Fluorescence-Quenching Study of Glucose Binding by Yeast Hexokinase Isoenzymes†

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ABSTRACT: A study of the effect of varying ionic strength on the glucose-induced quenching of tryptophan fluorescence of hexokinase isoenzymes A(P-I) and B(P-II) was carried out at pH 8.3 and pH 5.5. At pH 8.3 both isoenzymes gave apparently linear Scatchard-type data plots even with protein concentrations and ionic strengths for which both dimeric and monomeric forms of hexokinase coexist in significant amounts. Taking into account a 1% accuracy in the experimental measurements, we concluded that the intrinsic dissociation constants, K_M and K_D, for the binding of glucose to the monomeric and dimeric forms of HkB, are within a factor of two of each other, i.e., $K_D/K_M \leqq 2$. The values of K_M, estimated from the apparent K, were so greatly influenced by ionic strength that it is clear that it is meaningless to compare K_M and K_D values measured at different ionic strengths as has been done in the literature. Curvature in the pH 5.5 fluorescence-quenching plots for relatively low ionic strengths demonstrates cooperativity for glucose-binding to the dimer, positive for HkA but negative for HkB. In contrast, the binding is relatively noncooperative at high ionic strength at this pH. These results were attributed to the well known effect of salt-neutralization of side chain electrical charges on the flexibility and compactness of proteins.

Being an integral part of glycolysis, the interaction of glucose with hexokinase (HK)[1] is of great interest. The yeast Hk isoenzymes A (or P-I) and B (or P-II) exist in dimer form with molecular weight 104 000 at pH 5.5 (i.e., near the isoelectric pH of 5.25 and 4.93, respectively (Schmidt & Colowick, 1973b)), when the ionic strength, I, is 0.5 or less and the enzyme concentration, E_0, is at least 1.0 mg/mL (Derechin et al., 1972). Dissociation of the dimer into two identical monomers is promoted by elevating either the pH or I and by decreasing E_0 (Schachman, 1960; Derechin et al., 1972; Schulze & Colowick, 1969).

The early evidence for dissociation of Hk by its substrate glucose (Schachman, 1960; Ramel et al., 1963) is now known to be invalid, because of protease impurity in the Hk preparations used (Lazarus et al., 1966; Schulze & Colowick, 1969). Subsequent work with highly purified, protease-free Hk has led to considerable disagreement (Schulze & Colowick, 1969; Derechin et al., 1972; Hoggett & Kellett, 1976; Shill & Neet, 1974; Williams & Jones, 1976).

Hoggett & Kellett (1976) claimed to have confirmed an earlier suggestion of Gazith et al. (1968) that glucose-induced dissociation of Hk is due to stronger glucose binding by monomeric Hk than by the dimer, which, of course, is consistent with the classical thermodynamic explanation (Weber, 1975). The former authors reported that the intrinsic dissociation constant, K, for the glucose-HkB complex is 8- to 20-fold smaller for monomeric HkB than for dimeric HkB. However, their monomer constant, K_M, was measured at pH 8 and ionic strength 1.0, while their dimer constant, K_D, was measured at pH 6.5 and 7 and at the very low ionic strength of 0.001, i.e., the I of 0.02 M Tris buffer at pH 6.5 and 7. As we shall show in this paper, such a comparison is *not* valid, because both I and pH strongly influence the compactness and flexibility of proteins (Tanford, 1961; Lumry & Biltonen, 1969; Von Hippel & Schleich, 1969), and, further, there is good evidence that a conformation change occurs in some proteins specifically in the pH 7–8 region (Steinhardt & Reynolds, 1970). In fact, Hoggett & Kellett themselves reported a 2.4-fold decrease in the K_D of HkB between pH 6.7 and 7. Consequently, these factors should influence the enzyme's affinity for glucose.

It is believed that glucose binds mole per mole to monomeric Hk (Colowick, 1973; Noat et al., 1969), but there is conflicting evidence as to whether the dimer binds one mole of glucose (Anderson & Steitz, 1975) or 2 mol of glucose (Colowick, 1973; Hoggett & Kellett, 1976).

We report here our study of the effect of ionic strength on glucose-binding by the isoenzymes at pH 8.3, where the net charge of the protein is highly negative and at pH 5.5, i.e., near the isoelectric pH, where the net charge should be relatively small.

