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EFFECTS OF GLUCOSE AND MAGNESIUM ION ON THE QUENCHING OF YEAST HEXOKINASE FLUORESCENCE BY ACRYLAMIDE

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Summary

To probe the effects of the substrate, glucose, and the cofactor, Mg^{2+}, on the structure of hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1), titrations of the tryptophan fluorescence of yeast hexokinase isozyme P-II(B) were performed. Acrylamide was used as a quenching titrant in the absence and in the presence of glucose and Mg^{2+} singly and together at pH 5.5 and 8.3 at 20° C. The four tryptophan residues of the monomeric subunit of yeast hexokinase may be classified as two surface residues, one being highly accessible to dissolved I^- and one with restricted accessibility to I^-, one glucose-quenchable residue in the cleft, and one buried (Kramp, D.C. and Feldman, I. (1978) Biochim. Biophys. Acta 537, 406—416). The acrylamide data were analyzed by least-squares computer analysis for quenching constants and fractional fluorescence values of the tryptophan residues. The quenching constants measure the accessibilities of the residues to the quencher, while the fractional fluorescences are related to the microenvironments of the fluorophores. At each pH value, glucose altered the quenching constants, but not the fractional fluorescence, of the tryptophan residues. Mg^{2+} greatly accentuated this glucose effect, especially for the surface residue near the cleft opening.

Comparison of acrylamide- and I^--quenching data shows that this particular residue has a positively charged microenvironment. A pH change from 5.5 to 8.3 increased the acrylamide-accessibility of the cleft tryptophan but did not seem to influence accessibility of the surface residues or the buried residue significantly, thus strengthening our previous conclusion that the cleft opening is small enough at pH 5.5 to partially restrict entrance of organic molecules

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and negative ions. However, with saturating glucose present there was a pH effect on the surface residue accessibility. Titrations in 55 vol.% glycerol suggest the presence of transient channels (not just holes) in the hexokinase structure, which allows penetration of the protein by the solution. Consequently, the buried tryptophan residue is quenched more strongly by dissolved acrylamide than is attributable to diffusion of quencher through the protein matrix.

Introduction

Two studies recently appeared, almost simultaneously, with information concerning the locations of the four tryptophan residues of the monomeric subunit of yeast hexokinase (ATP:D-hexokinase 6-phosphotransferase, EC 2.7.1.1) isozyme P-II(B) *. Anderson et al. [3] published an X-ray-derived amino acid sequence, which they considered to be 60 to 70% correct. If we correlate this sequence with their polypeptide-backbone map presented previously [4] it appears that: Trp-51 is located in an α-helix portion close to the subunit/subunit interface of the dimer; Trp-109 is located on the surface in a β-sheet near the cleft opening; Trp-219 is located in the interior; and Trp-414 is very close to the glucose-binding site in the cleft, which separates the subunit into two lobes. The results of fluorescence-quenching titrations of this isozyme with I^- as titrant led Kramp and Feldman [5] to classify the tryptophan residues as follows: Trp-S_1, a surface residue highly accessible to solvent; Trp-S_2, a surface residue with restricted accessibility (possibly in a crevice); Trp-G, a glucose-quenchable residue in the cleft; and Trp-B, a buried tryptophan in the hydrophobic interior. Thus, the latter four residues, respectively, seem to correspond to residue 109, 51, 414 and 219 of Anderson's X-ray-derived sequence. These residues are responsible, respectively, for about 9, 22, 28 and 41% of the total 350 nm fluorescence at pH 8.3 and 20°C [5].

Despite the apparent agreement between these two studies, the electrostatic interaction between the charged I^- and the protein leaves some uncertainty in equating I^--accessibility, as measured by fluorescence-quenching, with the solvent exposure of a tryptophan residue. Eftink and Ghiron [6—8] have found that the neutral acrylamide molecule is a very efficient quencher of tryptophan fluorescence and is very discriminating in sensing the degree of exposure of a tryptophan residue in proteins. These authors showed that even buried tryptophan residues are collisionally quenched by acrylamide due to nanosecond fluctuations in the protein matrix, which produce transient interstitial openings in the tertiary structure. However, the collision rate constants of well-buried residues (e.g., 0.1 and 0.3 $M^{-1} \cdot ns^{-1}$, respectively, for aldolase and ribonuclease T_1 at 25°C) are at least 10-fold smaller than those of exposed residues (4 $M^{-1} \cdot ns^{-1}$ for adrenocorticotropin and glucagon, respectively), and partially exposed residues have intermediate values.

