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EFFECTS OF FREE MAGNESIUM AND ALKALI IONS ON THE CONFORMATION AND GLUCOSE-BINDING STRENGTH OF YEAST HEXOKINASE ISOZYMES

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Summary

Titrations of the tryptophan fluorescence of yeast hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) isozymes P-I (A) and P-II (B) were performed with Mg^{2+}, Li^+, Na^+ and K^+ as titrant in absence and in presence of glucose, and vice versa, at pH 8.3 and 5.5 at 20°C. Mg^{2+} quenches the fluorescence of surface tryptophan primarily and does so by producing a conformational change which alters the microenvironment of the tryptophan. For both isozymes Mg^{2+} exerts a specific ion effect, i.e. significantly larger than the ionic strength (I) effect, which enhances the glucose quenching by causing a conformation change which increases the glucose-binding constant. For the P-I isozyme glucose binding exhibits positive cooperativity at both pH 8.3 and 5.5 when the ionic strength (I) is low, i.e. 0.04 or less, regardless of which of the above four cations is present. For P-II, however, glucose binding is non-cooperative at pH 8.3 regardless of I or the cation species and at pH 5.5 and low I with K^+ or Mg^{2+} as the predominant cation present, but there is apparent negative cooperativity at pH 5.5 and low I when Na^+ or Li^+ predominates. These results are discussed in terms of known structural characteristics of the isozymes.

Introduction

This paper describes a fluorescence-quenching titration study of the interactions between the yeast hexokinase (ATP:D-hexose 6-phosphotransferase,

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EC 2.7.1.1) isozymes P-I (A) and P-II (B) * and the metal ions Mg^{2+}, Li^+, Na^+, K^+ and their effects on glucose binding by the isozymes at pH 5.5, i.e. near the isoelectric pH [2] and at pH 8.3, where the isozyme has a large negative net charge.

Both isozymes exist in a dimeric and monomeric state with the monomer/dimer ratio increasing with pH and ionic strength [3,4]. The two isozymes have somewhat similar tertiary structures in that each binds glucose in a deep cleft which separates the monomeric subunit into two lobes and glucose binding causes the two lobes to move together closing the cleft [5–7]. The binding is accompanied by some quenching of the enzyme's fluorescence [8] because of an induced conformational change which alters the microenvironment of a tryptophan residue situated in the cleft [9].

We have recently shown [9] that the four tryptophan residues [2] in the monomeric subunit of the P-II isozyme can be classified as (i) one glucose-quenchable residue in the cleft, (ii) two surface residues, one highly accessible to solvent and one with restricted accessibility (possibly in a surface crevice), and (iii) one residue 'buried' in the hydrophobic interior. In view of the similarity in their tertiary structures and the fact that each isozyme contains the same number of tryptophan residues, it is probable that this classification is applicable also to the P-I isozyme.

Bachelard [10] concluded that the essential in vivo cofactor, Mg^{2+}, can act either as an allosteric activator or as an inhibitor depending on the Mg^{2+}/ATP ratio and the glucose concentration. This view was contested by Purich and Fromm [11], who reinterpreted Bachelard's data in terms of a non-allosteric role for both Mg^{2+} and $MgATP^{2-}$. More recently, Peters and Neet [12] decided that the metal ion is involved only in the catalysis, i.e. as $MgATP^{2-}$, and not in the regulatory conformational change. Despite the obvious importance of knowing whether free Mg^{2+} has a role in the hexokinase reaction, there seems to be only one report in the literature concerning the interaction of Mg^{2+} with hexokinase in absence of an adenine nucleotide, namely, an erroneous 1964 statement of Zewe et al. [8] that neither Mg^{2+} nor Mn^{2+} affects the fluorescence of hexokinase.

Enzyme activation by monovalent cations has been reviewed [13,14]. Lithium is now of special interest because of its use in psychotherapy. Because of its small ionic radius Li^+ frequently behaves more like the divalent Mg^{2+} (diagonal relationship) than like Na^+ or K^+ [15,16]. It has been reported that Li^+ activates hexokinase slightly in rat brain and erythrocytes presumably by competing with Mg^{2+} [17].

Materials and Methods

The hexokinase isozymes were prepared from fresh baker's yeast by the method of Rustum et al. [18] with slight modification [19]. Assay by the method of Darrow and Colowick [20], as modified by Lazarus et al. [21], gave

* Isozymes A and B isolated in Barnard's laboratory [1] and isozymes P-I and P-II purified in Colowick's laboratory [2] are believed to be identical. In this paper we will be using the latter two symbols to conform with usage of the present day majority.

specific activities of 215 and 725 I.U./mg at 25°C and fructose/glucose phosphorylation ratios of 2.2 and 1.1 for P-I and P-II, respectively. Polyacrylamide gel electrophoresis (without sodium dodecyl sulfate) indicated that both products were essentially pure, i.e. greater than 99%, as measured with a Beckman CDS-100 computing densitometer. The stock isozymes were kept in a 0.1 M KH_2PO_4/1.0 M NaCl (pH 5.5) buffer at 4°C. Just before each experiment an aliquot was desalted on a Sephadex G-25 column and the eluate's absorbance at 280 nm was measured. Using Barnard's values [1] of 1.2 ml/mg cm for the specific activity of P-I and 0.98 ml/mg cm for P-II, we then diluted the eluate with buffer to the desired concentration.

All fluorescence measurements were made at 20°C with 300 nm excitation and 350 nm emission. An Aminco-Bowman spectrofluorimeter equipped with light source and phototube output correction modules, a magnetic arc stabilizer for the xenon lamp, and a digital multimeter readout gave better than 0.25% reproducibility.

