THE COOPERATIVE BINDING OF GLUCOSE TO YEAST HEXOKINASE PI DIMER

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SUMMARY

The binding of glucose to yeast hexokinase PI isozyme has been measured by micro scale equilibrium dialysis using a double labelling technique. It was found that under conditions where the enzyme exists exclusively as dimer glucose bound to one site per monomer with strong cooperativity (Hill coefficient = 1.6). Under the same conditions, the PII isozyme dimer binds glucose non-cooperatively (1). The possibility exists therefore that the yeast hexokinase PI and PII isozymes may differ markedly in their regulatory properties.

The binding of sugars to yeast hexokinase induces a large conformational change in the enzyme resulting in the strong enhancement of nucleotide binding (2-5). It is therefore of interest to study directly the binding of sugars to hexokinase and Colowick and Womack have reported results obtained using the rapid and versatile dialysis rate technique which they developed specifically for this purpose (2,6). They showed that the proteolytically modified, monomeric SI and SII forms of yeast hexokinase bound glucose about an order of magnitude more strongly than the corresponding unmodified, dimeric PI and PII forms. However, the binding of glucose to the P forms was so weak that the dialysis rate technique could not provide precise information about the binding constant nor about any possible cooperativity. Colowick and coworkers had previously shown that the binding of sugars to the P forms results in the dissociation of dimer to monomer under many conditions (2), an observation consistent with stronger binding to monomer than to dimer (1,2).

In this note, we describe the results of a micro scale equilibrium dialysis study of the binding of glucose to yeast hexokinase PI. We have avoided the complications of the monomer-dimer association-dissociation equilibrium by choosing conditions under which the enzyme is dimeric even at saturating glucose.

MATERIALS AND METHODS

Materials: Yeast Hexokinase PI was prepared as described by Hoggett and Kellett (1). D-[^{14}C] glucose and L-[^{3}H] glycine with specific activities of 284mCi mmol^{-1} and 5.3 Ci mmol^{-1} respectively were obtained from Radiochemicals, Amersham, England. D-glucose and L-glycine were purchased from Sigma and Fisons respectively. All other reagents were of Analar grade.

Micro Equilibrium Dialysis: The protein chamber used in these experiments was a 50 µl perspex chamber of the type normally used for protein crystallisation studies (7). The membrane was Visking dialysis tubing (^{24}/32", average pore radius 24Å) which had been treated by boiling in dilute EDTA/bicarbonate solution followed by rinsing in distilled water three times and then finally in a buffer. The chamber was sealed with dialysis tubing by means of a rubber "O" ring. Because of the very small volume involved, a double labelling technique with L-glycine as the second non-binding label was used to improve the accuracy of the distribution measurements.

A 50 µl chamber was filled with a 10 mg cm^{-3} solution of hexokinase PI in 50 mM tris.HCl buffer, pH 6.7, and sealed with dialysis tubing. The chamber was placed in a vial containing 2.5 cm^3 of glucose solution consisting of a given concentration of D-glucose, D-[^{14}C] glucose (8 x 10^{-2} µCi cm^{-3}), L-[^{3}H] glycine (2.5 µCi cm^{-3}), L-glycine (0.05 mM) and EDTA (0.1 mM) in 50 mM tris.HCl, pH 6.7 at 5°C. The sealed vial was then left on a rotating disc (2 rpm) at 5°C until dialysis was complete after approximately 18 hours.

20 µl samples from inside and outside the dialysis chamber were counted for both ^{14}C and ^{3}H using a Beckman Scintillation Counter LS 230. Samples were dispersed in 8 cm^3 of toluene/Triton X scintillation fluid (667 cm^3 toluene, 333 cm^3 Triton X, 5.5 g PPO and 0.1 g POPOP) and 1 cm^3 of water. ^{14}C standards were counted to determine the percentage of ^{14}C spillover into the ^{3}H channel. A further 20 µl sample was taken from inside the dialysis chamber to determine enzyme concentration using an extinction coefficient of E = 0.88 cm^2 mg^{-1} at 280 nm.

The results were calculated as follows. After the normal background corrections, the ^{3}H counts were corrected for the spillover of ^{14}C counts into the ^{3}H channel by means of the ^{14}C spillover factor obtained directly from the counting of ^{14}C standards in both channels. To minimise the effect of this correction factor, the solutions contained a higher ^{3}H activity than ^{14}C (2.5 and 8 x 10^{-2} µCi cm^{-3} respectively). The ^{3}H spillover into the ^{14}C channel was negligible. The difference between the ^{14}C to ^{3}H ratio inside (R_i) and outside (R_o) the protein chamber was equivalent to the amount of glucose bound to the enzyme. Further, since the outside volume (2.5 cm^3) was much greater than the

inside volume (50 µl), the free glucose concentration outside (c_f) remained effectively unchanged. Hence, the concentration of bound glucose (c_b) was given by the expression:

$$c_b = \frac{(R_i - R_o)}{R_o} \times c_f$$

Enzyme concentration is expressed as the molar concentration of monomer units of molecular weight 50,000.