Materials and Methods

Hexokinase Purification and Assay. Hexokinase isoenzymes A and B were purified by the method of Rustum et al. (1971), with slight modification and extra care which we deemed essential. Only very fresh yeast (obtained from a local bakery immediately upon delivery) with hexokinase activity of 50–100 IU/g wet weight at 25 °C was used. This assay was performed on the supernate of a yeast slurry (ca. 10 g/50 mL) in which the cell walls had been ruptured by shaking the slurry with ca. 10 g of 0.45-mm glass beads in a Braun homogenizer cooled to 4 °C by CO_2 gas. Phenylmethanesulfonyl chloride was used instead of diisopropyl fluorophosphate as a protease inhibitor. We have found it necessary to carry out the DEAE chromatography twice and the DE52 chromatography three times to obtain a product sufficiently pure and active. Our final products, HkA and HkB, had specific activities of 250 IU/mg

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[1] Abbreviations used: Hk, hexokinase; HkA and HkB, hexokinase isoenzymes A(P-I) and B(P-II) respectively; K, apparent dissociation constant for the glucose-HK complex; K_M, intrinsic dissociation constant for complex with monomeric Hk; K_D, intrinsic dissociation constant for complex with dimeric HK; I, ionic strength; L, association constant of HK; $NaDodSO_4$, sodium dodecyl sulfate.

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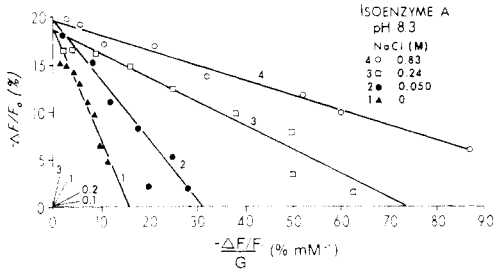

FIGURE 1: Ionic strength dependence of glucose-induced quenching of hexokinase A fluorescence at pH 8.3 and 20 °C. $E_0 = 42$ μg/mL; 300-nm excitation; 350-nm emission; 0.05 M Gly-Gly buffer. Short lines in lower left corner indicate glucose concentrations (see text for explanation).

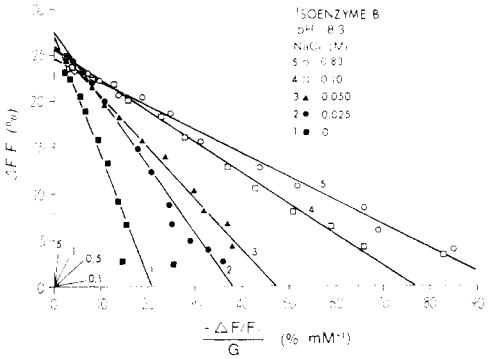

FIGURE 2: Ionic strength dependence of glucose-induced quenching of hexokinase B fluorescence at pH 8.3 and 20 °C. $E_0 = 50$ μg/mL; 300-nm excitation; 350-nm emission; 0.05 M Gly-Gly buffer.

and 750 IU/mg, respectively, and values of 2.0 and 1.1 for F/G, the ratio of enzymic phosphorylation of fructose to that of glucose. The method of Darrow & Colowick (1962), as modified by Lazarus et al. (1966), was employed for the assay. Protein concentrations were measured with the aid of a Beckman spectrophotometer using Barnard's values of 1.2 and 0.98 mL/(mg cm) for the specific absorptivities of HkA and HkB, respectively, at 280 nm (Barnard, 1975). The purity of our products was found to be >99% by the use of polyacrylamide non-NaDodSO$_4$ disc gel electrophoresis and employing a Beckman CDS-100 computing densitometer system.

Fluorescence Titrations. An Aminco-Bowman spectrofluorimeter with an attachment to correct for the wavelength dependencies of the lamp and phototube output was employed for the fluorescence titrations. The accuracy of the fluorescence measurements was maximized, i.e., to 1%, by the use of a digital multimeter capable of millivolt readings. The cell housing was equipped for circulation of constant-temperature water. Increments (usually 5 μL or 10 μL at a time) of glucose in buffer were added to the cuvette containing initially 1.0 mL of hexokinase in buffer at concentrations indicated in the figure legends and the solution was stirred gently. The fluorescence intensity, F, for the indicated wavelength for each point was multiplied by the dilution factor, so that each ΔF is referred to the original F_0 value of the titration. Almost invariably the dilution factor was less than 1.1.

Sedimentation Equilibrium Measurements. A Spinco Model E ultracentrifuge equipped with a photoelectric scanning UV absorption photometer and with 3-mm Yphantis cells was used to determine the monomer:dimer ratio in solutions of interest. For each solution the data was initially analyzed from log c vs. r^2 plots, the slope of which contains the apparent molecular weight of the macromolecule as a factor, providing that only one macromolecular species is present (Tanford, 1961). When the MW_{app} thus obtained was not within 10% of the molecular weight of either dimeric Hk (104 000) or monomeric Hk (52 000) the data was analyzed by the method of Hoggett & Kellett (1976) to obtain the association constant L of the monomer $\rightleftharpoons$ dimer equilibrium, i.e., $L = D/M^2$, where D and M represent the dimer and monomer concentrations, respectively. Knowing L and the total hexokinase concentration, T, one can calculate the D:M ratio.

