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ACTIVATION OF *THERMUS* PHOSPHOFRUCTOKINASE BY MONOVALENT CATIONS

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Summary

The presence of the monovalent cations Tl^+, NH_4^+, K^+, Rb^+ or Cs^+, in decreasing order of potency, produce a marked equivalent increase in the specific enzyme activity of phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) purified from extreme thermophile, *Thermus* X-1. By contrast, the monovalent cations Li^+, Na^+ or $CH_3NH_3^+$ produce no detectable catalytic activation at concentrations up to 100 mM. The relative potency of these cations suggests that each polypeptide chain in the tetrameric enzyme possesses a cation binding site having tetragonal symmetry and that the protein ligands are principally hydroxyl or carboxylate oxygens.

Only the enzyme-cation complex and not the enzyme by itself exhibits cooperativity with respect to the dependence of catalytic rate on the concentration of the substrate, fructose 6-phosphate. In the presence of subsaturating but not saturating concentrations of substrate, the catalytic activation produced by monovalent cations is also cooperative. Exclusion chromatographic measurements indicate that the enzyme remains tetrameric at catalytic concentrations in the presence or absence of an activating monovalent cation.

Introduction

We have previously shown that phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) purified from the extreme thermophile *Thermus* X-1 is representative of the tetrameric allosteric phosphofructokinases found in bacteria as contrasted with the larger self-associating phosphofructokinases found in eucaryotic organisms [1]. The phosphofructokinases obtained from *Thermus* X-1 [1], *Escherichia coli* [2—4], *Bacillus*

stearothermophyllus [5] and *Clostridium pasteurianum* [6] are each tetramers having identical polypeptide chains of molecular weight $3.4 \pm 0.1 \cdot 10^4$. The polypeptide chains of these bacterial enzymes have closely related amino acid compositions with no substantive differences in hydrophobicity, hydrogen bonding potential or average residue size. These enzymes all exhibit K-type allosteric kinetics in which the substrate, fructose 6-phosphate, and the product, ADP, function as positive effectors while phospho*enol*pyruvate is an avid negative effector. By contrast, eucaryotic phosphofructokinases [7,8] consist of larger (greater than $8 \cdot 10^4$) polypeptide chains which form an interconvertible range of polymers. Fructose 6-phosphate, NH_4^+, and either ADP, 5′-AMP or 3′,5′-cyclic AMP are positive effectors and ATP is a negative effector, while most of the remaining monovalent cations stimulate the rate of catalysis non-allosterically. In this report, we demonstrate that the bacterial phosphofructokinases represented by the *Thermus* enzyme are also stimulated by certain monovalent cations, that this stimulation itself can be cooperative and that complexation of the enzyme with monovalent cations causes the binding of fructose 6-phosphate to be cooperative.

Materials and Methods

Phosphofructokinase was purified from *Thermus* X-1 as described by Cass and Stellwagen [1] and had a specific activity of 94 μmol/min per mg. Enzyme activity was measured at 25° C using the discontinuous coupled assay procedure [1], to avoid stimulation of phosphofructokinase by the NH_4^+ present in the auxiliary enzymes. The cation for all reagents in the assay solution was Tris-H^+. All phosphofructokinase solutions were exhaustively dialyzed in 50 mM Tris-HCl (pH 7.5), 1 mM EDTA prior to use.

Exclusion chromatography was done at 25° C using a 40 × 2 cm Sephadex G-200 column equilibrated with 50 mM Tris-HCl buffer (pH 8.5), 1 mM $MgCl_2$. Samples of protein (13—380 μg/ml) and known activity were applied in 1 ml equilibration buffer (1 ml fractions). The protein dilution experienced by the elution fraction having maximal protein concentration was found to be 67 times, using aldolase as standard. Eluate fractions were assayed for enzymic activity in the presence of KCl. Elution profiles were related to molecular weight using ovalbumin, bovine serum albumin, rabbit muscle aldolase and blue dextran for calibration.

