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A pH Sensitive Colorometric Assay for the High-Throughput Screening of Enzyme Inhibitors and Substrates: A Case Study Using Kinases

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Abstract—We have developed an uncoupled, pH sensitive kinase assay that can be used for high-throughput screening of potential inhibitors or for determining substrate specificity. Kinases catalyze the transfer of a γ-phosphoryl group from ATP to an appropriate hydroxyl acceptor with the release of a proton. This assay is based on the detection of this proton using an appropriately matched buffer/indicator system. The assay was used to measure the activity of four readily available kinases, including hexokinase, glucokinase, glycerokinase, and pyruvate kinase, which was run in the reverse direction. We also went on to screen a small series of mono- and diphosphonucleotides for inhibition of hexokinase as well as a modest set of potential hexokinase substrates. We determined sucrose to be a modest substrate for hexokinase with a K_m of 1.8 ± 0.2 mM, a k_{cat} of 142 ± 3 min^{-1}, and a V_{max} that is 15% of that for glucose. Given the importance of kinases in a diverse array of biological functions and disease states, there is a need for a simple, rapid assay system. We feel this assay will lend itself well to meet this end. This method should be applicable to many other enzymatic reactions which involve a change in pH.

Introduction

Kinases are involved in a large number of biologically relevant processes including neurobiology, immunology, metabolite processing, embryonic development, and cancer. For this reason, there is great impetus to develop small molecule inhibitors, specific to a particular kinase, for both therapeutic reasons as well as for functional analysis.[1,2] A quick search of the literature reveals thousands of references concerned with the design of small molecule inhibitors of kinases and the elucidation of substrate specificity. Both rational and combinatorial approaches to inhibitor design have been utilized in the search for therapeutic agents.[3–9] A chemical-genetics approach has also been devised in order to elucidate down stream signaling events.[10]

The use of a combinatorial approach to either inhibitor design or substrate elucidation requires a viable assay amenable to automation. There are a number of different assays reported in the literature, but many require the use of radioactivity, increasing cost, or involve coupling the kinase of interest to another kinase, complicating inhibitor screening.[3,4,7,11–13] We sought to develop an assay that is uncoupled, required no radioactivity, and could be used in a high-throughput manner.

The general reaction catalyzed by kinases involves the transfer of the γ-phosphoryl moiety of ATP to an acceptor hydroxyl group with the release of a proton (Fig. 1). A great deal of work has been done with fluorescent and colorimetric sensors for detection of binding events, screening of inhibitor and substrate libraries, and searches for catalytic activity.[14–16] Among these studies, there are a number of reports that indicate a pH indicator could be used to detect a proton released in a qualitative manner, thus making it possible to screen for enzyme activity colorimetrically.[17] An assay of this type would eliminate the need for radioactivity, would be a direct measure of the kinase of interest, and would be readily adapted to rapid screening.

In this report, we demonstrate a colorimetric, uncoupled, pH sensitive kinase assay. We were able to use our assay for both qualitative screening of kinases, substrates, and inhibitors, as well as quantitatively to measure kinetic parameters. One of the requirements for such a system is that the pK_a of the buffer and the pK_a of the indicator be as close to equal as possible. This will

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ensure that the color change is proportional to the number of protons released.[17] For our system, we chose to use Cresol Purple and bicine both with pK_a's of 8.3 at 25 °C. This allowed us to run our reactions at a pH that is useful, although not optimal, for all of the kinases screened. This system should be tunable to other pH values, to match other enzymes or to optimize activity, with the correct choice of buffer and indicator (Table 1).

For our purposes, we chose to use four readily available and inexpensive kinases that used inexpensive substrates as a model system. We were able to establish a linear relationship between the production of protons and the change in absorbance for each of the kinase assay conditions by performing titrations with either HCl or NaOH. We also screened a set of potential hexokinase substrates and a small set of potential hexokinase inhibitors as a proof of principle. All of the inhibitors were mono- and di-phospho nucleotides. After the initial screen of hexokinase substrates, we measured V_{max}, k_{cat}, and K_m for ATP, D-glucose, and sucrose. We believe this system can be used for other kinases as well as other enzyme systems (Fig. 2).