Titrations were performed by adding 5 μl increments of titrant to a cuvette containing initially 1.0 ml of 0.1 mg/ml hexokinase in buffer. A calibrated Unimetrics micropipette good to 1% reproducibility was employed. The glucose titration curves were analyzed with the fluorescence equivalent of the Scatchard binding equation [19,22]:

$$(\Delta F/F_0)G^{-1} = k_G F_{GQ} - k_G(\Delta F/F_0) \quad (1)$$

where ΔF is the change in the relative fluorescence intensity F produced by glucose concentration G, F_0 is the relative fluorescence intensity before addition of either glucose or metal ion, k_G is the glucose-binding constant, and F_{GQ} is the percentage of F_0 quenchable by saturating glucose. Each experimental F value was multiplied by the dilution factor, never greater than 1.095, to normalize ΔF to F_0. Eqn. 1 may be applied to a multispecies system, such as one containing both monomeric and dimeric forms of free and complexed macromolecules, if the binding sites of the macromolecule are independent and if the intrinsic ligand-binding constants are constant, but not necessarily the same, throughout the titration [19]. Consequently, a linear plot demonstrates non-cooperative binding; a concave plot indicates 'apparent' negative cooperativity, i.e. either true negative cooperativity or binding-site heterogeneity, while positive cooperativity is evidenced by a convex plot, which theoretically contains a maximum whose location is determined by the degree of cooperativity [23]. If the degree of cooperativity is very small or very large the maximum may be undetected, because it may occur closer to the beginning or the ending of the titration than the experimental error warrants measuring, but the curve should still be convex. For this reason and because the maximum may be quite broad, the degree of positive cooperativity frequently can be determined more accurately from a Hill plot than from a Scatchard plot [23], although the latter is a better qualitative indicator.

Titrations with metal ions as the titrant have been represented simply by $(\Delta F/F_0)$ vs. metal ion concentration, because quenching by prior added glucose may change during the metal titration (see explanation of Fig. 1 in Results).

Results

Mg^{2+} quenching of fluorescence of hexokinase isozymes

Although unstated, the report of Zewe et al. [8] that Mg^{2+} does not quench hexokinase fluorescence must refer only to the low Mg^{2+} concentration used in their work with $MgATP^{2-}$, i.e. $6.25 \cdot 10^{-4}$ M or less. Actually, their highest Mg^{2+} concentration is the point at which we can just detect Mg^{2+} quenching of hexokinase fluorescence (i.e. approx. 0.25%). At higher Mg^{2+} concentration quenching is definite, being more than 3-fold stronger than is attributable to a simple ionic strength effect. For instance, 0.033 M $MgCl_2$ quenches the 350 nm emission of the P-I isozyme 4.9% in 0.02 M glycylglycine buffer at pH 8.3 and 20°C, while the alkali chlorides, LiCl, NaCl and KCl at 0.1 M concentration (i.e. all four salts at same I) quench 2.5, 3.0, and 3.1% of F_0, respectively. P-II gave fairly similar results. Further, $MgCl_2$ quenches P-II as well as a 3-fold higher concentration of CsCl, which is known to be a good heavy-atom collisional quencher of tryptophan residues [24].

0.1 M $MgCl_2$ does not quench aqueous indole, tryptamine, or *N*-acetyltryptophanamide, but it enhances the intensity of the 350 nm emission of aqueous tryptophan 7.6%. Hence, it is improbable that Mg^{2+} quenching of hexokinase fluorescence is a direct electromagnetic interaction between Mg^{2+} and a tryptophan residue of the enzyme. Rather, like glucose [19], Mg^{2+} must quench by causing a conformation change which alters the microenvironment of one or more tryptophan residues. However, whereas glucose quenches only the tryptophan residue located in the cleft [9], Mg^{2+} quenches surface tryptophan primarily. This is evident from the coincidence of curves 1 and 4 of Fig. 1, which represent $MgCl_2$ titrations of P-II fluorescence in absence of glucose and in presence of a nearly saturating glucose concentration, 50 mM, respectively, at pH 8.3.

Isozyme P-II

It is also seen in Fig. 1 that curves 2 and 3, which, respectively, represent $MgCl_2$ quenching titrations with undersaturating glucose concentrations, 1.0 mM and 5.0 mM, initially present, lie considerable higher than the no-glucose curve. From curves 1 and 2 alone one might conclude that glucose enhances the ability of Mg^{2+} to quench P-II fluorescence. However, curve 3 would then be higher than curve 2 and the 50 mM glucose curve 4, would lie even higher, whereas in fact there is inversion in the sequence of the curves and curve 4 virtually coincides with the no-glucose curve.

Obviously, the reverse is true, i.e. Mg^{2+} enhances the ability of the prior added glucose to quench P-II fluorescence. Thus, Mg^{2+} binding produces a conformational change which results in an increase in the glucose-binding strength of P-II accompanied by enhanced glucose quenching. The F_{GQ} value of this isozyme at pH 8.3 is about 25% of F_0 [19]. Prior addition of 1.0 mM glucose at this pH initially quenches 7% of F_0, or 28% of F_{GQ}, while 5.0 mM glucose quenches 16.8% of F_0, or 67% of F_{GQ}. Thus, there is much less glucose-quenchable fluorescence available for the Mg^{2+} effect in the latter case, and consequently curve 3 lies below curve 2. 50 mM glucose, which is 95% saturating at pH 8.3 (see curve 1, Fig. 3), leaves only 1% of F_{GQ} unquenched, so that the

