Kinetics and thermodynamics of the binding of forskolin to the galactose–H^+ transport protein, GalP, of *Escherichia coli*

Giles E. M. MARTIN,* Nicholas G. RUTHERFORD,* Peter J. F. HENDERSON* and Adrian R. WALMSLEY†‡

*Department of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT, and †Krebs Institute for Biomolecular Research, Department of Molecular Biology and Biotechnology, University of Sheffield, P.O. Box 594, Firth Court, Western Bank, Sheffield S10 2UH, U.K.

The binding of the transport inhibitor, forskolin, to the galactose–H^+ symporter, GalP, of *Escherichia coli* was evaluated by equilibrium and time-resolved fluorescence measurements. A quench in protein fluorescence of 8–12% was observed upon the binding of forskolin. The overall dissociation constant (K_d) for forskolin determined by fluorescence titration ranged between 1.2 and 2.2 μM, which is similar to that reported from equilibrium dialysis measurements of the binding of [^{3}H]forskolin (K_d = 0.9–1.4 μM). The kinetics of forskolin binding were measured by stopped-flow fluorescence methods. The protein fluorescence was quenched in a biphasic manner; the faster of these two rates was dependent on the concentration of forskolin and was interpreted as the initial binding step from which both the association (k_{on}) and dissociation (k_{off}) rate constants were determined. The association and dissociation rate constants were 5.4–6.2 $\mu M^{-1} \cdot s^{-1}$ and 5.1–11.5 s^{-1} respectively, and the K_d was calculated to be 1.5 μM. The binding of forskolin was inhibited by D-galactose, but not by L-galactose, and displacement by sugar provided an additional method to calculate the dissociation rate constant for forskolin (k_{off} = 12.4–13.0 s^{-1}). The rate of the slow change in protein fluorescence (3–5 s^{-1}) was independent of the forskolin concentration, indicating an isomerization of the transporter between different conformations, possibly outward- and inward-facing forms. These kinetic parameters were determined at a series of temperatures, so that the thermodynamics of forskolin binding and transporter re-orientation could be analysed. The binding process was entropically driven (ΔS = 83.7 $J \cdot K^{-1} \cdot mol^{-1}$; ΔH = 8.25 $kJ \cdot mol^{-1}$), similar to that for cytochalasin B, which is also an inhibitor of GalP. Measurements of the binding of [^{3}H]forskolin by equilibrium dialysis revealed competitive displacement of bound forskolin by cytochalasin B, possibly suggesting that the sugar, forskolin and cytochalasin B binding sites are overlapping; the K_ds for forskolin and cytochalasin B were calculated to be 0.85 μM and 4.77 μM respectively, and the concentration of binding sites was 10.2 $nmol \cdot mg^{-1}$.

INTRODUCTION

GalP is the protein responsible for galactose–H^+ symport in the Gram-negative bacterium *Escherichia coli* [1]. It is a member of a superfamily of homologous transport proteins from organisms as diverse as cyanobacteria and humans [1–3]. The level of amino acid identity within this family (22–64%) suggests that these proteins adopt similar three-dimensional structures and employ similar mechanisms for substrate recognition and translocation. Indeed, GalP is thought to be the bacterial equivalent of the mammalian glucose transporter GLUT1 [1], since these proteins share a remarkably similar sugar specificity [4,5] and are both subject to inhibition by the antibiotics cytochalasin B [6–8] and forskolin [9–12]. GalP is therefore an ideal model for studies of the structure/function relationship of GLUT1 and sugar transporters in general. The binding of inhibitors and sugars to these proteins needs to be characterized quantitatively in order to understand both the recognition and translocation processes. In this study we have characterized the kinetics of the interaction of forskolin with the GalP transport protein of *E. coli*. This was made possible by the overexpression of the protein to levels constituting 40–60% of the total bacterial inner membrane protein [13], which has allowed ligand binding to this protein to be studied by direct physical measurements on isolated membrane preparations.

Forskolin is a potent reversible inhibitor of both passive glucose transport and proton-driven galactose transport [9–12]. It is thought that inhibition results from binding of forskolin with an internal sugar binding site of the transporter, since part of the structure of forskolin is superimposable on to that of the hexose D-galactose (Figure 1); this similarity led to the suggestion that the transporter recognizes forskolin by a mechanism similar to that by which it recognizes sugars [11].

Here we report that forskolin quenches the inherent fluorescence of the GalP protein. By measuring the rates and extent of fluorescence change with various concentrations of

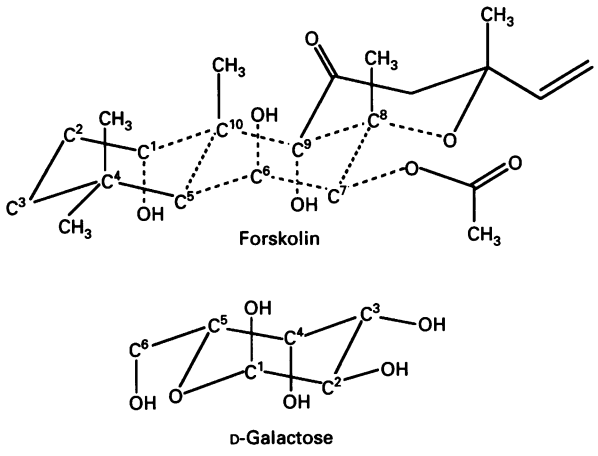

Figure 1 Structures of forskolin and D-galactose

Abbreviations used: GalP, D-galactose–H^+ symporter of *Escherichia coli*; GLUT1, human erythrocyte glucose transporter.

‡ To whom correspondence should be addressed.

forskolin and at different temperatures, we were able to determine the kinetic and thermodynamic constants describing the binding of forskolin to GalP.

EXPERIMENTAL

Materials

Sugars and sugar analogues (glucose-free where appropriate) were obtained from Sigma Chemical Co. The transport inhibitors forskolin and cytochalasin B were also purchased from Sigma. D-[1-^{3}H]Galactose was purchased from Amersham Radiochemicals Ltd. [12-^{3}H]Forskolin was supplied by NEN Research Products, Du Pont (U.K.) Ltd. All other chemicals were of analytical reagent grade.

