The contribution of two isozymes to the pyruvate kinase activity of Vibrio cholerae: One K⁺-dependent constitutively active and another K⁺-independent with essential allosteric activation

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Abstract

In a previous phylogenetic study of the family of pyruvate kinase EC (2.7.1.40), a cluster with Glu117 and another with Lys117 were found (numbered according to the rabbit muscle enzyme). The sequences with Glu117 have been found to be K⁺-dependent, whereas those with Lys117 were K⁺-independent. Interestingly, only γ-proteobacteria exhibit sequences in both branches of the tree. In this context, it was explored whether these phylogenetically distinct pyruvate kinases were both expressed and contribute to the pyruvate kinase activity in Vibrio cholerae. The main findings of this work showed that the isozyme with Glu117 is an active K⁺-dependent enzyme. At the same substrate concentration, its $V_{\text{max}}$ in the absence of fructose 1,6 bisphosphate was 80% of that with its effector. This result is in accordance with the non-essential activation described by allosteric ligands for most pyruvate kinases. In contrast, the pyruvate kinase with Lys117 was a K⁺-independent enzyme displaying an allosteric activation by ribose 5-phosphate. At the same substrate concentration, its activity without the effector was 0.5% of the one obtained in the presence of ribose 5-phosphate, indicating that this sugar monophosphate is a strong activator of this enzyme. This absolute allosteric dependence is a novel feature of pyruvate kinase activity. Interestingly, in the K⁺-independent enzyme, Mn²⁺ may “mimic” the allosteric effect of Rib 5-P. Despite their different allosteric behavior, both isozymes display a rapid equilibrium random order kinetic mechanism. The intracellular concentrations of fructose 1,6-bisphosphate and ribose 5-phosphate in Vibrio cholerae have been experimentally verified to be sufficient to induce maximal activation of both enzymes. In addition, Western blot analysis indicated that both enzymes were co-expressed. Therefore, it is concluded that VcIPK and VcIIPK contribute to the activity of pyruvate kinase in this γ-proteobacterium.
**Introduction**

*Vibrio cholerae* is a prokaryotic bacterium that contains two unique circular chromosomes [1]. It has three copies of genes (*pykF*, *pykA*-1 and *pykA*-2) encoding three distinct pyruvate kinases (PKs) EC (2.7.1.40), respectively here referred to VcIPK, VcIIPK and VcIIIPK. Two of these genes (*pykF* and *pykA*-1) are present in chromosome 1 (genome size 2.96 Mb) and the other gene (*pykA*-2) is located in chromosome 2 (1.07 Mb) [2]. A previous phylogenetic analysis of the PK family showed a dichotomic tree structure separating the family of the PKs into two clusters [3]. Cluster I groups 52.6% of the total sequences analyzed, and is characterized by having a glutamate at position 117 (numbered according to the rabbit muscle enzyme (RMPK)) and cluster II with 46% of total sequences having a lysine at the same position. To date, all characterized PKs harboring Glu117 exhibit K⁺-dependent activity, whereas those with Lys117 are K⁺-independent. VcIPK is located in cluster I, and VcIIPK in cluster II, suggesting that they should exhibit K⁺-dependent and K⁺-independent activity, respectively. The feature of having isozymes in both branches of the phylogenetic tree is thought to be unique to γ-proteobacteria. On the other hand, most PKs are regulated by heterotropic effectors whose nature depends on the cell metabolism [4, 5]. Cluster I is mainly comprised of PKs modulated by bi-phosphorylated sugars such as fructose 1,6 bisphosphate (Fru 1,6-BP) [6, 7] or fructose 2,6 bisphosphate (Fru 2,6-BP) [8,9] whereas cluster II includes enzymes largely regulated either by AMP or monophosphorylated sugars [10–12]. Members of the γ-proteobacteria group such as *Escherichia coli* [10], *Salmonella typhimurium* [13] and *Yersinia enterocolitica* [14] also have both type I and type II PKs which are located, respectively in Cluster I and Cluster II of the phylogenetic tree. These enterobacteriaceae type I PKs are modulated by Fru 1,6-BP, and the type II are regulated principally by AMP. In this vein, we were interested in elucidating whether VcIPK and VcIIPK, which are phylogenetically distant, were expressed, and contributed to the pyruvate kinase activity of *Vibrio cholerae*. Since there are no previous studies on PKs from this γ-proteobacterium, we determined their kinetic and regulatory properties and explored their expression in *V. cholerae*. The data show, that VcIPK is a constitutively active enzyme, whereas VcIIPK is mediated by an allosteric activation. However, since its allosteric effector was present in *V. cholerae* and both enzymes were co-expressed, it was concluded that both VcIPK and VcIIPK contribute to the activity of pyruvate kinase in this γ-proteobacterium.

**Materials and methods**

Cloning, expression and purification of VcIPK and VcIIPK

The PK genes from *Vibrio cholerae* chromosome 1 were amplified from genomic DNA extracted from the *V. cholerae* N16961 strain. The genes were amplified by PCR using the following primers for VcIPK: FW 5’-TACTTCCAATCCAATGCTAAA AAGACCAAAATCGTATGTACGATT-3’ and RV 5’-TTATCCACTTCCAATGTTACAGTAGGTGTACTGATGCAGTT-3’ and for VcIIPK: FW 5’-TACTTCCAATCCAATGCTTAGCTAGGTGTACTGATGCAGTT-3’ and RV 5’-TTATCCACTTCCAATGTTACAGTAGGTGTACTGATGCAGTT-3’. Each insert was introduced into the pMCSG7 vector as described in [15]. The plasmids were isolated and sequenced to verify the absence of mutations. The resulting pMCSG7/VcIPK or pMCSG7/VcIIPK plasmids were used to transform E. coli BL21-CodonPlus (DE3)-RIL competent cells. Both proteins were then overexpressed in four liters of Luria Bertani (LB) medium then growth, induced, harvested and lysed as described previously [15]. The suspensions were centrifuged at 13,250 × g, the supernatants loaded on a His Trap FF column (GE Healthcare), and the enzymes eluted with a linear gradient of imidazole (10–500 mM). The fractions with
the highest activity of PK were pooled (200 mM imidazole), concentrated by membrane filtration (Centricon 100,000 molecular weight cutoff), and loaded on a Hi Trap desalting column (GE Healthcare). Both enzymes were precipitated with ammonium sulfate at 80% saturation and stored at 4°C. Unfortunately, despite several attempts, the poly-histidine tag was not able to be removed using TEV protease for either enzyme, indicating that the cleavage site is not accessible to the TEV protease. Therefore, in subsequent purifications the incubation with TEV protease was omitted for both enzymes. The VcIPK and VcIIPK were 90 and 88% percent pure, respectively, as determined by SDS-PAGE (Laemmli [16] at 12.5% polyacrylamide; 4°C and 160 V) by densitometry analysis after application of Coomassie brilliant blue stain. The yield for both enzymes was ~40 mg per 4 liters of cell culture. Both proteins were positively identified by mass spectrometry using a BRUKER microflex MALDI-TOF mass spectrometer (spectra not shown) to obtain the grade of purity and molecular mass of the isozymes. To determine the enzyme oligomeric state, 50 μg of VcIPK and VcIIPK were loaded onto a blue native-PAGE (BN-PAGE) and separation was carried out overnight at 4°C as described in [17] with a gradient gel of 4–11% acrylamide, 100 μg of solubilized extract of lauryl maltoside inverted membranes of Paracoccus denitrificans and 100 μg of mitochondrial extract of rat liver were used as molecular weight markers.