Results and Discussion

Titration with HCl or NaOH to establish a linear relationship between proton production and absorbance change

In order to be able to calculate the rate of a particular reaction, there needs to be a linear relationship between the change in absorbance and the production of protons.[17] We established this relationship by titrating each of the reaction conditions with HCl (hexokinase, glucokinase, and glycerokinase) or NaOH (pyruvate kinase). We chose to titrate the pyruvate kinase assay buffer with NaOH because we were running the reaction in the reverse direction instead of the forward direction, thus consuming rather than producing a proton. For each of the assay buffer conditions, we were able to establish a linear relationship, although the relationship was different for the different buffer conditions. The glucokinase/hexokinase buffer system showed a larger change in absorbance as a function of titrant added than the other two systems. For the glucokinase/hexokinase system, a change in OD_{577} of -1.647 corresponded to the addition of 1 μmol of protons; for the glycerokinase system, an OD_{577} change of -0.3927 corresponded to a 1 μmol addition of protons; and for the pyruvate kinase system, a change of $+0.1768$ in OD_{577} corresponding to the loss of 1 μmol of protons. In each case, we were able to correlate the change in absorbance of the enzyme catalyzed

Table 1. Common indicators with their Δε values, pK_a values, and potential buffers

Indicator	Δε ($M^{-1}\,cm^{-1}$)	pK_a	Buffer
Bromocresol Green	52 000 (612 nm)	4.7	Acetate
Methyl Red	38 000 (575 nm)	5.0	Tricitrate
Chlorophenol Red	27 800 (410 nm)	6.0	MES
Bromothymol Blue	43 000 (616 nm)	7.1	MOPS
p-Nitrophenol	17 300 (404 nm)	7.2	BES
Phenol Red	56 000 (557 nm)	7.4	TES
Cresol Red	100 500 (570 nm)	7.9	HEPPS
Cresol Purple	47 300 (577 nm)	8.3	Bicine
Thymol Blue	61 700 (594 nm)	8.9	Bis-tris Propane

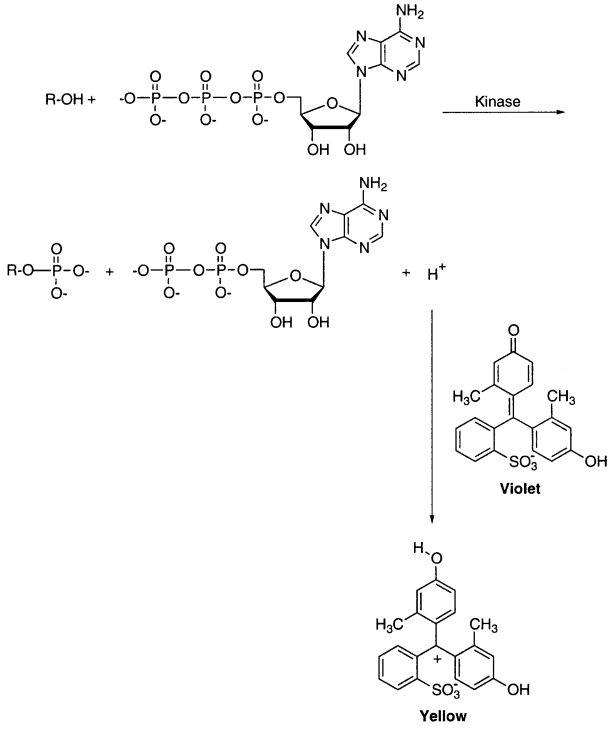

Figure 1. The general reaction catalyzed by kinases. The indicator color change can be measured colorimetrically to rapidly screen for activity or inhibition.

