Native mass spectrometry gives insight into the allosteric binding mechanism of M2 pyruvate kinase to fructose-1,6-bisphosphate

Author(s):
Gavriilidou, Agni F.M.; Holding, Finn P.; Meyer, Daniel; Coyle, Joseph E.; Veprintsev, Dmitry B.; Zenobi, Renato

Publication date:
2018-03-20

Permanent link:
https://doi.org/10.3929/ethz-b-000252576

Originally published in:
Biochemistry 57(11), https://doi.org/10.1021/acs.biochem.7b01270

Native mass spectrometry gives insight into the allosteric binding mechanism of M2 pyruvate kinase to fructose-1,6-bisphosphate

Agni F. M. Gavriilidou[1], Finn P. Holding[2], Daniel Mayer[3], Joseph E. Coyle[2], Dmitry B. Veprintsev[3‡] Renato Zenobi[1*]

[1]ETH Zurich, Department of Chemistry and Applied Biosciences, CH-8093 Zurich, Switzerland

[2]Astex Pharmaceuticals, 436 Cambridge Science Park, Milton Road, Cambridge, CB4 0QA, United Kingdom

[3]Paul Scherrer Institute, 5232 Villigen PSI (Switzerland)

‡Current address: Centre of Membrane Proteins and Receptors, School of Life Sciences, University of Nottingham, NG7 2UH, UK

SUPPORTING INFORMATION

Experimental information

All solvents and fructose-1,6-bisphosphate (FBP) were purchased from Sigma Aldrich (Buchs, Switzerland). Recombinant wild type M2 pyruvate kinase isoform (PKM2) and the S437Y mutant proteins were provided by Astex Pharmaceuticals. Recombinant wild type PKM2 was also expressed and purified at the Paul Scherrer Institute based on the following protocol:

The gene encoding human PKM2 (vector pET28b)

MGSSHHHHHH-
SSGLVPRGSKPHSEAGTAFIQTQQLHAAMADTFLEHMCRLDIDSPPITARNTGI
ICTIGPASRSVETLKEMIKSGMNVARLNFSHGTHEYHAETIKNVRTATESFAS
DPILYRPVAVALDTKGPEIRTGLIKGSGTAEVELKKGATLKITLDNAYMEKCD
ENILWLDYKNICKVVEVGSKIYVDDGLISLQVKQKGADFLVTEVENGGSLGS
KKGVNLPGAAVDLPAVSEKDIQDLKFGVEQDVDMVFASFIRKASDVHEVRK
VLGEKGKNIKIISKIENHEGVRRFDEILEASDGIMVARGDLGIEIPAEKVFLAQK
MMIGRCNRAGKPVICATQMLESMIKKPRPTRAEGSDVANAVLDGADCIMLS
GETAKGDYPLEAVRMQHLIAREAEAAIYHLQLFEELRRLAPITSDPTEATAVG
AVEASFTKSGRSAHQVARYRPRAPIIAVTRNPQTARQAHLYRGIFPVLCKDPV
QEAWAEDVDLRVNFAMNVGKARGFFKKGDVVIVLTGWRPGSGFTNTMRVV
PVP (theoretical molecular weight = 59382 Da)was expressed in *Escherichia coli* BL21(DE3) cells.

A starting culture of 500 ml was grown overnight at 30 °C and 40 ml of this culture were used to inoculate 12 x 1 L of expression culture in 2 L Erlenmeyer flasks. The cells were grown at 37 ºC for ~4h until reaching an OD_{600} of 0.6. The temperature was decreased upon reading to 18 ºC and 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added for induction after 1h. The cells were harvested after 18h by centrifugation at 5000 rpm for 20 min. The pellets were stored at -20 ºC until purification. The cells were resuspended in 10 mM Tris base, pH 7.5, 500 mM NaCl, 5% (v/v) glycerol and protease inhibitor complex EDTA-free (Roche) buffer and

