Changes in the Kinetic Behaviour of Threonine Transport into *Trypanosoma brucei* Elicited by Variation in Hydrogen Ion Concentration

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1. The dependence of V and V/K_m for threonine transport into *Trypanosoma brucei* upon the external concentration of H^+ was studied. 2. Two ionizing groups, the α -amino group of the substrate and a group at the substrate-binding site of the carrier, were found to influence the observed kinetic behaviour of transport. 3. The pK of the group at the substrate-binding site on the free carrier was found to be 6.95 at 30°C and to be temperature-dependent; its heat of ionization was -63.8 kJ, which is outside the range for most proton dissociations and suggests a significant contribution from some other source, possibly the remainder of the carrier or the membrane environment. 4. Binding of substrate caused the pK of its α -amino group to shift to a higher value, whereas that of the carrier group shifted to a lower value (6.65 at 30°C). 5. The ionic interaction between substrate and carrier appeared to be involved in the stabilizing of the protonated substrate and the species of the carrier–substrate complex required for the membrane-translocation step. 6. The same ionic species of carrier–substrate complex is required for both substrate dissociation and translocation of the substrate through the membrane. 7. H^+ symport or antiport did not occur during threonine uptake.

In 1943 Edsall drew the attention of biochemists to the possibility of identifying functional chemical groups in proteins by determining their characteristic ionization constants and heats of ionization (Cohn & Edsall, 1943). Dixon (1953) developed these ideas into a comprehensive theory on the effect of H^+ concentration on the kinetic behaviour of enzymes, largely based upon the original ideas of Michaelis & Davidsohn (1911) and Michaelis & Rothstein (1920), which were unified by von Euler *et al.* (1924) and further developed by Waley (1953).

Many studies subsequently appeared in the literature reporting the investigation of the behaviour of individual enzymes in response to variation in H^+ concentration, and predicting the chemical identity of a functional group at the active site of an enzyme, characterizing the environment of an ionizing group which affects the rate of catalysis or delineating the role of an ionic group on the substrate (see for example Massey & Alberty, 1954; Laidler, 1955; Dodgson *et al.*, 1955; Herries *et al.*, 1962; Chase, 1967; Kaplan & Laidler, 1967; Kasserra & Laidler, 1969; Fersht & Requena, 1971; Brocklehurst, 1974).

The limitations of such studies are numerous. Adams (1916) first pointed out the distinction between the observed stages of ionization and the actual extent of ionization of the participating groups. This difference was experimentally demonstrated by Benesch & Benesch (1955). A mirage pK can

result from a change in rate-limiting step of a reaction with a change in H^+ concentration (e.g. Jencks, 1969; Dixon, 1973). The effect of co-operativity in H^+ or other ligand binding (Dixon & Tipton, 1973) due to the presence of another group with a pK close to that of the observed group has been described. The involvement of equilibria, other than simple ionization, that contribute to an apparent pK has been reported in the experimental studies by Fersht & Requena (1971) and Fersht (1971).

In the present study the theory describing changes in enzyme behaviour as a result of variation in H^+ concentration is applied to the experimental study of threonine transport into *Trypanosoma brucei*. Progress towards the chemical identification of a functional group at the active site of a neutral-amino acid-transporting carrier (system N_1) (Voorheis, 1971), as well as the role of some ionic forms of the substrate in the interaction between the active site of the carrier and the substrate, are reported. Some evidence for an interaction between a group at the active site of the carrier and its environment has also been obtained.

Materials

L-Threonine (allo-free) was from Koch-Light Laboratories, Colnbrook, Bucks., U.K. L-[U- ^{14}C]-Threonine (10mCi/nmol) was from The Radio-

chemical Centre, Amersham, Bucks., U.K. All laboratory reagents were of AnalaR grade from BDH Chemicals, Poole, Dorset, U.K., or from Sigma (London) Chemical Co., Kingston-upon-Thames KT2 7BH, U.K.

The scintillation mixture for radioactivity counting contained 10g of butyl-PBD [5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole], 500g of Triton X-100 and sufficient toluene to make a final volume of 2.5 litres.

Trypanosoma (Trypanozoon) brucei sp. (Hoare, 1967) was from the Lister Institute, Kings Road, London, U.K., cloned by Dr. R. W. F. Le Page and given the strain number 427-12/1C1.

Methods

Growth, harvesting and preparation of cells

The techniques for obtaining cells have been described previously (Owen & Voorheis, 1976). Inocula of *T. brucei* were stored frozen in liquid N₂ and then grown in rats for each day's use. Blood-stream forms of trypanosomes were separated from blood elements by a minor modification of the method of Lanham (1968) and were washed twice in Krebs-Ringer phosphate (KRP) buffer containing 2mM-NaH₂PO₄, 20mM-Na₂HPO₄, 98mM-NaCl, 2mM-KCl, 1mM-MgSO₄ and 10mM-D-glucose, adjusted to the appropriate pH with either 1M-NaOH or 1M-HCl.