We have employed acrylamide as the quencher in fluorescence titrations of

* Isozyme B isolated in Barnard's laboratory [1] is believed to be identical with isozyme P-II of Colowick's laboratory [2]. Although we used Barnard's procedure, we employ the latter symbol to conform with usage of the present day majority.

yeast hexokinase isozyme P-II to probe the effects of the substrate, glucose, and the cofactor, Mg^{2+}, on specific areas of the isozyme, i.e., surface, cleft and buried, at the same pH values of earlier I^--quenching studies [5], namely, pH 5.5, near the isoelectric pH of the isozyme, and pH 8.3, where it carries a large net negative charge. We showed earlier [9] that Mg^{2+} causes conformational changes in hexokinase isozymes both in the absence and in the presence of glucose, especially in the latter case, which result in increased glucose-binding strength and in alteration of the degree of cooperativity of glucose-binding by the P-I isozyme and of anticooperative glucose-binding by P-II. A regulatory role for Mg^{2+} in the hexokinase reaction was suggested, apart from its established function in the substrate $MgATP^{2-}$ chelate.

Materials and Methods

The hexokinase isozyme P-II was prepared from baker's yeast, analyzed for activity and purity, stored, and desalted before experiment exactly as described earlier [9]. Our product was approx. 99% pure, with a specific activity of 725 I.U./mg at 25°C and a fructose : glucose phosphorylation ratio of 1.1. Barnard's value of 0.98 $ml \cdot mg^{-1} \cdot cm^{-1}$ was employed for the specific absorbance of this isozyme [1].

Fluorescence measurements reproducible to about 0.15% were made with an Aminco-Bowman spectrofluorimeter equipped with light-source and phototube-output correction modules, a magnetic arc stabilizer for the Xenon lamp, a digital multimeter readout, and a jacketed cell compartment through which constant-temperature liquid was pumped. The excitation wavelength was always 300 nm. This eliminated both tyrosine absorption and tryptophan-tryptophan energy transfer [10].

Titrations of the 350 nm emission of the isozyme were carried out at 20°C by sequential addition of aliquots (5—25 µl) of a 5 M acrylamide solution to 1.0 ml of 0.1 mg/ml isozyme solution. Both the titrant and the isozyme solution were buffered; 0.05 M sodium acetate (pH 5.5) or 0.05 M glycylglycine (pH 8.3). A Unimetrics syringe with a non-wettable, disposable Teflon tip was used. Pipetting with better than 1% reproducibility was routine, except when the titrant was in glycerol solution. In this case, the protein-containing cuvette was weighed before and after each addition of titrant, and the added volume of titrant was calculated from the weight difference and the density, 1.1308 g/ml, of the titrant, 5 M acrylamide in buffered glycerol/water (55 : 45, v/v). The fluorescence reading would usually begin to drift with time when the acrylamide concentration reached about 1 M, probably due to protein denaturation. Consequently, each titration was stopped as soon as drifting was detected.

The fluorescence titrations were analyzed by least-squares computer analysis using the equation:

$$\frac{-\Delta F}{F_0} = \sum_i \frac{f_i K_{qi} Q}{1 + K_{qi} Q} \tag{1}$$

for selective collisional quenching of i equally absorbing, independent fluorophores of a macromolecule in the absence of energy transfer [11]. Q is the quencher concentration, and f_i is the fraction of the initial fluorescence, F_o,

attributable to the ith fluorophore. K_{qi}, the Stern-Volmer quenching constant, is related to the collision rate constant, k_{qi}, by the equation:

$$K_{qi} = k_{qi}\tau_{oi} \tag{2}$$

where τ_{oi} is the fluorescence lifetime in the absence of quencher.

$$\Delta F = F_{corr} - F_o \tag{3}$$

$$F_{corr} = F_{obs} \times 10^{A/2} \times 10^{A'/2} \times V/V_o \tag{4}$$

F_{obs} and F_{corr} represent the relative values of the observed intensity of the 350 nm emission and the corrected value, respectively. The exponents A and A' in the inner filter corrections [12] are the 300 nm absorbance values of the acrylamide, at points of interest in each titration, and of 55 vol.% glycerol. For our 55 vol.% glycerol (Eastman Spectroquality) A' was 0.010 at 300 nm. Our acrylamide (Eastman grade, recrystallized twice from ethyl acetate) had a molar absorbance of 0.100 at the same wavelength and obeyed Beer's Law in aqueous solution, so that A was calculable for each point. V_o is the solution volume before titration, usually 1.0 ml, and V is the solution volume for a given point in the titration. The V/V_o ratio corrects for the dilution of the enzyme during the titration, and it seldom exceeded 1.1.