To probe if the His<sub>6</sub> tag effects the kinetic behavior of V. cholerae PKs. VcIPK was subcloned into pTrc99A and transformed into E. coli PB25Δ-PKI Δ-PKII. Cells were lysed by sonication. The crude extract was precipitated with 50 and 90% ammonium sulfate. After overnight dialysis, the sample was loaded in a size-exclusion column (Sephacryl 300) equilibrated with 25 mM HEPES containing 50 mM KCl (pH 7.0). Fractions with the highest activity were collected and loaded to a cation-exchange column (Carboximethyl-Sephar ose) in 25 mM HEPES pH 7.0. The protein eluted in the front, and fractions with the highest activity were then loaded into an anion-exchange column (Diethylaminoethyl-Sepharose), and both PKs eluted with a linear gradient of 25 mM HEPES containing 1M KCl (pH 7.0). SDS-PAGE consistently showed that PK co-purified with other proteins. Assays of activity of this preparation revealed that the kinetic constants for PEP<sup>3-</sup> and ADP-Mg of VcIPK with and without the His<sub>6</sub>tag were almost identical. To further compare enzymatic activity, the same ionic strength was used (150 mM). $K_{0.5}$ for PEP$^{3-}$ was 0.55 ± 0.015 mM and 0.68 ± 0.016 mM for the His<sub>6</sub> tagged and non His<sub>6</sub> tagged protein, respectively. $K_{0.5}$ for ADP-Mg was 0.26 ± 0.03 mM and 0.27 ± 0.02 mM for the His<sub>6</sub> tagged and non His<sub>6</sub> tagged protein, respectively. The response to the allosteric effector was similar. In the presence of 2 mM Fru 1,6-BP, the $K_{0.5}$ for PEP$^{3-}$ was 0.069 ± 0.004 and 0.060 ± 0.004 for the His<sub>6</sub> tagged and non His<sub>6</sub> tagged protein, respectively. The $V_{max}$ was 40% of that of the enzyme with the His<sub>6</sub> tag. Considering that VcIPK is unstable, this difference may be due to the longer purification procedure of the enzyme. Also, the presence of contamination with other proteins, the most abundant being the tryptophanase of E. coli (identified by sequencing protein fragments by mass spectrometry, data not shown), can explain the lower $V_{max}$. Tryptophanase has similar molecular weight and pI as those of VcIPK. Therefore, the studies of VcIPK and VcIIPK were performed with the His<sub>6</sub> tag.

**Preparation of chromium-ADP**

Chromium-ADP was prepared according to [18].

**Pyruvate kinase assays**

Ammonium sulfate suspensions of lactate dehydrogenase (EC 1.1.1.27) (LDH) were obtained from Roche Applied Science. Desalted enzymes were prepared as described in Kasahara and
Penefsky [19]. Reaction mixtures contained less than 10 μM of contaminating NH$_4^+$, Na$^+$, and K$^+$ were present as indicated in [20]. The formation of pyruvate was measured at 25˚C in a coupled system with LDH and NADH [21]. The reaction mixtures contained 50 mM HEPES, pH 7.0 and the concentrations of monovalent cations (Li$^+$, Na$^+$, K$^+$, NH$_4^+$, Rb$^+$ and Cs$^+$), divalent cations (Mg$^{2+}$ and Mn$^{2+}$), the substrates phosphoenolpyruvate (PEP) and ADP, allosteric modulators Fru 1,6-BP and ribose 5 phosphate (Rib 5-P) and inhibitors (oxalate and ADP-Cr$_2^{2+}$) as indicated under “Results and Discussion”. The software CHELATOR [22] was used to calculate ADP-Mg complexes and Mg$^{2+}$ free concentrations. In order to determine ADP-Mn complexes and Mn$^{2+}$ free concentrations, the $K_d$ of Mn$^{2+}$ was used [23]. The ionized PEP (PEP$^3-$) concentrations were calculated considering a pK value of 6.3 [24]. To adjust for the different ligand concentrations, tetramethylammonium chloride (TMACl) was used to maintain constant ionic strength. Sufficient quantities of LDH were added to counteract the inhibitory effect caused by oxalate in the inhibition assays. A 5-fold inclusion of LDH did not result in an increase in the specific activities of PK. The experiments were carried out at 25˚C and the reaction was initiated with the addition of either VcIPK or VcIIPK.

**Kinetic studies**

The initial velocities of VcIPK and VcIIPK activities were determined in absence or presence of dead-end inhibitors (oxalate or ADP-Cr$_2^{2+}$). In the former condition, the velocity patterns were obtained at various PEP$^3-$ concentrations and several fixed concentrations of ADP-Mg or ADP-Mn. In the latter condition, the inhibition patterns were obtained by varying the concentration of one substrate with the second substrate fixed and at different fixed concentrations of the inhibitor. All these experiments were conducted in the presence of Fru 1,6-BP and Rib 5-P for VcIPK and VcIIPK, respectively.

**Fluorescence experiments**

Intrinsic fluorescence emission spectra and anisotropy measurements of VcIPK and VcIIPK were performed in an ISS PC1 photon counting spectrofluorometer (ISS, Urbana, IL) at 25˚C. Fluorescence was measured at an excitation wavelength of 280 nm and emission was collected from 290 to 400 nm with 8-nm excitation and emission slits in one milliliter cells. Spectra without protein were subtracted from those containing the enzymes. Fluorescence anisotropy determinations of PKs in the presence of various ligands, emission at $\lambda_{max}$ was recorded upon excitation at 280 nm. Excitation and emission slits were 8-nm.

**Polyclonal anti-VcIPK and anti-VcIIPK antibodies**

Peptides from VcIPK and VcIIPK were synthesized by Genescript. (NJ, USA). These peptides comprised from residue 419 to 436 of VcIPK and from residue 427 to 444 of VcIIPK. Both peptides were coupled to keyhole limpet hemocyanin (KLH). 200 μg of each peptide were mixed with 500 μl (phosphate-buffered saline) PBS buffer and 500 μl complete Freund’s adjuvant (SIGMA) to form a stable emulsion. One rabbit was immunized with each antigen by subcutaneous injection in the large muscle of the rear legs with 200 μl of the emulsion. Three weeks later, a blood sample was collected to determine the serum antibody titers, followed by a booster injection with a similar dose of the antigen and the Freund’s incomplete adjuvant. After two more weeks, the animals were bled out and the sera was separated by centrifugation at 4˚C at 3000 rpm for 30 minutes without further purification. Aliquots of 50 μl were stored at -70˚C.
Western blots against VcIPK and VcIIPK

*Vibrio cholerae* (strain CVD 103), *E. coli* DH5α, and *E. coli* PB25 cell extracts (0.5 ml) were diluted 1:20 in a buffer that contained 20 mM Tris-HCl, 10% glycerol, 50 mM sucrose, 1 mM EDTA, 1 mM ATP, 1 mM PMSF, 5 mM benzamidine and a cocktail of proteases, Complete (Roche) 4 tablets/l (pH 7.6) and lysed by sonication. Protein concentrations were determined by the Lowry method [25] and separated using denaturing SDS-PAGE overnight at 10˚C (Laemmli gels [16] at 8% polyacrylamide) in parallel to purified recombinant VcIPK or VcIIPK. The gels were then transferred to PVDF membranes. Western blots were carried out with anti-VcIPK and anti-VcIIPK polyclonals antibodies at a 1:30,000 dilution factor. Secondary goat anti-rabbit-HRP (Santa Cruz Biotechnology) against primary antibodies and Streptactin-HRP against Western-C standards were mixed at 1:30,000 and 1:120,000 dilutions, respectively. Blots were then developed using the chemiluminescent HRP substrate detection kit of Millipore (Immoblion™ Western). An estimation of the amount of expressed VcIPK and VcIIPK was calculated by densitometry using GelQuant software of DNR Bio-Imaging Systems, Ltd.