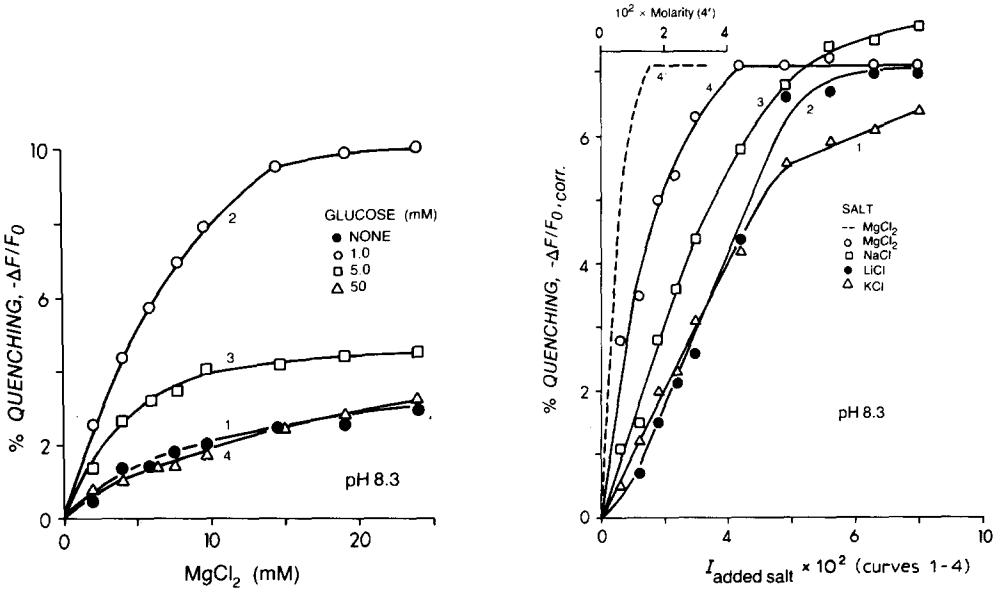

Fig. 1. Mg^{2+}-induced quenching of P-II fluorescence in absence and in presence of varying prior added glucose concentrations at pH 8.3 and 20°C. 0.02 M glycylglycine buffer (I = 0.01). ΔF refers to quenching after the initial glucose quenching.

Fig. 2. Quenching of P-II fluorescence induced by various salts in presence of 1.0 mM prior added glucose at pH 8.3 and 20°C. 0.02 M glycylglycine buffer (I = 0.01). Bottom abscissas (I_{salt} ($\times 10^2$)) refer to curves 1—4; top abscissas (molarity) refer to curve 4′ only. ΔF refers to quenching after the initial glucose quenching. Ordinate ($-\Delta F/F_0$ (corrected)) is $-\Delta F/F_0$ in presence of glucose minus $-\Delta F/F_0$ in absence of glucose.

effect of added Mg^{2+} is almost negligible.

Similar experiments with alkali chlorides as titrants also demonstrated this inversion, but enhancement of the quenching was much less than with $MgCl_2$. This difference is seen in Fig. 2, which compares the metal ion titrations of P-II at pH 8.3 when 1.0 mM glucose was initially present. This glucose concentra-

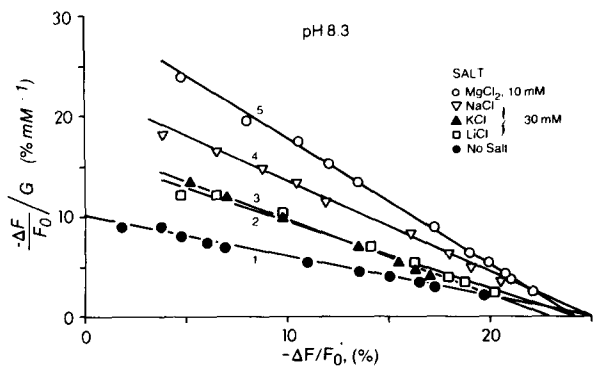

Fig. 3. Metal ion dependence of glucose-induced quenching of P-II fluorescence at pH 8.3 and 20°C. 0.02 M glycylglycine buffer (I = 0.01). Abscissa/ordinate quotient at any point gives the glucose concentration (mM) for that point. ΔF refers to quenching after the initial quenching by the salt.

tion was chosen because it gave the highest (i.e. most Mg^{2+}-sensitive) curve in the previous Figure. The Mg^{2+} curve, 4, converges with the Na^+ and Li^+ curves, 3 and 2, near 0.06 *I* and with the K^+ curve, 1, near 0.1 *I*. On a molar basis (curve 4′), however, the Mg^{2+} effect is considerably larger than that of the alkali ions, even at 0.1 *I*.

The larger Mg^{2+} effect, compared to that of any of the alkali ions, seen at *I* values below 0.06 indicates that it is to a large extent a specific ion effect rather than a simple ionic strength effect. This difference is quantified for 0.03 *I* by Fig. 3, which depicts glucose titrations of P-II at pH 8.3 in absence of metal ion (except for the 0.01 M Na^+ in the glycylglycine buffer) and in the presence of prior added metal chlorides all at 0.030 *I*. All of these plots are linear within experimental error, thus demonstrating non-cooperative binding. The slopes give k_G values of 0.41 mM^{-1} in absence of added salt and 0.68 mM^{-1}, 0.72 mM^{-1}, 0.93 mM^{-1} and 1.3 mM^{-1} when the added salt is LiCl, KCl, NaCl, and $MgCl_2$, respectively.