The f_i and K_{qi} values so calculated, and listed in Table I, are good to within ±1% (S.E.) and ±0.1 (S.E.), respectively, for duplicate experiments in aqueous buffer and twice these values in 55 vol.% glycerol.

Results

Fig. 1 depicts acrylamide titrations of the 350 nm emission of hexokinase P-II in aqueous pH 8.3 buffer in the absence of added salt, in the presence of 23 mM $MgCl_2$, and in the presence of 70 mM KCl. The titration is seen to be insensitive to ionic strength, I, up to 0.073 (i.e., salt I + buffer I) even when I was due to the Mg^{2+} salt. The concavity of the curve demonstrates that selective collisional quenching of the several tryptophan residues, due to their different

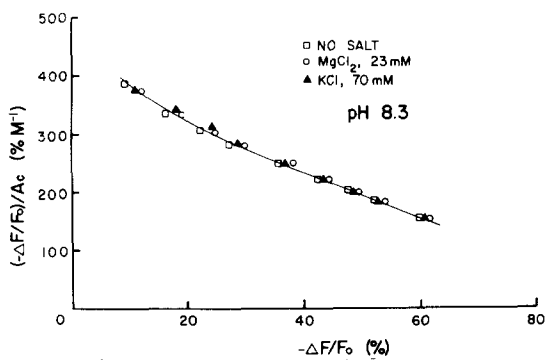

Fig. 1. Acrylamide titrations of hexokinase P-II fluorescence in the absence and in the presence of $MgCl_2$ (23 mM) and KCl (70 mM) at pH 8.3 and 20°C. 0.05 M glycylglycine buffer (I = 0.03). Abscissa/ordinate quotient at any point gives the acrylamide concentration for that point. Abscissa includes initial metal quenching.

degrees of exposure to the acrylamide, strongly outweighs any static component [6,7]. In this figure the abscissa values include the initial quenching by the metal salts, 3% for $MgCl_2$ and 2% for KCl. The curve extrapolates to 100% quenching, signifying accessibility of all four tryptophan residues to the acrylamide. A similar result was obtained at pH 5.5.

The computer analysis of the acrylamide titrations of the isozyme gave very good two component fits at both pH values. The calculated f_i and K_{qi} values are shown in Table I. At pH 8.3 the fractional fluorescences, f_1 and f_2, for the first and second components of the analysis, were 30 and 70%, respectively. The corresponding quenching constants were 6.67 and 3.03 M^{-1}. In view of the earlier finding [5] that the two surface residues, Trp-S_1 and Trp-S_2, account for 9 and 22% of F_o, respectively, at pH 8.3, it is evident that the 6.67 M^{-1} value is a weighted average, or effective value K_{q1}, of unresolved K_{qi} values of the two surface residues, while the 3.03 M^{-1} value is also an effective value, K_{q2}, for unresolved K_{qi} values of the cleft and buried tryptophans.

The f_i values, 40 and 60%, calculated for pH 5.5 are sufficiently close to the pH 8.3 values to indicate that the corresponding K_{qi} values, 10.0 and 2.54 M^{-1}, are also weighted averages of the same residue pairs. In fact, erroneous inclusion of some of the second component in the calculation of K_{q1} is less likely at pH 5.5 than at pH 8.3, because the calculated K_{q1} and K_{q2} values are further apart for the lower pH.

Fig. 2 shows the acrylamide titrations at pH 5.5 when saturating glucose (272 mM) was initially present in the hexokinase solution, with and without added salts. The f_i and K_{qi} values for curves 1 ($MgCl_2$ present) and 4 (no added salt) are given in Table I. A similar set of curves was obtained at pH 8.3, and the corresponding f_i and K_{qi} values are also in Table I.