lysed by sonication (3 x 5 min, 1s pulse/0.5s pause). The cell debris was removed by centrifugation at 4 ºC for 1h at 35,000 rpm. The collected supernatant was filtered through four 0.45 µm syringe filters. PKM2 protein was subsequently purified by Ni^{2+}-NTA affinity chromatography on a 5 mL HisTrap FF crude column (GE Healthcare, Little Chalfont, Bucks, UK). The column was washed with ten column volumes of 10 mM Tris base, pH 7.5, 500 mM NaCl, 30 mM imidazole, 5 mM $MgCl_2$, 5% (v/v) glycerol and eluted by increasing the imidazole concentration to 250 mM. The protein-containing fractions were combined and concentrated with a 30 kDa spin concentrator (Millipore). An analytical size-exclusion on a Superdex 200 16/600 column (GE Healthcare, Little Chalfont, Bucks, UK) in 25 mM Tris base, pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 10% (v/v) glycerol was performed and the protein-containing fractions were pooled, concentrated, flash frozen in liquid nitrogen and stored at -80 ºC until use. The purity of the samples was analyzed with SDS-PAGE and mass spectrometry.

ITC measurements were performed at 25 ºC on a Microcal VP-ITC instrument (MicroCal, Northampton, MA, USA) in 25 mM Tris base, pH 7.5 100 mM KCl, 5 mM $MgCl_2$ and on a Microcal iTC200 microcalorimeter instrument (Malvern Instruments Ltd, Malvern, UK) in 200 mM ammonium acetate (NH_4Ac), pH 7.5, in order to determine the effect of buffer composition on the binding of FBP to PKM2. Both ITC experiments were configured with protein in the sample cell and compound in the injection syringe. In the VP-ITC the monomeric PKM2 concentration was 10 µM and the FBP concentration was 100 µM and in the iTC200 the monomeric protein concentration was 17 µM and the FBP concentration was 94 µM. Slightly different ionization enthalpies were determined in the two solutions due to different ionization enthalpies of the buffers[1]. ITC peaks were integrated with the NITPIC software[2,3] in order to obtain an unbiased integration of the peaks and the Affinimeter software (S4SD, Santiago de Compostela, Spain) was used to fit the ITC data. However, a definitive fit of the isotherms was not possible due to the complexity of the model and the high number of degrees of freedom.

The thermal shift assay was performed on a Rotor-Gene Q machine. The fluorescence signal of Sypro orange® was measured three times for each sample with an excitation wavelength of 470 nm and emission wavelength of 610 nm. The temperature was incrementally raised from 25 °C to 95 °C in 0.5 °C steps with set autogain optimization.

All MS experiments were carried out under native-like conditions using 200 mM NH_4Ac adjusted to pH 7.5. Prior to mass spectrometric analysis, PKM2 protein stock solutions were desalted and buffer exchanged by centrifugal filtration (Amicon Ultra-0.5 ml 10K, Merck Millipore, Darmstadt, Germany) or by dialysis (Slide-A-Lyzer Mini Dialysis Device, 10K MWCO) against aqueous NH_4Ac. FBP solutions were prepared in the same NH_4Ac solution. The stock concentration of monomeric PKM2 after desalting was ~70 µM. Prior to the MS experiments the protein was diluted from the stock solution to a minimum of 1.25 µM monomeric PKM2. Each stock solution and diluted samples were kept for a maximum of 48hrs. After acquiring 5-10 spectra,

a control for the protein quality was acquired, by spraying 5 μM of monomeric PKM2 and comparing the spectra and the relative oligomeric populations. No change in the relative oligomeric distribution and abundance was observed.

Additional buffer additives were tested to attempt to gain additional insight into binding of FBP to PKM2. 10mM imidazole was added to NH_4Ac and EDDA was also tested, but no other ligation states of FBP to PKM2 were observed.

ESI spectra were acquired on a Synapt G2-S HDMS (Waters, Manchester, UK) in positive ion mode. Sample solutions were directly infused with gold/palladium-coated borosilicate glass nano ESI emitters (Thermo Fisher Scientific, Reinach, Switzerland) using the NanoLock Spray ionization source (Waters, Manchester, UK). Instrumental conditions were controlled in order to minimize dissociation of the noncovalent complexes being studied but were sufficiently robust to ensure complete desolvation and desorption of adducts which could adversely affect the accuracy of peak quantitation. MS source parameters were therefore adjusted to maximize data quality: capillary voltage was set to 1.2 kV, backing pressure of 0.3 bar was applied to assist the liquid sample flow whilst the source was maintained at ambient temperature and the sampling cone voltage and source offset were set to 50 and 30 V, respectively. All voltages were set to the lowest possible values to minimize disruption of oligomers in vacuo. The default values for capillary voltage, sampling cone voltage and source offset were set to 2 kV, 80 and 50 V respectively. The trap gas flow was set to 7 ml/min (from a default value of 0.4 ml/min) which facilitated the detection of highly resolved peaks. Hernandez et al.[4] indicated the importance of gas pressure for the detection of ions in the high m/z region as increased gas pressure maximizes the probability of collisions between gas and analyte molecules resulting in enhanced desolvation and minimization of oligomeric dissociation as a result of collisional cooling. Therefore, ion suppression due to nonvolatile adducts will be decreased and the intensity of the ion peaks will increase.

