Glucose-Induced Thermal Stabilization of the Native Conformation of GLUT 1

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ABSTRACT: The glucose transporter, GLUT 1, was purified from erythrocyte membranes and incorporated into vesicles of erythrocyte lipids. These protein-containing vesicles were studied with differential scanning calorimetry. It was found that the protein underwent an irreversible denaturation at 68.5 ± 0.2 °C (at a scan rate of 0.25 °C/min) which was shifted to 72.6 ± 0.2 °C in the presence of 500 mM D-glucose, while 500 mM L-glucose or 10 μM cytochalasin B did not produce a significant shift. The calorimetric enthalpy was found to be 150 kcal/mol, independent of the presence of D-glucose. On a weight basis this value is lower than that for soluble proteins, but it is comparable to values obtained with other integral membrane proteins. The van't Hoff enthalpy is similar to the calorimetric enthalpy, within the experimental error, indicating that the transition is not likely to be cooperative. The activation energy is estimated from both the scan rate dependence of the transition temperature and from the shape of the DSC curve. The presence of 500 mM D-glucose slightly decreases the activation energy. It is concluded that the shift to a higher denaturation transition temperature in the presence of D-glucose is not a result of increased kinetic stability of GLUT 1.

Facilitated diffusion of polar substances through membranes occurs because of the presence of specific transporter proteins. In the case of glucose, the major energy and carbon source in all animal cells, the process is mediated by a family of integral membrane proteins with 12 transmembrane helices, known as facilitative glucose transporters or GLUTs. Five isoforms are known in this family. GLUT 4 is expressed in the classical insulin sensitive cells. GLUT 1, an isoform expressed in many cells, is abundant in erythrocytes. GLUT 1 has a K_M of 5 mM for glucose, comparable with that for GLUT 4. The GLUT 2 isoform, present in liver and pancreas, has a significantly higher K_M for glucose (15−20 mM), to function optimally under high glucose load. There are binding sites on the transporter for glucose on both the extracellular and cytoplasmic side of the membrane. GLUT 1 is the only isoform of this family that is available as a functional pure protein.

The variation of the thermal stability of proteins with temperature is dependent on the sign of the enthalpy of denaturation. In many cases ΔH is temperature dependent; i.e., the reaction is accompanied by a ΔC_p. At high temperatures ΔH is always positive, requiring that the protein becomes less stable with increasing temperature. The position of equilibrium between folded and unfolded states of the protein can be shifted by the binding of small molecules to one or the other of the conformational states through a mass action effect. There will be more groups of the protein exposed in the unfolded state allowing for greater nonspecific binding. However, in the folded native state there is likely to be a specific binding site for glucose in GLUT 1 since the facilitation of transport is specific for D-glucose. The strength of this binding is not very large since the glucose must be rapidly exchanged on and off this binding site in order for its transport through GLUT 1 to be very rapid.

A convenient way to study the thermal stability of proteins is with differential scanning calorimetry (DSC). Recently a new generation of DSC instruments have been introduced which allow the measurement of thermal transitions with very much greater sensitivity (*1, 2*). We have employed this method to study the characteristics of the thermal denaturation of GLUT 1 and the effect of glucose binding on the stability of the folded protein. GLUT 1 is an integral membrane protein which contains approximately 82% α-helical structure (*3*). The helices of GLUT 1 are oriented within 38° of the bilayer normal, and this arrangement is slightly affected by glucose binding (*4*). A specific model for the structure of GLUT 1 has been proposed (*5*). In general, integral membrane proteins have a high degree of thermal stability, probably in large part because the transmembrane helices are particularly stable in the largely anhydrous environment of the membrane (*6*). The denaturation enthalpy of only a few membrane proteins has been studied (*7*).

MATERIALS AND METHODS

Materials. GLUT 1 in endogenous lipids was isolated from human erythrocytes as described (*8*). The preparation showed a D-glucose and displaceable cytochalasin B binding of 14−16 μmol/mg of protein and a protein−lipid mass ratio of typically 1:6.

Differential Scanning Calorimetry (DSC). Measurements were made using a Nano differential scanning calorimeter (Calorimetry Sciences Corp., Provo, UT). The features of the design of this instrument have been described (*1*).