As shown by the sum of the f_i values, the no-salt curve of Fig. 2 extrapolates to an abscissa of 84% and a similar curve, i.e., with saturating glucose but no added salt, for pH 8.3 extrapolates to 75%. The 16 and 25% deviations from 100% are due to the initial quenching of the cleft tryptophan, Trp-G, by the saturating glucose and are in good agreement with glucose-titration values [13]. In these cases the second component of the computer fit is, then, due solely to

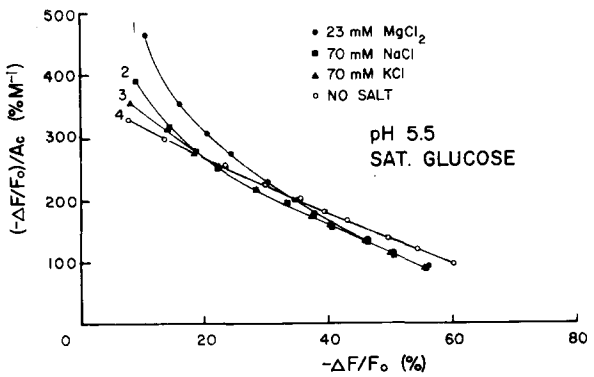

Fig. 2. Acrylamide titrations of hexokinase P-II fluorescence with saturating glucose (272 mM) initially present at pH 5.5 and 20°C. 0.05 M sodium acetate buffer (I = 0.04). $\Delta F/F_o$ does not include initial quenching by glucose or metal ions.

TABLE I

EFFECTIVE ACRYLAMIDE-QUENCHING CONSTANTS (K_{qi}) AND FRACTIONAL FLUORESCENCE VALUES (f_i) FOR HEXOKINASE P-II 350 nm EMISSION AT 20°C

The solutes listed were in addition to enzyme and buffer. Saturating glucose concentration: 272 mM at pH 5.5, 95 mM at pH 8.3. Mg^{2+} where indicated was added as 23 mM $MgCl_2$ plus saturating glucose.

Solvent	Solute	pH 5.5						pH 8.3			
		f_1 (%)	K_{q1} (M^{-1})	f_2 (%)	K_{q2} (M^{-1})	f_3 (%)	K_{q3} (M^{-1})	f_1 (%)	K_{q1} (M^{-1})	f_2 (%)	K_{q2} (M^{-1})
H_2O		39.7	10.0	59.8	2.54			30.0	6.67	70.0	3.03
H_2O	Saturating glucose	40.0	5.92	44.0	2.95			30.0	10.3	44.5	2.98
H_2O	Mg^{2+} + glucose	11.0	26.3	20.0	11.5	46.0	2.18	25.0	16.0	49.8	2.63
55% glycerol	—	42.0	3.62	62.0	1.10			30.0	2.50	70.0	0.86
55% glycerol	Saturing glucose	33.0	4.00	52.0	1.82			75.0	2.60		

the buried residue, Trp-B, of which the fractional fluorescence, f_2, was 44% at both pH values, in good agreement with the 41% value calculated in the I^--quenching study [5]. It is also seen that f_1 was also unchanged by the glucose at either pH. Nevertheless, there were significant glucose-induced changes in both quenching constants at each pH. At pH 5.5 saturating glucose caused K_{q1} to decrease considerably, from 10.0 to 5.92 M^{-1}, while K_{q2} increased slightly, from 2.54 to 2.95 M^{-1}. At pH 8.3 the corresponding changes were in the opposite direction, K_{q1} increased from 6.67 to 10.3 M^{-1} and K_{q2} decreasing from 3.03 to 1.98 M^{-1}. Thus, although the extensive glucose-induced conformational change of the isozyme (rotation of one lobe of the monomeric subunit by 12° relative to the second lobe, thereby closing the cleft between the lobes [14]) does not seem to alter the microenvironments of any of the tryptophan residues, it does affect their accessibilities to the acrylamide.

Although $MgCl_2$, NaCl, KCl at I 0.07 did not affect the acrylamide titration significantly in the absence of substrate (Fig. 1), they all did so when saturating glucose was present initially (Fig. 2). $MgCl_2$ produced a greater change in the titration curve with glucose present than did the alkali salts. As is evident in Table I, curve 1 of Fig. 2 gave an excellent three-component computer fit, while all other titrations in this study, including the titration at pH 8.3 with glucose and Mg^{2+} initially present, gave only two-component fits.