Measurements of H⁺ concentration

All measurements were made with a W. G. Pye model 290 pH-meter, by using an Ag/AgCl H⁺ electrode with a KCl liquid junction and a calomel reference electrode. All values were measured at 20°C and have been reported uncorrected.

After the termination of each experiment the H⁺ concentrations of media in control suspensions of trypanosomes were checked and not found to differ from the values measured before the beginning of each experiment.

Assay of amino acid transport

Samples (0.1 ml) of cells (2.0 × 10⁹/ml) at 0°C were added with rapid mixing to 15 ml conical centrifuge tubes containing KRP buffer and [U-¹⁴C]-threonine (0.1 μCi and a concentration between 0.1 and 10 mM) at either 31.5° or 42°C. The mixture (2 ml) was incubated at either 30° or 40°C respectively for 1 min. The incubation was stopped by addition of 10 ml of ice-cold buffer. The cells were centrifuged (650g for 10 min) at 4°C. The supernatant was discarded and the cells were resuspended into 10 ml of ice-cold KRP buffer.

After three washes with ice-cold buffer the pellet of cells was transferred to a scintillation vial with 10 ml of scintillation mixture and the radioactivity was measured in a liquid-scintillation counter with the channels-ratio method of quench correction. The uptake reaction was found to be linear for the first 5 min and initial rates were always based on measurements made over the first 1 min.

H⁺ movements across the plasma membrane

H⁺ movements were measured by using the procedure of Henderson (1974) and a pH-meter built by Dr. P. J. F. Henderson to a design of Professor P. B. Garland (Department of Biochemistry, University of Dundee, Dundee, Scotland, U.K.). The measurement assembly contained a 6 ml water-jacketed cell and a Jena glass micro combination electrode (Pye-Unicam model 401 M5). Measurements were made under aerobic conditions at 37.5°C with 0.1 mM-Na₂HPO₄, 120 mM-NaCl, 2 mM-KCl, 1 mM-MgSO₄ and 10 mM-D-glucose as the supporting medium, adjusted to the appropriate pH with 0.1 M-HCl.

Calculation of kinetic constants from experimental data

The kinetic constants, K_m and V , along with their standard errors, were obtained by linear regression of $1/v$ against $1/[S]$; then $\log V$ and $\log(V/K_m)$ were plotted as functions of pH and the results transferred from graph-paper to transparent overlays. A series of theoretical curves for each of these functions (eqns. 16 and 22; see Appendix) were plotted by using a range of values for each constant; the data on the overlay were superimposed on the series of theoretical curves and the curve fitting best by eye was selected.

Results

In studying the effect of H⁺ concentration on the observed values of the kinetic constants of an enzyme activity it is essential first to establish the validity of the following important assumptions: (1) that the effects of H⁺ concentration on the system are reversible within the pH range used, and (2) that none of the ionic forms of the buffer present inhibit the enzyme-catalysed reaction. There is every reason to believe that this practice also should apply to studies of the pH-dependence of carrier-mediated transport reactions. Experience with transport systems suggests that it is necessary to verify two additional assumptions: (3) that the variation in alkali metal-ion concentration or in ionic strength resulting from pH adjustments are without effect on the transport reaction, and (4)

Fig. 1. Reversible effect of H^+ concentration on threonine uptake

Two sets of cell samples were incubated (10^8 /ml, 37.5°C) at various H^+ concentrations for 10 min in a final volume of 2 ml. After washing, all samples in the first set were re-incubated at the same H^+ concentration with 1 mM-[U- ^{14}C]threonine ($0.05 \mu\text{Ci}/\mu\text{mol}$) for 1 min (○). All members of the second set were similarly re-incubated, but at pH 7.0 (●). Each result is the mean $\pm$ S.E. of five determinations.

Fig. 2. Effect of phosphate on threonine uptake. Cells (10^8 /ml) were incubated (final volume 2 ml) for 1 min (37.5°C) with 1 mM-[U- ^{14}C]threonine ($0.05 \mu\text{Ci}/\mu\text{mol}$) at various P_i concentrations with Cl^- replacing the P_i removed. Each result is the mean $\pm$ S.E. of four determinations.

that the variation in H^+ concentration does not alter the transport rate in an indirect fashion, for example by changing the transmembrane potential or affecting a coupled H^+ porter or antiporter, which is physically distinct from the carrier active site. If the preceding assumptions are not found to be valid, then the interpretation of the results from such experiments requires additional care.