Results

We first observed that the presence of 100 mM KCl as opposed to the same concentration of NaCl produces two striking changes in the dependence of the catalytic rate of *Thermus* phosphofructokinase on the concentration of fructose 6-phosphate. As shown in Fig. 1, the presence of KCl increased the observed maximum velocity about 25-fold. Secondly, the hyperbolic dependence of velocity on substrate seen in NaCl, Hill coefficient, n, of 1.1, became positively cooperative in the presence of KCl, $n_{max} = 2.0$. It should be noted that the Hill plot in KCl has the profile 0, +, 0 in the membrane nomenclature of Cornish-Bowden and Koshland [9], indicating that the association constant

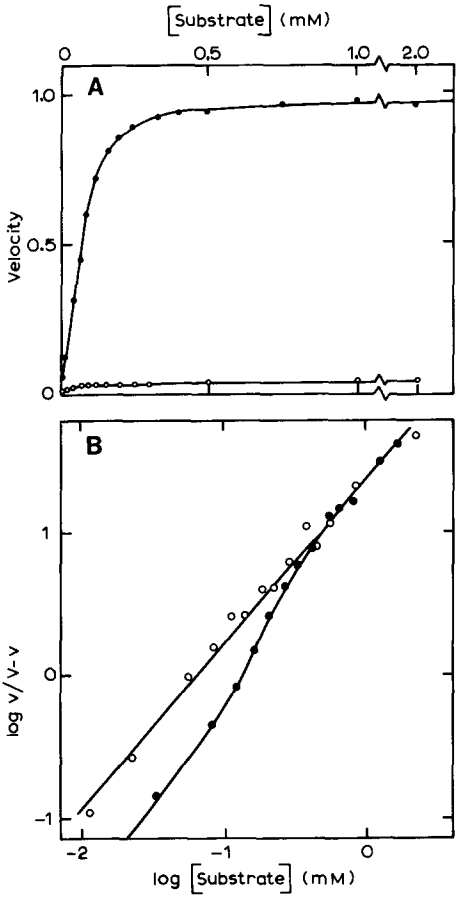

Fig. 1. (A) Dependence of the catalytic rate on the concentration of the substrate, fructose 6-phosphate. The catalytic rate or velocity has the units ΔA_{340}/min. ○, assay solutions containing 100 mM NaCl; ●, assay solutions containing 100 mM KCl. (B) Hill plot of the experimental values in (A).

for binding the third and fourth substrate molecules to the tetrameric enzyme are larger than the association constants for binding the first two substrate molecules. Extrapolation of the limiting slope of the Hill plot in KCL to a $v/V - v = 1$ facilitates estimation of the high association constant for substrate, $1.7 \cdot 10^4$ M^{-1}. It should also be noted that the single association constant for substrate detected in NaCl has a value of $1.7 \cdot 10^4$ M^{-1}, identical with the high association constant observed in KCl. In contrast to the results obtained with fructose 6-phosphate, the dependence of the catalytic rate on the other substrate, MgATP, remains hyperbolic in the presence of either 100 mM KCl or NaCl.

We next examined the dependence of the catalytic rate on the concentration of KCl. At a saturating concentration (2.5 mM) of fructose 6-phosphate, this dependence is strictly hyperbolic, giving a K_D for KCl of 5.9 mM. However, in the presence of a subsaturating concentration (0.1 mM) of this substrate the catalytic rate exhibits a more complex dependence on the concentrations of KCl (Fig. 2). The Hill plot of the stimulation produced by the KCl under these