The magnitude of f_3, 46%, calculated from curve 1 of Fig. 2 is sufficiently close to the f_2 value, 44%, calculated from curve 4 (no Mg^{2+}) to demonstrate that this f_3 value also refers solely to the buried tryptophan residue. Clearly, the glucose-induced conformational change in the presence of Mg^{2+} at pH 5.5 is so extensive that the acrylamide quenching constants of the two surface tryptophans are resolvable. That is, in this case the K_{q1} and K_{q2} values, 26.3 and 11.5 M^{-1}, refer to Trp-S_1 and Trp-S_2, respectively. Considering that acrylamide is neutral, this K_{q1} value is very high indeed, being roughly equal to the acrylamide quenching constants of small indole derivatives, e.g., 17.5 M^{-1} for acetyltryptophanamide and 26.6 M^{-1} for acetyltryptophan [15]. Even if the fluorescence lifetime of Trp-S_1 were doubled by the glucose effect, a large increase in solvent accessibility would still be indicated for this case. A similar phenomenon at pH 8.3 is also evident, since K_{q1} was increased from 10.3 to 16.0 M^{-1} by the glucose plus Mg^{2+}, but the K_{qi} values of the surface residues were still not resolvable.

The combination of glucose plus Mg^{2+} also caused a considerable decrease in the percentage of F_o attributable to the two surface residues compared to the value in the absence of Mg^{2+}. At pH 5.5 these two residues together emitted 40% of F_o when glucose, but no Mg^{2+}, was present. However, when Mg^{2+} was also present initially they emitted 31% of F_o (i.e., $f_1 + f_2$ in the latter case), while at pH 8.3 their combined emission was lowered from 30% of F_o to 25% of F_o by Mg^{2+}. Thus, with Mg^{2+} also present the glucose-induced conformational change altered the microenvironments of the surface residues and their acrylamide accessibilities, whereas in the absence of Mg^{2+} glucose did not seem to affect their microenvironments.

The K_{q2} value, 2.18 M^{-1}, of the buried residue at pH 5.5 obtained with both glucose and Mg^{2+} present was lower than the value, 2.95 M^{-1}, obtained in the absence of Mg^{2+}, despite the constancy of its fractional fluorescence. This

implies that Mg^{2+}, in combination with glucose, affects the acrylamide accessibility of Trp-B, as well as that of the surface residues. The sum of the f_i values in this case was only 77%. The difference between this value and the 84% value for the case with no Mg^{2+} is due in part (4%) to the Mg^{2+}-induced increase in the maximum amount of glucose-quenchable fluorescence, F_{GQ}, and in part (2%) to direct Mg^{2+} quenching of surface tryptophan [9]. The Mg^{2+} effect on F_{GQ} at pH 5.5 results from a change in f_i for one or more of the fluorophores, since ΔF is always normalized to F_o. There is no Mg^{2+}-induced change in F_{GQ} at pH 8.3 [9], although there is about 3% direct quenching by Mg^{2+} at this pH.

The viscosity of 55 vol.% glycerol is 10.7 times as large as that of water at 20°C [16]. Consequently, the quenching constant of a protein tryptophan residue should be significantly reduced by inclusion of glycerol as a solvent constituent when the quenching rate depends upon movement of the quencher through the solvent, but not if the quenching rate is limited by diffusion of the quencher through a solvent-impermeable protein matrix [8]. As expected, titration curves (plotted as in Figs. 1 and 2) for acrylamide quenching of the fluorescence of the isozyme in 55 vol.% glycerol were much less concave than the corresponding curves obtained in absence of glycerol, primarily in the first half because of the viscosity effect on the quenching of the surface residues. The computer-calculated values for f_i and K_{qi} at pH 5.5 and 8.3 are included in Table I. The plot for pH 8.3 with saturating glucose present was linear within experimental error with a slope, i.e., effective K_q, equal to 2.60 M^{-1}.

It can be seen in Table I that when glucose was absent the glycerol did not alter the f_i values significantly at either pH, but it did cause about a 3-fold decrease in each K_{qi} value at each pH. However, with glucose initially present the glycerol changed the relative values of the fractional fluorescences, f_2/f_1, from 1.1 to 1.6 at pH 5.5. Surprisingly, K_{q1} and K_{q2} were decreased by about the same amount, approx. 35%. Indeed, since with saturating glucose present K_{q2} refers solely to the buried residue, one might have expected K_{q2} to remain essentially constant.