Reversible effect of H^+ concentration on threonine uptake

An experimental design similar to that first presented by Hare (1928) was used. Duplicate sets of cell suspensions were incubated (37°C) in KRP buffer for 10 min at a series of pH values between 6.0 and 10.0. Then both sets of cell suspensions were centrifuged ($650g$ for 10 min) and washed (0°C) in KRP buffer, which had been previously adjusted to whatever pH would be used in the second incubation. One set of cell suspensions was incubated further in KRP buffer at the same H^+ concentration used in the first incubation, but for only 1 min (37°C) with 1 mM-[U- ^{14}C]threonine ($0.05 \mu\text{Ci}/\mu\text{mol}$). The H^+ concentration of each sample in the second set of cell suspensions was adjusted to pH 7.0 before incubation with labelled threonine. The transport reaction in the second incubation was stopped and the cells were washed and analysed for their content of radioactivity as described in the Methods section.

Within the range pH 6.5–10.0 the effect of incubating cells at different H^+ concentrations on the subsequent uptake of [U- ^{14}C]threonine was

reversible. At the highest H^+ concentration used (pH 6.0) some degree of irreversible inhibition of [U- ^{14}C]threonine uptake occurred (Fig. 1). This result was corroborated by the observation that cell motility was well maintained for periods in excess of 30 min within the range pH 6.5–10.0, but that outside this range loss of cell motility rapidly occurred.

Effect of phosphate buffer concentration on threonine uptake

The possibility that one of the ionic forms of the buffer present inhibited threonine transport was checked by measuring the initial rates of [U- ^{14}C]threonine uptake at different P_i concentrations from below to well above those generally used, while maintaining both pH and I constant. The rate of uptake was independent of P_i concentration over the range studied (Fig. 2).

Effect of Na^+ concentration on threonine uptake

The initial rate of [U- ^{14}C]threonine uptake was found to be independent of the concentration of Na^+ (Fig. 3). No detectable effect was found when Na^+ (as NaCl) was replaced with equimolar amounts of the choline ion (as choline chloride) or with iso-osmotic amounts of sucrose. In addition to the lack of a specific effect of Na^+ , [U- ^{14}C]threonine uptake was independent of ionic strength within the range studied.

Fig. 3. Effect of Na^+ concentration on threonine uptake. Cells ($10^8/\text{ml}$) were incubated (final volume 2 ml) for 1 min (37.5°C) with 1 mM-[$\text{U}-^{14}\text{C}$]threonine ($0.05 \mu\text{Ci}/\mu\text{mol}$) at various Na^+ concentrations with choline ($\circ$) or sucrose ($\bullet$) replacing the Na^+ removed. Each result is the mean $\pm$ S.E. of five determinations.

H^+ movements associated with threonine uptake

Several experiments were performed in which suspensions of cells received pulses of unlabelled threonine. Rapid recording of H^+ concentration in the supporting medium failed to demonstrate any change in pH induced by threonine during or subsequent to the substrate pulse; no evidence for the presence of an H^+ porter or antiporter coupled to threonine uptake could be found (Fig. 4).

Kinetic behaviour of threonine uptake

Lineweaver-Burk plots of threonine uptake into blood-stream forms of *T. brucei* were non-linear when plotted over a wide range of substrate concentration (Fig. 5). This behaviour was thought to be due to the existence of two uptake systems of differing affinity for neutral amino acids (Voorheis, 1971, 1976). Although it is theoretically possible to determine the pH-dependent variation in the kinetic constants for both systems simultaneously, this proved impractical for logistic reasons since the use of non-linear regression analysis (see legend to Fig. 5) is dependent upon determinations made at a very large number of substrate concentrations at each pH. Therefore the system of highest affinity (N_1 system), which was estimated to account for 80% of the uptake of threonine into blood-stream forms of *T. brucei* under physiological conditions, was selected for further investigation. Accordingly substrate concentrations in the range 0.1–10 mM were used. Under these conditions Lineweaver-Burk plots were more nearly linear and V and K could be

Fig. 4. H^+ movements associated with threonine uptake. Cells (5×10^8) were added to 0.1 mM-phosphate medium with 10 mM-D-glucose (37.5°C) and the pH change of the external medium was recorded (see the Methods section) as glucose was metabolized to pyruvate under aerobic conditions. (a) Control without additions; (b) addition of threonine ($50 \mu\text{mol}$) indicated by arrow; (c) addition of NaOH (50 nmol) indicated by arrow. In 1.5 min 5×10^8 cells would be expected to take up or excrete 58 nmol of H^+ at pH 7.1 if H^+ movements were stoichiometric with threonine uptake (see, for example, Fig. 1; 10^8 cells $\equiv$ 0.56 mg of protein). Final volume in all cases was 5 ml.

determined directly by a regression analysis. Even with this simpler approach each point in Figs. 6 and 7 relies upon 35 separate determinations of the initial rate of threonine uptake. Dixon & Webb (1958) has shown that graphical analysis of the data in such a situation leads to a large error in determining the K_m of the low-affinity system, but a much smaller error in the case of the high-affinity system.