Results

Like previous workers (Zewe et al., 1964; Higgins & Easterby, 1976; Hoggett & Kellett, 1976; Menezes & Pudles, 1976), we have utilized the glucose-induced quenching of the intrinsic fluorescence of Hk as a measure of the extent of glucose binding. However, to avoid compression of the high-glucose data points near the Y axis, we have not employed their double-reciprocal equation

$$\Delta F^{-1} = \Delta F_s^{-1} + (K/\Delta F_s)G_f^{-1} \quad (1)$$

but have used the rearranged form,

$$\Delta F = \Delta F_s - K(\Delta F/G_f) \quad (2)$$

These two equations may be considered to be the Klotz (1946) and Scatchard (1949) types of binding equations in terms of fluorescence quenching. Here, ΔF is the fluorescence quenching, i.e., $F - F_0$, where F is the fluorescence in presence of glucose relative to the fluorescence F_0 in absence of glucose, and G_f is the free glucose concentration. Since the initial molar ratio of glucose to enzyme always exceeded 60 and usually exceeded 100, the total glucose concentration was substituted for G_f in the calculations. ΔF_s is the extrapolated value of ΔF for complete saturation of the glucose-binding sites. K is the apparent intrinsic dissociation constant for glucose binding. It was convenient to use the negative of ΔF in both coordinates of our graphs, since ΔF is a negative quantity. Like previous workers, we corrected for the slight inconstancy of F_0 by normalizing ΔF to $\Delta F/F_0$ for each experiment, so that $\Delta F_s = (\Delta F/F_0)_s$.

The ionic strength dependences of the glucose-induced quenching of the tryptophan fluorescence of isoenzymes A and B in 0.05 M glycylglycine buffer at pH 8.3 are shown by Figures 1 and 2, respectively. The glucose concentration (mM) corresponding to any point equals the ordinate/abscissa quotient for that point. For the convenience of the reader we have indicated several glucose concentrations by the short lines in the lower left corner of these figures (and also in Figures 4 and 5). Extending one of these short lines to intersect a given data plot indicates the glucose concentration at the point of intersection. In each of these graphs, which represent the I range 0.03 (i.e., the buffer I at pH 8.3) to 0.86 (buffer plus 0.83 M NaCl), all curves appear to be linear. In each figure they converge to the same ΔF_s value, $-20 \pm 1\%$ in Figure 1 and $-26.5\% \pm 1.5\%$ in Figure 2, but in both figures K varies with I. These values are presented in Table I. It is seen that an increase in I from 0.03 to 0.86 caused K to decrease from 1.3 mM to 0.15 mM for HkA and from 1.3 mM to 0.26 mM for HkB. Also listed in Table I are K_M values estimated for HkB by a method described later in the Discussion Section.

Our ΔF_s value, −26.5%, in Figure 2 is considerably larger in magnitude than the value, −17%, reported by Hoggett & Kellett (1976). Since they employed 285-nm excitation and 325-nm emission, while we used 300-nm excitation and 350-nm emission, the obvious reason for the discrepancy in these ΔF_s values seemed to be related to the fact that they were observing both tyrosine and tryptophan fluorescence, while we measured only the latter. To study the effect of including tyrosine

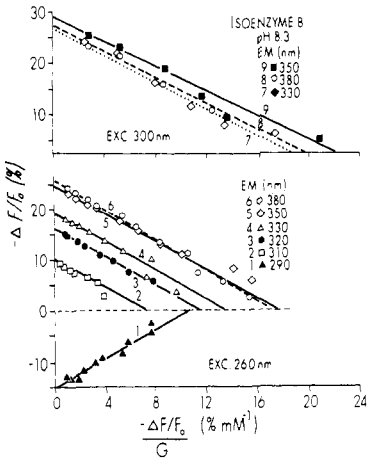

FIGURE 3: Dependence of glucose-induced quenching of hexokinase B fluorescence at pH 8.3 on emission wavelength with 260-nm excitation and with 300-nm excitation, 0.05 M Gly-Gly buffer, E_0 = 88 μg/mL.

TABLE I: Ionic Strength Dependence of Apparent Dissociation Constant, K, of Glucose Complexes of HkA and HkB at pH 8.3 and 20 °C.

[NaCl][a] (M)	K (mM)[b] HkA (100 μg/mL)	HkB (50 μg/mL)	K_M of HkB (est.)[c]
0	1.2	1.3	1.1
0.025		0.71	0.62
0.050	0.64	0.56	0.49
0.10		0.31	0.27
0.24	0.25		
0.83	0.15	0.26	0.26

[a] Salt added to 0.05 M glycylglycine buffer; $I \cong 0.03$ + added NaCl concentration. [b] Values taken from slopes of curves in Figures 1 and 2. [c] See text for method of estimation.

emission, we performed titrations with both 260-nm and 300-nm excitation and various emission wavelengths over the range 290 nm to 380 nm. The results are presented in Figure 3 for HkB. Similar plots were obtained for HkA. All the plots in Figure 3 appear to be linear and correspond to the same K, within experimental error.

