Label-free fluorescence detection of kinase activity using a gold nanoparticle based indicator displacement assay

Cristian Pezzato,a Davide Zaramella,a Massimiliano Martinelli,a Grégory Pieters,a Mario A. Pagano*b and Leonard J. Prins*a

A straightforward indicator-displacement assay (IDA) has been developed for the quantitative analysis of ATP→ADP conversion. The IDA relies on the use of gold nanoparticles passivated with a monolayer of thiols terminating with a 1,4,7-triazacyclononane (TACN)·Zn2+ head group. The analytes ATP and ADP compete to a different extent with a fluorescent probe for binding to the monolayer surface. In the presence of ATP the fluorescent probe is free in solution, whereas in the presence of ADP the fluorescent probe is captured by the nanoparticles and its fluorescence is quenched. The linear response of the fluorescence signal towards different ratios of ATP:ADP permitted the detection of protein kinase activity simply by adding aliquots of the enzyme solution to the assay solution followed by measurement of the fluorescence intensity. The assay poses no restrictions on the target kinase nor does it require labeling of the kinase substrate. The assay was tested on the protein kinases PIM-1 and Src and validated through a direct comparison with the classical radiometric assay using the γ-32P-labeled ATP.

Introduction

Protein kinases (PKs) are a class of enzymes that play critical roles in a variety of cellular functions including cell growth, development, differentiation, membrane transport, and cell death.1 Their function is exerted by transferring the γ-phosphate group from ATP to a serine, threonine or tyrosine residue in specific substrate proteins. These phosphorylation events modulate the activity of a vast number of proteins, including ion channels, transcription factors and phosphatases.2 PKs act primarily as components of signalling pathways in which extra- and intracellular stimuli are transduced by the cell into a series of phosphorylation events that ultimately bring about a cellular response, such as cell growth, development, differentiation, membrane transport, and cell death.3,4 Abnormalities in signalling pathways can lead to various pathological conditions including many forms of cancer. For this reason, PKs are highly important targets for both basic research and drug development.5

Traditionally, PK assays rely on the use of 32P-labeled ATP ensuring a high sensitivity.6 However, the application of these assays is hampered by the generated radioactive waste and the short half-life of 32P-labeled ATP. Besides, the laborious protocols are not compatible with high-throughput screening. Therefore non-radioactive methods are rapidly gaining interest.7,8 In particular, various fluorescence techniques (fluorescence intensity endpoint measurement,9 fluorescence resonance energy transfer (FRET),10 fluorescence polarization (FP),11 and fluorescence lifetime imaging (FLIM))12 have emerged as alternatives. Nonetheless, for a large part these techniques rely on elaborate constructs involving fluorophores conjugated to enzymes or antibodies. Consequently, there is a strong drive to develop new kinase assays using simple and stable components.13–17

Previously, we have studied the interaction between small anionic molecules, such as nucleotides and peptides, and Au MPC1-Zn2+ (d = 1.8 ± 0.4 nm), which are gold nanoparticles (d = 1.8 ± 0.4 nm) passivated with thiols terminating with a 1,4,7-triazacyclononane (TACN)·Zn2+ head group (Fig. 1).18,19 It was found that such molecules bind to the monolayer surface under saturation conditions even at low micromolar concentrations in aqueous buffer. Detailed studies have revealed that the interactions originate from a combination of electrostatic and hydrophobic interactions and coordination bonds, the relative contributions depending on the structure of the molecules.20 Recently, we have shown that such subtle changes in the binding affinity permitted discrimination of the eight
di- and trinucleotides (XDP and XTP, with X = A, T, G, C) by Au MPC 1·Zn2+. In that study, fluorescence output signals were generated by the displacement of fluorescent probe molecules from the surface of Au MPC 1 upon the addition of analytes. The observed large difference in the affinity between ATP and ADP prompted us to investigate whether this sensing system would be able to serve as a PK assay by quantitatively reporting on the conversion of ATP into ADP (Fig. 1).