The values of the kinetic constants for system N_1 will be in error if uncorrected values of observed v are used in their calculation while assuming a simple Michaelis-Menton model. This is true because the term $V_2[S]/K_{m_2} + [S]$ has not been subtracted from observed v . The magnitude of the error introduced will be dependent upon the concentration of S and upon the relative size of V_2 and K_{m_2} . Since the value of each of these kinetic constants was determined in the experiment shown in Fig. 5 (see legend for values) it is possible to estimate the error introduced by the assumption of the simpler model. For example, V_1 will be in error by 30% at 10 mM-substrate and only 6% at 0.1 mM-substrate. Since a range of substrate concentration values between these limits was

used in the pH-dependence experiments, a final error of about 20% will result. The error introduced into the calculations of the kinetic constants was a systematic one, since the low-affinity system (designated system N_2) is relatively insensitive to variation in H^+ concentration (Voorheis, 1971). Therefore the errors introduced into the values of the negative logarithms of the apparent ionization constants, pK_1 , pK_2 , pK_3 and pK_4 , are smaller still and most of this error is present in the pH-independent components of these constants (see the Appendix).

Effect of $[H^+]$ on the values of the kinetic constants for threonine transport

It is apparent (Figs. 6 and 7) that four dissociations influence the transport of threonine into *T. brucei*. Each may be identified by the position of an inflexion in these curves. Data were not obtained at high enough pH values to accurately specify the exact position of the two inflexions in the upper pH range. However, in one instance (Fig. 7a) an estimate could be made.

Two dissociations (pK_1 and pK_4) affect the relationship between $\log V$ and pH (Fig. 6). Their apparent pK values correspond to the positions on the pH axis of the two inflexion points in the $\log V$ -versus-pH plot (Fig. 6); however, only the inflexion point at lowest pH can be determined with confidence.

Fig. 5. Effect of substrate concentration on threonine uptake

Cells (10^8 /ml) were incubated (final volume 2ml) for 1 min ($37.5^\circ C$) with various threonine concentrations between 0.2 and 20mM. In all cases $0.1 \mu Ci$ of $[U-^{14}C]$ -threonine was present. Each value is the result of a single determination, and four determinations were performed at each substrate concentration. —, Theoretical curve resulting from a computer-calculated least-squares fit of a two-carrier model to the data using an iterative procedure. The final kinetic constants were found to be $V_1 = 27.0$ nmol/min per mg of protein, $K_{m1} = 17.4$ mM, $V_2 = 22.9$ nmol/min per mg of protein and $K_{m2} = 0.075$ mM.

Fig. 6. Effect of H^+ concentration on V

Cells (10^8 /ml) were incubated (final volume 2ml) for 1 min [(a) $40^\circ C$; (b) $30^\circ C$] with various threonine concentrations between 0.1 and 10mM at pH values ranging from 6.5 to 9.0. Each value is the mean $\pm$ s.e. of seven experiments and each value contains 35 separate velocity measurements. See the Methods section for details of the treatment of the data. —, Theoretical curve fitted to the data; ----, calculated positions of the tangents predicted by the treatment of Dixon (1953). The positions of pK_4 lie above the range of the data and are not accurately determined.

Fig. 7. Effect of H^+ concentrations on V/K_m

Details were as shown in the legend to Fig. 6. The position of pK_3 at 30°C has shifted above the range of the data and is not accurately determined. (a) 40°C ; (b) 30°C .

The apparent first-order rate constant, V/K_m , also changes with varying H^+ concentrations (Fig. 7). Two dissociations influence these curves (pK_2 and pK_3); pK_3 can only be estimated at 40°C . At 30°C the position of the upper inflexion has shifted beyond the range of the data. The apparent pK values of these dissociations differ from those affecting the $\log V$ -versus-pH curves.

Thus four dissociations influence these curves; only two of which affect V (pK_1 and pK_4), whereas the remaining two (pK_2 and pK_3) only influence V/K_m .

Location of the dissociating groups

Only the protonation of groups on the carrier-substrate complex produce inflexion points in the $\log V$ -versus-pH curves, and only the protonation of groups on the free substrate or the free carrier affect the $\log(V/K)$ -versus-pH relationship (Dixon & Webb, 1958; Cleland, 1970). For these reasons it follows from the data in Figs. 6 and 7 that pK_5 and pK_4 result from protonations on the carrier-substrate complex, whereas pK_2 and pK_3 correspond to apparent pK values for protonations on either the free substrate or the free carrier.