Discussion

The fact that the $K_{Trp\text{-}S_1}$ value, 20 M^{-1}, for pH 8.3 obtained earlier by resolution of I^--titration data [5] is 3-fold larger than the K_{q1} value, 6.67 M^{-1}, of the two surface residues measured with neutral acrylamide as quencher indicates that, despite the net negative macromolecular charge, Trp-S_1 of hexokinase P-II has a positively-charged microenvironment, which facilitates quenching by negative ions. This explains why the I^- $K_{Trp\text{-}S_1}$ value is twice as large as the highest I^- K_q value so far reported for any other protein.

This conclusion is strengthened by the fact that this high $K_{Trp\text{-}S_1}$ value is not significantly influenced by saturating glucose [5]. That is, fluorescence quenching of Trp-S_1 by neutral acrylamide depends primarily on the steric accessibility of the fluorophore, which is glucose dependent, but its accessibility to I^- quenching is determined primarily by the net positive charge of its microenvironment, which apparently is unaltered by glucose at pH 8.3.

The Mg^{2+}-induced changes in the f_i and K_{qi} values of the surface tryptophans and the buried residue when glucose is also initially present, plus the fact that Mg^{2+} enhances the glucose-binding constant at both pH values [9], establishes that Mg^{2+} significantly enhances the extent of the conformational change which glucose normally causes in isozyme P-II.

The F/A ratio for the isozyme is 1.67 times larger at pH 5.5 than at 8.3. Since acrylamide quenching of protein tryptophan fluorescence is predominately collisional [5], this ratio may be taken to be the ratio of the average fluorescence lifetimes for these two pH values. Thus, the lifetime is pH sensitive and one cannot compare K_{qi} values obtained at different pH values. However, if we assume that K_{qi} is roughly equal to $k_{qi}\tau_o$, where τ_o is the average lifetime in the absence of quencher, then the ratio of the pH-independent quenching rate constants, k_{qi}, for the two pH values can be estimated by dividing the corresponding K_{qi} ratio by 1.67. Eftink and Ghiron [6] applied a similar assumption to obtain k_q(eff) from K_q(eff), where K_q(eff) was obtained from the initial slopes of Stern-Volmer plots, with apparent success relating k_q(eff) to the relative 'average' exposures of tryptophan residues in different proteins.

The k_{q1} and k_{q2} ratios, 1.1 and 2.0, thus calculated for the cases with no glucose seem to show that pH change from 5.5 to 8.3 does not alter the steric accessibility (i.e., to the neutral quencher) of the surface tryptophans significantly, but that there is a large effect on the cleft residue and/or the buried residue. That this change in k_{q2} is almost entirely related to the cleft residue is suggested by the fact that with saturating glucose initially quenching this residue's fluorescence, k_{q2} remained virtually constant (i.e., only 1.1-fold increase) when the pH was raised, while k_{q1} was increasing 2.9-fold. This conclusion that the cleft residue is much less accessible to acrylamide at pH 5.5 than at 8.3 implies that the cleft opening at the lower pH is small enough, because of tighter coiling, to partially restrict entrance of this quencher. The same conclusion was reached earlier for quenching by glucose [13] and by I^- [5]. It should, thus, not be surprising if Mg^{2+} binding near the cleft opening does indeed have a regulatory role, as we suggested earlier [9].

The k_{q1} ratios, 1.1 in the absence of glucose and 2.9 in the presence of glucose, lead to the conclusion that the steric accessibility of the surface tryptophan is pH dependent only after the glucose-induced conformational change has taken place. This is compatible with Steitz's schematic drawing of the isozyme structure [4], in which one can see that Trp-109, i.e., Trp-S_1, lies very close to the cleft opening. It may be that the glucose-induced conformational change does not involve as close an association of the subunit lobes at pH 8.3 as at the lower pH because of repulsion by the (net) negative surface charge on the lobes at the higher pH.