Results and discussion

In order to set up the indicator-displacement assay (IDA)22 a fluorescent indicator was selected based on the following criteria: (1) excitation and emission wavelengths far away from the (near-) UV region to avoid interference with natural chromophores/fluorophores (e.g. Trp- or Tyr-residues), (2) the ability to bind Au MPC 1·Zn2+ under saturation conditions at low micromolar concentrations in aqueous buffer, and (3) the capability to respond in a very different manner to ATP and ADP in competition experiments. The fluorescent tripeptide A, equipped with coumarin 343, satisfied the first two points with λex = 450 nm and λem = 493 nm and a surface saturation concentration (SSC) equal to 3.7 ± 0.2 μM at a head group concentration (i.e. [TACN·Zn2+]) of 10 μM at pH 7.5. The third point was verified by performing two displacement experiments in which the fluorescence intensity of A was measured upon the addition of increasing amounts of ATP or ADP to a solution of A@Au MPC 1·Zn2+ (Fig. 2a). Initial fluorescent intensities in these experiments started at around 9% of the maximum signal expected for a full release of probe A, due to the presence of a small amount of free A in solution. In agreement with our previous results,21 much larger concentrations of ADP were required to displace the same amount of A from Au MPC 1·Zn2+ resulting from the lower number of negative charges in ADP compared to ATP. The maximum difference in signal intensity was observed at an analyte concentration of 3.0 μM.
Having confirmed the ability of the system to discriminate in a straightforward manner between ATP and ADP, we next investigated whether the system could respond quantitatively to different ratios of ATP and ADP. As a test case we chose Src, the namesake of the Src-family kinases (SFKs), a group of tyrosine kinases, physiologically involved in the early steps of signal transduction, the increased activity of which is associated with several types of cancer. Binding of ATP to Src is characterized by a $K_M$-value of 8.6 μM. As a substrate, we used a specific peptide (here renamed Src-tide, H-KVEKIGEGTYGVYK-H, 250 μM) derived from the cyclin-dependent kinases cdc2 (residue 6 through 20) and used in vitro for testing the activity of SFKs in the presence of the co-factors Mn$^{2+}$ and Mg$^{2+}$ (5 mM). In order to minimize the possible interferences of the enzyme, substrate and co-factors with the displacement assay we opted for a discontinuous approach in which the enzyme solution was sampled and diluted to the assay concentration for read out. Initially, we performed a calibration of the system by artificially simulating enzyme activity. Twelve independent solutions were prepared, each of them containing all the enzyme assay components except for ATP:ADP at a constant overall concentration of 50 μM. Aliquots of these solutions were added to the IDA assay solution containing A@Au MPC 1·Zn$^{2+}$ in 96-well plates to reach an overall concentration of ATP and ADP equal to 3.0 μM. The fluorescence intensities originating from displaced A were measured after a signal stabilization time of 15 minutes (Fig. 2b). After that time, the signal intensity remained constant indicating that the ATP:ADP ratio is not altered in the assay solution. Importantly, the fluorescence intensity decreased linearly as a function of the relative amount of ADP added (simulating up to 40% conversion). This illustrates that the ATP→ADP conversion can be quantitatively assessed and that the enzyme assay components do not interfere with the displacement assay.

At this point we validated the system as a true protein kinase assay by following the Src induced conversion of ATP to ADP as a function of time (Fig. 3a). In order to demonstrate unambiguously the reliability of the new assay, enzyme activity was also measured in parallel using a conventional radiometric assay with the $[^{32}P]$-labeled ATP. In order to assure right comparison a common reaction medium was prepared containing all the components (except for ATP) at the same concentrations used for the calibration, but this time with Src-kinase (30 μL of a stock solution with a specific activity of $\approx 2.5$ pmol min$^{-1}$ μL$^{-1}$). The mixture was split into two portions, to one of which a small amount of $[^{32}P]$-ATP was added. To the second portion the same volume of MQ water was added. The resulting mixtures were incubated at 37 °C and the phosphorylation reaction was initiated by adding ATP (50 μM). Every 4 minutes samples were taken and quenched by pouring the aliquots in 0.1% phosphoric acid (radiometric assay) or by freezing them in dry ice (displacement assay). After a time course of 35 minutes the aliquots were processed using conventional techniques (radiometric assay) or the protocol described above (displacement assay). Both assays gave a nearly superimposable result in terms of ATP conversion as a function of time indicating the reliability of the displacement assay (Fig. 3b). Our assay measures ATP→ADP conversion which may, in principle, have origins other than kinase activity. However, the fact that the results of our assay match in a quantitative manner those of the conventional radiometric assay (which measures substrate conversion) indicates that other ATP consuming-processes do not occur in the enzyme solution.