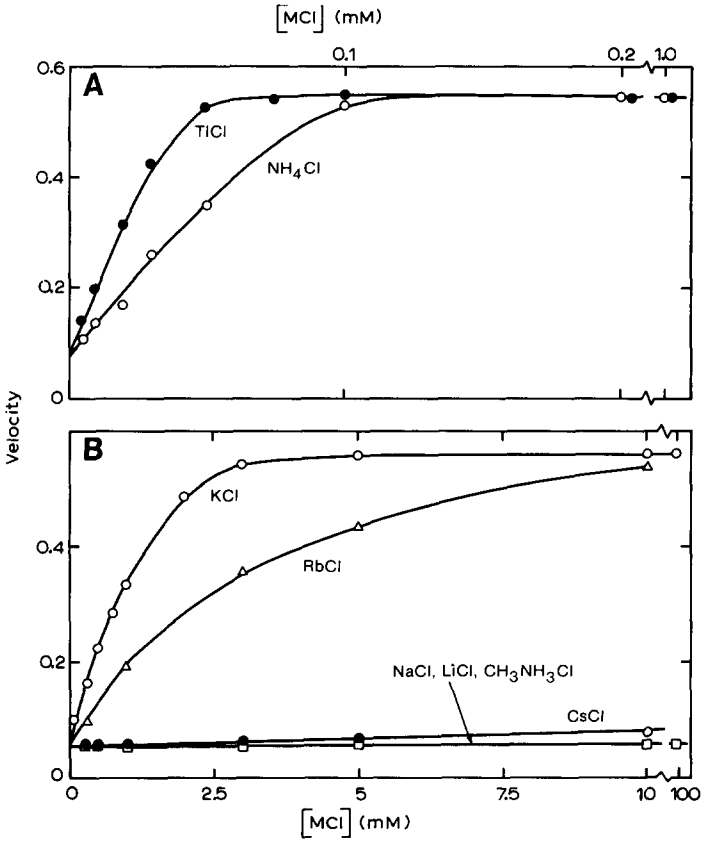

Fig. 2. Dependence of the catalytic rate on the concentration of monovalent cations chlorides. All measurements were made in the presence of 0.1 mM fructose 6-phosphate and (A) •, TlCl, or ○, NH_4Cl. (B) ○, KCl; △, RbCl; •, CsCl; □, NaCl, LiCl or CH_3NH_3Cl.

conditions is biphasic with an initial slope of about 1.2 and a final slope of about 4 (Fig. 3).

We then compared the capability of a variety of monovalent cations to stimulate the catalytic rate of *Thermus* phosphofructokinase. Several monovalent cations in addition to K^+ stimulate the enzyme with an order of potency of $Tl^+ > NH_4^+ > K^+ > Rb^+ > Cs^+$ (Fig. 2). No stimulation by Li^+, Na^+, or $CH_3NH_3^+$ was detected. At subsaturating concentrations of fructose 6-phosphate, the activation produced by each of the effective monovalent cations generated biphasic Hill plots of similar profile (Fig. 3). An apparent association constant was calculated for each enzyme-monovalent cation complex from the monovalent cation concentration producing half-maximal activation, $v/V - v = 1$. Each apparent association constant is related to the radius of the unhydrated monovalent cation (Fig. 4). It can be seen that only cations having unhydrated radii between 1 and 2 Å appear to be effective catalytic stimulants.

Finally, we examined the effect of KCl as a representative stimulatory monovalent cation on the size of the enzyme at low protein concentration. Exclusion chromatographic measurements indicate that all the enzyme activity chroma-

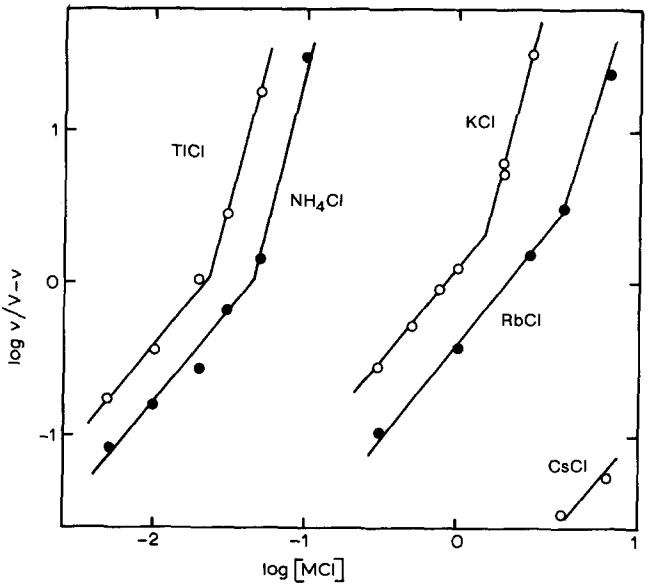

Fig. 3. Hill plot of the experimental values shown in Fig. 2.

tographs as a single symmetrical component having a molecular weight of about $1.3 \cdot 10^5$ in the presence of either 100 mM KCl or NaCl over the protein concentration range 0.2—6 μg/ml.