Growth of the GalP-producing *E. coli* strain

The *E. coli* strain JM1100 (genotype *ptsG ptsM ptsF mgl galP* Δ(*his-gnd*) *thyA galK*) containing plasmid pPER3 (*bla*^{c}, *tet*^{c}, *galP*^{c}) was used for the expression of GalP protein [13].

Overexpression of the GalP protein to levels constituting 40–60% of the total inner membrane protein was achieved as described by Walmsley et al. [8]. The activity of the protein was assessed by measuring energized transport of radioisotope-labelled sugar into intact cells, carried out as described by Henderson et al. [14].

Preparation of inner membranes containing overexpressed protein

Subcellular vesicles were prepared from intact cells by explosive decompression in a French press [15] to yield predominantly inside-out vesicles [16]. Inner membranes were purified from the French press vesicles by sucrose density gradient centrifugation [17].

Determination of protein concentration

Protein concentrations were determined by the method of Schaffner and Weissman [18].

Quantification of overexpressed protein

The level of GalP expression was examined by SDS/PAGE and staining of separated proteins with Coomassie Brilliant Blue [19]. The amounts of overexpressed GalP protein were measured by quantitative densitometry of the Coomassie-stained gel using a Molecular Dynamics 100A Computing Densitometer and were expressed as a percentage of the total membrane protein as analysed by the Imagequant program. The yield of GalP in typical preparations was 40–60% of the inner membrane proteins.

Steady-state fluorescence measurements

Equilibrium fluorescence measurements were made with a Jasco FP777 spectrofluorimeter. The temperature of the sample compartment of the instrument was maintained at 20 °C (unless otherwise stated) using a circulating water bath. The protein was excited at a wavelength of either 280 nm or 297 nm and the resulting fluorescence was measured between wavelengths 310 and 450 nm. The band widths of the excitation and emission beams were set to 3 nm and 10 nm respectively. Inner membrane vesicles were resuspended in phosphate buffer (50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.4) to a concentration of 250 $\mu g \cdot ml^{-1}$ and were examined in a quartz cuvette with a path length of 1 cm. Titrations of the transporter with forskolin were carried out by making sequential additions from a concentrated ligand solution, such that the total increase in sample volume was less than 15%. After each addition, the contents of the cuvette were mixed using a microspatula and allowed to equilibrate for 5 min before the fluorescence emission was monitored. The effect of dilution on the fluorescence values was determined by titrating protein with aliquots of buffer, instead of ligand solution. Although dilution was found to produce only a very small change in fluorescence, the values obtained in the presence of ligands were corrected for dilution. Data were analysed using an unweighted non-linear least squares fit of the data to a hyperbola using the Biosoft program Ultrafit 2.1.

Stopped-flow fluorescence measurements

Rapid changes in fluorescence were monitored using an Applied Photophysics (London, U.K.) spectrofluorimeter (stopped-flow), coupled to an Archimedes 420/1 microcomputer for data collection and analysis. The protein was excited at a wavelength of 297 nm, selected by focusing a 150 W Xenon arc lamp on to an f/3.4 monochromator. The emitted light was selected using a WG335 Schott filter which was positioned in front of the observation photomultiplier tube.

Inner membrane vesicles (100 $\mu g \cdot ml^{-1}$ in phosphate buffer) were mixed rapidly with ligand and the resulting change in fluorescence was collected over a split time base, so that half of the data were recorded in one-fifth of the time taken for the second half. This allowed collection of adequate data points both for the initial exponential phase of fluorescence change and for the subsequent approach of the signal to its steady-state level, giving a more accurate determination of the rate and amplitude of an exponentially changing signal [20].

The stopped-flow records of at least eight mixings were averaged and the rate of change calculated. Data were analysed routinely using the non-linear least-squares regression program supplied with the Applied Photophysics S.F.17 MV stopped-flow equipment.

Equilibrium binding of forskolin and cytochalasin B

Equilibrium dialysis was carried out as described by Walmsley et al. [17]. The binding of [^{3}H]forskolin to inner membranes (0.5 $mg \cdot ml^{-1}$) containing GalP was measured over a range of 1–30 μM forskolin at 8 °C, in the presence of cytochalasin B (0–20 μM). Ratios of bound to free forskolin were calculated from the equilibrium distribution of the radiolabelled ligand and used to determine the K_d, the apparent K_d (i.e. in the presence of cytochalasin B) and the number of forskolin binding sites by an unweighted non-linear least-squares fit of the data to an hyperbola using the Biosoft program Ultrafit 2.1. The competitiveness of cytochalasin B and forskolin binding was examined by fitting the data to an equation for competitive interaction (see the Results and Discussion sections).

RESULTS

Forskolin induces changes in the equilibrium fluorescence of GalP

The fluorescence emission spectrum of inner membranes enriched in the GalP protein is shown in Figure 2 (curve A); maximal fluorescence was centred between 325 and 330 nm. The addition of forskolin reduced the peak height (curve B), and 10 μM forskolin diminished protein fluorescence ($\lambda_{ex.}$ 280 nm; $\lambda_{em.}$ 329 nm) by between 6 and 12%, depending on the level of

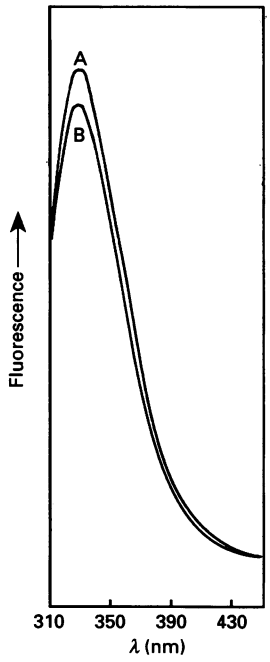

Figure 2 Fluorescence emission spectra of membranes containing overexpressed levels of GalP

The upper trace (curve A) represents the emission spectrum of membranes (250 $\mu g \cdot ml^{-1}$) in the absence of ligand. The lower trace (curve B) was recorded in the presence of 10 μM forskolin. The protein was excited at 280 nm.