Comparing the Mg^{2+} value with the Na^+ and K^+ values, respectively, indicates a value of 40 or 86% for the specific Mg^{2+} effect. The latter calculation seems more reasonable, since it seems highly unlikely that two monovalent ions with such a large difference in radius (1.33 Å for K^+, 0.60 Å for Li^+) would decrease the k_G of P-II by the same amount, i.e. from 0.93 to 0.7 mM^{-1} if k_G for the NaCl case is the reference. On the other hand, two large ions like K^+ and the strongly hydrated Li^+ would be expected to bind very weakly if at all. In this case, it would appear that 30 mM Na^+ also has a small specific effect in that it raises k_G measurably, i.e. 33%.

The dependence of the glucose titrations of P-II at pH 5.5 on the various metal ions is shown by Fig. 4. Only the KCl and $MgCl_2$ plots, 3 and 5, of Fig. 4 are sufficiently linear to indicate non-cooperative glucose binding. Their slopes, 0.063 and 0.089 mM^{-1}, respectively, are 10—15 times smaller than the pH 8.3 values, 0.72 and 1.3 mM^{-1}. We previously found a similar large effect on k_G

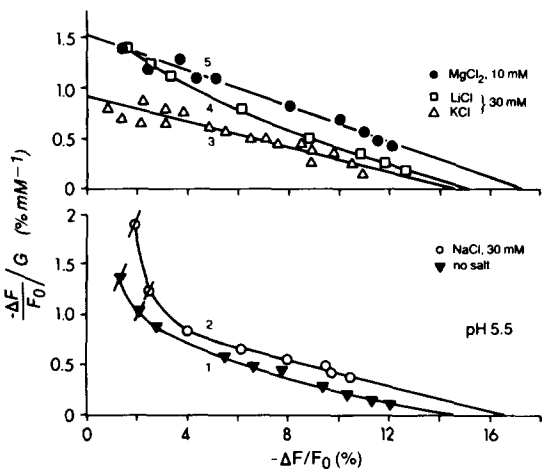

Fig. 4. Metal ion dependence of glucose-induced quenching of P-II fluorescence at pH 5.5 and 20°C. 0.02 M sodium acetate buffer (*I* = 0.017). See legend to Fig. 3 for further details.

when 0.8 M NaCl was present, ensuring complete monomerization, and suggested that the cleft size is smaller at the lower pH because of the tighter coiling and smaller molecular volume [19]. The concavity of the NaCl and LiCl curves, 2 and 4, and the buffer-only curve, 1, demonstrate apparent negative cooperativity for the glucose binding in these cases. Although the curvature in curve 1 is close to experimental error, it is reproducible. Anyway, the validity of the biphasic nature of this particular curve is not important to this paper. The biphasic nature of curve 2 suffices. This curvature decreases when the NaCl concentration is raised, probably because of salt neutralization of the side-chain electrical charges, and it disappears when $I = 0.8$ where only monomeric isozyme exists [19].

It is also seen in Fig. 4 that F_{GQ} is not the same for each curve, i.e. 14.6% in buffer only and in presence of 30 mM KCl, while it is 15.2, 16.4, and 17.2% in presence of 30 mM LiCl, 30 mM NaCl, and 10 mM $MgCl_2$, respectively. These differences reflect the different specific ion effects on the P-II conformation, which controls the microenvironments of the tryptophan residues. A change in F_{GQ} may result from a change in any one, or more, of the tryptophan residues, not just the cleft tryptophan residue, since F_{GQ} is normalized to the total F_0 at the start of the glucose titration. Since (i) K^+ is the only one of these four cations that did not alter F_{GQ} from the buffer-only value at pH 5.5, (ii) the KCl curve at this pH is linear and has the smallest slope, and (iii) along with Li^+, it enhances k_G the least at pH 8.3, it would seem that K^+ has the least effect of these four ions on the conformation of P-II.

Fig. 5 shows the Mg^{2+}-induced quenching of P-II fluorescence at pH 5.5 as a function of prior added glucose. The occurrence of the inversion in the curve sequence, i.e. $3 < 4 > 5$, demonstrates that it is due to Mg^{2+} enhancement of glucose binding as at pH 8.3 (Fig. 1). However, because Mg^{2+} also increases

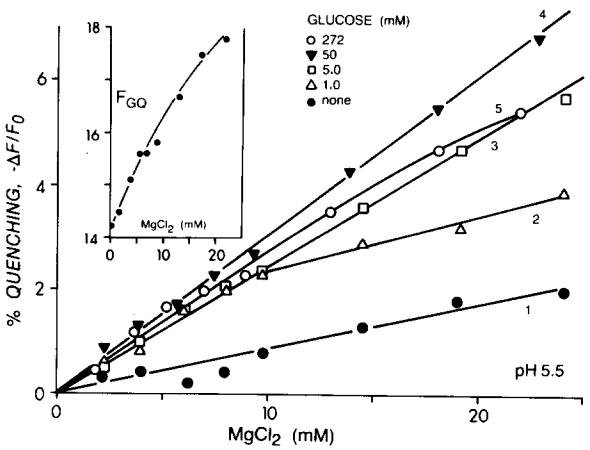

Fig. 5. Mg^{2+}-induced quenching of P-II fluorescence in absence and in presence of varying prior added glucose concentrations at pH 5.5 and 20°C. 0.02 M sodium acetate buffer ($I = 0.017$). ΔF refers to quenching after the initial glucose quenching. The values for the maximum glucose-quenchable 350 nm emission, F_{GQ}, plotted in the inset graph were obtained by subtracting $-\Delta F/F_0$ produced by a given $MgCl_2$ concentration from the total $-\Delta F/F_0$ of 272 mM glucose plus the same $MgCl_2$ concentration and then dividing by 0.96, since 272 mM glucose quenches 96% of F_{GQ} at pH 5.5.