Sampling of the enzyme solution and dilution of the aliquots in the assay buffer has the advantage that the initial experimental conditions for the enzyme (pH, buffer, substrate, cofactors, etc.) hardly affect signal generation by the displacement assay. This, together with the fact that signal generation is a consequence of ATP→ADP conversion rather than substrate-phosphorylation, makes it a universal assay for kinases. To demonstrate this, the protocol was repeated using PIM-1, the Proviral Integration site for Moloney murine leukemia virus kinase 1, a PK that is overexpressed in a number of tumors with a role in the resistance to apoptosis. PIM-1 binds ATP with a lower affinity compared to Src ($K_M = 90$ μM
In contrast to Src, which requires one or more acidic amino acids in the vicinity of the phosphorylation site, PIM-1 targets serine or threonine residues surrounded by basic amino acids. The substrate used in the PIM-1 assay was synthesized according to Bullock et al. who had designed a highly specific substrate for PIM-1 (here renamed Pim-tide = H-ARKRRRHPSGPPTA-NH₂, 100 µM) using a positional scanning peptide library approach. Also for this enzyme, both the radiometric assay and the displacement assay gave virtually identical results (Fig. 3c).

The assay volume is an important issue for a practical application of the assay, since they determine how much enzyme is required to measure its activity. In the assays described above, considerably larger aliquots were taken for the displacement assay compared to the radiometric assay (30 vs. 5 µl, respectively). A straightforward way to reduce the required volume is by using 384-well plates rather than 96-well plates. For each well a working volume of 24 µl was used containing 23.2 µl of the assay solution (A@Au MPC·Zn²⁺) and just 0.8 µl of the enzyme solution. This allowed us to perform 4 readings from a single aliquot of 5 µl, which is similar to that used for the radiometric assay, maintaining a reliable detection of enzyme degradation under the experimental conditions.

The assay volume is an important issue for a practical application of the assay, since they determine how much enzyme is required to measure its activity. In the assays described above, considerably larger aliquots were taken for the displacement assay compared to the radiometric assay (30 vs. 5 µl, respectively). A straightforward way to reduce the required volume is by using 384-well plates rather than 96-well plates. For each well a working volume of 24 µl was used containing 23.2 µl of the assay solution (A@Au MPC·Zn²⁺) and just 0.8 µl of the enzyme solution. This allowed us to perform 4 readings from a single aliquot of 5 µl, which is similar to that used for the radiometric assay, maintaining a reliable detection of enzyme degradation under the experimental conditions.

The assay volume is an important issue for a practical application of the assay, since they determine how much enzyme is required to measure its activity. In the assays described above, considerably larger aliquots were taken for the displacement assay compared to the radiometric assay (30 vs. 5 µl, respectively). A straightforward way to reduce the required volume is by using 384-well plates rather than 96-well plates. For each well a working volume of 24 µl was used containing 23.2 µl of the assay solution (A@Au MPC·Zn²⁺) and just 0.8 µl of the enzyme solution. This allowed us to perform 4 readings from a single aliquot of 5 µl, which is similar to that used for the radiometric assay, maintaining a reliable detection of enzyme degradation under the experimental conditions.

The assay volume is an important issue for a practical application of the assay, since they determine how much enzyme is required to measure its activity. In the assays described above, considerably larger aliquots were taken for the displacement assay compared to the radiometric assay (30 vs. 5 µl, respectively). A straightforward way to reduce the required volume is by using 384-well plates rather than 96-well plates. For each well a working volume of 24 µl was used containing 23.2 µl of the assay solution (A@Au MPC·Zn²⁺) and just 0.8 µl of the enzyme solution. This allowed us to perform 4 readings from a single aliquot of 5 µl, which is similar to that used for the radiometric assay, maintaining a reliable detection of enzyme degradation under the experimental conditions.
synthesizer. The tyrosine kinase Src was a kind gift from Prof. A. M. Brunati at the University of Padova; PIM-1 was purchased from Sigma-Aldrich.

Src-tide (H-KVEKIGE†GTVVVYK-H). HPLC (Agilent RRHD Zorbax Eclipse Plus C18 (2.1 × 150 mm 1.8 μm), gradient: 5–95% B (A: H₂O + 0.1% HCOOH, B: CH₃CN + 0.1% HCOOH) in 5 minutes, λ = 280 nm): 5.35 min. MS (ESI+, CH₃CN + 0.1% HCOOH) m/z: 835.5 ([M + 2H]+, calcld: 835.5), 557.4 ([M + 3H]+, calcld: 557.3), 418.4 ([M + 4H]+, calcld: 418.2).