At both pH values the F/A ratio is 1.08 times larger for the isozyme in 55 vol.% glycerol than in aqueous solution. Assuming, as above, that F/A is proportional to the fluorescence lifetime, so that this number is a rough estimate of the ratio of the average lifetimes in the two solvents, we calculated the ratios of the k_{qi} values in water and glycerol-water from the corresponding K_{qi} values of Table I. These ratios, $k_{qi,w}/k_{qi,g}$, are shown in Table II. The 3-fold diminution of both quenching rate constants at both pH values due to glycerol

TABLE II

GLYCEROL DEPENDENCE OF ACRYLAMIDE QUENCHING OF HEXOKINASE P-II 350 nm EMISSION AT 20°C

Subscripts, w and g, refer to the solvents, water and 55 vol.% glycerol, respectively. The $K_{qi,w}/K_{qi,g}$ ratios were obtained from the K_{qi} values in Table I and were divided by the $(F/A)_w/(F/A)_g$ ratio, 0.93, to give the $k_{qi,w}/k_{qi,g}$ ratios.

pH	Glucose (mM)	$K_{q1,w}/K_{q1,g}$	$K_{q2,w}/K_{q2,g}$	$k_{q1,w}/k_{q1,g}$	$k_{q2,w}/k_{q2,g}$
8.3	0	2.7	3.5	2.9	3.8
5.5	0	2.8	2.3	3.0	2.5
5.5	272	1.5	1.6	1.6	1.7

when glucose was absent is the amount expected for solvent-exposed tryptophan residues. For example, using 50% glycerol Eftink and Ghiron [8] obtained $k_{q,w}/k_{q,g}$ ratios of 3.5 and 3.6 for adrenocorticotropin and glucagon, respectively, both of which have $k_{q,w}$ values expected for fully exposed residues, i.e., about 4 $M^{-1} \cdot ns^{-1}$ at 25°C. For proteins with $k_{q,w}$ (1—2 $M^{-1} \cdot ns^{-1}$) for partially exposed residues, such as pepsin, monellin and nuclease, they found $k_{q,w}/k_{q,g}$ ratios equal to 2.6—3.0.

The almost equal values, 1.6 and 1.7, which we calculated for the glycerol effect on k_{q1} and k_{q2} at pH 5.5 with saturating glucose present seem especially interesting, since in this case k_{q2} refers only to Trp-B. A $k_{q,w}/k_{q,g}$ value near unity is expected for a buried residue, if its quenching is a result of stepwise diffusion of the quencher through the protein matrix via transient free volume elements or 'holes' produced by thermal conformational fluctuations proceeding on a nanosecond time scale, as in aldolase and ribonuclease T_1, which have $k_{q,w}$ values of only 0.1 and 0.25 $M^{-1} \cdot ns^{-1}$, respectively, and $k_{q,w}/k_{q,g}$ ratios of 1.1 and 1.2 [8]. Our Trp-B value of 1.7 for this ratio should indicate at least partial solvent exposure, since this value was obtained [8] for β-trypsin, which has a $k_{q,w}$ value of 1.1 $M^{-1} \cdot ns^{-1}$. Apparently, quenching solution reaches Trp-B of hexokinase through channels (i.e., not just matrix 'holes') extending to the surface, at least after the glucose-induced conformational change. The relatively low wavelength, 328 nm, for the isozymes's tryptophan emission maximum, which is due to Trp-B [13], in both the absence and the presence of glycerol implies that the channels are transient, being produced by the thermal conformational fluctuations. Transient channels, rather than fixed channels, could prevent the water and the buried tryptophan from remaining in contact for sufficient time to form the fluorophore-water exciplex responsible for the more red emission of an exposed residue.

Since glycerol has about the same size as acrylamide, the glycerol-water solvent should be able to move through these channels. However, the transient nature, and probably the small size, of these channels coupled with the high solvent viscosity would still lead to the smaller quenching constant for Trp-B than for the surface residues seen in Table I for pH 5.5, i.e., 1.82 M^{-1} compared to 4.00 M^{-1}. The fact that $k_{q1,w}/k_{q1,g}$ and $k_{q2,w}/k_{q2,g}$ were the same value when glucose was present is, thus, fortuitous and results from the fact that saturating glucose reduced K_{q1} by almost a half, i.e., from 10.0 to 5.92 M^{-1}, in

aqueous solution, while in glycerol solution K_{q1} was not influenced significantly by the glucose.

Such channels were not demonstrated at pH 8.3, because K_{q1} and K_{q2} were not resolved at this pH for the case in which saturating glucose was initially present.

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