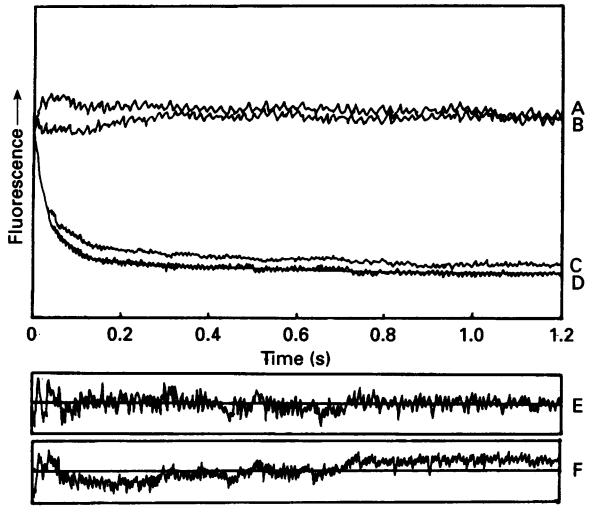

Figure 4 Time course for the binding of forskolin to subcellular vesicles

Representative stopped-flow traces showing the quench in protein fluorescence (excitation 297 nm; emission > 335 nm) upon forskolin binding to membranes enriched in GalP. Trace B shows the baseline fluorescence when membranes (100 $\mu g \cdot ml^{-1}$) were mixed with buffer (50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.4), whereas trace D shows the large quench in fluorescence produced when membranes were mixed with forskolin (5 μM in the same buffer). The rate of quench was biphasic and best fitted by a double-exponential function as shown by the random scatter of the residual variance of the data about the best-fit double exponential (trace E), compared with that of a single exponential (trace F). The binding of forskolin, and the resulting quench, was inhibited when the substrate D-galactose (50 mM) was included in the buffer (trace A) but not by L-galactose (trace C). The total fluorescence change on the ordinate axis is 7.5%.

overexpression achieved (40–60% of total inner membrane protein). When the tryptophan residues were excited preferentially ($\lambda_{ex.}$ 297 nm) the fluorescence quench was reduced to 4–10%. The binding of forskolin did not produce any significant shift in the wavelength of maximal fluorescence emission. No changes in fluorescence were observed when forskolin was added to membranes prepared from the *galP*$^{\Delta}$ *E. coli* strain JM1100, harbouring the control plasmid pBR322 (results not shown).

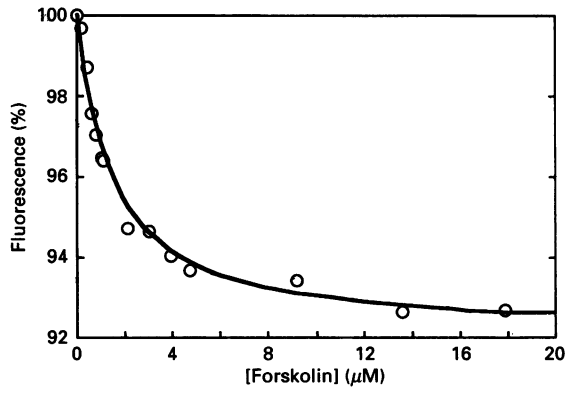

Figure 3 Fluorescence titration of GalP with forskolin

The quench in protein fluorescence was monitored (excitation 280 nm; emission 330 nm) as forskolin was added in small increments to membranes (250 $\mu g \cdot ml^{-1}$) until no further change was observed. The dissociation constant for forskolin binding to GalP was determined from the titration curve. The smooth line represents the fit to an hyperbolic equation with a K_d of 1.2 μM. The total fluorescence decrease was 8.3%.

Determination of the dissociation constant (K_d) of the GalP–forskolin complex at 20 °C

The overall dissociation constant (K_d) for forskolin was determined by titration of the protein fluorescence. The net change in protein fluorescence was measured over a range of forskolin concentrations spanning 0.2 K_d–10 K_d (Figure 3). The titration of intrinsic fluorescence resulted in a decrease in the fluorescence that could be fitted to the following hyperbolic relationship:

$$\Delta F = \frac{\Delta F_{max.} \times [\text{forskolin}]}{K_d + [\text{forskolin}]}$$

where [forskolin] is the concentration of forskolin, ΔF is the percentage quench in fluorescence, $\Delta F_{max.}$ is the maximal quench in fluorescence and K_d is the dissociation constant for forskolin. The average K_d from three titrations was 1.83 ± 0.56 μM, which is very similar to that determined from equilibrium dialysis (1.3–1.4 μM [12]).

Determination of the kinetics of forskolin binding to GalP at 20 °C

The kinetics of forskolin binding to GalP were investigated by stopped-flow fluorescence spectroscopy ($\lambda_{ex.}$ 297 nm; $\lambda_{em.}$ > 335 nm). Rapid mixing and data recording allowed the rate of change in protein fluorescence resulting from the interaction of forskolin with GalP to be resolved. The rate of change in fluorescence was biphasic, and the respective rates of signal change were obtained from a fit of the sum of two exponentials to the data (Figure 4); the goodness of fit was supported by the

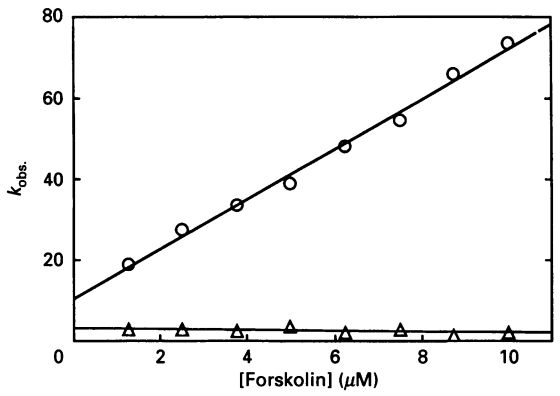

Figure 5 Concentration-dependence of the apparent binding rates of forskolin to GalP

The faster of the two rates determined from fitting to a double exponential was found to increase linearly with increasing concentration of forskolin. Linear regression analysis of the data yielded an apparent association rate constant (k_{on}) from the slope, a dissociation rate constant (k_{off}) from the ordinate intercept and an apparent dissociation constant (K_d) from k_{off}/k_{on}. For the data shown the values for k_{off}, k_{on} and K_d are 6.2 $\mu M^{-1} \cdot s^{-1}$, 10.4 s^{-1} and 1.7 μM respectively. The slower rate was independent of concentration, indicative of an isomerization step, and the rate ranged from 1.5–3.6 s^{-1}.