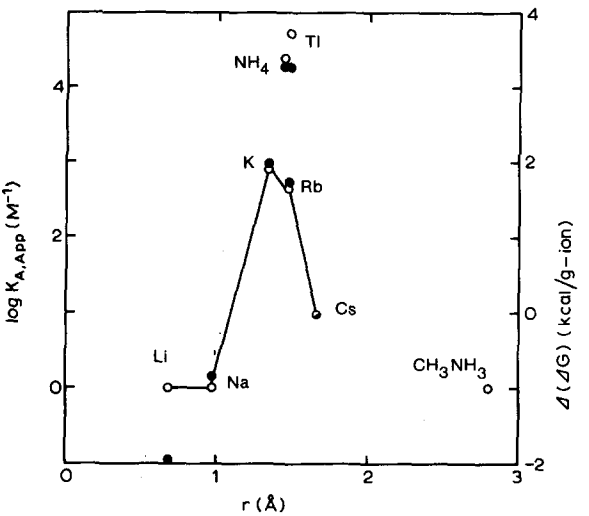

Fig. 4. Dependence of monovalent cation selectivity on the radius of unhydrated monovalent cation. ○, apparent association constants for phosphofructokinase-cation complexes; ●, difference between the relative free energy of binding of the cations to nonactin and the relative free energy of hydration of the alkali cations [11].

Discussion

Cumulative experience [10] indicates that the enhancement of enzyme catalytic rates by monovalent cations results from formation of enzyme-cation rather than substrate-cation complexes. Since the large quantities of purified *Thermus* phosphofructokinase required for direct binding measurements are not available, we assume that this generalization also pertains to the measurements described herein as well. Since each of the stimulating monovalent cations has an equivalent effect with respect to cooperativity and maximal catalytic rate enhancement, they most likely have a common site on the enzyme. The identity of the polypeptide chains of the enzyme [1], together with the cooperativity of the monovalent cation binding, indicates that the enzyme has at least one monovalent cation binding site/polypeptide chain.

As shown in Fig. 4, the relative values of the association constants for the enzyme-alkali earth cation complexes has the order $K^+ > Rb^+ > Cs^+ >> Na^+$, Li^+. The same order of selectivity has been observed for some other enzymes [10] and ion carriers, eg. nonactin [11]. Szabo et al. [11] argue that this selectivity results from the net difference between the affinity of the ion for H_2O as opposed to the complexation agent, with the distance of separation of the charge centers in the ligands of the complexation agent as the principal contributor. In the case of NH_4^+ and Tl^+, which often digress from the alkali earth monovalent cation series, the tetragonal geometry of the former and the deformability of the latter must also make significant contributions to the K_A values. Indeed, the dependence of the relative K_A values for all the stimulatory phosphofructokinase-cation complexes on the unhydrated ion radii is remarkably similar to that reported [11] for the nonactin-cation complexes (Fig. 4). This profile suggests that the monovalent cation binding site on *Thermus* phosphofructokinase has tetragonal symmetry and that the majority of the protein ligands are not carbonyl oxygens but more likely hydroxyl and/or carboxylate oxygens.

The simplest model which occurs to us for accomodating the catalytic response of the *Thermus* enzyme to changes in substrate and cation concentrations consists of the tetrameric enzyme itself E_4^R, and two different conformations of its monovalent cation complex, $(EM)_4^R$ and $(EM)_4^T$, related by the accessible equilibria,

$$E_4^R \overset{4M}{\longrightarrow} (EM)_4^R \rightharpoondown (EM)_4^T .$$

This model requires that the maximum catalytic velocity of $(EM)_4^T$ and $(EM)_4^R$ are equal and greater than that of E_4^R, that the association constants of E_4^R and $(EM)_4^R$ for the substrate fructose 6-phosphate are equal and greater than that of $(EM)_4^T$, and that the association constant for a given monovalent cation M of $(EM)_4^T$ is greater than that of $(EM)_4^R$. In the presence of excess monovalent cation $(EM)_4^T$ predominates and the catalytic rate exhibits a cooperative response to substrate because the association constant of $(EM)_4^R$ for substrate is greater than that of $(EM)_4^T$. In the absence of an activating monovalent cation E_4^R is the only species present and the catalytic rate dependence on substrate is hyperbolic. In the presence of excess substrate the R conformations are maintained because of

their stronger affinity for substrate and the activation of catalysis by monovalent cation is hyperbolic. However, in the presence of less substrate, continued addition of cation can now cause isomerization of $[EM]_4^R$ to $[EM]_4^T$ producing a cooperative dependence of catalytic rate on the activator concentration.

Acknowledgements

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