Monospecificity of anti-VcIPK and anti-VcIIPK was probed against VcIPK, VcIIPK and VcIIIPK. Purified recombinant *V. cholerae* enzymes were separated overnight at 10˚C (Laemmli gels [16] at 8% polyacrylamide). The gel was transferred to PVDF and the membrane cut in the middle as indicated in the corresponding figure legend. Blots probing anti-VcIPK and anti-VcIIPK were carried out separately with each half-PVDF membrane as described above.

Determination of metabolites in *Vibrio cholerae* extracts

Bacteria were cultured in 500 ml of LB inoculated with 1 ml of an overnight pre-inoculum culture. The cells were grown overnight at 37˚C and 200 rpm. Afterwards, the absorbance at 600 nm was determined by serial dilutions to determine the final number of cells in the culture assuming that A600 of 0.7 = 6 X 10^8 cells/ml [26]. Cells were harvested by centrifugation at 2,930 × g for 15 min at 4˚C and washed twice with PBS (pH 7.4). The cell pellet was resuspended in 2–3 ml of PBS and 0.5 ml of a 30% perchloric acid and 10 mM EDTA solution was added and the cell vigorously vortexed to induce lysis. The cell lysate was incubated for 45–60 min on ice and centrifuged at 12,062 × g for 10 min. The supernatant was recovered and neutralized by addition of a buffer containing 0.1 M Tris and 3M KOH. The final sample volume was registered and immediately used. Just before metabolite determination, the samples were centrifuged to remove any particulate material.

Metabolites were determined by spectrophotometry at 340 nm following the NADPH production or NADH consumption using coupling enzymatic systems as described in [27]. The initial reaction mix contained 1.74 ml of 50 mM HEPES supplemented with 1 mM EGTA at pH 7.4, 10 mM MgCl2, 1 mM NADP⁺ and 10 mM glucose. 0.2–0.5 ml of neutralized cell extracts were added and the basal absorbance in the absence of added enzymes was recorded. Coupling enzymes were sequentially added to determine specific metabolites within the same sample as follows: for glucose 6-phosphate (Glc 6-P), 2 units of glucose 6-phosphate dehydrogenase (EC 1.1.1.49) from *Leuconostoc mesenteroides* (Roche, Mannheim Germany) were added; for fructose 6-phosphate (Fru 6-P), 7 U of glucose 6-phosphate isomerase (EC 5.3.1.9) (Roche) was added; for ATP, 3 U of hexokinase (EC 2.7.1.1) (Roche) were added. Finally, for Fru 1,6-BP, 10 mM Pi and 1 U of recombinant *Entamoeba histolytica* pyrophosphate-dependent phosphofructokinase (EC 2.7.1.90) [28] were also added. From the stepwise increase in NADPH formation, the metabolite content was determined. Rib 5-P was determined in a 1 ml final volume reaction. The reaction mixture contained 0.9 ml of 100 mM Tris pH 7.0, 0.15
mM NADH and 0.1–0.2 ml of neutralized and clarified extract. Afterwards, 3.4 U glycerol-3-phosphate dehydrogenase (EC 1.1.1.94) (Roche) were added, followed by 100 U triosephosphate isomerase (EC 5.3.1.1) (SIGMA). Further 0.7 mM D-xylulose-5-phosphate (Xul 5-P) lithium salt (SIGMA), 1 mM thiamine pyrophosphate and 10–20 U Saccharomyces cerevisiae transketolase (EC 2.2.1.1) (TK) (SIGMA) were also added. The formation of glyceraldehyde-3-phosphate (G 3-P) from Rib 5-P and Xul 5-P through TK was determined. Commercial Xul 5-P was contaminated with Rib 5-P, hence an assay was run in parallel, in the absence of cell sample and using different amounts of Xul 5-P. With this latter assay, it was determined that Xul 5-P contained 40–50% Rib 5-P, but not erythrose-4-phosphate (Ery 4-P). Therefore, for Rib 5-P determination this contamination was subtracted. Furthermore, TK can also form G 3-P and Fru 6-P from Xul 5-P and Ery 4-P, the latter may also be present in the cell sample. Therefore, another assay was prepared in parallel containing 0.8 ml 100 mM Tris-HCl pH 7.0, 1 mM NADP⁺, 0.2 ml of clarified extract, 7 U glucose-6-phosphate isomerase, 2 U glucose-6-phosphate dehydrogenase, 0.2 mM commercial Xul 5-P, 1 mM thiamine pyrophosphate and 10–20 U TK. The change in absorbance was minimal. For the purpose of ensuring the control of the enzymatic assay, commercial Ery 4-P was added, obtaining a high increase in NADPH production. This result indicated that the amount of Ery 4-P in the cells was below the limit of detection of the assay. The intracellular volume of V. cholerae was determined using the width and length values of the cell [29] using the equation described in [30]. The product of the intracellular volume multiplied by the number of cells (6 x 10⁸ cells/ml) at an A₆₀₀nm = 0.7 was used to obtain the total intracellular volume of the cell sample. This value was used to determine the concentration of the metabolites.

Results and discussion

The Vibrio cholerae genome contains two circular chromosomes [1], and genes pyk-F and pyk-A-1 are in chromosome 1, whereas pyk-A-2 is in chromosome 2 [2]. The products of these genes are referred as VcIPK, VcIIPK and VcIIIPK (not studied), respectively. Duplication of pyruvate kinase genes (PK) is frequently found in various γ-proteobacteria [13, 31]. It is of interest to understand if these enzymes are expressed under common culture conditions (LB and aerobiosis), and if the optimal conditions to express their activity are found in the bacterium. Since no information is available in the literature about PKs from V. cholerae, the kinetic characterization of the isolated enzymes is described here.

Purification of VcIPK and VcIIPK and molecular mass determination

VcIPK and VcIIPK were purified as described in Material and Methods. SDS PAGE revealed a single band of approximately 50 kDa, proving its high degree of purity (Fig 1A). BN-PAGE [16] was used to determine the oligomeric state of both enzymes. As in most PKs [32], a native band of approximately 200 kDa was observed, indicating that both isozymes are homotetramers (Fig 1B). Mass spectrometry of the VcIPK and VcIIPK yielded a molecular weight of 53,138 Da and 54,914 Da, respectively; 50,513 Da and 52,289 Da correspond to the monomers of VcIPK and VcIIPK, respectively when taking the size of 2,624.7 Da corresponding to the His₆ tag and the cleavage site of protease TEV (data not shown). The calculated molecular weights (protparam Expasy tools) of VcIPK and VcIIPK were, in concordance to mass spectrometry, 50,439 Da and 52,333 Da, respectively.