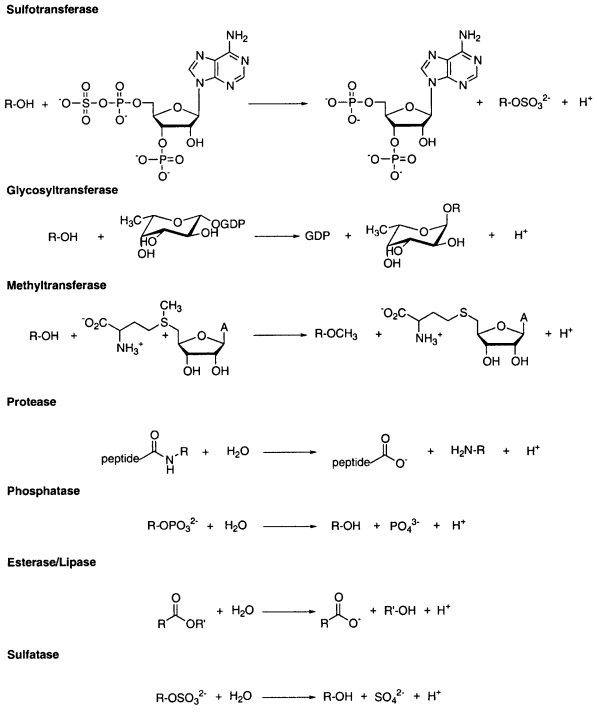

Figure 2. A series of important, enzymatic reactions that could be studied using a pH sensitive assay.

reactions with the number of protons released or consumed (Fig. 3). This allowed for the quantitative determination of kinetic parameters.

Establishing a relationship between the amount of enzyme used and the reaction rate

After an initial screen of a couple of different kinases showed a change in absorbance above a measured background, we wanted to show a linear relationship between the amount of enzyme added and the rate of the reaction. This was done for both hexokinase and for glucokinase. Rates were calculated by a previously described method[17] using eqs (1) and (2):

$$Q = \frac{C_B}{C_{\ln}} \times \frac{1}{\Delta\varepsilon_{577\,\mathrm{nm}} l} \quad (1)$$

$$rate(\mu mol/\min) = \frac{\mathrm{d}A}{\mathrm{d}t} \times Q \times volume \times 10^6 \quad (2)$$

Where C_B and C_{In} are buffer and indicator concentrations, respectively, $\Delta\varepsilon_{577\mathrm{nm}}$ is the difference between the protonated and deprotonated extinction coefficient at 577 nm, and l is the path length of the cuvette. The data from this are not shown.

Measuring the activity of a small set of kinases

In an effort to show the generality of this assay, we sought to screen a small series of kinases. We chose this group based on cost and availability considerations. The data for this series of measurements are shown in Figure 4. For each of the kinases assayed we were able to detect activity relative to a negative control without added enzyme. For each of the particular sets of reactions, a negative control was run and the results from three sets of measurements were averaged together. The rate differences between the particular kinases can be attributed to two things, one is the differing concentration of enzymes used and two is the differing pH requirements for the individual kinases.

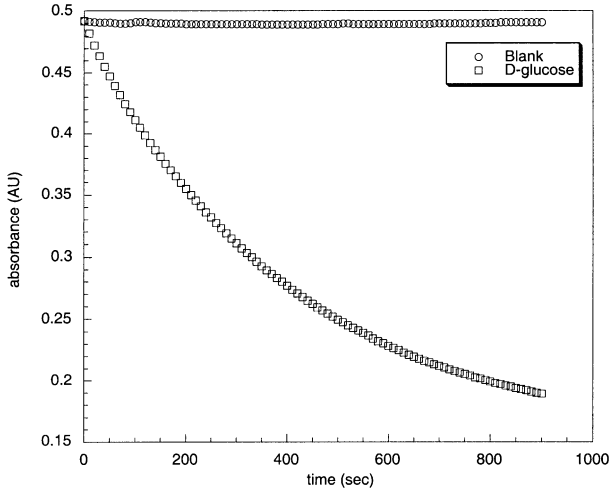

Figure 3. Plot of the change in absorbance as a function of time. Hexokinase was assayed in 20 mM bicine pH 8.3, 5 mM ATP, 5 mM $MgCl_2$, 5 mM D-glucose, and 0.2 mM Cresol Purple. Measurements were taken at 577 nmol for 15 min. The measurement was repeated in triplicate and the values averaged.

Screen of a small set of potential hexokinase substrates

We next sought to screen a small series of potential hexokinase substrates. A total of 15 substrates was chosen based on diversity of structure. Figure 5a shows a series of monosaccharides and Figure 5b shows a series of disaccharides and one pentasaccharide. Of the 15 potential substrates that were screened, D-mannose was the closest in activity to D-glucose, followed by sucrose. All of the active substrates had been reported previously[16] with the exception of sucrose. All of the other sugars showed less activity, many only slightly above background. Importantly, we were quickly able to identify active substrates using our assay conditions.