Additional information, such as the direct measurement of a pK value, is required to determine that a value derived from an activity-versus-pH relationship belongs to a group located on a carrier

or on a substrate once it is known that the group being protonated does reside on a carrier-substrate complex or on a free moiety. Those groups located on free substrate are easiest to identify, since independent measurements of their pK values usually can be found in the literature. The value of the pK of the amino group of threonine at 30°C was found to be 8.98 and at 40°C to be 8.76 by interpolation in a $1/pK$ -versus- $1/T$ plot of the data obtained by Smith *et al.* (1942). In the present study the value of the pK of the amino group of threonine (pK_3) at 40°C was estimated to be 8.90, whereas that at 30°C was shifted above the range of the data (Fig. 7).

The difference in absolute values for the pK of the amino group in free threonine obtained here, compared with those of Smith *et al.* (1942), reflect in my study a limited range of data, the absence of any correction for dielectric constant of the medium, the use of concentration values rather than activities, biological variability inherent in using multiple batches of cells and an approximation required in the derivation of theoretical curves to fit the data (see the Discussion section and Appendix). This information allows pK_4 (Fig. 7) to be identified as belonging to the amino group of bound threonine in the carrier-substrate complex. Although the pK of carrier-bound threonine is above the range of pH used, it can be predicted to exist from its effect on the data collected at lower pH values (Fig. 6).

It was concluded that the system N_1 amino acid-transporting carrier probably stabilizes the protonated form of the α -amino group of the substrate, since the pK of this amino group was shifted to a higher value upon binding of threonine to the carrier. It seems likely that this stabilization is accomplished by the formation of a bond with considerable ionic character.

The remaining two pK values (pK_1 and pK_2) identified (Figs. 6 and 7) cannot be due to the protonation of groups on the substrate because the other functional groups of threonine, carboxyl and hydroxyl, both have pK values so far removed in value from any pH within the range studied that the very small change in their state of protonation would have remained completely undetected. These pK values must belong to the other kinetic species, the carrier, and may be identified as resulting from the protonation of some group at its active site. The inflexion point (pK_1) at lowest pH in the $\log V$ -versus-pH plot (Fig. 6) results from the protonation of a group on the carrier at the active site of the carrier-substrate complex; pK_2 is the inflexion point at lowest pH in the $\log (V/K_m)$ -versus-pH curve (Fig. 7). The protonation of the group producing pK_2 is located at the active site of the free carrier. It is clear that binding of substrate to the system N_1 amino acid-transporting carrier stabilizes the unprotonated form of the group on the carrier at the active site (pK_2 shifting to pK_1) and that the unprotonated form of this group is required for transport.

Apparent heats of dissociation

The ΔpK_2 value (30–40°C) observed for the group dissociating on the free carrier at the active site (Fig. 7) was 0.35. The value of the apparent heat of dissociation of the free carrier group was therefore calculated to be –63.8 kJ by using the integrated form of the van't Hoff relationship, and this value became –73.1 kJ (pK_1) upon binding of substrate. It is unlikely that the negative heat of dissociation arose because of the variability of the data since a large negative value was observed in all seven experiments.

One word of caution in the interpretation of these apparent heats of dissociation must be mentioned. The values obtained are based upon two-point van't Hoff plots. Therefore only the sign and the relative magnitude of these values can be given significance. Even with this restriction in mind, the value obtained was so far outside the range of heats for most H^+ dissociations (–6.3–+63 kJ/mol) that it is likely that this dissociation may be accompanied by some other change such as an interaction with a group elsewhere in the carrier or perhaps a rearrangement in the membrane environment at the surface of the cell.

Discussion

The results of these experiments have been interpreted in terms of a rate-dependence of transport upon the major species of discrete proton-binding groups located on various forms of the carrier and the substrate. Although there is no evidence to suggest that a more complex model is needed, some caution must be exercised, since these results do not preclude a more complex interpretation. Our specific knowledge of the detailed steps in the transport event is meagre. If the active transport of amino acids by membrane carriers, which are not coupled to ion gradients, are ever found to involve the substrate in a chemical reaction, then more complex models doubtless will be required. Likely models might then involve an H^+ -dependent change in rate-limiting step (Dixon, 1973) or even a rate-dependence upon each of the minor species of two ionizing groups whose identity would then be opposite to that suggested in the present study.

If the simplest reasonable model of transport (Scheme 1) is conceived to fit the behaviour of the system N_1 amino acid carrier, then the steady-state rate equation may be written in the form:

$$v = \frac{V_o K_{m_i} [S]_o - V_i K_{m_o} [S]_i}{K_{m_i} [S]_o + K_{m_o} [S]_i + K_x [S]_i [S]_o}$$

where v is the net velocity inwards, V_o is the maximum velocity from outside to inside and V_i is measured from inside to outside. K_{m_i} and K_{m_o} are the Michaelis constants inside and outside the cell and K_x is a group of additional rate constants. This equation was first presented by Britton (1966) in a form containing all of the individual rate constants. As shown here the relationship of this equation to that of Haldane's (1930) for a reversible single-substrate enzyme-catalysed reaction may be particularly evident. The major difference resides in the presence of an additional denominator term, $K_x [S]_i [S]_o$, which appears due to the pathway

Scheme 1. Simple reversible transport

for translocation of free carrier. If this translocation is very fast with respect to all other steps the model becomes Haldane's and this term disappears. In either case, when $[S]_i$ is small terms containing it drop out, K_{m_1} cancels and the simplest Michaelis relationship remains. Use of this result has been made in the model for the present study (see Appendix).

