with four independent batches of cells, and was $132 \pm 52 \mu\text{mol O}_2 \text{ min}^{-1} (\text{g protein})^{-1}$.

Inhibitor	Concn	Relative rate of respiration in:		
		Na/HEPPS/glucose	K/HEPPS/glucose	Tris/HEPPS/glucose
CCCP	30 μM	83*	100	57
TCS	5 μM	62*	64	67*
Valinomycin	1 $\mu\text{g ml}^{-1}$	100	92	93*
Gramicidin	1 $\mu\text{g ml}^{-1}$	97	100	100*
Tetraphenylphosphonium chloride	20 μM	92*	ND	ND
	50 μM	80*	ND	ND
	100 μM	70*	ND	ND

ND, Not done.

phosphorylation and thus measurement of respiration provides a direct means of assessing glycolysis and hence ATP generation. The effect on O₂ consumption of the ionophores and protonophores used in this study was investigated in media of different ionic composition (Na⁺ K⁺ or Tris⁺) (Table 3). In some cases the values quoted may underestimate the degree of inhibition observed under the conditions of the transport assays since some of the inhibitors had time dependent effects depending on the buffer system. It was noteworthy that CCCP in the K/HEPPS system, and valinomycin or gramicidin in either the K/HEPPS or Na/HEPPS system brought about either no or very little inhibition of respiration yet these combinations depolarized the cytoplasmic membrane (Midgley, 1983a, b) and inhibit AIB transport.

DISCUSSION

Our observations concerning the characteristics of AIB uptake by bloodstream forms of *T. b. brucei* 8/18 suggest that AIB is a suitable probe to study the mechanism of energy-coupling to active transport of amino acids in this organism. Kinetic studies indicate that AIB enters by two processes. The transport component apparent at high concentrations of AIB was probably a second mediated system, despite the lack of saturation observed in the concentration range used. This was indicated by >90% inhibition of AIB transport (measured at 40 mM-AIB) by TCS, CCCP or 2-deoxyglucose (K. P. Coolbear & M. Midgley, unpublished observations).

By using AIB, any complications arising from the subsequent metabolism of the transported compound are avoided and this factor may explain the discrepancy between previous results obtained using threonine (Voorheis 1980; Klein *et al.*, 1980) and those presented here. Our observations support a scheme in which AIB enters via an H⁺-symporter, responding mainly to the transmembrane $\Delta\psi$ that has been demonstrated in this organism (Midgley, 1983a, b). Thus AIB transport was inhibited by the protonophores TCS and CCCP independently of the cation present, whereas valinomycin or gramicidin caused inhibition only in the presence of the appropriate cation. The lack of inhibition of AIB transport by gramicidin or valinomycin (in the absence of high external K⁺ or Na⁺ concentrations), which bring Na⁺ and K⁺ (gramicidin) or K⁺ (valinomycin) into passive flux equilibrium, indicates that AIB uptake can operate when $\Delta\mu_{\text{K}^+}$ and/or $\Delta\mu_{\text{Na}^+} = 0$. In contrast, the sensitivity of AIB transport to protonophores was independent of the cation present and indicates that when $\Delta\mu_{\text{H}^+} = 0$, AIB uptake is inhibited. Thus $\Delta\mu_{\text{H}^+}$ plays a key role in driving AIB transport. Direct attempts to measure AIB-induced H⁺ movements in non-metabolizing cells in the presence of a suitable counter-ion (e.g. K⁺ plus valinomycin) have proved negative (M. Midgley, unpublished observations). We regard this as insufficient to disprove the model we propose.

A major difficulty in interpreting such experiments is that the mechanism by which the membrane potential is maintained in these organisms is currently unknown. Eddy (1982) has discussed the importance of inhibiting proton recycling in order to demonstrate the true stoichiometry of a proton symport and has noted that it is not always feasible to make direct assays when a proton pump is not functioning, as the putative symport may not function sufficiently rapidly.

It could be argued that the crucial effects of inhibitors we have observed are in fact due to secondary or non-specific effects. Previous experiments (Midgley, 1983*a, b*) and data presented here have shown that the protonophores and ionophores ($\pm K^+$ or Na^+) are without sufficient effect on glycolysis (a process that both requires and generates ATP) in this organism to explain the inhibition of AIB transport by these compounds. The simplest hypothesis is that the inhibitory effects on AIB transport are directly related to the inhibitory effects on $\Delta\psi$ maintenance. Since the external pH value was 8.0 in these experiments, it is probable that the $\Delta\psi$ was the major component of the $\Delta\mu_{H^+}$ under the conditions studied. The magnitude of the observed $\Delta\psi$ (Midgley, 1983*b*) is sufficient to account for the accumulation ratio of AIB reported here.

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REFERENCES

- BROHN, F. H. & CLARKSON, A. B., JR. (1978). Quantitative effects of salicylhydroxamic acid and glycerol on *Trypanosoma brucei* glycolysis *in vitro* and *in vivo*. *Acta Tropica* **35**, 23-33.
- EDDY, A. A. (1978). Proton-dependent solute transport in micro-organisms. *Current Topics in Membranes and Transport* **10**, 279-360.
- EDDY, A. A. (1982). Mechanisms of solute transport in selected eukaryotic micro-organisms. *Advances in Microbial Physiology* **23**, 1-78.
- GUTTERIDGE, W. E. & COOMBS, G. H. (1977). *Biochemistry of Parasitic Protozoa*, p. 41. London: MacMillan Press.
- HAMILTON, W. A. (1975). Energy coupling in microbial transport. *Advances in Microbial Physiology* **12**, 1-53.
- KLEIN, R. A., ANGUS, J. M., AMADIFE, A. E. & SMITH, L. (1980). Stereospecificity of the threonine dehydrogenase from bloodstream forms of *Trypanosoma brucei*. *Comparative Biochemical Physiology* **66B**, 143-146.
- LUMSDEN, W. H. R., HERBERT, W. J. & MCNEILLAGE, G. J. C. (1973). *Techniques with Trypanosomes*. Edinburgh & London: Churchill Livingstone.
- MIDGLEY, M. (1978). The transport of α -aminoisobutyrate into *Crithidia fasciculata*. *Biochemical Journal* **174**, 191-202.
- MIDGLEY, M. (1983*a*). The interaction of a fluorescent probe with *Trypanosoma brucei brucei*: evidence for the existence of a membrane potential. *FEMS Microbiology Letters* **18**, 203-206.
- MIDGLEY, M. (1983*b*). Further evidence for the existence of a membrane potential in *Trypanosoma brucei brucei*. *Journal of General Microbiology* **129**, 2677-2679.
- STEPHENSON, M. C. & MIDGLEY, M. (1980). Measurement of the membrane potential component of the transmembrane proton electrochemical gradient in *Crithidia fasciculata*. *Biochemical Society Transactions* **8**, 307-308.
- VOORHEIS, H. P. (1971). Amino acid transport in *Trypanosoma brucei brucei*. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **65**, 241-242.
- VOORHEIS, H. P. (1977). Changes in the kinetic behaviour of threonine transport into *Trypanosoma brucei* elicited by variation in hydrogen ion concentration. *Biochemical Journal* **164**, 15-25.
- VOORHEIS, H. P. (1980). Energized amino acid transport by *Trypanosoma brucei* requires a glycolytic intermediate. *Biochemical Society Transactions* **8**, 273-275.