Determining K_m, k_{cat}, and V_{max} for D-glucose, ATP, and sucrose

After determining sucrose to be a substrate, we set out to test if our assay could be used quantitatively. We first determined the K_m of D-glucose and ATP by measuring the rate at 5.0, 2.5, 1.0, 0.5, and 0.1 mM D-glucose and ATP concentrations, respectively. The rates were calculated as above. We calculated a K_m of 0.420 ± 0.07 mM and a k_{cat} of $943 \pm 42\,\mathrm{min}^{-1}$ for D-glucose and a K_m 0.140 ± 0.013 mM for ATP, values within experimental error of previously published values.[18,19] We then calculated the K_m for sucrose by measuring the rate at 25, 12.5, 5.0, 2.5, and 1 mM. The calculated K_m was 1.8 ± 0.2 mM, k_{cat} was $142 \pm 3\,\mathrm{min}^{-1}$, and the V_{max} was 15% that of D-glucose.

Qualitatively measuring inhibition of a small set of potential inhibitors

Finally, we wanted to show that our assay could be used to rapidly screen for inhibition of a particular kinase. We used hexokinase as a model enzyme and picked potential inhibitors from a group of mono- and diphospho nucleotides. Of the four molecules screened, only the known inhibitor, ADP, showed inhibition, even at concentrations as high as 5 mM (data not shown). This provides a way to rapidly screen inhibitors. Molecules found to be inhibitors can then be scrutinized more carefully.

Conclusion

In the present study, we have developed a general pH assay for the high throughput screening of enzyme substrates and inhibitors. As a model system, we chose a series of readily available kinases, potential substrates, and inhibitors. We were able to qualitatively and rapidly screen both substrates and inhibitors and then to quantitatively characterize a novel hexokinase substrate, sucrose. We feel this assay will provide a useful

tool given the need for a rapid, uncoupled, inexpensive screen of a number of enzymes for both drug screening and biochemical studies.

Experimental

Materials

m-Cresol Purple was purchased from Aldrich (Milwaukee, WI). Hexokinase, glucokinase, glycerokinase, pyruvate kinase, phosphoenol pyruvate, D-glucose, D-galactose, D-mannose, L-mannose, L-fucose, D-arabinose, L-arabinose, sucrose, bicine, adenosine 5′-diphosphate, adenosine 5′-monophosphate, cytidine 5′-diphosphate, guanosine 5′-diphosphate, magnesium chloride, and bicine were purchased from Sigma (St. Louis, MO). The visible spectrophotometer (DU-650) was purchased from Beckman Instruments (Fullerton, CA).

Methods

Establishing a linear relationship between proton production and absorbance. Three separate sets of titrations were performed to establish linearity for each of the assay conditions used. Each of the assays was run on a 500 μL scale at 25 °C. Each of the stock assay solutions was prepared from the following stock solutions: 100 mM bicine pH 8.3, 100 mM D-glucose, 100 mM glycerol, 100 mM PEP, 100 mM ATP (prepared fresh daily), and 100 mM ADP (prepared fresh daily). The 10 mM HCl solution was prepared by the careful dilution of a titrated 1.6 M HCl stock solution. For hexokinase and glucokinase a stock solution containing 20 mM bicine pH 8.3, 5 mM glucose, 5 mM ATP, 5 mM $MgCl_2$, and 0.2 mM Cresol Purple (from a 10 mM stock in DMSO) was prepared fresh and added to a cuvette. This was then titrated with 1.0 μL aliquots of 10 mM HCl, after each addition, the solution was mixed with an inoculation loop and the absorbance at 577 nm was measured.