It is of the greatest importance to be aware that, although the forms of the rate equation when $[S]_i$ is small are the same as that of the simple Michaelis equation, the composition of V_0 (the maximum inward velocity), K_{m_0} (the Michaelis constant outside the cell) and V/K_{m_0} (the apparent first-order rate constant) in terms of individual rate constants is much more complex. For this reason the apparent first-order rate constant is not necessarily a true reflection of only the binding reaction as suggested by Cleland (1970).

Dixon & Webb (1958) has discussed the derivation by Waley (1953) of expressions for the pH-dependence of the kinetic constants for the Briggs-Haldane situation and a more complete theoretical treatment has been given by Laidler (1955). From these results it is clear that the simpler theory is also probably valid for more complex situations with the reservation that small errors may be introduced into the determination of the absolute values of the group pK values due to the possible slower reaction of other ionic forms of a reactant considered active in only one form.

If the simpler theory is applicable, at least in practice, then much valuable information can be obtained from investigations of the pH-dependence of steady-state kinetic constants in more complex situations such as the transport reaction; Cleland's (1970) conclusion then becomes a good approximation to the actual situation.

The observed pK of the dissociating group on the carrier also may include a contribution from other equilibria as has been shown with the observed pK from the α -amino group of the isoleucine-16 residue in chymotrypsin (Fersht & Requena, 1971; Fersht, 1971).

Although any chemical group in a protein that can be protonated could be conceived to be the dissociating group at the carrier active site, imidazole, amino, thiol and possibly carboxyl groups appear most likely from the pK values found. Chemical-modification studies, which are the subject of a further report, eliminate thiol groups and implicate the presence of both amino and imidazole groups at the active site.

The identity of the ionizing group at the active site of the carrier must await additional studies.

In the present study the H^+ -dependence of V/K_m indicates that the binding reaction requires participation of the protonated α -amino group of the

substrate and the unprotonated form of a group at the binding-site of the system N_1 carrier. The shift in pK of the substrate upon binding to the carrier reflects a stabilizing of the protonated form of the substrate's α -amino group. The reciprocal shift in the pK of the carrier group and the consequent stabilizing of its unprotonated form tempts the speculation that a bond of considerable ionic character is formed between substrate and carrier by the interaction of these groups during the binding reaction. Although the lack of an effect of changes in ionic strength of the supporting medium does not support this hypothesis directly, it is quite possible that high enough ionic strength was not reached for adequate detection. It is unlikely that the complex is inaccessible to external solvent or ions, since V was dependent upon the concentration of medium H^+ .

The translocation step reflected in the H^+ dependence of V requires a carrier-substrate complex with the same protonation state necessary in the free forms for the binding reaction. The exact nature of the translocation step through the membrane remains obscure.

The very large negative heat of dissociation of the group at the carrier binding site indicates that any reasonable value for the enthalpy term has been obscured by a large entropy contribution of an opposite sign. The environment of this group might be expected to produce such an effect either due to a temperature-dependent change in the degree of interaction of this group with another in the protein or because of a change in the membrane environment, such as an increase in the amount of structured water usual at such an interface consequent upon a greater exposure of hydrophobic surface with increasing temperature. A more extensive study of the temperature-dependence of pK₁ and pK₂ should reveal whether this environmental influence is of a continuous or a discontinuous nature.

Cuppoletti & Segel (1975) have analysed the kinetics of sulphate transport by *Penicillium notatum* in a very careful study. These authors have shown that H^+ and Ca^{2+} promote sulphate transport, binding in a random order but before sulphate binding. In contrast with the amino acid-transport system in *T. brucei* only K_m and not V is affected by $[H^+]$ in the sulphate-transport system of *P. notatum*. Although the hypothesis of an H^+ symport or OH^- antiport has been eliminated in *T. brucei*, this possibility requires further investigation in *P. notatum*.