Monovalent cation activation of VcIPK and VcIIPK

In a previous phylogenetic study from the family of pyruvate kinase [3], it was generalized that those PKs that have Glu at the position 117 (numbered according to RMPK) exhibited a
K⁺-dependent activity, whereas those with Lys in that position have been demonstrated to have a K⁺-independent activity. In agreement with the phylogenetic study [3] VcIPK containing Glu 117 (Fig 2A) and VcIIPK containing Lys 117 (Fig 2B) exhibited K⁺-dependent and K⁺-independent activity, respectively. The activation of VcIPK and VcIIPK by different concentrations of Li⁺, Na⁺, K⁺, NH₄⁺, Rb⁺ and Cs⁺ (Fig 2) was determined in media that contained a ten-fold greater concentration of the calculated K₉₅ for PEP³⁻, ADP-Mg complex and Mg²⁺ free of VcIPK. In the case of VcIIPK, due to the lack of saturation for PEP³⁻, the experiments were conducted at the highest substrate concentrations allowed (40 mM of total PEP). In agreement with other PKs [33, 34], VcIPK reached maximal activation in the presence of K⁺ (100%) followed by Rb⁺ (81%), NH₄⁺ (48%), Cs⁺ (38%), Na⁺ (7%) and Li⁺ (2%) (Fig 2A).

It is important to note that the assay with NH₄⁺ was performed from 0 to 12 mM because at higher concentrations an inhibitory effect was observed. On the other hand, Na⁺ and Li⁺ up to 100 mM did not exhibit a saturation curve, which is necessary for the calculation of kinetic parameters.

Consistent with the PKs that possess Lys in the position 117 [11, 35–39], VcIIPK exhibited a lack of activation by monovalent cations and a minor inhibitory effect (Fig 2B).

The kinetic parameters for the activation of VcIPK by K⁺, NH₄⁺, Rb⁺ and Cs⁺ are shown in Table 1, but kinetic parameters for Na⁺ and Li⁺ could not be calculated, because the lack of saturation to include in Table 1. In comparison with other K⁺-dependent PKs, VcIPK exhibited higher k₉₅ and 2 to 5-fold lower K₀.₅ for the monovalent cations [34, 40]. According to [41,42], VCIPK is a good example of Type Ib activation by monovalent cations. In such a mechanism, M⁺ coordination is absolutely required for catalysis, where both k₉₅ and k₉₅/Kₘ increase hyperbolically with [M⁺] (S1 Fig).
Activation of VcIPK and VcIIPK by allosteric effectors

It has been reported that K$^+$-dependent PKs are allosterically activated by bi-phosphorylated sugars [32] such as Fru 1,6-BP [7, 43] and Fru 2,6-BP [44], and K$^+$-independent PKs are mainly activated by AMP [13] and monophosphorylated sugars, such as Glc 6-P [45] and Rib 5-P [12]. In order to express maximal activities of both enzymes, various allosteric effectors of PKs were tested. The experiments with VcIPK were conducted at 0.3 mM of PEP$^3-$, a concentration that corresponds to 0.18-times the $K_0.5$ in the absence of Fru 1,6-BP, and at a concentration that corresponds to the $K_0.5$ obtained for the saturation curve of the substrate in the presence of 5 mM of the modulator (for the latter data, see Table 2). Due to the low activity that VcIIPK exhibited in the absence of the allosteric effector, the assays were performed with 3.61 mM PEP$^3-$, 6.5 mM ADP-Mg complex and 30 mM Mg$^{2+}$free where a reliable activity could be measured. Fig 3 shows that VcIPK was activated 31-fold by Fru 1,6-BP (from 9 to 279 μmol/min.mg), whereas VcIIPK was activated 200-fold (from 1.5 to 300 μmol/min.mg) by Rib 5-P and 159-fold (1.5 to 239 μmol/min.mg) by Glc 6-P. However, Rib 5-P is a better allosteric activator than Glc 6-P. In the presence of Glc 6-P, VcIIPK exhibited a $V_{max}$ of 229 ± 4 μmol/min.mg, a $K_0.5$ for PEP$^3-$ of 1 ± 0.02 mM and a Hill number of 2.4 ± 0.1. These data are 12% lower, 3-fold higher and 1.3-fold higher than those obtained with 5 mM of Rib 5-P, respectively (compared to the data for PEP$^3-$ of VcIIPK in Table 2).

Table 1. Kinetic parameters for the monovalent cations in VcIPK at saturating concentrations of PEP$^3-$, ADP-Mg complex and Mg$^{2+}$free. The data from Fig 2A were fitted to the Hill equation $v = V_{max}\ast [S]^{n}\ast K_0.5^{n}\ast [S]^{-n}$ (Origin version 7.0). The mean and standard deviation of three experiments are shown.

| M$^+$  | $K_0.5$ (mM) | $K_{cat}$ (s$^{-1}$) | $n$  |
|-------|-------------|---------------------|-----|
| K$^+$ | 8.5 ± 0.5   | 1361 ± 30           | 1.1 ± 0.1 |
| Rb$^+$| 5.9 ± 0.2   | 1067 ± 13           | 1.4 ± 0.1 |
| NH4$^+$| 2.7 ± 0.3  | 723 ± 38            | 1.3 ± 0.1 |
| Cs$^+$| 13.5 ± 0.5  | 515 ± 8             | 1.6 ± 0.1 |

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Table 2. Kinetic parameters for PEP3-, ADP-Mg and Mg\(^{2+}\)\_free in the absence or presence of 0.05 and 5 mM Fru 1,6-BP for VcIPK or of 0.05 and 5 mM Rib 5-P for VcIPK. The data of Fig 4 were fitted (nonlinear regression Origin version 7.0) to the Hill equation \(v = V_{\text{max}} \times [S]^n / K_{0.5} + [S]^n\). The mean and standard deviation of three to four experiments are shown. N. D. not determined.

| Effector (mM)     | VcIPK  | VcIPK  |
|-------------------|--------|--------|
| K\(_{0.5}\) (mM) | k\(_{\text{cat}}\) (s\(^{-1}\)) | n |
| PEP\(^3\)         | 0      | 1.7 ± 0.4 | 1114 ± 21 | 3.7 ± 0.2 | 1.0 ± 0.1 | 0.7 ± 0.03 | 792 ± 12 | 2.9 ± 0.1 |
|                   | 0.05   | 0.9 ± 0.02 | 1205 ± 14 | 3.6 ± 0.2 | 0.9 ± 0.02 | 1.7 ± 0.03 | 959 ± 14 | 1.8 ± 0.1 |
|                   | 5      | 0.3 ± 0.02 | 1402 ± 29 | 1.0 ± 0.1 | 0.3 ± 0.02 | 0.7 ± 0.03 | 836 ± 18 | 1.2 ± 0.1 |
| ADP-Mg            | 0      | 0.6 ± 0.02 | 1294 ± 18 | 1.0 ± 0.03 | 0.4 ± 0.02 | 0.7 ± 0.03 | 917 ± 21 | 1.1 ± 0.1 |
|                   | 0.05   | 0.4 ± 0.02 | 1348 ± 20 | 1.2 ± 0.1 | 0.2 ± 0.02 | 0.7 ± 0.03 | 814 ± 17 | 2.3 ± 0.2 |
|                   | 5      | 0.5 ± 0.02 | 1313 ± 19 | 1.3 ± 0.1 | 0.3 ± 0.02 | 0.7 ± 0.03 | 849 ± 11 | 2.9 ± 0.2 |
| Mg\(^{2+}\)       | 0      | 0.2 ± 0.01 | 1228 ± 15 | 1.5 ± 0.1 | 33 ± 6 | 206 ± 22 | 1.1 ± 0.1 |
|                   | 0.05   | 0.3 ± 0.02 | 1260 ± 31 | 1.4 ± 0.1 | 2.0 ± 0.1 | 814 ± 17 | 2.3 ± 0.2 |
|                   | 5      | 0.3 ± 0.01 | 1282 ± 21 | 1.4 ± 0.1 | 1.4 ± 0.03 | 849 ± 11 | 2.9 ± 0.2 |