Data were smoothed with the MassLynx 4.0 software and data processing was performed using MATLAB (2010a, The MathWorks, Natick, MA, USA). FBP bound and unbound peak areas were integrated and measured by using a Gaussian model to fit the peaks and the Hill equation was applied to determine the binding constant (K_d) and Hill coefficient (n) which describes the extent of cooperativity, where L is the ligand concentration:

$$\frac{\mathbf{Bound}}{\mathbf{Total}} = \frac{\mathbf{L^n}}{\mathbf{K_d + L^n}} = \frac{\frac{\mathbf{L^n}}{\mathbf{K_d}}}{\mathbf{1} + \frac{\mathbf{L^n}}{\mathbf{K_d}}}$$

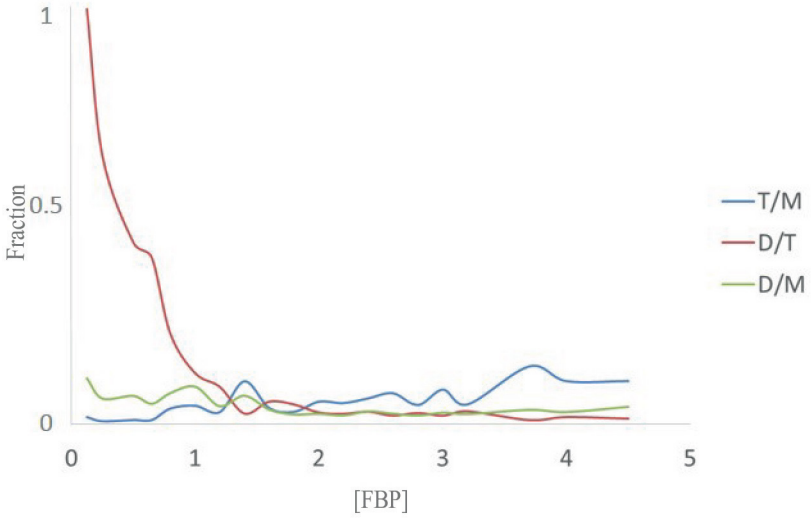

Figure S1: Plot of the ratio of the peak areas representing monomer (M), dimer (D) and tetramer (T) populations versus the FBP concentration. The effect of FBP concentration on the D/T areas are represented in red, D/M area in green and T/M in blue. The monomer population was low abundance compared to the other oligomer populations and was not affected by the presence of FBP. On the other hand, the D/T was clearly influenced in the presence of FBP, dimeric PKM2 decreased with increasing FBP concentration.