F_{GQ} significantly at pH 5.5 (see Fig. 5 inset) the saturating-glucose curve, 5, lies above the no-glucose curve, 1, in Fig. 5, e.g. by approx. 3.5% at the 22 mM Mg^{2+} point. The Mg^{2+}-induced increase in F_{GQ} is also the primary reason that the highest curve in Fig. 5 is the 50 mM curve, whereas the 1.0 mM glucose curve is highest in Fig. 1. As we pointed out earlier, when F_{GQ} is unchanged Mg^{2+} enhances the quenching of hexokinase primarily by increasing the percent glucose saturation, so that the Mg^{2+} effect is small when a prior added glucose concentration is relatively high (e.g. 50 mM) leaving only a small fraction of the enzyme unbound initially. However, when it increases F_{GQ}, the Mg^{2+} enhances the quenching of the initially glucose-bound enzyme also, so that even with an initially high but undersaturating glucose concentration the Mg^{2+} effect on the quenching is relatively large. In fact, the Mg^{2+} effect would be highest with saturating glucose present if k_G were unaffected by the Mg^{2+}, but not if k_G is increased. Thus, the sequence of curves in Fig. 5, $3 < 4 > 5$ demonstrates that Mg^{2+} increases both F_{GQ} and k_G of P-II at pH 5.5.

The specificity of the Mg^{2+} effect at pH 5.5 is evident at much higher I than at pH 8.3. This is seen in Fig. 6. As we observed at pH 8.3 (Fig. 2), LiCl gave the lowest curve in Fig. 6 and $MgCl_2$ gave the highest.

Isozyme P-I

The effects of Mg^{2+} on the fluorescence quenching of P-I at pH 8.3 and 5.5 in presence of prior added glucose are shown by Figs. 7 and 8, respectively. As in Figs. 1 and 5, both Figs. 7 and 8 show the curve inversion due to Mg^{2+}

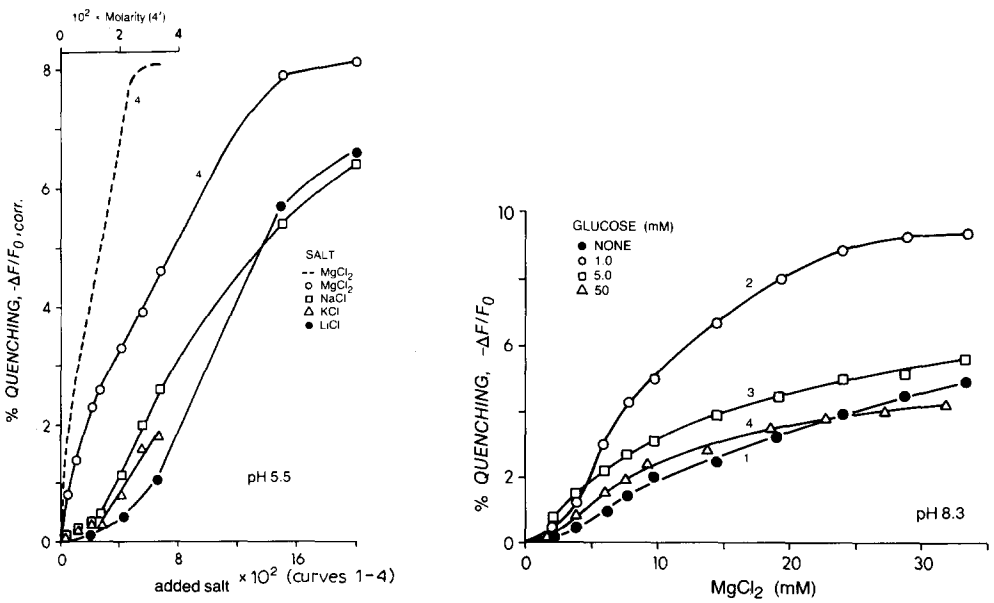

Fig. 6. Quenching of P-II fluorescence induced by various salts in presence of 50 mM prior added glucose at pH 5.5 and 20°C. 0.02 M sodium acetate buffer. See legend to Fig. 2 for details of abscissa and ordinate.

Fig. 7. Mg^{2+}-induced quenching of P-I fluorescence in absence and in presence of varying prior added glucose concentration at pH 8.3 and 20°C. See legend to Fig. 1 for details.

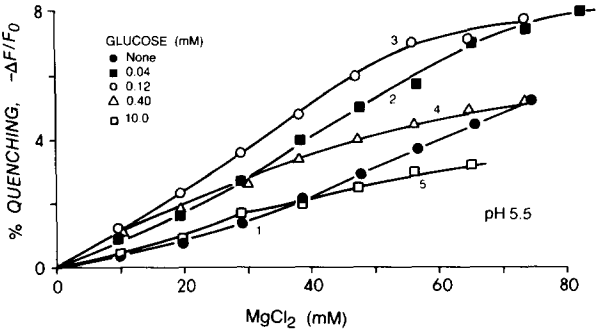

Fig. 8. Mg^{2+}-induced quenching of P-I fluorescence in absence and in presence of varying prior added glucose concentrations at pH 5.5 and 20°C. ΔF refers to quenching after the initial glucose quenching. 0.02 M sodium acetate buffer.

enhancement of glucose binding. In Figs. 7 and 8, however, the saturating-glucose curve and the no-glucose intersect with the former lying higher before the intersection. An explanation will be offered in the Discussion.