Assays of VcIPK and VcIPK in the presence of Mg\(^{2+}\) and Mn\(^{2+}\)

PKs exhibit an absolute dependence on divalent cations for activity, because they are essential for phosphate transfer [46]. Usually Mg\(^{2+}\) or Mn\(^{2+}\) are used and may be exchanged due to their similarities in the chelate structures of these ions [47]. However, Mg\(^{2+}\) is generally the most efficient physiological cation for PKs, with notable exception of Corynebacterium glutamicum PK, that has a preferential use of Mn\(^{2+}\) or Co\(^{2+}\) [38], Bradyrhizobium japonicum PK which only uses Mn\(^{2+}\) [48], and Sporolactobacillus inulinus PK [49] which strongly prefers Mn\(^{2+}\) over Mg\(^{2+}\) for activity. Thus, the effects of Mg\(^{2+}\) and Mn\(^{2+}\) on the activity of VcIPK and VcIPK were studied.

Kinetic parameters for VcIPK and VcIPK in the presence of Mg\(^{2+}\)

Saturation curves for PEP\(^{3}\) (Fig 4A and 4B), ADP-Mg (Fig 4C and 4D) and Mg\(^{2+}\)\_free (Fig 4E and 4F) of VcIPK and VcIPK are shown, respectively. All experiments were conducted in the absence or presence of 50 μM or 5 mM of Fru 1,6-BP or Rib 5-P for VcIPK and VcIPK, respectively. As shown in Fig 4A, 4C, 4D and 4E, VcIPK exhibited high activity with or without the effector indicating that Fru 1,6-BP was a non-essential activator. As in other PKs [13], Fru 1,6-BP activated VcIPK by decreasing the K\(_{0.5}\) for PEP\(^{3}\) with minor or no effect on the kinetic constants for ADP-Mg complex or Mg\(^{2+}\)\_free. The allosteric activator, decreased the K\(_{0.5}\) for PEP\(^{3}\) by seven-fold, increased the k\(_{\text{cat}}\) by 20%, and decreased the Hill number from 3.7 to 1 (Fig 4A and Table 2). On the other hand, Fig 4B, 4D and 4F show that VcIPK in the presence of non-physiologically high concentrations of the substrates (40 mM PEP, 39 mM Mg\(^{2+}\) and 9.7 mM ADP) exhibited low activities (25 μmol/min.mg) in the absence of the Rib 5-P. Even at the highest PEP concentration tested no saturation kinetics was observed without the allosteric effector. In contrast, in the presence of Rib 5-P, a saturation curve for PEP\(^{3}\) in VcIPK was observed indicating that Rib 5-P was an essential activator. The allosteric effector also decreased the K\(_{0.5}\) for all the substrates (PEP\(^{3}\), ADP-Mg and Mg\(^{2+}\)\_free) by at least one order of magnitude, increased the k\(_{\text{cat}}\) in the range of 400 to 700% and varied the Hill number in a different manner for each substrate (Table 2). Moreover if the low substrate concentrations (0.091–0.300 mM PEP, 0.55–0.823 mM ADP and 1–3 mM Mg\(^{2+}\)\_free) found in vivo in other γ- proteobacteria [50–53] were used in the assays, the activity of VcIPK would absolutely rely on Rib 5-P. This allosteric regulation is particular for this PK.
Kinetic parameters for VcIPK and VcIIPK in the presence of Mn$^{2+}$. Saturation curves for PEP$^3$ (Fig 5A and 5B), ADP-Mn (Fig 5C and 5D) and Mn$^{2+}$free (Fig 5E and 5F) of VcIPK and VcIIPK are shown, respectively. All the experiments were conducted in the absence or presence of 5 mM of Fru 1,6-BP or of 5 mM Rib 5-P for VcIPK and VcIIPK, respectively. VcIIPK exhibited lower activities in the presence of Mn$^{2+}$ (Fig 5A, 5C and 5E) compared to those in the presence of Mg$^{2+}$ (Fig 4A, 4C and 4E) either with or without Fru 1,6-BP. This result is in agreement with other PKs [15, 46, 54, 55]. Although the maximum activity for VcIPK was 1.4-fold higher with Mg$^{2+}$ than with Mn$^{2+}$, the $K_{0.5}$ for Mn$^{2+}$free was 17-fold lower than the $K_{0.5}$ for Mg$^{2+}$free, with no significant change in the constants for the PEP$^3$ and ADP-Mn (see Tables 2 and 3). In agreement with the results in the presence of Mg$^{2+}$, Fru
1,6-BP activated VcIPK by decreasing the $K_{0.5}$ for PEP$^3$ by 7-fold (Fig 5A) with minor or no effect on the kinetic constants for ADP-Mg complex or Mn$^{2+}$ free (Fig 5C and 5E). Remarkably, in the absence of Rib 5-P and presence of Mn$^{2+}$, VcIIPK exhibits a saturation curve for PEP$^3$ (Fig 5B). This is in contrast to the non-saturation kinetics observed in the presence of Mg$^{2+}$.

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Fig 5. The effect of Fru 1,6-BP in VcIPK and Rib 5-P in VcIIPK on the saturation curves for PEP$^3$, ADP-Mn and Mn$^{2+}$ free. In (A, C and E) the effect of Fru 1,6-BP (closed symbols) is shown in VcIPK and in (B, D and F) the effect of Rib 5-P (open symbols) is shown in VcIIPK. The concentrations of the allosteric activators were (■, □) 0 mM, (●, ▲) 0.05 mM and (▲, △) 5 mM. The reaction mixtures contained 25 mM HEPES pH 7.0, 0.2 mM NADH, 8 μg/ml LDH, TMACl in order to maintain the ionic strength at 300–350 mM, and also with and without 90 mM KCl in VcIPK and VcIIPK, respectively. Kinetics for PEP$^3$ and ADP-Mg were performed at saturating concentrations of the other substrate. The concentrations of Mn$^{2+}$ free for the saturation curves of PEP$^3$ and ADP-Mg were 2 mM and 15 mM for VcIPK and VcIIPK, respectively. For the saturation curves of Mn$^{2+}$ free, the concentration of PEP was varied from 5 to 40 mM and those of ADP-Mg from 3 to 9 mM. Assays were performed at 25°C and the reactions were started by the addition of 0.1 to 5 μg/ml of PK. The data were fitted to the Hill equation. Standard deviation bars of three to four experiments are shown.