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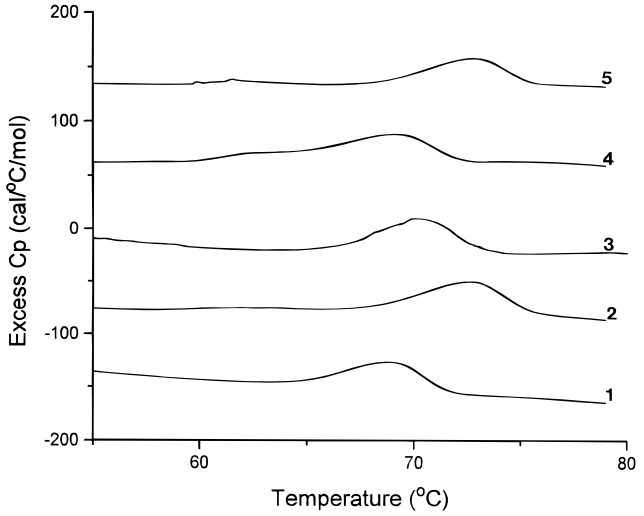

FIGURE 1: DSC curves for the thermal denaturation of GLUT 1 reconstituted in erythrocyte lipids. Key: protein concentration 0.8 mg/mL; protein/lipid ratio approximately 1:6; scan rate 0.25 K/min; curve 1, no addition; curve 2, in the presence of 500 mM D-glucose; curve 3, in the presence of 500 mM L-glucose; curve 4, in the presence of 10 μM cytochalasin B; curve 5, in the presence of 500 mM D-glucose and 10 μM cytochalasin B. Curves are arbitrarily displaced along the ordinate for ease of presentation.

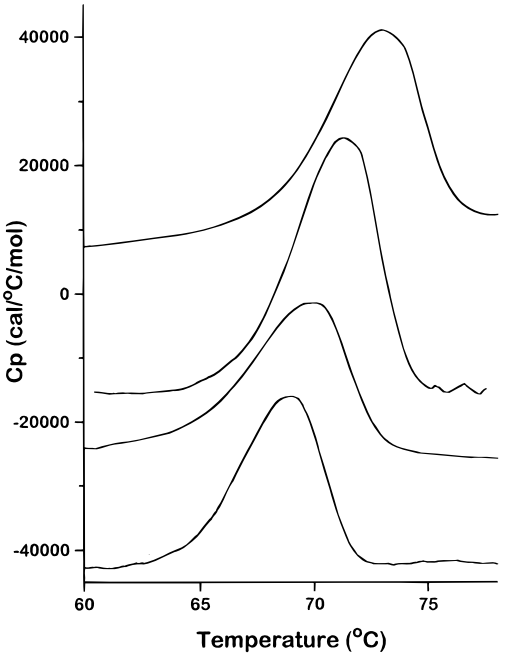

FIGURE 2: DSC curves for the thermal denaturation of GLUT 1 reconstituted in erythrocyte lipids. Key: protein concentration 0.8 mg/mL; protein/lipid ratio approximately 1:6. Scan rates for curves from bottom to top are 0.25, 0.5, 1 and 1.5 K/min. Curves are arbitrarily displaced along the ordinate for ease of presentation.

Solutions were degassed under vacuum prior to loading in the calorimeter cells. The buffer used was 10 mM Tris, pH 7.4.

Analysis of DSC Data. DSC curves were analyzed in several different ways. By using the fitting program, DA-2, provided by Microcal Inc., developed for reversible thermal transitions, values for the calorimetric (ΔH_{cal}) as well as the van't Hoff enthalpy (ΔH_{VH}) were obtained. The Arrhenius energy of activation (ΔE) of thermal unfolding was calculated from the shape of the DSC transition using a program kindly provided to us by Drs. Lepock and Senisterra (*9*),