Figure 3 shows that the magnitude of $-\Delta F_s$ with 260 nm excitation at pH 8.3 decreases continually with decreasing emission wavelength below 350 nm, and that ΔF_s is positive for 290-nm emission. However, as depicted by curves 7-9, with 300-nm excitation $-\Delta F_s$ for 350-nm emission is larger than for either 330-nm or 380-nm emission.

The fluorescence-quenching curves for HkA and HkB at pH 5.5 are given in Figures 4 and 5, respectively. Only curves 3 and 4 in Figure 4, representing the 0.45 M and 0.65 M NaCl cases, appear to be linear throughout, while only curve 5, 0.83 M salt, seems linear in Figure 5. The remaining curves are definitely nonlinear, being concave downward in Figure 4 and concave upwards in Figure 5. These all seem to have a linear portion on the high-glucose (left) side, although it is extremely short in curve 1 of Figure 5. For each isoenzyme the curvature is greatest in absence of salt, and it decreases as the added salt concentration increases.

Although the ΔF_s values for HkA at pH 5.5 show no trend, but lie within the range −20% to −23%, there is a definite, reproducible trend in the HkB value, from −18% at the lowest I to −28% at the highest I. The numerical results were less reproducible for HkA than for HkB, but the various curve

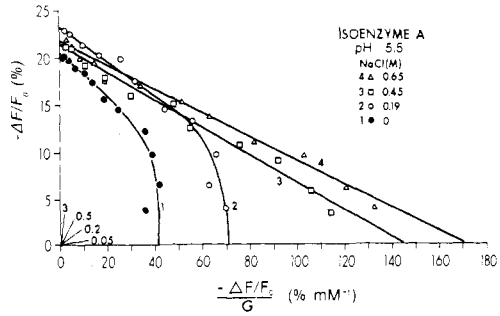

FIGURE 4: Ionic strength dependence of glucose-induced quenching of hexokinase A fluorescence at pH 5.5 and 20 °C. E_0 = 11 μg/mL; 300-nm excitation; 350-nm emission; 0.05 M NaOAc buffer.

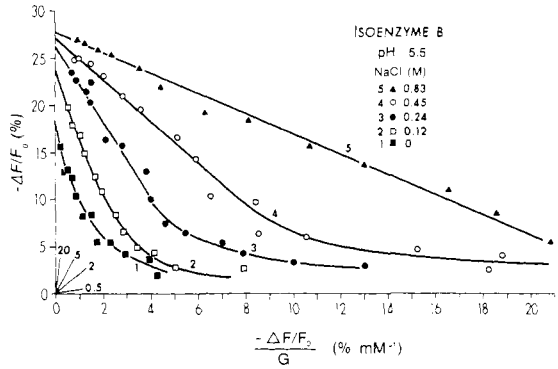

FIGURE 5: Ionic strength dependence of glucose-induced quenching of hexokinase B fluorescence at pH 5.5 and 20 °C. E_0 = 100 μg/mL; 300-nm excitation; 350-nm emission; 0.05 M NaOAc buffer.

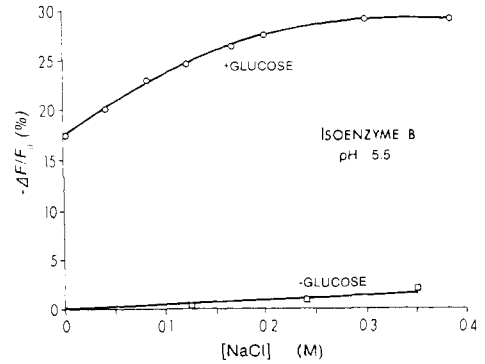

FIGURE 6: Ionic strength dependence of saturation-quenching value of glucose-induced quenching of hexokinase B fluorescence at pH 5.5. E_0 = 84 μg/mL; 300-nm excitation; 350-nm emission; 0.05 M NaOAc buffer. Glucose concentration = 254 mM.

shapes were reproducible. For a given titration, the HkA results were usually reproducible to within 2%, while for HkB they were within 1% reproducibility. That the trend in ΔF_s of HkB in Figure 5 is real is demonstrated by Figure 6, which shows the effect of NaCl addition to a HkB solution which initially contained a completely saturating glucose concentration, 254 mM.

Tables II and III show the pH and ionic strength dependencies of the sedimentation equilibrium results for the two isoenzymes over the glucose concentration range employed in our fluorescence experiments.