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APPENDIX

It is the purpose of this Appendix to show the derivation of the theoretical equations that specify the dependence of V , K_m and V/K_m for the transport of neutral amino acids into *Trypanosoma brucei* via the N_1 system amino acid-transporting carrier. The derivation of these equations was made with the aid of the rapid-equilibrium assumption, which was justified by the good fit of the final relationships to the experimental data that this simple model produced [see the Methods section of the main paper (Voorheis, 1977)]. Theoretical equations fitting more complex models exist in the literature; they are based on theoretical considerations additional to those used here. They are difficult to apply to this particular case because of their complexity and because they consider different ionic species of the substrates and of the enzyme-bound intermediate(s). Nevertheless, they do possess some relevance to the situation investigated here, and interested readers are referred to the reports by Dixon (1953), Laidler (1955), Ottolenghi (1971) and Brocklehurst & Dixon (1976).

Concentration of the ionic form of the free substrate that binds to the free carrier outside of the cell

Let S_A represent the substrate. On the substrate there is a group A, which can bind a proton to form S_{AH^+} outside of the cell. The following equilibrium will occur:

The group ionization constant, K_3 , will be given in terms of concentration as the expression:

$$K_3 = \frac{[S_A][H^+]}{[S_{AH^+}]} \quad (1)$$

The total concentration of the substrate outside of the cell is given by:

$$[S_A]_0 = [S_A] + [S_{AH^+}] \quad (2)$$

substituting for $[S_A]$ and rearranging yields the relationship:

$$[S_{AH^+}] = [S_A]_0 \left/ \frac{K_3}{[H^+]} + 1 \right. \quad (3)$$

Scheme 1.

where S_{AH}^{+} is the ionic form of the free substrate, which binds to the free carrier.

Concentration of the ionic form of the free carrier that binds the free substrate

Let C_B represent the carrier. On the carrier at its active site there is a group B, which can bind a proton to form C_{BH}^{+} at the outer surface of the cell. The following equilibrium will occur:

The group ionization constant, K_2 , will be given in terms of concentration as the expression:

$$K_2 = \frac{[C_B][H^{+}]}{[C_{BH}^{+}]} \quad (4)$$

The total concentration in the suspending medium of the free carrier at the outer surface of the cell is given by:

$$[C_B]_0 = [C_B] + [C_{BH}^{+}] \quad (5)$$

Substituting for $[C_{BH}^{+}]$ and rearranging yields the relationship:

$$[C_B] = [C_B]_0 \left/ \left(\frac{[H^{+}]}{K_2} + 1 \right) \right. \quad (6)$$

where C_B is the ionic form of the free carrier, which binds free substrate.

Concentration of the ionic form of the carrier-substrate complex that is responsible for the transport reaction

Let C_BS_A represent the carrier-substrate complex, where B and A have the same meaning as they do on the free reactants. The equilibria shown in Scheme 1 will exist.

The group ionization constants, K_1 etc., will be given in terms of concentrations as the expressions:

$$K_1 = \frac{[C_BS_{AH}^{+}][H^{+}]}{[C_{BH}S_{AH}^{+}]} \quad (7)$$

$$K'_1 = \frac{[C_{BH}S_A][H^{+}]}{[C_{BH}S_{AH}^{+}]} \quad (8)$$

$$K_4 = \frac{[C_BS_A][H^{+}]}{[C_BS_{AH}^{+}]} \quad (9)$$

$$K'_4 = \frac{[C_BS_A][H^{+}]}{[C_{BH}S_A]} \quad (10)$$

The total concentration in the suspending medium of the carrier-substrate complex at the outer surface of the cell is given by:

$$[C_BS_A]_0 = [C_{BH}S_{AH}^{+}] + [C_{BH}S_A] + [C_BS_A] + [C_BS_{AH}^{+}] \quad (11)$$

Substituting for $[C_{BH}S_{AH}^{+}]$, $[C_{BH}S_A]$ and $[C_BS_A]$ and rearranging yields the relationship:

$$[C_BS_{AH}^{+}] = [C_BS_A]_0 \left/ \left(\frac{[H^{+}]}{K_1} + \frac{K_4}{[H^{+}]} + \frac{K'_1}{K_1} + 1 \right) \right. \quad (12)$$

If $[C_{BH}S_A]$ is very small then $K'_1 \ll K_1$, and $K'_4 \gg K_4$; the expression (eqn. 12) simplifies to:

$$[C_BS_{AH}^{+}] = [C_BS_A]_0 \left/ \left(\frac{[H^{+}]}{K_1} + \frac{K_4}{[H^{+}]} + 1 \right) \right. \quad (13)$$

where $C_BS_{AH}^{+}$ is the ionic form of the carrier-substrate complex, which is responsible for transport of the substrate into the cell. Because of this simplification the molecular and the group constants will have identical values.

The reason that $[C_{BH}S_A]$ is very low in this particular case is because this ionic species involves unfavourable protonation states for both substrate and carrier in the complex at any pH between the pK for the ionizing group on the carrier and that on the substrate. In the general case this situation is found only when these pK values are widely separated.