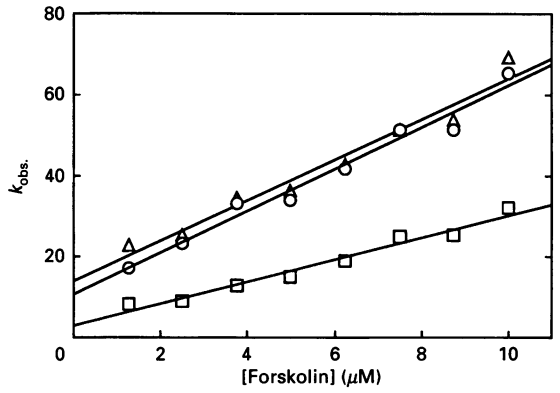

Figure 6 Rate of binding of forskolin in the presence of D- and L-galactose

The forskolin binding rate is plotted as a function of the concentration of forskolin in the absence of sugar (○), and in the presence of 100 mM D-galactose (□) or 100 mM L-galactose (△).

random scatter of the residual variance around the best-fit line. The addition of forskolin to membranes not containing GalP gave no change in fluorescence (results not shown), confirming that the observed transient was due to binding to the transporter. No quench in fluorescence was recorded when forskolin was mixed with membranes pre-equilibrated with the substrate D-galactose, which suggests that the sugar can compete with the binding of forskolin (Figure 4). In contrast, pre-equilibration with L-galactose did not inhibit the quench in fluorescence.

The faster of the two observed binding rates was linearly dependent upon the forskolin concentration (Figure 5). This behaviour is consistent with a simple one-step association of ligand and protein:

$$\text{GalP} + \text{forskolin} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \text{GalP–forskolin}$$

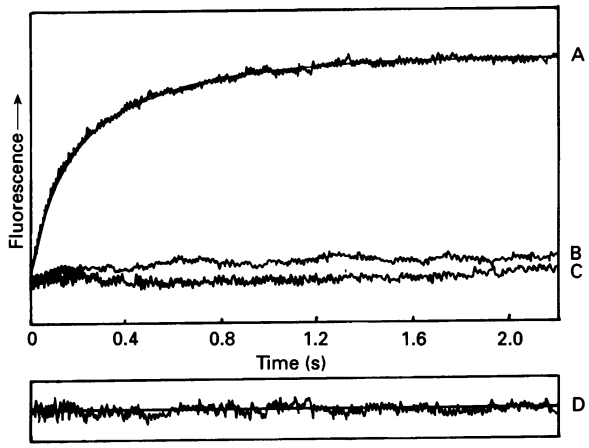

Figure 7 Dissociation rate constant for forskolin calculated from the rate of forskolin displacement from GalP by D-galactose

Trace A shows the increase in protein fluorescence (excitation 297 nm; emission > 335 nm) when membranes (100 $\mu g \cdot ml^{-1}$) equilibrated with forskolin (10 μM) were mixed in the stopped-flow with D-galactose (200 mM). The resulting change in protein fluorescence was fitted to a double-exponential function, revealing dissociation rate constants of 12.3 s^{-1} and 2.4 s^{-1} (D). Traces B and C show that mixing with buffer + L-galactose (200 mM) or buffer alone respectively do not displace forskolin from the protein. The total fluorescence change on the ordinate axis is 7.5%.

with the observed rate ($k_{obs.}$) under pseudo-first-order conditions approximated by:

$$k_{obs.} = k_{on}[\text{forskolin}] + k_{off}$$

where k_{on} is the second-order association rate constant and k_{off} is the first-order dissociation rate constant. The dissociation constant (K_d) for this step would be given by k_{off}/k_{on}. The average (of five experiments) association rate constant for this step was 5.95 ± 0.36 $\mu M^{-1} \cdot s^{-1}$ and the average dissociation rate constant was 8.62 ± 2.49 s^{-1}. This indicates a dissociation constant (K_d) of 1.45 ± 0.43 μM, which is similar to that obtained from equilibrium fluorescence and dialysis measurements. The slow phase, which accounted for 20–25% of the total fluorescence change, occurred at a rate of 2–4 s^{-1} (average of 10 measurements = 2.55 ± 0.60 s^{-1}); it was independent of the concentration of forskolin, indicative of a relatively slow isomerization. The rate of the fast phase was reduced in the presence of D-galactose but not L-galactose (Figure 6).

D-Galactose reverses the quenching of fluorescence caused by forskolin

An alternative measure of the dissociation rate constant was obtained by using D-galactose to displace bound forskolin from GalP [8]. This process can be monitored as an increase in the protein fluorescence, because the binding of D-galactose on its own causes little change in the fluorescence of the protein. Although the traces could be fitted to a single exponential ($k_{obs.} = 7.40 \pm 1.06$ s^{-1}), a double exponential gave a marginally better fit ($k_{obs.} = 12.0 \pm 0.84$ s^{-1} and 1.88 ± 0.77 s^{-1}; Figure 7). However, the better fit might result from the increased degree of freedom of the double-exponential equation. A clear distinction between the two fits is necessarily difficult when the two phases are not well resolved in time. Both of these dissociation rate constants are very similar to those calculated by extrapolation of the observed rate of binding ($k_{off} = 8.62 \pm 2.49$ s^{-1}). As would be