Discussion

Derivation of Fluorescence Quenching Equation for Multispecies System. The original derivation of the fluorescence quenching eq 1 by Zewe et al. (1964) was sufficient for the case

TABLE II: Dependence of Dissociation of HkA Dimer on pH, Ionic Strength, and Glucose Concentration Determined by Sedimentation Equilibrium at 20 °C.

pH 5.5 (0.025 M NaOAc buffer)					pH 8.3 (0.05 M Gly-Gly buffer)			
[NaCl] (M)	[Glucose] (mM)	Mol wt	L (L/g)	Monomer[a] (wt %)	[NaCl] (M)	[Glucose] (mM)	L (L/g)	Monomer[a] (wt 5)
0	0	105 000		0	0	0	84	29
	46	108 000		0		38	54	35
0.19	0		470	14	0.24	0	2.5	83
	46		18	52		0.33	1.0	92
						38	1.2	90
0.83	0		6.3	70				
	0.18		4.6	74	0.83	0	0.6	95
	46		2.2	84		0.24	1.1	92
						38	0.7	94

[a] Calculated from L for $E_0 = 100$ μg/mL.

TABLE III: Dependence of Dissociation of HkB Dimer on pH, Ionic Strength, and Glucose Concentration Determined by Sedimentation Equilibrium at 20 °C.

pH 5.5 (0.025 M NaOAc buffer)					pH 8.3 (0.05 M Gly-Gly buffer)			
[NaCl] (M)	[Glucose] (mM)	Mol wt	L (L/g)	Monomer[a] (wt %)	[NaCl] (M)	[Glucose] (mM)	L^b (L/g)	Monomer[c] (wt %)
0	0	115 000		0	0	0	10	73
	140	101 000		0		25	3	88
0.12	0	106 000		0	0.025	0	15	67
	38		20	50		25	3	88
0.45	0		100	27	0.050	0	13	69
	38		1.4	89		25	3	88
0.83	0		3.1	80	0.10	0	12	70
	0.5		1	92		25	3	88
	5.0		2	85				
	140	50 000		100	.83	0	0.2	99
						25	0.3	99

[a] Calculated from L for $E_0 = 100$ μg/mL. [b] Average of two separate runs (±1%). [c] Calculated from L for $E_0 = 50$ μg/mL.

where the system contains only one free enzyme species and only one enzyme-substrate complex. Thus, eq 1 and 2 can be derived by simply equating the right sides of eq 3 and 4 below. However, the application of either of these quenching equations to the case where both monomeric and dimeric forms of free and complexed enzyme species are present is too restricted to leave to one's intuition. The derivation for this case therefore follows:

For multiple binding of ligand, G, by a macromolecule, P, which has n independent, indistinguishable and identical binding sites (i.e., each with the same intrinsic association constant, k, in concentration units), the average number of ligand molecules bound per macromolecule, $\bar{\nu}$, is given by eq 3 (Tanford, 1961).

$$\bar{\nu} = \frac{G_b}{P_T} = \frac{nkG_f}{1 + kG_f} \tag{3}$$

where G_b and P_T are the molar concentrations of bound ligand and total protein (i.e., bound plus free), respectively.

If the fluorescence of this macromolecule is decreased to the same extent by each ligand molecule (or ion) that binds, regardless of the identity of the site, then

$$\bar{\nu} = \frac{\Delta F/c}{P_T} \tag{4}$$

where c is the fluorescence quenching constant, $\Delta F/G_b$.

Now consider the case where the system contains both monomeric and dimeric enzyme, i.e., $(M_f + MG)$ and $(D_f + DG + DG_2)$, respectively, where M_f and D_f refer to the molar concentrations of free monomer and dimer, respectively, MG and DG to 1:1 complexes, and DG_2 to the 1:2 dimer–ligand complex. Obviously, we are adhering to the belief that there is only one glucose-binding site per monomeric unit (Anderson & Steitz, 1975; Hoggett & Kellett, 1976).

The average number of ligands bound per monomer, $\bar{\nu}_M$, is then obtainable directly from eq 3, i.e.

$$\bar{\nu}_M = \frac{k_M G_f}{1 + k_M G_f} \tag{5}$$

If we *assume* no site–site interaction for the dimer, the average number of ligands bound per dimer, $\bar{\nu}_D$, is (Tanford, 1961):

$$\bar{\nu}_D = \frac{k_{D1} G_f}{1 + k_{D1} G_f} + \frac{k_{D2} G_f}{1 + k_{D2} G_f} \tag{6}$$

where k_{D1} and k_{D2} are the intrinsic association constants for the first and second sites on the dimer (i.e., one on each subunit).

ΔF is an additive quantity, so that:

$$\Delta F = c_M G_M + c_{D1} G_{D1} + c_{D2} G_{D2} \tag{7}$$

where the c_i values are the individual quenching constants and the G_i values are the molar concentrations of ligand bound to the indicated sites.