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Table 3. Kinetic parameters for PEP\(^{3-}\), ADP-Mn and Mn\(^{2+}\)\(_{\text{free}}\) in the absence or presence of 5 mM Fru 1,6-BP or of 5 mM Rib 5-P for VclIPK and VclIPK, respectively. The data of Fig 5 were fitted (nonlinear regression Origin version 7.0) to the Hill equation \(v = V_{\text{max}}^* \left[ S \right]^{n} K_{0.5}^* + \left[ S \right]^{n} \). The mean and standard deviation of three to four experiments are shown.

| Effector (mM) | PEP\(^{3-}\) | ADP-Mn | Mn\(^{2+}\) |
|--------------|-------------|---------|-----------|
|              | \(K_{0.5}\) (mM) | \(k_{\text{cat}}\) (s\(^{-1}\)) | \(n\) | \(K_{0.5}\) (mM) | \(k_{\text{cat}}\) (s\(^{-1}\)) | \(n\) | \(K_{0.5}\) (mM) | \(k_{\text{cat}}\) (s\(^{-1}\)) | \(n\) |
| 0            | 2.8 ± 0.1   | 778 ± 11 | 3.1 ± 0.2 | 0.5 ± 0.01 | 381 ± 9 | 3 ± 0.2 |
| 5            | 0.4 ± 0.03  | 740 ± 21 | 1.5 ± 0.1 | 0.08 ± 0.003 | 435 ± 9 | 1.3 ± 0.1 |
| 0            | 1.7 ± 0.3   | 919 ± 71 | 1.0 ± 0.1 | 0.3 ± 0.01 | 333 ± 3 | 1.5 ± 0.1 |
| 5            | 1.2 ± 0.2   | 876 ± 48 | 1.2 ± 0.1 | 0.2 ± 0.02 | 348 ± 14 | 1.1 ± 0.1 |
| 0            | 0.01 ± 0.0004 | 776 ± 7 | 1.6 ± 0.1 | 0.07 ± 0.01 | 310 ± 11 | 1.4 ± 0.1 |
| 5            | 0.01 ± 0.0002 | 836 ± 6 | 1.4 ± 0.1 | 0.02 ± 0.001 | 287 ± 11 | 2.9 ± 0.04 |

Initial velocity studies of VclIPK and VclIPK

To date initial velocity studies of PK have been focused on RMPK. This enzyme follows a random order rapid equilibrium kinetic mechanism at saturating concentrations of K\(^+\), as first demonstrated by Boyer's group [58], and confirmed later by others [18, 59, 60]. In the absence of K\(^+\), RMPK changes its kinetic mechanism to ordered rapid equilibrium with PEP being the first substrate [18]. Similar studies have been conducted in the K\(^+\)-dependent PK of the muscle of the sea mollusk *Concholepas concholepas* [61], and in the K\(^+\)-independent PK of the euryarchaeota *Thermophilum pendens* [15]. It was found that both enzymes follow a rapid equilibrium random order kinetic mechanism. However, when the *C. concholepas* enzyme was studied in the presence of Mn\(^{2+}\) the results demonstrated an ordered sequential mechanism, with ADP-Mn being the first substrate [61]. In this work, initial kinetic studies were carried out both in the presence and absence of K\(^+\) for VclIPK (Fig 6A and 6B) and VclIPK (Fig 6C and 6D), respectively. These experiments were performed at various concentrations of one of the substrates and at fixed concentrations of the other; however, the assays for VclIPK were performed in the presence of Mg\(^{2+}\) and Fru 1,6-BP, whereas those for VclIPK were performed with Mn\(^{2+}\) and Rib 5-P. The experimental conditions chosen for VclIPK allowed hyperbolic kinetics and maximal activities, whereas those for VclIPK were chosen because the enzyme showed a sigmoidal response for PEP\(^{3-}\) even in the presence of Rib 5-P either with Mg\(^{2+}\) or
However, this cooperative effect was lower with Mn$^{2+}$ ($n_H = 1.3$) than with Mg$^{2+}$ ($n_H = 1.8$). Additionally, oxalate inhibition was not observed in VcIPK in the presence of Mg$^{2+}$.

In VcIPK (Fig 6A) and VcIIPK (Fig 6C), the double reciprocal plots of the initial velocities versus the ionized PEP concentrations intersected on the 1/S axis and to the left on the 1/v axis. When the concentrations of ADP-Mg in VcIPK (Fig 6B) or ADP-Mn in VcIIPK (Fig 6D) were varied, the lines intersected on the 1/S axis and to the left on the 1/v axis. These results indicate either an ordered steady state or a rapid equilibrium random-order kinetic mechanism. These data were globally fitted to the equation described in Table 4, and the kinetic constants obtained are shown. It is observed that both isozymes in the presence of their respective allosteric effectors exhibit similar catalytic efficiencies (Table 4).

**Dead-end inhibition studies of VcIPK and VcIIPK**

The use of dead-end inhibitors provide a strong advantage in the determination of the kinetic mechanisms of enzymes [62]. In this work, oxalate was used as dead-end analog of PEP [63].
Table 4. Intersecting patterns, kinetic mechanisms and kinetic constants of VcIPK and VcIPK. Intersecting patterns were taken from the double reciprocal plots of the initial velocity data. The data shown in Fig 6 were globally fitted (nonlinear regression Origin version 7.0) to the equation describing a rapid equilibrium random order mechanism \( v = \frac{V_{max} [A][B]}{K_a K_b + K_b [A] + [A][B]} \), where \( v \) represents the initial velocity, \( A \) is PEP, \( B \) is ADP-Mg\(^{2+}\) and \( K_a \) and \( K_b \) are the Michaelis-Menten constants for PEP and ADP-Mg\(^{2+}\), respectively. Standard deviation values are shown. Catalytic efficiency values, \( k_{cat}/K_m \) (M\(^{-1}\)s\(^{-1}\)) are expressed in log form.

| Condition          | Initial velocity intersecting patterns | Kinetic mechanism | \( V_{max} \) | \( K_{m, PEP} \) | \( K_{m, ADP-Mg^{2+}} \) | \( k_{cat} \) | Log \( k_{cat}/K_m \) | Log \( k_{cat}/K_m \) ADP-Mg\(^{2+}\) |
|--------------------|---------------------------------------|-------------------|------------|---------------|----------------|-------------|-----------------|-----------------|
| VcIPK with Mg\(^{2+}\) | Intersects to the left of the \( 1/v \) axis and on the 1/S axis | Intersects to the left of the \( 1/v \) axis and on the 1/S axis | Random rapid equilibrium | 476±20 | 0.3 ±0.02 | 0.5±0.04 | 1684 | 6.76 | 6.54 |
| VcIPK with Mn\(^{2+}\) | Intersects to the left of the \( 1/v \) axis and on the 1/S axis | Intersects to the left of the \( 1/v \) axis and on the 1/S axis | Random rapid equilibrium | 147±2 | 0.06 ±0.02 | 0.13 ±0.02 | 541 | 6.95 | 6.62 |

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Table 5. Dead-end inhibition patterns and inhibition constants for oxalate and ADP-Cr\(^{2+}\) in VcIPK and VcIPK. Inhibition patterns were taken from the double reciprocal plots of the inhibition experiments. Simple inhibition patterns were confirmed from linear replotts of the slopes or intercepts versus the inhibitor concentrations (not shown). The inhibition constants were calculated from the fits of the complete data set to the corresponding equations from linear competitive inhibition (C) \( v = \frac{V_{max} [S]}{K_m([1+I]/K_i)+[S]} \), linear noncompetitive inhibition (NC), or linear mixed inhibition (MT) \( v = \frac{V_{max} [S]}{[1+I]/K_i} \), where \( \alpha = 1 \) and \( \alpha < 1 \) for NC and MT, respectively; \( K_i \) is the inhibition constant.