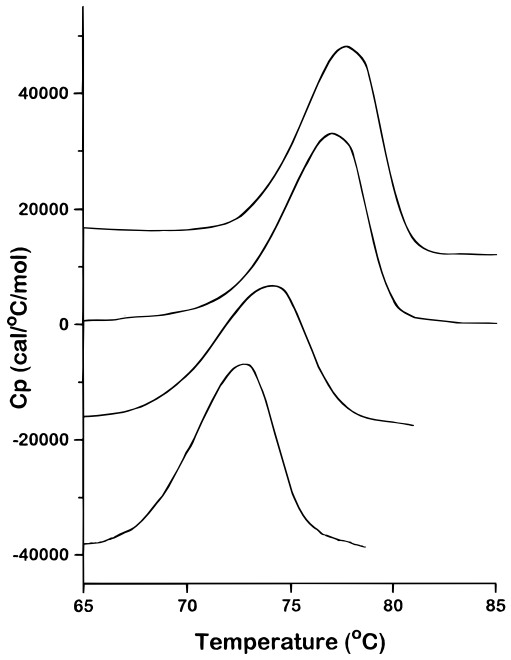

FIGURE 3: Same as Figure 2 but in the presence of 500 mM D-glucose.

Table 1: Characteristics of the Denaturation of GLUT 1[a]

[glucose] (mM)	scan rate (°C/min)	T_m (K)	ΔH_{cal} (kcal/mol)	ΔH_{VH} (kcal/mol)	ΔE (kcal/mol)
0	0.25	342	120	210	152
500	0.25	346	157	172	123
0	0.5	343	129	166	127
500	0.5	347	124	167	128
0	1	344	188	204	150
500	1	350	161	189	136
0	1.5	346	160	183	125
500	1.5	351	173	200	131

[a] ΔH_{cal} was determined from area under transition in DSC curve. ΔH_{VH} and ΔE were calculated from the shape of the DSC curves as described in the text. All scans are at 0.8 mg/mL GLUT 1.

incorporated into Origin, version 2.9. In addition we obtained estimates for ΔE, the activation enthalpy (ΔH^*), and ΔH_{VH} from the variation of the transition temperature (T_m) with scan rate (a) (*10*, *11*). The slope of a plot of $1/T_m$ versus $\ln(a/T_m^2)$ is equal to $-\Delta E/R$, where R is the gas constant. The variation of transition temperature (T_m) with scan rate (a) can also be used to determine ΔH_{VH} (*10*), from the equation

$$T_m = \theta + (\ln \alpha - 0.3665)R\theta^2/\Delta H^* \quad (1)$$

where T_m is the temperature at which half of the protein is unfolded, θ is obtained from the y-intercept, and ΔH^* is related to ΔH_{VH} by the equation

$$\Delta H_{VH} = 4\Delta H^*/e \quad (2)$$

RESULTS

The thermal denaturation of GLUT 1 in liposomes was studied by DSC. An endothermic transition at 68.5 ± 0.2 °C was observed for three independent preparations using a heating scan rate of 0.25 K/min. No transition was observed on recooling from temperatures just above the observed

Table 2: Summary of Parameters Characterizing the Denaturation of GLUT 1[a]

[glucose] (mM)	ΔH_{cal}[b] (kcal/mol)	ΔH_{VH}[b] (kcal/mol)	ΔE[c] (kcal/mol)	ΔE[d] (kcal/mol)	ΔH^*[e] (kcal/mol)	ΔH_{VH}[e] (kcal/mol)
0	149 ± 31	191 ± 20	139 ± 15	102 ± 14	136 ± 15	200 ± 22
500	154 ± 21	182 ± 15	130 ± 6	76 ± 7	103 ± 17	152 ± 25

[a] Errors given are the standard deviation. [b] Calculated for a reversible transition from the area under the curve and the shape of the curve. [c] Average of data from Table 1 for all scans. [d] From plot shown in Figure 5. [e] From plot shown in Figure 4.

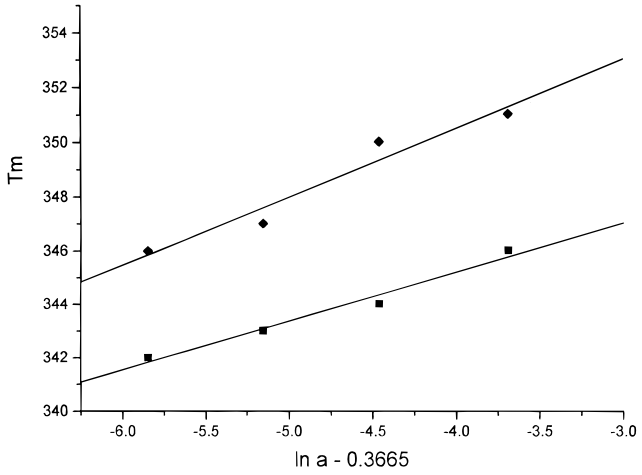

FIGURE 4: Denaturation temperature of GLUT 1 (T_m) as a function of scan rate (a). The van't Hoff enthalpy is estimated from the slope of this plot. Points correspond to data obtained in the presence (diamonds) and in the absence (squares) of 500 mM D-glucose.