Figs. 9 and 10 depict titrations of P-I at pH 8.3 and 5.5, respectively, in absence of added salt and in presence of prior added $MgCl_2$ (10 mM) and alkali chlorides (30 mM). Fig. 11 shows similar titrations with three different prior added $MgCl_2$ concentrations. The convexity seen in all those curves which represent titration with added salt contributing 0.03 to I shows that glucose binding exhibits positive cooperativity in each of these cases. However, the glucose binding is non-cooperative at pH 8.3 when I is at least 0.15. This is

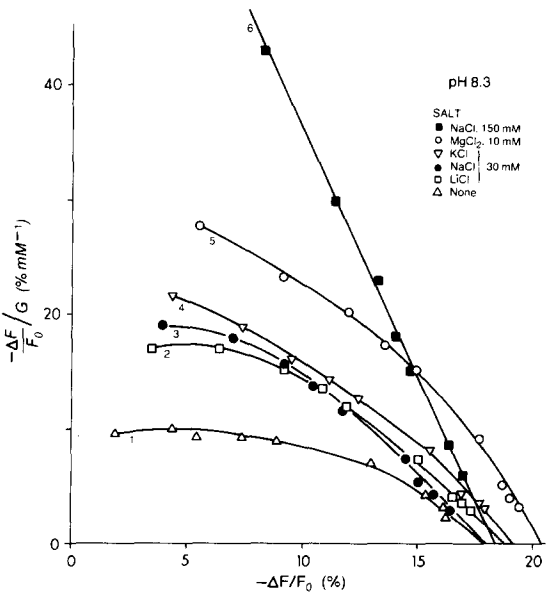

Fig. 9. Glucose-quenching titrations of P-I in presence of different prior added metal ions at pH 8.3 and 20°C. 0.02 M glycylglycine buffer. See legend to Fig. 3 for further details.

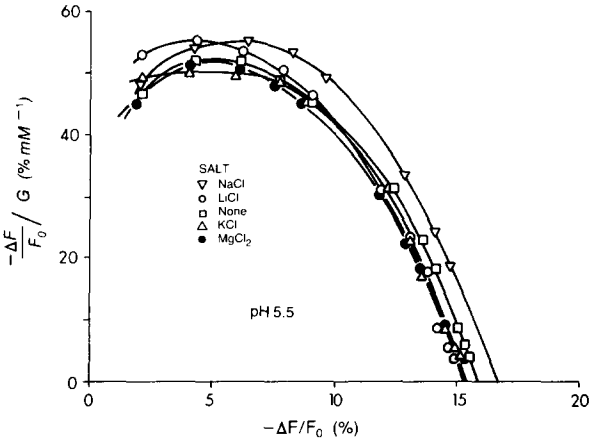

Fig. 10. Glucose-quenching titrations of P-I in presence of different prior added metal ions at pH 5.5 and 20°C. 0.02 M sodium acetate buffer. See legend to Fig. 3 for further details.

evident from the unmistakeable linearity of curve 6, Fig. 9 and curve 3, Fig. 11, i.e. with 150 mM NaCl or 50 mM $MgCl_2$, respectively, present. The slight curvature of curve 2, Fig. 11, is probably within experimental error. In a previous paper [19] we reported curve linearity at pH 8.3 for P-I when the buffer, 0.05 M glycylglycine, contained no added salt, as well as with prior added NaCl (50 mM or more). In retrospect, we believe that this previously published buffer-only curve may indeed be slightly convex.

Hill coefficients, n_H, determined for the curves of Figs. 9—11 are presented in Table I. The constant n_H value, 1.4, at pH 5.5 is evidence that at this pH and low I the degree of positive cooperativity for glucose binding to P-I is unaffected by any of the four salts studied at $I = 0.03$. From the theoretical

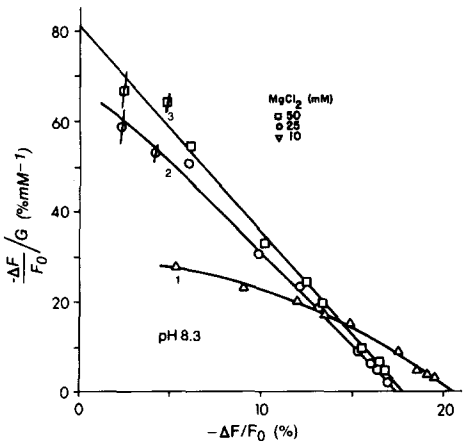

Fig. 11. Glucose-quenching titrations of P-I in presence of different $MgCl_2$ concentrations added prior to the titration at pH 8.3 and 20°C. See legend to Fig. 3 for further details.

TABLE I

HILL COEFFICIENTS AND RELATED PARAMETERS FOR GLUCOSE QUENCHING OF HEXOKINASE P-I IN PRESENCE OF Mg^{2+}, Li^+, Na^+, AND K^+

pH 5.5 and 8.3 at 20°C. n_H was calculated from Hill plots of data in Fig. 4 for pH 5.5 values and of data of Figs. 3 and 5 for pH 8.3 values. F_{GQ} values are the X-intercepts in Fig. 4 for pH 5.5 values and the X-intercepts in Figs. 3 and 5 for pH 8.3 values. $(-\Delta F/F_0)_{max}$ values are from Fig. 4. $n_{H,S}$ values were calculated with Eqn. 2, assuming $(-\Delta F/F_0)_{max}/F_{GQ} = p_{max}$.