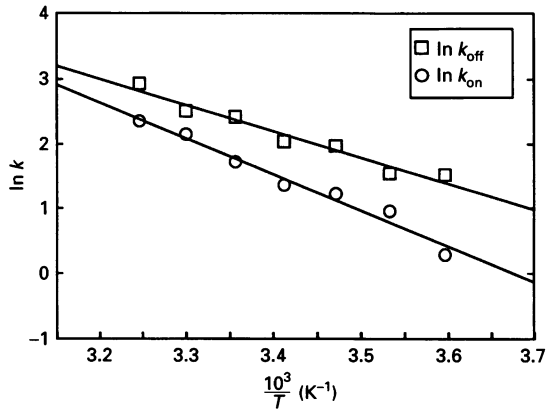

Figure 8 Arrhenius plots for the temperature-dependencies of the association (k_{on}) and dissociation (k_{off}) rate constants for the interaction of forskolin with GalP

The association (k_{on}) and dissociation (k_{off}) rate constants for the interaction of forskolin with GalP were determined at a series of temperatures (5–35 °C). Values for k_{on} were calculated from the concentration-dependence of the rate of forskolin binding, whereas those for k_{off} were measured by the displacement of forskolin by D-galactose. The activation energy for each process was calculated directly from fits to the Arrhenius equation by non-linear regression, yielding values of 44.9 ± 8.2 kJ·mol^{-1} for k_{on} and 36.7 ± 9.5 kJ·mol^{-1} for k_{off}.

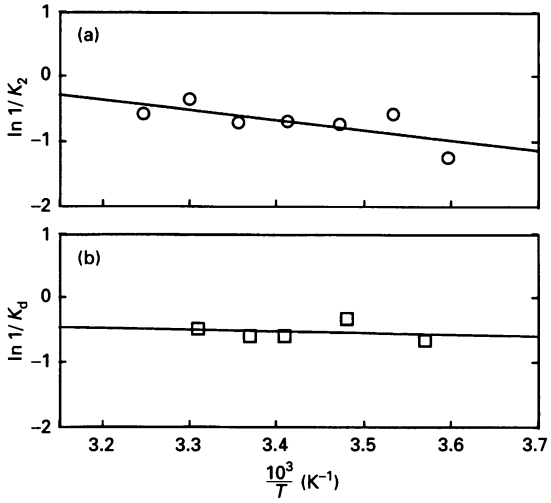

Figure 9 van't Hoff plots of the temperature-dependency of (a) $1/K_2$ and (b) $1/K_d$ for the GalP–forskolin complex

The dissociation constant for the binding step (K_2) and the overall dissociation constant (K_d) were determined at a series of temperatures. K_2 was calculated from the rate constants k_{off} and k_{on}, and K_d was calculated from titrations of protein fluorescence. The shallow slopes of the van't Hoff plots indicate that there is very little enthalpy change associated with forskolin binding. The enthalpies for (**a**) the binding step (K_2) and (**b**) the overall interaction (K_d) were determined by linear regression to be -12.8 kJ·mol^{-1} and -0.75 kJ·mol^{-1} respectively.

expected, L-galactose was unable to displace bound forskolin from GalP (Figure 7).

Determination of ΔG, ΔH and ΔS for the binding of forskolin to GalP

The association and dissociation rate constants for the GalP–forskolin complex were determined at a series of temperatures (5–35 °C), so that the thermodynamics of the binding process could be analysed. The k_{on} values were calculated from the concentration-dependence of the binding of forskolin and the k_{off} values from the displacement of forskolin by D-galactose. The resulting data gave linear Arrhenius plots for both

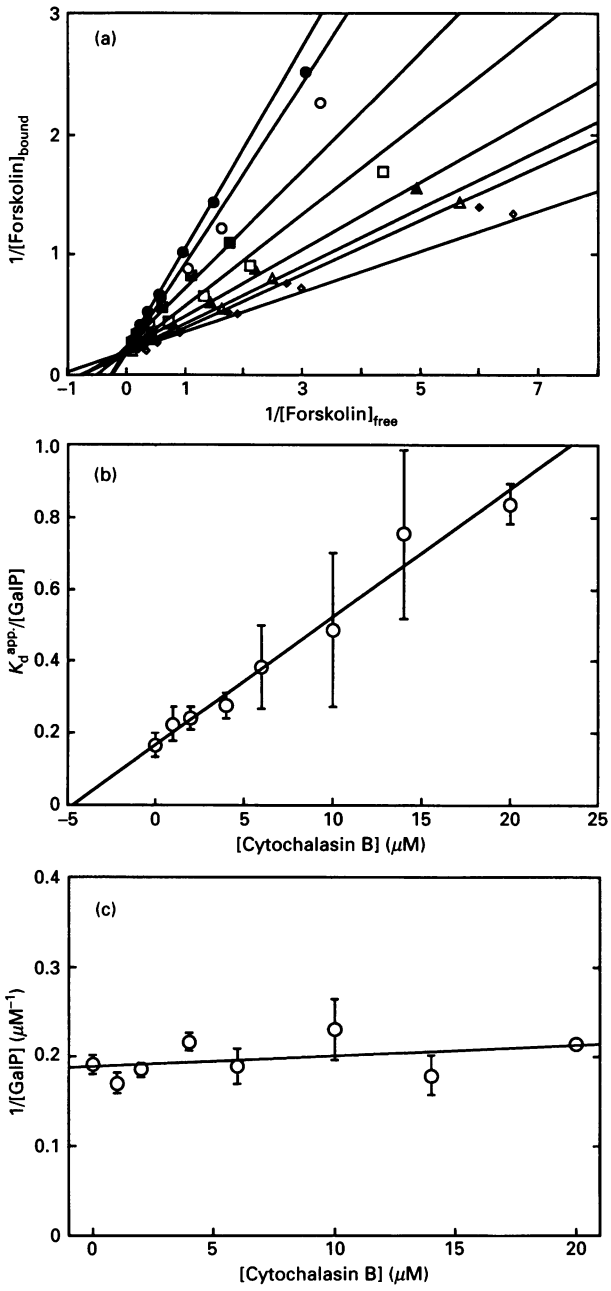

Figure 10 Binding of forskolin to GalP in the presence of cytochalasin B

Inner membrane vesicles enriched with GalP were subjected to microdialysis against a range of solutions of different forskolin concentrations at fixed concentrations of cytochalasin B, and the proportions of forskolin bound to GalP and free in solution were assayed by using [^{3}H]forskolin. The dissociation constant K_d ($K_d^{app.}$ in the presence of a competing ligand) for the GalP–forskolin complex and the concentration of binding sites ([GalP]) were determined from a fit of the data to a hyperbola (bound versus free forskolin). (**a**) Reciprocal plots of the amount of bound versus free forskolin in the presence of 0 μM (◇), 1 μM (◆) 2 μM (△) 4 μM (▲), 6 μM (□), 10 μM (■), 14 μM (○) and 20 μM (●) cytochalasin B. (**b**) and (**c**) are replots of the slope ($K_d^{app.}$/[GalP]) and intercept (1/[GalP]) respectively of each reciprocal plot versus the concentration of cytochalasin B.