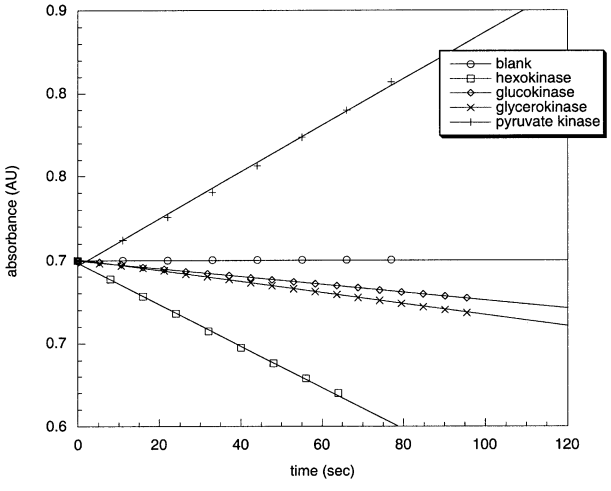

Figure 4. Measurement of various kinase activities at 25 °C. Four different kinase activities were measured in 20 mM bicine pH 8.3, 5 mM appropriate substrate and co-substrate, 5 mM $MgCl_2$, and 0.2 mM Cresol Purple. All reactions were measured at 577 nmol. A negative control was run for each of the kinases measured. All measurements were done in triplicate and the starting absorbance was normalized to 0.7.

For glycerokinase, a stock solution containing 20 mM bicine pH 8.3, 5 mM glycerol, 5 mM ATP, 5 mM $MgCl_2$, and 0.2 mM cresol purple was prepared fresh and added to a cuvette. This was then titrated in a manner similar to the one above, except that 2.0 μL aliquots were used. For pyruvate kinase, a stock solution containing 20 mM bicine pH 8.3, 5 mM PEP, 5 mM ADP, 5 mM $MgCl_2$, and 0.2 mM Cresol Purple was prepared fresh and added to a cuvette. Because this reaction was assayed in the reverse direction, 2.0 μL aliquots of 10 mM NaOH were used in place of HCl. Measurements of the absorbance at 577 nm were carried out after each addition. For each of the titrations, three sets of data points were collected and the results were averaged.

Glucokinase and hexokinase activity at varying enzyme concentrations. A stock assay solution was prepared as detailed above. Varying concentrations of glucokinase (0.1, 0.2, and 0.4 U, where one unit is defined as the amount to convert 1.0 μmole D-glucose to glucose-6-

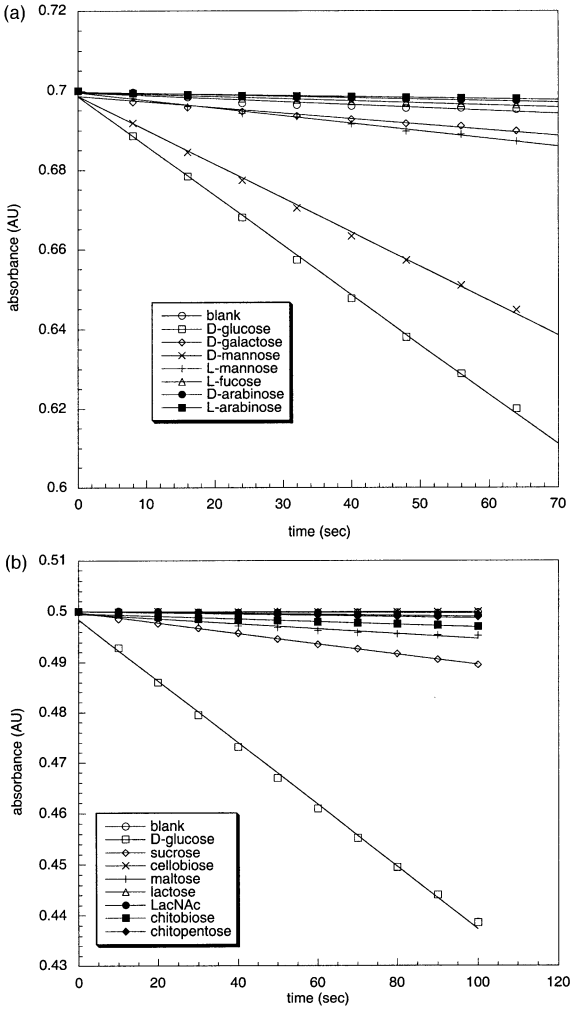

Figure 5. Assay of hexokinase substrate activity. All assays were carried out in 20 mM bicine pH 8.3, 5 mM substrate, 5 mM ATP, 5 mM $MgCl_2$, and 0.2 mM Cresol Purple. Measurements were made at 25 °C and the OD_{577} was measured. Each measurement was repeated in triplicate and an average taken. Figure 5a shows a series of monosaccharides and 5b shows a series of disaccharides and one pentasaccharide. All lines were normalized to a starting absorbance of 0.7 for 5a and to an absorbance of 0.5 for 5b.