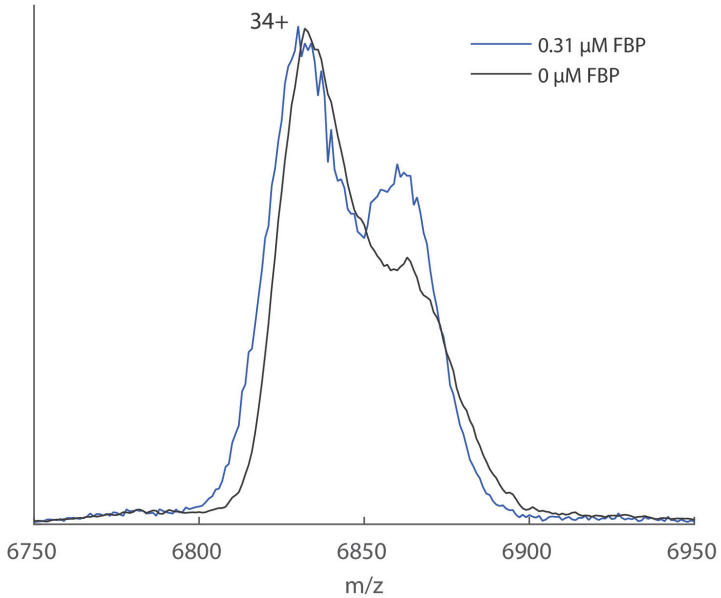

Figure S2: Spectra of the 34+ ion peak PKM2 with 0 µM (black line) and 0.31 µM (blue line) FBP. Both peaks possess a distinct shoulder, which attains greater prominence at increased FBP concentration.

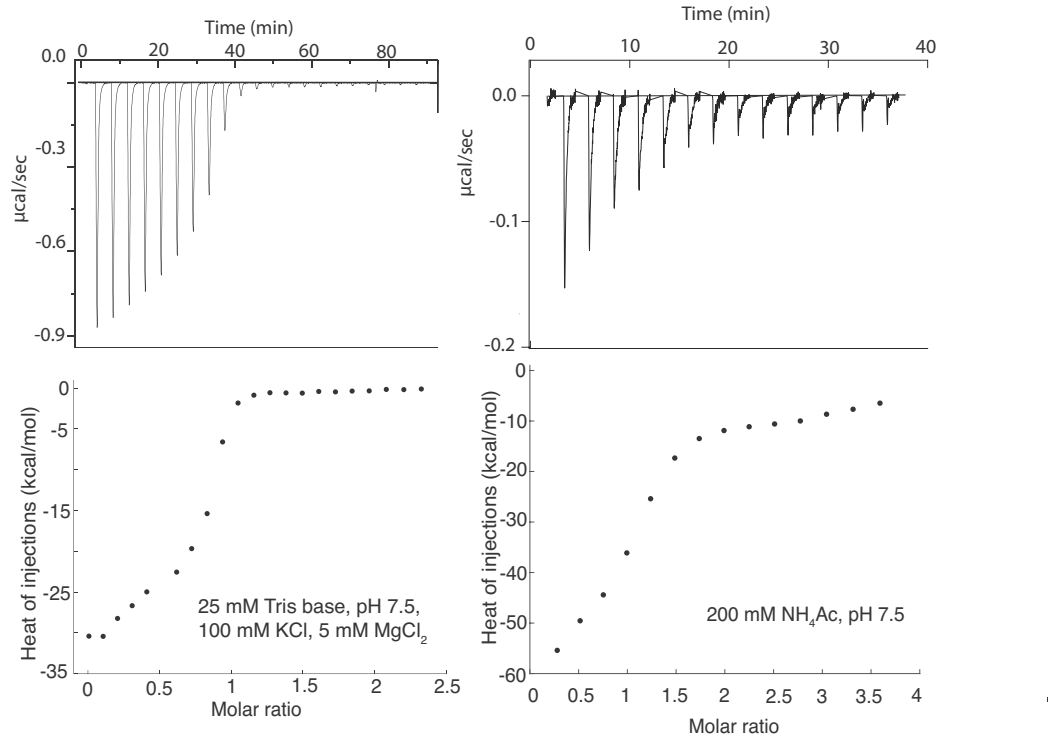

Figure S3: FBP binding to WT PKM2 was measured by ITC in Tris buffer and in NH_4Ac. Biphasic isotherms were generated in both cases indicating a complex binding mode which prevented fitting of the data and calculation of a definitive K_d value.