association and dissociation rate constants, and the activation energies were determined directly from fits of the data to the Arrhenius equation (Figure 8). In addition, the effect of temperature on the K_d, determined both by equilibrium titration of fluorescence and from k_{off}/k_{on} values, were examined and shown in the form of a van't Hoff plot (Figure 9). The results of the thermodynamic study are given below.

Displacement of bound forskolin from the GalP protein by cytochalasin B

It is of considerable interest to determine whether forskolin and cytochalasin B bind to GalP at the same site. Both inhibitors quench the fluorescence of GalP in a similar qualitative manner (cf. [8]), an initial indication that they bind at the same site, albeit with moderately different values for the binding constants. To investigate this further the direct binding of a range of concentrations of [^{3}H]forskolin was measured by equilibrium dialysis at systematically varied concentrations of unlabelled cytochalasin B, all in a single experiment. In the absence of cytochalasin B, forskolin bound with a K_d of $0.87 \pm 0.16\ \mu$M, similar to the values determined by fluorescence measurements and by separate equilibrium dialysis experiments [12]. The concentration of GalP was measured at $5.23 \pm 0.30\ \mu$M (10.5 ± 0.6 nmol·mg^{-1}). Increasing concentrations of cytochalasin B systematically displaced the bound [^{3}H]forskolin, and replots of the slopes of the lines ($K_d^{app.}$/[GalP]) or the intercepts (1/[GalP]) of the reciprocal lots were linear, with no significant intercept effect. This pattern is clearly indicative of a competitive, mutually exclusive process (Figure 10). A fit of all the data was made to the equation for competitive interaction given below,

$$[F]_{bound} = \frac{[GalP][F]_{free}}{K_d^F(1+[CB]/K_d^{CB})+[GalP]}$$

where [F] and [CB] are the concentrations of forskolin and cytochalasin B respectively; [GalP] is the concentration of GalP; and K_d^F and K_d^{CB} are the dissociation constants for the two inhibitors. The deduced dissociation constants for forskolin and cytochalasin B were calculated as $0.87 \pm 0.16\ \mu$M and $4.77 \pm 1.84\ \mu$M respectively, and the number of binding sites as $5.12 \pm 0.53\ \mu$M (10.2 ± 1.0 nmol·mg^{-1}). The simplest explanation of all these data is that forskolin and cytochalasin B bind at the same site.

DISCUSSION

The binding of forskolin to GalP is characterized by fast and slow changes in protein fluorescence. The faster of these two phases was found to be linearly dependent on forskolin concentration, the simplest interpretation of which is a one-step binding process.

$$\text{GalP} + \text{forskolin} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \text{GalP–forskolin}$$

To account for the slow phase in fluorescence and its independence of antibiotic concentration, we propose that an isomerization occurs. From the experiment using the radiolabelled forskolin, the binding of forskolin and cytochalasin B to the GalP symporter appears to be competitive, which suggests that these inhibitors bind to the same site. We now compare the non-equilibrium kinetics of binding of both inhibitors using the data in this paper and those on cytochalasin B in [8] to reveal those similarities and differences that are informative of the molecular events involved in their recognition.

Like forskolin, the binding of cytochalasin B to GalP is characterized by fast and slow changes in protein fluorescence [8]. The fast phase was attributed to the binding of cytochalasin B to the inward-facing conformation of the transporter, while the slow phase was attributed to the binding of cytochalasin B to those transporters that were originally in the outward-facing conformation and which underwent slow re-orientation to the inward-facing conformation. This was represented by the following model:

$$T_1 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} T_2 \underset{k_{-2}}{\overset{k_2[CB]}{\rightleftharpoons}} T_2(CB)$$

in which T_1 and T_2 are, respectively, the outward- and inward-facing forms of the unloaded transporter, and T_2(CB) is the transporter–cytochalasin B complex. For such a model, the overall dissociation constant of the transporter–cytochalasin B complex is given by the following function:

$$K_d = K_2(1+K_1)$$

with K_1 and K_2 defined as $K_1 = k_{-1}/k_1$ and $K_2 = k_{-2}/k_2$. Assuming that this defines the complete mechanism, then K_1 can be calculated as 3.63. This value will be independent of the nature of the ligand bound to the inward-facing conformation (T_2).

We have considered more complex scenarios of a two-step model in which the measured fast transient, induced by antibiotic binding, was due to the transition between T_1 and T_2(CB). If k_1 and k_{-1} are fast relative to k_2[CB] and k_{-2}, then the measured dissociation constant (k_{off}/k_{on}) for the binding step (K_2) would approach $K_2(1+K_1)$. This is clearly not the case for cytochalasin B binding, since the value of k_{off}/k_{on} (0.41 μM) is not the same as the overall K_d (1.9 μM) [8]. If the T_1 to T_2 isomerization is slow relative to antibiotic binding, then the T_1 to T_2 transition would become rate-limiting and one would expect the observed rate constant to decrease with increasing antibiotic concentration such that:

$$k_{obs.} = k_1 + k_{-1}(K_2/([CB]+K_2))$$

Since neither situation occurs then the simplest explanation for the observed kinetics is that k_1 and k_{-1} are slow and the T_2 conformation is favoured. Accordingly, the fast phase induced by antibiotic binding is directly attributable to the binding of the antibiotic to T_2 and the slow phase is due to the T_1 to T_2 transition. The fact that the amplitudes and the rates of the slow phases for the binding of cytochalasin B and forskolin to GalP are identical is consistent with this interpretation, because the re-orientation process should be independent of the ligand bound.