If we now *assume* that all c_i equal the same value, c, then:

$$\frac{\Delta F}{c} = G_M + G_{D1} + G_{D2} = \bar{\nu}_M M_T + \bar{\nu}_D D_T \quad (8)$$

If we now substitute eq 5 and 6 into eq 8 and apply $k = K^{-1}$, the resulting equation is:

$$\frac{\Delta F}{c} = \frac{G_f}{K_M + G_f} M_T + \left(\frac{G_f}{K_{D1} + G_f} + \frac{G_f}{K_{D2} + G_f}\right) D_T \quad (9)$$

In the special case where each of the three association constants, K_M, K_{D1}, and K_{D2}, equals the same constant, K, we get:

$$\frac{\Delta F}{c} = (M_T + 2D_T)\frac{G_f}{K + G_f} \quad (10)$$

The quantity $c(M_T + 2D_T)$ is, by definition, the (unnormalized) ΔF_s. Making this substitution in eq 10 gives eq 1 and 2.

Mechanism of Glucose-Induced Quenching of Hk Fluorescence. There is evidence that glucose binds to Hk by hydrogen bonding of amino acid side chains with glucose hydroxyl groups (Crane, 1962; Steitz et al., 1977). The glucose-binding site in the crystalline BIII monomer is similar to those in the crystalline BII dimer (Anderson & Steitz, 1975), one for each monomeric unit, in that it lies in a deep cleft that separates each monomeric unit into two lobes. The glucose-binding site should be somewhat similar in dissolved Hk, except for the fact that in solution the size of the cleft should vary with the ionic strength and pH. The specificity of Hk for hexoses and the relative affinities for these may be, at least partially, sterically determined, as seems to be true for bovine serum albumin (Giles & McKay, 1962)

The mechanism of the glucose-induced fluorescence quenching is very *unlikely* to involve a direct collisional reaction, because significant quenching occurs with a glucose concentration which is much too low for a diffusion-controlled process. For instance, 0.2 mM glucose causes more than 10% quenching for both HkA and HkB at pH 8.3 in presence of 0.83 M salt (Figures 1 and 2), although one can calculate from the Stern–Volmer equation (Parker, 1968) that this amount of quenching by a collision process should require more than ten times as high a glucose concentration. In fact, 250 times as much (i.e., 50 mM) iodide ion, a good collisional quencher, is required to quench HkB by 10%.

Higgins & Easterby (1976) have suggested that the apparent insensitivity of λ_{max}, the wavelength of maximum Hk tryptophan emission, 328 nm, to glucose addition implies that the quenching is "caused by interaction of tryptophanyl residues with other amino acid side chains with an approximately constant polarity". This is a reasonable mechanism, but it should be noted that its initiation by glucose-binding requires a conformation change in the enzyme to change the relative positions of fluorophores and quenching side chains. There is considerable evidence for such a conformation change (Colowick, 1973).

The insensitivity of λ_{max} to glucose addition may be due to the fact that it is determined by one or more tryptophan fluorophores buried in hydrophobic regions with a combined quantum yield much larger than that of a "redder" glucose-quenchable tryptophan residue. Consistent with this suggestion is the fact that with 300-nm excitation the $-\Delta F_s$ value for 350-nm emission (curve 8, Figure 3) is larger than for either 330-nm emission (curve 7) or 380-nm emission (curve 9). This signifies that for HkB there are at least three overlapping fluorescence spectra. Assuming the reasonable λ_{max} values 327 nm, 334 nm, and 350 nm for buried, cleft (i.e., glucose-quenchable), and surface tryptophan residues, respectively, one can construct composite spectra for Hk in absence and presence of glucose which differ by only 1 nm if the buried residue accounts for about 50% of the total tryptophan emission.

Ionic Strength Effect on K at pH 8.3. The fact that all of the curves in Figures 1 and 2 appear to be linear might lead one to invoke eq 10 and conclude that $K_M = K_{D2} = K$. However, this would be inconsistent with thermodynamics, according to which glucose-induced dissociation of dimeric Hk is due to stronger binding of glucose by monomeric Hk than by dimeric Hk, i.e., that $K_M < (K_{D1}K_{D2})^{1/2}$ (Weber, 1975). This apparent paradox may well be due to the fact that the glucose-induced lowering of the dissociation energy of dimeric Hk is too small to be reliably determined by the fluorescence measurements which have an accuracy of 1% for HkB and 2% for HkA.

Consider the HkB association constants, L, for pH 8.3 presented in Table III. The values in the absence of glucose, L_0, and in the presence of glucose, L_g, are 10–15 and 3 L/g, respectively, over the ionic strength range 0.03–0.13. High salt, 0.83 M, produces almost complete dissociation. Saturating glucose decreases the dissociation energy of Hk dimer by an amount equal to $RT \ln(L_0/L_g)$, i.e., 840 ± 120 cal/mol for HkB at 20 °C. By thermodynamics, this should equal the difference between the energy of binding two glucose molecules to one HkB dimer, $RT \ln(K_{D1}K_{D2})$, and twice the energy of binding one glucose molecule to a HkB monomer, $2RT \ln K_M$. Accordingly, $RT \ln(K_{D1}K_{D2})^{1/2}/K_M$ is 420 ± 60 cal/mol, and $(K_{D1}K_{D2})^{1/2}/K_M \cong 2$.