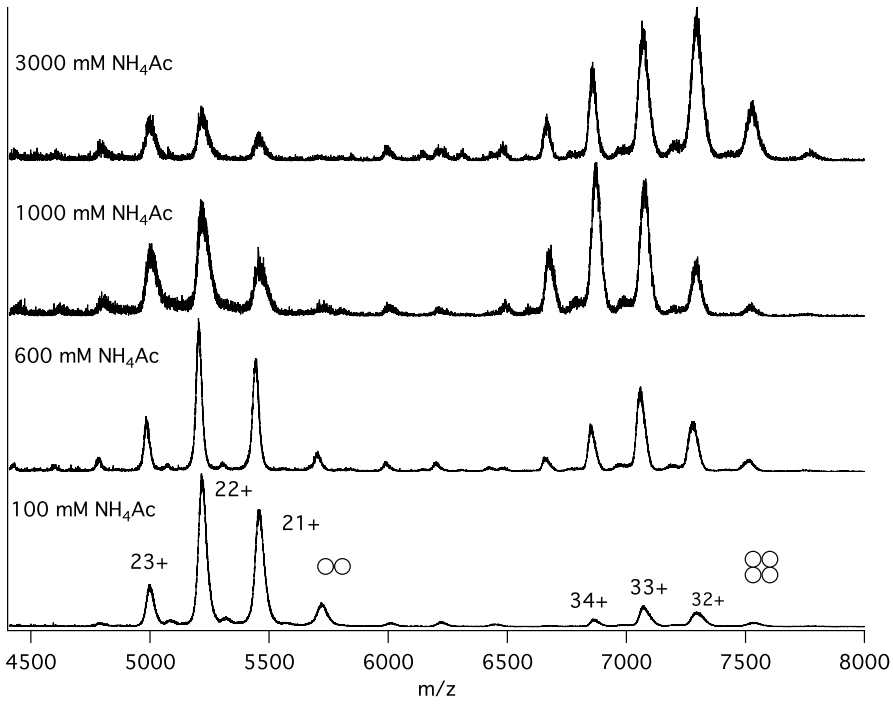

Figure S4: Representative spectra of 2.5 µM dimeric concentration PKM2 titrated against NH_4Ac. The intensity of the dimer (two circles) decreased and the tetramer (four circles) increased with increasing NH_4Ac concentration.

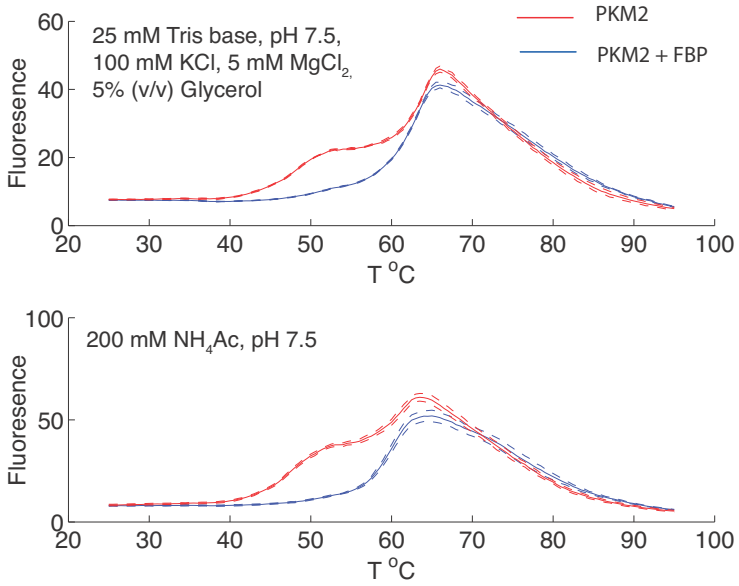

Figure S5: Melting curves of PKM2 in Tris buffer and in NH_4Ac. The thermal stability of the protein was measured in the presence and absence of FBP and a shift to higher melting temperature was observed in the presence of FBP. No difference between the two solutions was observed indicating that the stability of the protein was not compromised by the different buffer solutions.

References

(1) Goldberg, R. N., Kishore, N., and Lennen, R. M. (2002) Thermodynamic quantities for the ionization reactions of buffers. *J. Phys. Chem. Ref. Data 31*, 231–370.
(2) Brautigam, C. A., Zhao, H., Vargas, C., Keller, S., and Schuck, P. (2016) Integration and global analysis of isothermal titration calorimetry data for studying macromolecular interactions. *Nat. Protoc. 11*, 882–894.
(3) Keller, S., Vargas, C., Zhao, H., Piszczek, G., Brautigam, C. A., and Schuck, P. (2012) High-precision isothermal titration calorimetry with automated peak-shape analysis. *Anal. Chem. 84*, 5066–5073.
(4) Hernández, H., and Robinson, C. V. (2007) Determining the stoichiometry and interactions of macromolecular assemblies from mass spectrometry. *Nat. Protoc. 2*, 715–26.