Analytical Ultracentrifugation: Sedimentation equilibrium experiments were performed with a Spinco Model E Ultracentrifuge equipped with a photoelectric scanning absorption photometer. Scans were made at 280 nm after equilibrium had been established in solution columns of 3 mm length. Molecular weights were calculated by least squares analysis of ln c versus r^2 plots and the partial specific volume of the enzyme was taken as 0.740 $cm^3\ g^{-1}$.

TABLE 1

Molecular weights of Hexokinase PI in 50 mM tris. HCl at pH 6.5 and 10°C.

Glucose (mM)	Molecular Weight
0	104,000
0.2	100,000
2.0	109,000

RESULTS

Molecular Weight Determinations: Table 1 shows the molecular weights determined by sedimentation equilibrium of hexokinase PI in 50 mM tris. HCl buffer, pH 6.5 at 10°C. The molecular weight corresponded to that of dimer, approximately 100,000, both in the absence and in the presence of glucose. Even at saturating glucose, plots of ln c versus r^2 were linear over the whole range of 0.1 to 1 mg cm^{-3} implying that dissociation to monomer was not detectable under these conditions.

The Double Labelling Technique: Preliminary tests in the absence of enzyme and using only ^{14}C glucose showed that sampling errors were

TABLE 2 Double-labelling micro equilibrium dialysis data for the binding of glucose to hexokinase PI dimer

Sample	^{14}C cpm	^{3}H cpm	^{3}H cpm corrected for spillover	^{14}C : ^{3}H ratio	free glucose mM	bound glucose mM	PI mM	$\bar{\nu}$
outside	3974	27695	26701	0.149	0.200	0.016	0.191	0.091 ±0.008
inside	3974	25562	24569	0.162				
outside	5836	41539	40080	0.146	1.000	0.183	0.368	0.61 ±0.02
inside	6936	40621	38887	0.178				
outside	3858	29568	28603	0.135	2.000	0.157	0.197	0.87 ±0.08
inside	4163	29492	28451	0.146				

significant. In order to correct them, a second label, L-[^{3}H]-glycine, was used which did not bind to hexokinase and was therefore uniformly distributed across the membrane. Table 2 illustrates the advantage of double labelling.

Row 1 of Table 2 contains data obtained at a free glucose concentration of 0.20 mM. Inspection of the ^{14}C counts suggests that there is no binding of glucose to the enzyme at 10 mg cm^{-3}. However, the ^{3}H counts, corrected for ^{14}C spillover into the ^{3}H channel, show an unusually large sampling error of 8%, equivalent to 1.6 µl in the 20 µl sample. In fact, the difference between the ^{14}C:^{3}H ratio inside and outside the membrane reveals binding to the extent of 0.091 moles glucose per mole enzyme ($\bar{\nu}$). Counting of data in Row 1 was stopped at the pre-set error level of two standard deviations corresponding to 1% in ^{14}C counts, and the final error of 8.8% in $\bar{\nu}$ arises as a result of the statistical accumulation of errors in the several subsequent simple calculations. In the experiment at 1.0 mM glucose, Row 2, the error in $\bar{\nu}$ was reduced to 3.0% by counting ^{14}C to 0.5% pre-set error level and raising enzyme to twice the normal concentration at 20 mg cm^{-3}, giving larger counting differences between inside and outside. However, these measures would have been of no avail but for the correction by double labelling of the more normal sampling error of 3% seen in this case.

The Binding of Glucose to Yeast Hexokinase PI Dimer: Figure 1 shows the dependence of the number of moles of glucose bound per mole of hexokinase PI (50,000 daltons), $\bar{\nu}$, on glucose concentration, in 50 mM tris. HCl buffer, pH 6.7 at 5^{o}C. As shown above, PI under these conditions exists exclusively as dimer. The dependence of binding is clearly sigmoidal with $\bar{\nu}$ approaching a value of one near saturating glucose concentrations (see also Table 2, Row 3). It was not possible to make accurate measurements closer to saturation because the difference between bound and free glucose was relatively too small. Nor was it possible

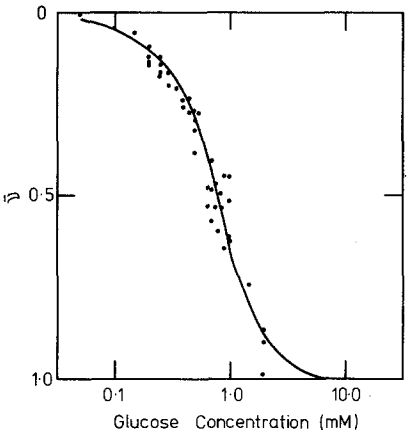

Figure 1. The binding of glucose to hexokinase PI dimer. 50 mM tris. HCl buffer, pH 6.7 at 5°C.