The two-step model is sufficient to explain the observations with cytochalasin B, but does not explain fully the binding of forskolin. It implies that the overall K_d is equal to $K_2(1+K_1)$, but the measured values of K_d, K_2 and K_1 (calculated for GalP from cytochalasin B data [8]) of 1.83 μM, 1.45 μM and 3.63 μM respectively do not satisfy this equation. The simplest explanation is that the binding of forskolin induces a further conformational change in GalP, and we suggest that T_2(F) isomerizes to T_3(F) as shown in the following scheme:

$$T_1 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} T_2 \underset{k_{-2}}{\overset{k_2[F]}{\rightleftharpoons}} T_2(F) \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} T_3(F)$$

For such a model, the dissociation constant of the transporter–antibiotic complex is given by the following function:

$$K_d = \frac{K_2(1+K_1)}{(1+1/K_3)}$$

with K_1, K_2 and K_3 defined as $K_1 = k_{-1}/k_1$, $K_2 = k_{-2}/k_2$, and $K_3 = k_{-3}/k_3$. For such a model a minimal value of 0.37 can be

calculated for K_3 from K_2 and the K_d. A similar model is used to explain the binding of cytochalasin B to the erythrocyte glucose transporter GLUT1 [8].

How would this third step influence the observed transient? If it is due to the formation of $T_3(F)$, this transition must be fast, or at least faster than the dead time of the stopped-flow, otherwise the observed rate constant would reach a plateau dictated by the rate of the $T_2(F)$ to $T_3(F)$ isomerization:

$$T_2 \underset{k_{-2}}{\overset{k_2[F]}{\rightleftharpoons}} T_2(F) \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} T_3(F)$$

$$k_{obs.} = k_3([F]/([F]+K_2))+k_{-3}$$

Consistently, the rate constant for the observed transient does not reach a plateau level, suggesting that k_3 and $k_{-3} \gg k_2[F]$ and k_{-2}. Under these circumstances, the slope of the concentration-dependence of $k_{obs.}$ (Figure 5) would still give a true measure of the association constant (k_{on}); however, the true dissociation constant would be greater than that deduced by extrapolating $k_{obs.}$ to zero [F] such that

$$k_{(intercept)} = k_{-2}/(1+K_3)$$

where $K_3 = k_3/k_{-3}$ [21]. The dissociation rate constant (k_{-2}) was also determined by sugar displacement. These displacement experiments yielded a value of $12.3 \pm 0.9\ s^{-1}$, compared with a value of about $8.6 \pm 2.5\ s^{-1}$ by extrapolation of $k_{obs.}$ which, although not very different, is consistent with the latter being an underestimate. The biphasicity of the displacement traces may reflect the fact that, in the presence of sugar, some of the transporters adopt the T_1-sugar conformation (e.g. a slow phase due to a T_2-sugar to T_1-sugar isomerization) in addition to sugar binding directly to T_1.

The binding of forskolin to GalP is therefore best described by a three-step model. The first step involves an isomerization between the outward- and inward-facing forms of the transporter, which is independent of the antibiotic bound and is thought to be the measured slow conformational change. The second is the actual binding step, for which the association rate constant can be measured from the concentration-dependence of $k_{obs.}$ (Figure 5) and the dissociation rate constant from the displacement experiments (Figure 7). The third step is an isomerization between forms of the transporter with bound ligand. The rates of association of forskolin and cytochalasin B with the GalP protein are similar, although forskolin dissociates at a rate that is 3–4-fold faster than that for cytochalasin B. This indicates that the inward-facing conformation of GalP has about a 3-fold higher affinity for cytochalasin B than for forskolin. In contrast, results from both the steady-state titration of the protein fluorescence and equilibrium dialysis with each antibiotic indicate that, overall, the GalP protein has a higher affinity for forskolin ($K_d^{forskolin}$ 0.9–2.2 μM and $K_d^{cytochalasinB}$ 1.9–4.7 μM).

The difference in the activation energies for the association and dissociation processes for forskolin indicates that the binding process is slightly endothermic and $\Delta H = 8.25\ kJ \cdot mol^{-1}$. The van't Hoff plot of K_2 (Figure 9a) also shows there is only a small change in the enthalpy upon binding, but the data are indicative of a slightly exothermic process. Clearly though, the binding process is entropically driven, since ΔS was calculated to be $83.7\ J \cdot K^{-1} \cdot mol^{-1}$ at 20 °C [$\Delta G = -RT\ln K$; $\Delta G = \Delta H - T\Delta S$]. This is similar to the thermodynamics for the binding of cytochalasin B to GalP; the binding of cytochalasin B is characterized by a small exothermic change in enthalpy ($\Delta H = -0.5\ kJ \cdot mol^{-1}$) and a large entropy change ($\Delta S = 120\ J \cdot K^{-1} \cdot mol^{-1}$).

The overall enthalpy change for forskolin binding was determined from the van't Hoff plot of the K_d (Figure 9b), which yielded a value of $-0.75\ kJ \cdot mol^{-1}$. Since the minimal enthalpy change for the outward–inward T_1–T_2 transition of GalP has been determined previously ($\Delta H = -5.1\ kJ \cdot mol^{-1}$), from studies of the binding of cytochalasin B, the enthalpy change associated with the T_2(forskolin)–T_3(forskolin) transition can be calculated from the following relationship [8]:

$$\Delta H(\text{overall}) = \Delta H(1) + \Delta H(2) + \Delta H(3)$$

yielding a value of $-3.9\ kJ \cdot mol^{-1}$. The overall entropy change for forskolin binding was calculated as $107.3\ J \cdot K^{-1} \cdot mol^{-1}$ from ΔH(overall) and ΔG(overall). Similarly, using a value of $-37.9\ J \cdot K^{-1} \cdot mol^{-1}$ for $\Delta S(1)$, $\Delta S(3)$ can be calculated as $61.5\ J \cdot K^{-1} \cdot mol^{-1}$.