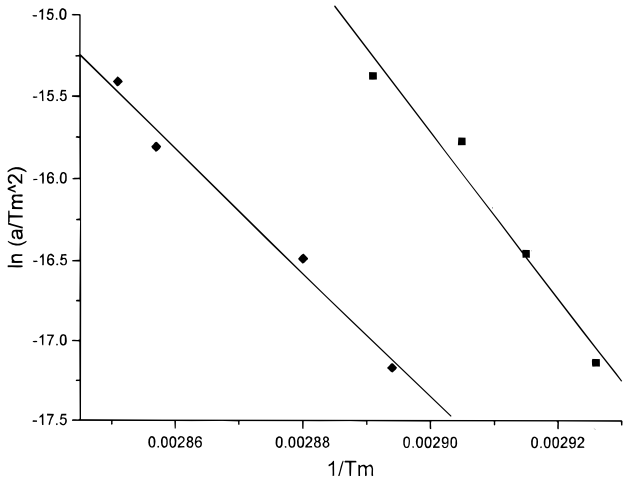

FIGURE 5: Evaluation of activation energy from the variation of transition temperature (T_m) with scan rate (a). Points correspond to data obtained in the presence (diamonds) and in the absence (squares) of 500 mM D-glucose.

endotherm or upon a second heating scan after recooling from the unfolded state. Addition of 500 mM D-glucose raised the denaturation temperature from 68.5 ± 0.2 to 72.6 ± 0.2 °C at a scan rate of 0.25 K/min. The shift of denaturation temperature with 500 mM L-glucose was much less, to only 69.4 ± 0.3 °C (Figure 1). In no case was a transition observed in the cooling direction, and a second heating scan did not result in a detectable endotherm.

Cytochalasin B binds strongly to GLUT 1 and inhibits its function (*12*). The addition of 10 μM cytochalasin B had little effect on the unfolding transition as observed by DSC either in the absence or presence of 500 mM D-glucose (Figure 1). It should be noted that although the interaction of glucose and cytochalasin B with GLUT 1 are mutually affected, the substrate and inhibitor each produce different conformational changes (*13*−*15*).

We have also measured the dependence of the thermal transition on heating scan rate (Figures 2 and 3). Although the transition is irreversible, we have analyzed the DSC curves to calculate the calorimetric transition enthalpy (ΔH_{cal}) as well as the van't Hoff enthalpy (ΔH_{VH}). This approach is common to the study of protein denaturation and has been justified by assuming the existence of an initial reversible unfolding step, followed by irreversible denaturation.

From the area under each DSC curve we obtain ΔH_{cal}, and from the shape of each curve ΔH_{VH} was estimated. In addition, for irreversible transitions, an analysis of the shape of the curve yielded ΔE. These values are summarized in Table 1, both in the presence and in the absence of 500 mM D-glucose. Furthermore, from eq 1, the shift of T_m with scan rate (a) can be used to calculate the activation enthalpy ΔH^* (Figure 4). ΔH_{VH} can be independently evaluated for a series of scans at different scan rates, applying eq 2. Also, ΔE can be obtained from the slope of a plot of $1/T_m$ versus $\ln(a/T_m^2)$ (*10*, *11*), as described in the methods section. The linearity of this plot shows that the transition can be well described as a two-state process (Figure 5). The parameters obtained by the various methods of analysis are summarized in Table 2, both in the presence and absence of D-glucose.