The apparent linearity of the pH 8.3 curves relative to the large curvature in the pH 5.5 curves (Figures 4 and 5) implies sufficiently negligible site–site interaction in the dimer at the higher pH to allow us to conclude that K_{D1} and K_{D2} are approximately equal, so that $K_D/K_M \cong 2$.

By applying eq 9 one can construct quenching curves which show that a K_D/K_M ratio of this magnitude gives curvature which is just at the edge of detectability when the experimental error of a fluorescence measurement is 1%, as in our HkB experiments. The curvature in these calculated plots is so small that a least-squares straight line gives a K value (i.e., slope) that is only slightly larger than K_M. Specifically, for $K_D/K_M = 2$, K/K_m is 1.3, 1.23, 1.16, 1.14, and 1.09, when the weight percent of monomeric HkB is, respectively, 50, 60, 70, 75, and 85, so that small corrections can be applied to the experimental K values to obtain a good estimate of K_M. For instance, the correction would be 16% or less for the HkB values over the ionic strength range 0.03–0.13 in Table I, since the wt% monomer (% M) is 70% or more throughout (Table III). In fact, the correction factor would be the same for each entry in this ionic strength range, since % M varies to the same extent in each case, i.e., ~70–88%.

The K_M values listed in Table I were obtained by making a 13% correction to the K values from $I = 0.03$ to 0.13, with no correction being required for the high salt value, since virtually all of the HkB is monomeric in this case. A fourfold decrease in K_M between $I = 0.03$ and 0.13 is seen, but K_M seems to be constant above $I = 0.13$. It is evident, therefore, that it is meaningless to compare K_M and K_D values measured at different ionic strengths, when at least one of these values is obtained at very low I, as Hoggett & Kellett (1976) have done. In fact, since glucose causes significant dissociation of the HkB dimer when $I = 0.1$, even at pH 5/5 (Table III), the two polymeric forms of HkB cannot be studied separately under conditions which allow comparison of their properties, i.e., at the same I and pH.

The enhancement of the glucose-binding strength (decreasing K_M) with increasing salt concentration is opposite to the expected effect of salt on the strength of the hydrogen bonds by which glucose attaches to this isoenzyme. However, one can explain the salt effect by considering the action of salt on protein structure at pH 8.3. Weber (1965) has pointed out that discrete disruption or modification of the ligand-binding region ought to produce a conspicuous change in the binding properties. Such an effect should result from addition of salt, since the mutual electrostatic repulsions of the large excess of negative charges at pH 8.3 cause some expansion of the macromolecule relative to the more compact form at the isoelectric pH (Tanford, 1961). The tendency of the negatively charged side chains to maximize their separation distance would restrict their flexibility, so that they would resist a specific glucose-induced conformational change and would, thereby, have a negative effect on the strength of glucose binding. Addition of NaCl should diminish the mutual repulsions of the fixed negative charges, thereby making the polypeptide chains more flexible (Tanford, 1961). As a result, conformational change should require less energy and the strength of the glucose binding should be increased. Accordingly, in Table I we see that for both isoenzymes at pH 8.3 there is a gradual decrease in K with increasing ionic strength. It is noteworthy that K is approximately the same for both isoenzymes at pH 8.3 throughout the ionic strength range studied, in great contrast to the pH 5.5 results discussed below.

Our high ionic strength K value, 0.26 mM, which is K_M, for HkB at 8.3 agrees well with the 0.33 mM value of Hoggett & Kellett (1976), even though different buffers were used, glycylglycine in our case, Tris in their case.

The upper limit of our lowest ionic strength K_D value, 2.2 mM, i.e., from our previously developed relation that $K_D/K_M \leqq 2$, is approximately equal to their K_D value, 2.5 mM, at pH 7. In addition to adding some support to our calculations, this modest agreement suggests that neither glycylglycine nor Tris ions have a specific effect on the strength of glucose binding to Hk in their presence, even though they do exert an ionic strength effect. It is obvious, however, that these buffer ions do have specific effects on the dissociation of the dimer in addition to their electrical charge effect. For instance, where Hoggett & Kellett measured an L_0 of 33 L/g in Tris buffer at pH 8, we obtained an L_0 of 10–15 L/g in glycylglycine buffer at pH 8.3. This difference represents about 600 cal/mol of dimer.

Tyrosine Contribution to the Hk Fluorescence Spectrum. Figure 3 shows clearly that at pH 8.3 glucose enhances the tyrosine fluorescence of the HkB isoenzyme even though it decreases the tryptophan fluorescence, so that the net ΔF varies with both excitation and emission wavelength, thus explaining the discrepancy between our value, −26%, for $-\Delta F_s$ and the value, −17%, of Hoggett & Kellett.