The nature of the T_2(forskolin) to T_3(forskolin) transition is not known, but one possibility is that it represents a partial re-orientation of the transporter. The re-orientation of the transporter would be entropically driven, with the inward and outward re-orientation of the unloaded transporter involving an entropy change of $37.9\ J \cdot K^{-1} \cdot mol^{-1}$ [8]. However, the postulated partial re-orientation of the originally inward-facing transporter involves a greater entropy change [$\Delta S(3) = 61.5\ J \cdot K^{-1} \cdot mol^{-1}$]. One interesting possible explanation for this difference is that the closure of the inward-facing substrate binding site involves the expulsion of water, but the adoption of the outward-facing conformation involves a partial re-hydration of the binding site. The binding of forskolin may induce the closure of the binding site, by acting as a transition-state analogue, which does not allow complete re-orientation. Interestingly, this situation is reversed for the binding of cytochalasin B to GLUT1, where the entropy change for the T_2–T_1 transition is larger [$\Delta S(1) = 145.0\ J \cdot K^{-1} \cdot mol^{-1}$] than that for the T_2(CB) to T_3(CB) transition [$\Delta S(3) = 64.5\ J \cdot K^{-1} \cdot mol^{-1}$] [8]. Perhaps the re-orientation process proceeds to a smaller extent with cytochalasin B than with forskolin. Consistent with this view, the data did not require postulation of any additional transition of the GalP–cytochalasin B complex [8].

Part of forskolin does resemble a sugar, and the proposed larger conformational change induced by this ligand may reflect its binding to the sugar binding site. In this model, forskolin would lack the potential for forming one hydrogen bond, compared with that of a sugar, because it contains a carbon at forskolin C-5 where the ring oxygen is the proposed equivalent. This ring oxygen, in glucose, appears to be essential for transport, since the 5-thio derivative binds with reasonable affinity, but is not transported [5]. Perhaps forskolin is not transported for similar reasons. Since forskolin and cytochalasin B are displaced by sugar (the present paper; [8,12]), it seems likely that all three can bind to the same place in the GalP protein.

These reported measurements provide the benchmark data for examining the forskolin binding site by a programme of mutagenesis; ultimately, such comparisons will illuminate the molecular basis of ligand recognition by this family of proteins.

This research was supported by research grants from the Wellcome Trust and the Medical Research Council (M.R.C.) (to A.R.W. and P.J.F.H.) and the Science and Engineering Research Council (S.E.R.C.) (to P.J.F.H.), and by equipment grants from the University of Sheffield (to A.R.W.), the Royal Society (to A.R.W.) and the University of Leeds (to P.J.F.H.). A.R.W. is an M.R.C. Senior Fellow. A.R.W. is a member of the Krebs Institute at Sheffield, and P.J.F.H. of the Leeds Centre for Molecular Recognition in Biological Systems, which are designated centres for molecular recognition studies supported by the S.E.R.C. We are also grateful to Mr. J. O'Reilly for isolating and characterizing the inner membrane preparations from *E. coli*.

REFERENCES

1 Henderson, P. J. F. (1993) Curr. Opin. Cell Biol. **5**, 708–721
2 Griffith, J. K., Baker, M. E., Rouch, D. A., Page, M. G. P., Skurray, R. A., Paulsen, I. T., Chater, K. F., Baldwin, S. A. and Henderson, P. J. F. (1992) Curr. Opin. Cell Biol. **4**, 684–695
3 Baldwin, S. A. (1993) Biochim. Biophys. Acta **1154**, 17–49
4 Barnett, J. E. G., Holman, G. D. and Munday, K. A. (1973) Biochem. J. **131**, 211–221
5 Walmsley, A. R., Martin, G. E. M. and Henderson, P. J. F. (1994) J. Biol. Chem. **269**, 17009–17019
6 Jung, C. Y. and Rampal, A. L. (1977) J. Biol. Chem. **252**, 5456–5463
7 Cairns, M. T., McDonald, T. P., Horne, P., Henderson, P. J. F. and Baldwin, S. A. (1991) J. Biol. Chem. **266**, 8176–8183
8 Walmsley, A. R., Lowe, A. G. and Henderson, P. J. F. (1994) Eur. J. Biochem. **221**, 513–522
9 Laurenza, A., Sutkowski, E. M. and Seamon, K. B. (1989) Trends Pharmacol. Sci. **10**, 442–444
10 Sergeant, S. and Kim, H. D. (1985) J. Biol. Chem. **260**, 14677–14682
11 Joost, H. G., Habberfield, A. D., Simpson, I. A., Laurenza, A. and Seamon, K. B. (1988) Mol. Pharmacol. **33**, 449–453
12 Martin, G. E. M., Seamon, K. B., Brown, F. M., Shanahan, M. F., Roberts, P. E. and Henderson, P. J. F. (1994) J. Biol. Chem. **269**, 24870–24877
13 Roberts, P. E. (1992) Ph.D. Thesis, University of Cambridge
14 Henderson, P. J. F., Giddens, R. A. and Jones-Mortimer, M. C. (1977) Biochem. J. **162**, 309–320
15 Osborn, M. J., Gander, J. E., Parisi, E. and Carson, J. (1972) J. Biol. Chem. **247**, 3962–3972
16 Futai, M. (1978) in Bacterial Transport (Rosen, B. P., ed.), pp. 7–41, Marcel Dekker, New York
17 Walmsley, A. R., Petro, K. R. and Henderson, P. J. F. (1993) Eur. J. Biochem. **215**, 43–54
18 Schaffner, W. and Weissman, C. (1973) Anal. Biochem. **56**, 502–514
19 Henderson, P. J. F. and Macpherson, A. J. S. (1986) Methods Enzymol. **125**, 387–429
20 Walmsley, A. R. and Bagshaw, C. R. (1989) Anal. Biochem. **176**, 313–318
21 Vermersch, P. S., Tesmer, J. J. G., Lemon, D. D. and Quiocho, F. A. (1990) J. Biol. Chem. **265**, 16592–16603

Received 4 August 1994/22 December 1994; accepted 10 January 1995