| V. cholerae enzyme | Dead end analog of PEP: oxalate | Dead end analog of ADP-Mg\(^{2+}\): ADP-Cr\(^{2+}\) | \( K_i \) (oxalate) \( \mu \)M | \( K_i \) (ADP-Cr\(^{2+}\)) mM |
|--------------------|--------------------------------|---------------------------------|-----------------|-----------------|
| 1/\( v \) versus 1/PEP, fixed ADP-Mg\(^{2+}\) | 1/\( v \) versus 1/ADP-Mg\(^{2+}\), fixed PEP | 1/\( v \) versus 1/PEP, fixed ADP-Mg\(^{2+}\) | 1/\( v \) versus 1/ADP-Mg\(^{2+}\), fixed PEP | 1/\( v \) versus 1/ADP-Mg\(^{2+}\), fixed PEP | 1/\( v \) versus 1/ADP-Mg\(^{2+}\), fixed PEP |
| VcIPK               | C                             | NC                             | C               | 7.8 ± 0.3       | 1.1 ± 0.1     |
| VcIPK               | C                             | MT                             | MT              | 71 ± 6          | 1.3 ± 0.1     |

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and ADP-Cr\(^{2+}\) as that of ADP-Mg\(^{2+}\) [64]. In both enzymes, the patterns of oxalate inhibition versus PEP and ADP-Mg\(^{2+}\) were competitive and mixed-type with \( \alpha < 1 \) (factor affecting \( K_i \) for oxalate inhibition versus ADP-Mg\(^{2+}\)), respectively. This indicates that the enzyme-oxalate binary complex has a higher affinity for ADP-Mg\(^{2+}\) than that of the free enzyme. In VcIPK assays with ADP-Cr\(^{2+}\), the inhibition was non-competitive for PEP and competitive for ADP-Mg. VcIPK followed the same patterns as those of VcIPK, with the exception that a mixed type pattern with \( \alpha > 1 \) for ADP-Cr\(^{2+}\) inhibition versus PEP was observed. This was interpreted, that the free enzyme has higher affinity for PEP than the enzyme-ADP-Cr\(^{2+}\) binary complex. Collectively, the data for both enzymes show that oxalate is a competitive inhibitor with respect to PEP, whereas ADP-Cr\(^{2+}\) is a competitive inhibitor with respect to ADP-Mg\(^{2+}\). These results demonstrate that the analogs and the substrates bind to the same site. If it is considered that oxalate is a mixed type inhibitor with respect to ADP-Mg\(^{2+}\), it may be concluded that oxalate forms a non-productive ternary complex and thereby diminishes the \( V_{max} \). The same argument holds for ADP-Cr\(^{2+}\) versus PEP. In VcIPK, whereas in VcIPK, the non-competitive inhibition of ADP-Cr\(^{2+}\) versus PEP decreases the \( V_{max} \) without altering the binding of PEP. In all cases, replotts of the slopes or intercepts of the double reciprocal plots against inhibitor concentration were linear (not shown). All the data were globally fitted to the equations that describe linear competitive inhibition, linear non-competitive inhibition or linear mixed inhibition. The inhibition patterns and inhibition constants for VcIPK and VcIPK are shown in Table 5. Therefore the data with dead-end inhibitors suggest that VcIPK and VcIPK follow a rapid equilibrium random order kinetic mechanism, in spite of
having E74 or K75, respectively (corresponding to the E117 in the sequence of RMPK). These results are in agreement with the mechanism reported earlier for RMPK in the presence of K$^+$ and in the mutant E117K of RMPK [18].

Fluorescence studies of VcIPK and VcIIPK

For fluorescence studies, an excitation at 280 nm for both enzymes was selected due to their lack of tryptophan residues. VcIPK and VcIIPK have 5 and 8 tyrosines per monomer, respectively. Since there is no crystallographic structure reported for these PKs, the distribution of the tyrosines in their sequences, was based on the alignment of the sequences of E. coli PK type I with VcIPK (78% identity), and on that of the sequences of E. coli PK type II with VcIIIPK (69% identity) [32]. Therefore VcIPK appears to have one Tyr in domain B and two in domain A and C, respectively. VcIIIPK appears to harbor one Tyr in domain B, three in domain A and four in domain C. Thus, global conformational changes were determined by tyrosines fluorescence and intrinsic fluorescence anisotropies ($r$) induced by different ligands. As observed in Fig 7A fluorescence intensities, $\lambda_{max}$ and $r$ of VcIPK were similar with or without divalent cations (Mn$^{2+}$ and Mg$^{2+}$). However, the addition of Fru 1,6-BP quenched the intensity 50%, shifted $\lambda_{max}$ 2–3 nm to the red spectrum, and decreased the $r$ of the enzyme in the presence or absence of Mn$^{2+}$ or Mg$^{2+}$. In contrast, VcIIIPK exhibited no conformational changes in all the experimental conditions assayed, except in the presence of Mn$^{2+}$, where a 20% increase in the fluorescence intensity, a blue shift of 3 nm, and an increase of $r$ were observed (Fig 7B). These results indicate that the binding of Fru 1,6-BP to VcIIIPK caused the tyrosines to become more exposed to the solvent phase, increasing the global protein flexibility. These results are in agreement with the structural effect of Fru 1,6-BP in yeast PK [65, 66] human liver PK [67] and in E. coli PK [68]. On the other hand, no conformational change induced by its allosteric effector was detected in VcIIIPK by fluorescence measurements. Therefore, it might be that

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Fig 7. Effect of various ligands on the intrinsic fluorescence emission spectra at 280 nm, $\lambda_{max}$ and the anisotropy of VcIPK (A) and VcIIIPK (B). Closed symbols are for VcIPK and open symbols are for VcIIIPK. The symbols represent: PK + HEPES (■), PK + Mg$^{2+}$ (●), PK + Mn$^{2+}$ (▲), PK + Effector (▼), PK + Effector + Mg$^{2+}$ (○), PK + Effector + Mn$^{2+}$ (▲). Fluorescence studies were performed at 25°C in mixtures that contained 200 μg ml$^{-1}$ of VcIPK or VcIIIPK in 50 mM HEPES pH 7.0. In VcIPK, the media contained either 2 mM Mg$^{2+}$, 0.2 mM Mn$^{2+}$ or 5 mM of Fru 1,6-BP. In the mixtures that contained Fru 1,6-BP and the divalent cation, the concentration of the effector was 0.5 mM and the concentrations of Mn$^{2+}$ or Mn$^{2+}$ were 2 or 0.2 mM, respectively. In VcIIIPK, the media contained either 30 mM Mg$^{2+}$, 0.5 mM Mn$^{2+}$ or 5 mM Rib 5-P. In the mixtures that contained Rib 5-P and Mg$^{2+}$, the concentrations were 1.5 and 15 mM, respectively. When the effector was added with Mn$^{2+}$, the concentration of Rib 5-P was 0.5 and that of the divalent cation was 0.2 mM. The inset shows the $\lambda_{max}$ and the anisotropy values ($r$). The anisotropy values were calculated at their respective maximal emission.
tyrosines present in the C domain of this enzyme were not applicable to monitor the movements of the enzyme when RSP was bound.