PIM-tide (H-AKRKRHPSGGPNT-A). HPLC (Phenomenex RP Jupiter 4 μm Proteo 90 Å, gradient: 5–55%B (A: H₂O + 0.1% TFA, B: CH₃CN + 0.1% TFA) in 60 minutes, λ = 226 nm): 14.95 min. MS (ESI+, CH₃CN + 0.1% HCOOH) m/z: 1628.0 ([M + H]+, calcld: 1628.5), 1651.0 ([M + Na]+, calcld: 1651.5).

Fluorescence measurements were performed on a TECAN M1000 PRO micro-plate reader using Greiner Bio-one micro-titer plates (polystyrene 96-WELL*F and 384-WELL*F). LC-MS measurements were performed on an Agilent 1290 Infinity UPLC, equipped with a diode array detector (DAD), connected to an ESI-MS detector.

Assay solution

The assay solution (50 mL) was prepared by adding the following volumes to a volumetric flask: 2 mL of HEPES buffer (pH 7.5, stock: 0.5 M, final concentration: 20 mM), 108 μL of Au MPC 1-Zn²⁺ (stock: 4.62 mM, final concentration: 10 μM – referring to [TACN·Zn²⁺]), 0.5 mL of Zn(NO₃)₂ (stock: 1 mM, final concentration: 10 μM), and 68.5 μL of A (final concentration: 3.7 μM). The solution was stored at 4 °C and could be used for at least 2 weeks without observing alteration of the signal.

Displacement studies

Displacement experiments were performed by adding consecutive amounts of a stock-solution of a competitor ([ATP] = 1.0 mM, or [ADP] = 5.6 mM) in mQ water to 3 mL of the assay solution. Each addition was followed kinetically, monitoring the emission of probe A at 493 nm.

Src-kinase assay

Calibration curve. Samples containing ATP and ADP in ratios varying from 1:0 to 0.6:0.4 were prepared at a constant concentration of 100 μM. Additionally, each sample contained MgCl₂ (10 mM), PIM-tide (0.1 mM), and HEPES (pH 7.5, 20 mM). Separate microtiter plate wells were loaded with 340 μL of the assay solution and 10.5 μL of the calibration solution. Measurements were performed by monitoring the emission of probe A at 493 nm for 30 min. Also here, signals stabilised after around 15 min.

Assay. The enzyme solution was prepared at 0 °C by mixing in the following order: mQ water (195 μL), MgCl₂ (300 mM, 10 μL), HEPES (pH 7.5, 500 mM, 12 μL), PIM-tide (10 mM, 3 μL) and PIM-1 (50 μL of a stock solution with a specific activity of ≈2.5 pmol min⁻¹ μL⁻¹). After rapid centrifugation a final volume of 300 μL was reached upon adding ATP (15 μL, 1 mM). After a second rapid centrifugation an aliquot of 30 μL was taken as “time zero point” maintaining the enzyme solution always at 0 °C. Later, the enzyme solution was put in an incubator at 37 °C. Every 5 min, 30 μL aliquots of the reaction mixture were taken out and immediately frozen in dry-ice to block the enzyme activity. The enzyme was then assayed in the same way as used for calibration, adding 21 μL to a 329 μL of the assay solution ([ATP]ₜₜₑₑₑₑ = 3.0 μM). The radiometric assay was performed using an identical reaction mixture, but in the presence of ATP [γ-³²P] (3% v/v). In this case, the kinase reaction was quenched by pouring the aliquots (5 μL) into 0.1% phosphoric acid (25 μL), which in turn were spotted on a 2 cm² piece of a Whatman P81 filtermat, washed three times for 4 minutes in 75 mM H₃PO₄, air dried, put in a vial with scintillation fluid, and assayed for ³²P in a beta-counter (Packard).

PIM1-kinase assay

Calibration curve. Samples containing ATP and ADP in ratios varying from 1:0 to 0.6:0.4 were prepared at a constant concentration of 100 μM. Additionally, each sample contained MgCl₂ (10 mM), PIM-tide (0.1 mM), and HEPES (pH 7.5, 20 mM). Separate microtiter plate wells were loaded with 329 μL of the assay solution, and 25 μL aliquots (5 μL) into 0.1% phosphoric acid (25 μL), which in turn were spotted on a 2 cm² piece of a Whatman P81 filtermat, washed three times for 4 minutes in 75 mM H₃PO₄, air dried, put in a vial with scintillation fluid, and assayed for ³²P in a beta-counter (Packard).
Acknowledgements

Financial support from the ERC (StG-239898) and the University of Padova (CPDR125182 and CPDA138148) is acknowledged.

References

23 By performing the displacement experiments exactly at the SSC rather than at 80% as in previous studies, the system is responsive also to the additions of small quantities of analyte.