Although there is negligible tyrosine fluorescence at 350 nm, tyrosine is probably responsible for the excitation dependence of the glucose-induced quenching of 350-nm emission demonstrated by the difference in the ΔF_s values for curves 5 and 9 of Figure 3. A ready explanation for this difference is that with 260-nm excitation there is some resonance energy transfer from tyrosine residues to one or more of those fluorescent tryptophan residues which are not quenchable by glucose. This energy transfer increases F_0 without changing ΔF and thereby decreases the magnitude of $(-\Delta F/F_0)s$, i.e., $-\Delta F_s$. Energy transfer is absent with 300-nm excitation (Weber, 1960).

Ionic Strength Effect Near Isoelectric pH. There is a significant difference between the pH 7 and pH 6.5–6.7 K_D values which Hoggett & Kellett reported for HkB at very low ionic strength (0.02 M Tris), namely, 2.5 mM and 6 mM, respectively. Not only does this difference imply a significant pH effect on K_D, but its abruptness suggests that there is a major change in the protein's structure in the pH 6.7–7 interval, i.e., near the pK of imidazole groups, which number five and eight for HkB and HkA monomer, respectively (Schmidt & Colowick, 1973a). We found a fourfold increase in the high-salt K value of HkB, from 0.26 mM to 1.1 mM, when the pH was lowered from 8.3 to 5.5, despite the fact that the isoenzyme was virtually all monomeric in both cases (Table III); i.e., these Ks are K_M values.

The type of salt effect operative at pH 8.3 should be relatively insignificant near the isoelectric pH, where there is negligible overall net electrical charge. Rather, near the isoelectric pH there are strong attractive forces between the oppositely charged side chains of a polyampholyte, which produce a network of interconnected polypeptide chains. Since it produces tighter coiling and a smaller molecular volume than at higher pH (Tanford, 1961), this network may contract the size of the cleft and, if it encompasses both subunits of the dimer, may enhance site–site interaction by more readily transmitting the effects of the glucose-induced conformational change from one subunit to the other. Accordingly, the ability of salt to decrease these attractive forces should be a major reason for any ionic strength effect on glucose binding near the isoelectric pH.

The occurrence of curvature and its ionic strength dependence for the pH 5.5 glucose-quenching curves in Figures 4 and 5 are consistent with this view. With HkA, apparent linearity is produced by the presence of 0.45 M added salt, but only the high salt, 0.83 M, curve seems linear for HkB. Since at pH 5.5 both isoenzymes exist only as dimers at low ionic strength (i.e., in 0.025 NaOAc buffer with no added salt) even in presence of saturating glucose (Tables II and III), the curvature in these two figures can*not* be attributed to any kind of glucose-binding competition between monomeric and dimeric enzyme. Neither can it be due to a possible difference in quenching constants of the different glucose-quenchable fluorophores. A derivation similar to that presented for eq 10 clearly shows that a straight line plot would result if only dimeric Hk were present (i.e., for curve 1, Figures 4 and 5) and if the two dimer sites had different quenching constants, c_1 and c_2, but the same binding constant. The only change would be that in this particular case ΔF_s would equal $(c_1 + c_2)D_T$ instead of $c(M_T + 2D_T)$.

Rather, the direction of the curvature indicates that at this pH there is positive cooperativity for glucose binding to dimeric HkA but negative cooperativity for HkB. The Hill coefficients (Wyman, 1964) calculated from these data are: 1.4, 1.2, 0.95, and 0.96 for HkA curves 1 to 4, and 0.73, 0.75, 0.80, 0.90, and 0.96 for HkB curves 1 to 5.

In addition to decreasing site–site interaction the salt may be causing enlargement of the cleft size. The latter effect would explain the variation of ΔF_s seen in Figure 6. The fact that $-\Delta F_s$ levels off at a constant value, 29%, which is approximately the same value as at pH 8.3 suggests that the tight coiling of the subunits at pH 5.5 in low salt decreases the accessibility of the glucose into the cleft binding site and that salt-induced expansion of the cleft decreases this steric restriction.

The slopes of the linear portions on the high-glucose side of the plots in Figures 4 and 5 should be rough approximations of the dissociation constant, K_{D2}, for the second ligand bound to the dimer at the indicated ionic strength. For both isoenzymes the trend of these values, from 0.22 mM to 0.13 mM for HkA and from ~10 mM to 1.1 mM for HkB, is toward stronger binding strength as the added salt concentration is

raised. This variation can also be due to salt-induced enlargement of the cleft containing the second binding site. The apparent linearity of the high-salt plot for HkA at pH 5.5 demonstrates that, just as at pH 8.3, the monomer and dimer at the lower pH have intrinsic binding constants which differ by a factor of only about two or less when there is sufficient salt to destroy the cooperativity for glucose binding. For HkB at pH 5.5 the high-salt plot gives only K_M, since the monomer predominates overwhelmingly (Table III).

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