In the presence of Mn\(^{2+}\) the enzyme adopts a more compact and less mobile conformation. This conformation corresponds to its active state as described above in the kinetics of the enzyme with this divalent cation. Likewise, other PKs adopt a compact active conformation induced by ligands in RMPK [18] and by Fru 2,6-BP in trypanosomatid PKs [69, 70].

### Expression of VcIPK and VcIIPK in Vibrio cholerae

Western blot analyses were performed using antibodies that specifically recognize VcIPK and VcIIPK, as shown in [Fig 8C and 8D](#). No cross-reactivity was found between the polyclonal antibodies and the other *V. cholerae* PKs. The main bands corresponded to the expected molecular mass of VcIPK (53.14 kDa) ([Fig 8A](#)) and VcIIPK (54.91 kDa) ([Fig 8B](#)). To show that the bands correspond to PKs, an SDS-PAGE gel including extracts of *E. coli* DH5α and PB25 strains was performed. *E. coli* DH5α strain contains the two endogenous PykF and PykA, whereas in PB25 strain, the gene of PykF has been interrupted with chloramphenicol and that of PykA with kanamycin resistance cassettes. The identification by Western blots of PK bands of *E. coli* extracts, *V. cholerae* extracts and recombinant VcIPK and VcIIPK recognized by anti-VcIPK and anti-VcIIPK display a similar migration pattern. It is relevant to mention that the recognition of *E. coli* PKs might be due to the high identity between these two \(\gamma\)-proteobacteria (78% identity of VcIPK vs. PykF and 69% between VcIIPK and PykA). In the case of PB25 strain, the band is of a higher molecular weight due to the insertion of the antibiotic resistance cassette (chloramphenicol 25.6 in [Fig 8A](#)) and kanamycin 29.17 in [Fig 8B](#)).

The amount of PKs on *V. cholerae* extract were estimated to be 14 ± 0.2 μg/ mg of *V. cholerae* extract protein (VcIPK) and 26 ± 0.2 μg/ mg of *V. cholerae* extract protein (VcIIPK) by comparing the densitometry data with the lane loaded with known amounts of the corresponding recombinant PK. Lower faint bands below the main PK band may correspond to limited degradation products of the PK proteins. These data indicate that *V. cholerae* cells express both PKs growing in LB medium under aerobiosis.

### Determination of metabolites in Vibrio cholerae extracts

As described above, VcIPK exhibits high activity without allosteric effector (Fru 1,6 BP), whereas VcIIPK with Mg\(^{2+}\) is inactive in the absence of Rib 5-P. However, in the absence of Rib 5-P, VcIIPK is capable of high activities when the divalent cation was Mn\(^{2+}\) instead of Mg\(^{2+}\). Hence, it was relevant to also consider the physiological concentrations of Fru 1,6-BP and Rib 5-P in *V. cholerae*. Therefore, the concentrations of these metabolites and other glycolytic intermediates were determined using cell extracts of *V. cholerae* (CVD 103 strain) harvested at stationary phase from LB broth ([Table 6](#)). The concentrations of Fru 1,6-BP and Rib-5P, were 0.97 mM and 4.3 mM, respectively. Other intermediates of glycolysis, G6-P and Fru 6-P, were lower than those reported in cells grown at exponential phase in glucose-fed cultures [52]; whereas Rib 5-P was higher than that reported by Bennett *et al.* [52]. However the ATP determined in the same cell extracts, reached the millimolar concentrations obtained in previous reports [52] supporting the validity of the other determinations. To elucidate if both recombinant VcPKs are functional under physiological metabolite concentrations found in \(\gamma\)-proteobacteria, experiments were performed containing 300 μM PEP [50], 550 μM ADP [52], 1.6 mM Mg\(^{2+}\)\(_\text{free}\) [53] and either 972 μM Fru 1,6-BP for VcIPK or 4.3 mM Rib 5-P for VcIIPK. In these experimental conditions the activity for VcIPK was 25% of \(V_{\text{max}}\) (122 μmol/min mg) and the activity for VcIIPK was 10% of \(V_{\text{max}}\) with Mg\(^{2+}\) (29 μmol/min mg). The two forms of the enzyme appear to co-exist, at least in this *in vitro* condition. These data are coincident...
Fig 8. Western blot analysis of VcIPK (A) and VcIIPK (B) in *Vibrio cholerae* cell extracts and cross-reactivity of polyclonal anti-VcIPK (C) and anti-VcIIPK (D) against recombinant VcIPK, VcIIPK and VcIIIPK. Western blot was performed with anti-VcIPK (A) and anti VcIIPK (B). MW are the Molecular Weight Standards (Western-C of Biorad) and lanes 2, 3 and 4 are 30 μg of cell extracts of *E. coli* DH5α, of *E. coli* PB25 and of *V. cholerae*, respectively. Lane 5 was loaded with purified recombinant 0.5 μg of VcIPK (A) and 1 μg of VcIIPK (B). In (C) and (D) 0.5 μg of purified recombinant enzymes were loaded. VcIIIPK was generously given by Dr. Gloria Hernández-Alcántara.

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with a report showing that under most physiological conditions, the total pyruvate kinase activity of *E. coli* cell results from the contribution of both forms of the enzyme in different proportions [6, 10, 71]. On the other hand, given the concentration of Mn$^{2+}$ in *E. coli* ($\sim 10 \mu$M) [72], it is difficult to establish the physiological role for Mn$^{2+}$ on VcIIPK activity. Therefore the expression of the catalysis of VcIIPK in cells at the stationary phase of growth, under aerobiiosis and LB broth cell culture seems to be mainly regulated by Rib 5-P.

In sum the data show that VcIPK and VcIIPK exhibited K$^{+}$-dependent and K$^{+}$-independent activity, respectively. The isozymes possess different allosteric effectors, VcIPK is regulated by Fru 1,6-BP (“K type” system for PEP), whereas VcIIPK is activated by Rib 5-P (“mixed type” system for PEP and ADP-Mg). In contrast to the high activities expressed in VcIPK in the absence of Fru 1,6-BP, VcIIPK was completely dependent on Rib 5-P to exhibit high activities. Despite the different allosteric regulation between VcIPK and VcIIPK, in the presence of their respective activators both PKs exhibit similar catalytic efficiencies. Since we found that in *V. cholerae*, VcIPK and VcIIPK were co-expressed, and their allosteric effectors were present, it may be concluded that both enzymes contribute to the activity of pyruvate kinase in this bacterium. However, it cannot be discarded that the expression of PKs in *Vibrio cholerae* will change in different growing conditions and different metabolic requirements.

### Supporting information

**S1 Fig.** Changes in $k_{\text{cat}}$ (A) and in $k_{\text{cat}}/K_{0.5}$ (B) vs [K$^+$] for PEP$^-$ of VcIPK. $k_{\text{cat}}$ and $K_{0.5}$ values were obtained from curves of variable concentrations of PEP$^-$ (0.04, 0.097, 0.29, 0.87, 2.33 mM) at fixed variable concentrations of K$^+$ (1.5, 3.5, 7.5, 20 and 50 mM). The assays were performed in the presence of 5 mM Fru 1,6-BP, 6.5mM ADP-Mg and 2mM Mg$^{2+}$

(PDF)

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