Added salt (mM)	pH 5.5				pH 8.3	
	n_H	F_{GQ} (%)	$(-\Delta F/F_0)_{max}$	$n_{H,S}$	n_H	F_{GQ} (%)
Buffer only	1.42	15.9	5.2	1.48	1.56	18.0
LiCl (30)	1.38	15.3	4.3	1.39	1.23	18.7
NaCl (30)	1.42	16.7	6.0	1.56	1.27	18.0
KCl (30)	1.42	15.4	5.0	1.48	1.27	19.3
$MgCl_2$ (10)	1.42	15.4	5.0	1.48	1.32	20.5
$MgCl_2$ (25)					1.01	17.5
$MgCl_2$ (50)					1.00	18.0
NaCl (150)					1.00	18.4

relationship [23]

$$n_H = (1 - p_{max})^{-1} \quad (2)$$

between n_H and the percent ligand saturation, p_{max}, at the maximum in a Scatchard binding curve, one might then expect $(-\Delta F/F_0)_{max}$ to be the same for all curves in Fig. 10, but it can be seen that this is not the case. This apparent discrepancy is mainly due to the variation in F_{GQ} values. Both parameters, n_H and F_{GQ} are listed in Table I. In applying the Hill and Scatchard equations to our quenching data we are assuming that $(-\Delta F/F_0) = pF_{GQ}$, where p is the percent glucose saturation for any given G. This requires that the quenching constants of the different glucose-binding sites remain constant throughout a titration, but they need not be equal. Obviously, $(-\Delta F/F_0)_{max}$ varies with F_{GQ}. The agreement seen in Table I between n_H and the parameter $n_{H,S}$ calculated from Eqn. 2 seems sufficient to indicate that F_{GQ} is, at least approximately, constant for the glucose titrations at $I = 0.03$ and pH 5.5.

Differences in the general height of the Scatchard plots for $I = 0.03$ at each pH also are indications of specific cation effects. These are more noticeable at the higher pH, probably because the enzyme structure is more resistant to ion-induced conformation change at the lower pH as a result of the tighter coiling. The specific effect of Mg^{2+} in the enhancement of glucose binding to P-I is particularly well demonstrated at pH 8.3 by the wide gap between curve 5 and curves 2—4 of Fig. 9. Nevertheless, there seems to be negligible specific Mg^{2+} effect on the degree of cooperativity of the glucose binding at I 0.03 and pH 8.3, since n_H is approximately the same (1.23—1.32) for all four of these curves (Table I), in contrast to the 1.56 value in absence of added salt. The proximity of the k_G values, 4.55 and 4.35 mM^{-1}, and of the F_{GQ} values, 18.0 and 17.7%, corresponding to the linear plots, Fig. 9 (curve 6) and Fig. 11 (curve 3), suggests that at I 0.15 and pH 8.3 both $MgCl_2$ and NaCl eliminate the glucose-binding cooperativity primarily as a result of I.

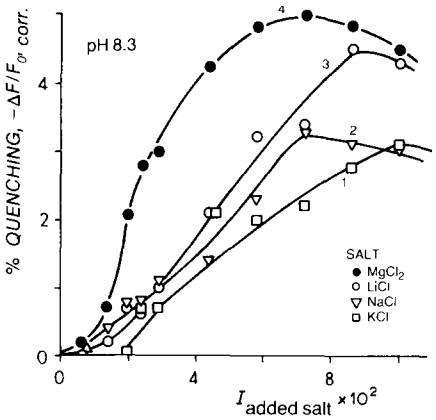

Fig. 12. Quenching of P-I fluorescence induced by various salts in presence of 1.0 mM prior added glucose at pH 8.3 and 20° C. 0.02 M glycylglycine buffer. ΔF refers to quenching after the initial glucose quenching. Ordinate ($-\Delta F/F0$ (corrected)) is $-\Delta F/F_0$ in presence of glucose minus $-\Delta F/F_0$ in absence of glucose.

The relative effects of the four cations on P-II were studied as a function of I by comparing the metal ion titrations in presence of an undersaturating (30—40%) glucose concentration, 1.0 mM at pH 8.3 (Fig. 12) and 0.12 M at pH 5.5 (Fig. 13). In both figures each curve except the K^+ curve contains a maximum and the Mg^{2+} curve is the highest one before the maximum. This demonstrates a specific Mg^{2+} effect in the lower I regions. Also, the positions of the maxima occur at lower I at the higher pH, i.e between 0.07 and 0.1 at pH 8.3 compared to approx. 0.14—0.2 at pH 5.5.

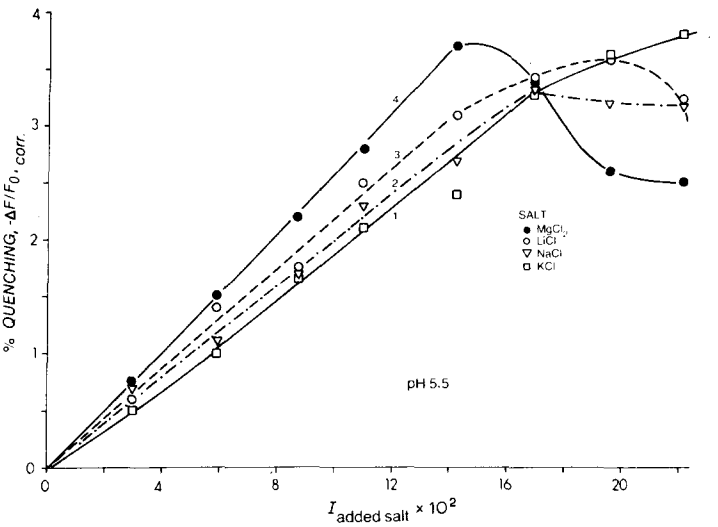

Fig. 13. Quenching of P-I fluorescence induced by various salts in presence of 0.12 M prior added glucose at pH 5.5 and 20° C. 0.02 M sodium acetate buffer. See legend to Fig. 12 for further details.