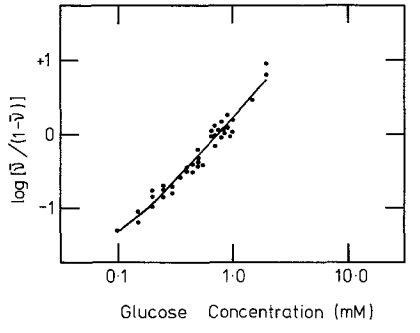

Figure 2. A Hill plot of the data in Figure 1. The Hill coefficient, n ≃ 1.6.

to extrapolate the data accurately to saturation with a Scatchard plot which was very highly curved. Figure 2 shows a Hill plot of the data assuming $\bar{\nu}$ = 1 at saturation. The value of the Hill coefficient, n, was approximately 1.6 demonstrating strong, positive cooperativity in the binding of glucose to PI dimer. Half saturation was achieved at approximately 0.75 mM glucose.

DISCUSSION

The micro scale equilibrium dialysis used in this work is very

economical on enzyme. The principal difficulty has been that, because of the very small sample volumes employed, pipetting errors are significant despite the use of microsyringes. The effect of these errors has nevertheless been successfully eliminated by corrections made possible with the use of a second radioactive label, [^{3}H]-glycine, which does not bind to hexokinase and is therefore uniformly distributed across the dialysis membrane. In this way, it has been possible to determine $\bar{\nu}$ with a high level of accuracy (Table 2). It is apparent that double labelling in this way could be profitably used in other related techniques, especially steady-state methods involving dialysis or ultrafiltration where the rate of transport of a small molecule across a membrane is very dependent on variations in membrane porosity or concentration layers (8).

Figures 1 and 2 show that in 50 mM tris. HCl buffer, pH 6.7 at 5^{o}C the binding of glucose to yeast hexokinase PI is highly cooperative ($n \simeq 1.6$), that half saturation is achieved at about 0.75 mM glucose and that there is one site per 50,000 daltons. The concentration of PI in dialysis experiments was at least 10 mg cm^{-3}. However, sedimentation equilibrium experiments under closely comparable conditions showed that even at concentrations as low as 0.1 to 1.0 mg cm^{-3} dissociation of dimer to monomer was negligible in the presence of either low or high glucose concentrations (Table 1). At the concentrations of the dialysis experiments, therefore, PI exists exclusively as dimer.

Colowick et al. found no great difference between PI and PII with glucose (2). In both cases, the binding was too weak for direct measurements to give useful information. We have also found this to be so for PII. However, our findings show clearly that PI binds glucose more strongly than PII (see below) and we can offer no obvious explanation for the discrepancy between our data and theirs. In fact our observations for glucose closely parallel those for mannose reported by Colowick et al. (2). They found that while mannose bound only weakly to PII, it bound strongly to PI and

displayed strong, positive cooperativity. In the latter case, because sugars bind more strongly to monomers than to dimers (2) and consequently cause dissociation of dimers under appropriate conditions (1,2), Colowick et al. attributed the strong cooperativity of mannose binding to PI to the monomer-dimer association-dissociation equilibrium. However, the results described above show that cooperativity persists even when dissociation of dimer to monomer is insufficient to be detectable at high glucose concentrations. The strong, positive cooperativity displayed in the binding of glucose to PI is thus clearly a property of the dimer and not solely of the monomer-dimer association-dissociation equilibrium.

In this respect, PI differs sharply from PII. We have recently reported data on the quenching of protein fluorescence by glucose which show that both sites on the PII dimer in solution are occupied and are equivalent with an intrinsic binding constant of 6.0 mM at pH 6.7, that is, binding to the PII dimer is non-cooperative (1). The association of subunits in the PII dimer in solution is therefore homologous in contrast to the heterologous situation observed in the crystal (4). We are at present extending our studies to the combined effects of glucose and nucleotide. However, the observation that the binding of glucose alone to the PII dimer is non-cooperative while that to the PI dimer is very strongly cooperative suggests that yeast hexokinase PI and PII isozymes may well display different regulatory properties.

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