DISCUSSION

D-Glucose significantly raises the T_m at all scan rates studied (Table 1). The effect of D-glucose in raising the T_m of GLUT 1 is not due to a nonspecific effect, such as solvation, since L-glucose has little effect. It thus seems likely that D-glucose shifts the denaturation equilibrium by preferentially binding to the folded state of the transporter. It is interesting that cytochalasin B does not affect the transition temperature of GLUT 1, although it also binds to the transporter. However, cytochalasin B binds at a different site on the protein from D-glucose (*13*−*15*).

Another example of noncovalent binding of a ligand to an integral membrane protein which results in a shift of the temperature of thermal denaturation is that of the binding of ouabain to Na^+,K^+-ATPase (*16*). Ouabain raises the denaturation temperature from 55.3 to 59.5 °C. However, the example presented in the present paper is different in that it is a natural ligand of a transporter, rather than an inhibitor drug. The temperature of unfolding of a domain of Ca^{2+}-ATPase is also shifted to higher temperatures upon activation with ATP and Ca^{2+} (*17*). There are also examples of very marked shifts of denaturation transitions with the covalent attachment of ligands to integral membrane proteins. Examples of this are the binding of retinal to rhodopsin (*18*) and of DIDS to the band 3 anion transporter of erythrocytes (*19*).

The state of association of GLUT 1 in detergent solubilized forms appears to depend on the detergent used (*20*). However, in a membrane there is evidence that GLUT 1 undergoes an equilibrium between dimer and tetramer forms which is affected by ATP (*21*). The ratio of $\Delta H_{VH}/\Delta H_{cal}$ gives the degree of cooperativity of the transition. The two independent estimates of ΔH_{VH} are in reasonable agreement (Table 2) and the ratio, $\Delta H_{VH}/\Delta H_{cal}$, is found to be within experimental error of one, indicating that the denaturation is not a cooperative process and that each monomer unfolds independently. However, in the analysis of individual scans (Table 1) it appears that ΔH_{VH} is somewhat larger than ΔH_{cal}, suggesting that a minor fraction of the GLUT 1 may be in oligomeric form which denatures cooperatively. However, there is no effect of D-glucose on the cooperativity of the transition.

It is interesting to note the similarity of the calorimetric transition enthalpy of GLUT 1 with that of other integral membrane proteins on a weight basis. GLUT 1 has a calorimetric transition enthalpy of 2.8 cal/g. This compares with 3.1 cal/g for unbleached rhodopsin (*18, 22*), 3.6 for Ca^{2+}-ATPase (*17*), and 3.7 cal/g for bacteriorhodopsin (*23*). Cytochrome oxidase from beef (*24*), yeast (*10*), and bacteria (*25*) are found to be 2.7, 2.4, and 2.9 cal/g, although subunit III of cytochrome oxidase is found to have a higher denaturation enthalpy of about 6 cal/g. A lower value of 1.3 cal/g was found for the denaturation enthalpy of Na^+,K^+-ATPase (*16*). Higher enthalpy values for the band 3 protein from erythrocytes had been reported; however, it has been found that if this protein is stripped of peripheral membrane proteins, the observed enthalpy of denaturation is 3.5 cal/g (*26, 27*), in line with values for other integral membrane proteins. The value of about 3 cal/g for integral membrane proteins compares with the much larger enthalpy for the denaturation of globular proteins at 67 °C, which is 7.8 ± 0.7 cal/g (*28*). The denaturation enthalpy is temperature dependent for globular proteins because ΔC_p is not zero and the observed denaturation enthalpy and temperature are different for different soluble proteins. However, the range of thermal transition temperatures found for different integral membrane proteins is narrower and is close to 70 °C for most cases studied. The lower value for the denaturation enthalpy of integral membrane proteins is indicative of the fact that a major portion of the protein structure is resistant to denaturation because it is buried within the membrane bilayer. We found no effect of D-glucose on ΔH_{cal}. Since the observed calorimetric transition was irreversible, we asked if it was possible that the effect of D-glucose was to increase the kinetic stability of the folded form of the protein. This does not appear to be the case, since the activation energy in the presence of D-glucose is equal or lower than in its absence. The results indicate that D-glucose does not increase the kinetic stability of the folded form of GLUT 1. Therefore, we conclude that the thermodynamics of the unfolding of GLUT 1 is typical of that of an integral membrane protein and that D-glucose binding stabilizes the native conformation of this protein.

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