Variation of V with pH

If only one form of the carrier-substrate complex, say $C_BS_{AH}^{+}$, can be translocated across the membrane:

$$V = k_{cat} \cdot [C_BS_{AH}^{+}] \quad (14)$$

and the value of V will vary with pH since $[C_BS_{AH}^{+}]$ is pH dependent. If all of the carrier-substrate complex were present as $C_BS_{AH}^{+}$ there would be an unique and maximal value of V , designated $\tilde{V}$, which will be given by the expression:

$$\tilde{V} = k_{cat} \cdot [C_BS_A]_0 \quad (15)$$

may be considered to be the H^{+} independent component of V .

Combining eqns. (14) and (15) and substituting for $[C_B S_{AH+}]$, the expression obtained from eqn. (13) yields the relationship:

$$V = \tilde{V} / \left(\frac{[H^+]}{K} + \frac{K_4}{[H^+]} + 1 \right) \quad (16)$$

Variation of K with pH

If the binding of substrate to the carrier requires a particular protonation state of the substrate and of the carrier, say S_{AH+} and C_B respectively, then a dissociation constant, K_S , may be defined by the expression:

$$\tilde{K}_S = \frac{[C_B][S_{AH+}]}{[C_B S_{AH+}]} \quad (17)$$

and the value of this ratio will be independent of pH even though the concentration of C_B , S_{AH+} and $C_B S_{AH+}$ will all be dependent upon pH . This assertion may be verified by substituting for the individual reactants in eqn. (17) their equalities derived from the relationships specifying the protonation equilibria of free substrate and free carrier as well as those of the carrier-substrate complex (eqns. 1 and 4, and 7–10). The resulting expression:

$$\tilde{K}_S = \frac{[C_B][S_{AH+}]}{[C_B S_{AH+}]} = \frac{K_1 K'_1 K'_4}{K_2 K_3 K_4} \cdot \frac{[C_B][S_A]}{[C_B S_A]^2} \quad (18)$$

in terms of all six proton dissociation constants is independent of H^+ .

The observed K_S is given as usual by the equation:

$$K_S = \frac{[C_B]_0 [S_A]_0}{[C_B S_A]_0} \quad (19)$$

in terms of the total concentrations of free carrier, free substrate and carrier-substrate complex. That this observed K_S will be dependent upon $[H^+]$ may be seen by combining eqns. (17) and (19) with the aid of the equalities set out in eqns. (3), (6) and (13):

$$K_S = \tilde{K}_S \cdot \frac{\left(\frac{[H^+]}{K_2} + \frac{K_3}{[H^+]} + \frac{K_3}{K_2} + 1 \right)}{\left(\frac{[H^+]}{K_1} + \frac{K_4}{[H^+]} + 1 \right)} \quad (20)$$

There are a number of circumstances in which K_S in eqn. (20) may be replaced with K_m :

$$K_m = \tilde{K}_m \cdot \frac{\left(\frac{[H^+]}{K_2} + \frac{K_3}{[H^+]} + \frac{K_3}{K_2} + 1 \right)}{\left(\frac{[H^+]}{K_1} + \frac{K_4}{[H^+]} + 1 \right)} \quad (21)$$

Among these circumstances are: (1) when the rapid-equilibrium assumption is valid and $K_m = K_S$; (2) when the steady-state assumption is valid and the rate equation is of the simple Michaelis-Menten type, and further provided that the same form of the carrier-substrate complex is required for translocation across the membrane and for substrate dissociation; (3) when the steady-state rate equation is more complex, but a special case applies such that the pH -dependences of additional terms either do not contribute significantly enough to cause serious error or contribute so that their effects largely cancel.

Variation of V/K_m with pH

Dividing eqn. (16) by eqn. (21) the following relationship is obtained:

$$V/K_m = \tilde{V} / \tilde{K}_m \cdot \left(\frac{[H^+]}{K_2} + \frac{K_3}{[H^+]} + \frac{K_3}{K_2} + 1 \right) \quad (22)$$

Final rate equation

Substitution of the expressions for V and for K_m obtained from eqns. (16) and (19) into the usual rate equation yields the relationships:

$$v = \frac{V / \left(\frac{[H^+]}{K_1} + \frac{K_4}{[H^+]} + 1 \right)}{\left\{ \frac{K_m}{S} \cdot \frac{\left(\frac{[H^+]}{K_2} + \frac{K_3}{[H^+]} + \frac{K_3}{K_2} + 1 \right)}{\left(\frac{[H^+]}{K_2} + \frac{K_4}{[H^+]} + 1 \right)} \right\} + 1} \quad (23)$$

The expressions:

$$\frac{[H^+]}{K_1} + \frac{K_4}{[H^+]} + 1$$

and

$$\frac{[H^+]}{K_2} + \frac{K_3}{[H^+]} + \frac{K_3}{K_2} + 1$$

will be recognized as having the same form as simple pH functions.

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