Discussion

The X-ray studies by Steitz's group [5—7] of the hexokinase structure and the glucose-induced conformation effects cited in the Introduction will be used to rationalize our results. Our data suggest that, although the many carboxyl groups furnish other Mg^{2+}-binding sites, Mg^{2+} attaches at, or close to, the cleft opening of hexokinase and causes a conformation change which enlarges this opening to a definite extent which is sufficient to facilitate access of glucose to its binding site, thereby increasing k_G. This occurs to a lesser extent at pH 5.5 than at 8.3 because of resistance by the tighter protein structure at the lower pH. If this Mg^{2+} site (site N) includes a nitrogen-containing group [25], e.g. surface arginine or histidine [26], then the Mg^{2+}-effect specificity is understandable, since Mg^{2+}, but not alkali ions, have a significant affinity for nitrogen-containing ligands [25].

The existence also of one or more oxygen-containing anionic ligands (site O) at, or near, the cleft opening is indicated by the various effects produced by the alkali cations at the two pH values. The differences in the magnitudes and sequences of these effects and their pH dependence, which we have pointed out in the Results, is consistent with the 'anionic field strength' theory of Eisenman [27,28] according to whom the electrostatic field strength of an anionic site is a variable which is dependent upon the site's total microenvironment and it governs the relative cation affinities of the site. Consequently, four of the six statistically possible selectivity sequences of the series Li^+, Na^+, K^+ occur biologically.

Of course, Mg^{2+} can also bind to site O. Thus, the fact that the Mg^{2+} effect is larger than the alkali effect on glucose binding at pH 8.3 suggests either that at this pH binding to site N is more effective in enlarging the cleft opening than is the binding to site O or that the extent of enlargement of the cleft opening is an additive resultant of the cation binding to both types of sites. There is a definite limit, however, to the extensiveness of the conformational change which Mg^{2+} can produce, so that even though the Mg^{2+} effect at pH 8.3 is larger than the effect of alkali cations at low I these effects become equal with a sufficiently high I (Figs. 2, 6, 12 and 13).

If in P-I site N were actually covered over by the glucose-induced rotation of the lobes, then there would be less quenching caused by Mg^{2+} binding to a glucose-hexokinase complex than by Mg^{2+} binding to uncomplexed enzyme. This should cause the saturating-glucose curves in Figs. 7 and 8 to lie lower than the no-glucose curves, with the difference between the curves getting larger as $MgCl_2$ is increased. However, at pH 8.3 this effect is less than that resulting from the Mg^{2+}-induced increase in the F_{GQ} of P-I when $MgCl_2$ is low, e.g. 2.5% with 10 mM $MgCl_2$ (Fig. 9), so that the saturating-glucose curve lies above the no-glucose curve in the lower $MgCl_2$ region of Fig. 7 and intersection of these curves at the 24 mM point results. At pH 5.5 Mg^{2+} does not increase F_{GQ} of P-I when $MgCl_2$ is low (Fig. 10) and, consequently, there is little difference between the two curves in the low $MgCl_2$ region of Fig. 8. If Mg^{2+} is bound before addition of glucose, the glucose-induced cleft-closing prevents dissociation of the metal ion so that the glucose-induced ΔF is a true indication of the glucose-binding strength. This is evidenced by the linearity of curve 3, Fig. 11.

The occurrence of the maxima in the $MgCl_2$ curves, 4, of Figs. 12 and 13 can then be attributed simply to our having made too large a correction when we subtracted $(-\Delta F/F_0)$ in absence of glucose from $(-\Delta F/F_0)$ in presence of glucose to get the ordinate value $(-\Delta F/F_0)_{corr}$. The same explanation would apply to the maxima in the alkali ion curves also if there is an alkali ion-binding site in the same region. This may be a separate O site or it may be an oxygen-containing group in the Mg^{2+}-binding N site. The fact that these two effects, i.e. intersection of the no-glucose and saturating-glucose curves and the occurrence of maxima in the I dependence curves, were not observed with P-II suggests that the N site may actually lie within the cleft opening of P-I but lies slightly outside of it in P-II.

There have been several conflicting reports concerning cooperativity of glucose binding to P-II and glucose-induced dissociation of the isozyme [19, 29,30]. Attention has been drawn particularly to the buffer dependence of the observations, e.g. Tris buffer vs. triethanolamine chloride [29]. Our results show that discrepancies between different investigations can arise simply by interchanging K^+ and Na^+ in the buffer employed. Perhaps, since K^+ is the overwhelmingly predominant monovalent cation in the biological cell and since it seems to have the least effect on the structure of both isozymes, this cation should be employed preferentially in in vitro studies of hexokinase.

Anderson et all [31] have concluded that the glucose-induced conformational change must be essential for subsequent catalytic steps. Our demonstration that free Mg^{2+} has a specific enhancing effect on the glucose-binding strength of both isozymes therefore suggests that in the in vivo hexokinase reaction the free Mg^{2+} has a regulatory role, as well as being involved in the catalysis. This is contrary to the conclusions of Peters and Neet [12].

Apropos this point, we can reexamine Fig. 4. From the two linear plots, 3 and 5, we see that at pH 5.5 k_G in the presence of 10 mM $MgCl_2$ is 41% greater than k_G in presence of 30 mM KCl. However, from the initial and final slopes of the biphasic curve 2 we can conclude that in presence of 30 mM Na^+ at pH 5.5 the k_G value for the first bound glucose molecule is much larger even than k_G in presence of Mg^{2+} while the second k_G in presence of Na^+ is close to the K^+ value. This suggests that cation binding to O-type sites, which can happen with either Na^+ or Mg^{2+}, may lead to enlargement of the cleft in one subunit but that simultaneous binding of another Mg^{2+} to the N-type site prevents the cleft from opening beyond a definite size. Thus, Mg^{2+} could regulate the size of the cleft opening.

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