phosphate per min at pH 9.0) and 30 °C and hexokinase (0.5, 1.0, and 2.0 where 1 U is defined as the amount to convert 1.0 μmole of D-glucose to glucose-6-phosphate per min at pH 7.6 and 25 °C) were added to a series of cuvettes, as well as water for a negative control. Enough of the stock assay solution was added to each of the cuvettes to bring the final volume to 500 μL, the reaction was stirred with an inoculation loop, and the absorbance at 577 nm was recorded for each sample at 5-s intervals for a total of 5 min. All measurements were carried out at 25 °C. The points corresponding to the steepest part of the curve were then plotted. Each of the measurements was repeated in triplicate and the values averaged.

Activity determination for kinases. A 0.5 U/μL stock hexokinase solution and a 0.1 U/μL stock glucokinase solution were prepared fresh daily. Glycerokinase (0.45 U/μL, where 1 U is the amount to convert 1.0 μmole glycerol to l-α-glycerophosphate per min at pH 9.8 and 25 °C) and pyruvate kinase (1.7 U/μL, with 1 U defined as the amount to convert 1 μmol of PEP to pyruvate per min at pH 7.6 and 37 °C) were used as purchased after determining there was no activity difference when the stock ammonium sulfate suspension was exchanged for a different buffer. Stock assay buffer solutions were prepared as reported above. The assays were performed by adding 1.0 μL of the appropriate enzyme stock solution or 1.0 μL of water to a cuvette, the appropriate stock assay solution was then added to bring the total volume to 500 μL, the reactions were mixed with an inoculation loop, and data was collected at 577 nm at 8 s intervals for a total of 5 min. The points corresponding to the steepest part of the line were then plotted. All of the measurements were repeated in triplicate and an average of each of the points taken.

Measuring substrate specificity for hexokinase. For these assays, six monosaccharides (D- and L-arabinose, D-galactose, D- and L-mannose, and L-fucose), six disaccharides (sucrose, cellobiose, maltose, lactose, galβ1,4GlcNAc, and chitobiose), and one pentasaccharide (chitopentose) were chosen. For each of the sugars, a 100mM stock solution was made. All assays were run using a 5mM final concentration for each of the different substrates. All other assay conditions were run as reported above.

K_m, k_{cat}, and V_{max} determination for hexokinase as a function of D-glucose concentration. All assays were run as listed previously, except that the concentration of D-glucose was varied; 5.0, 2.5, 1.0, 0.5, and 0.1 mM were used. The rate at each concentration was calculated and plotted as a function of substrate concentration. The data were then fit to the Michaelis–Menten equation and K_m, k_{cat}, and V_{max} were determined using non-linear regression.

K_m and V_{max} determination for hexokinase as a function of ATP concentration. Determined as above, except D-glucose was held constant and ATP concentrations of 5.0, 2.5, 1.0, 0.5, and 0.1 mM were used.

K_m, k_{cat}, and V_{max} determination for hexokinase as a function of sucrose concentration. Determined as above except 25, 12.5, 5.0, 2.5, and 1.0 mM concentrations of sucrose were used.

Qualitative measurement of hexokinase inhibition. A 100 mM stock solution of each of the compounds (ADP, AMP, CDP, and GDP) to be tested was made. Hexokinase (1 μL) was diluted with the appropriate molecule to be tested such that final reaction concentrations of 0, 0.5, 1.0, and 5.0 mM would result after dilution with the assay buffer solution. Each of the enzyme/inhibitor solutions was brought up to a final volume of 26 μL per reaction to be run. The enzyme/inhibitor solutions were then added to a cuvette and enough assay buffer solution was added to bring the final volume to 500 μL. The assay buffer solutions were adjusted with more buffer to compensate for changes in pH brought about by the potential inhibitors. Final buffer concentrations were as follows: 20 for 0 mM inhibitor, 21 for 0.5 mM inhibitor, 22 for 1.0 mM inhibitor, and 30 for 5.0 mM inhibitor. Measurements were carried out as reported above.

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