Simple fluorescence-based detection of protein kinase A activity using a molecular beacon probe

Changbei Ma¹b, Xiaoyuan Lv³, Kemin Wang³, Shunxin Jin¹, Haisheng Liu¹, Kefeng Wu¹, and Weimin Zeng¹

¹State Key Laboratory of Medical Genetics & School of Life Sciences, Central South University, Changsha, China; ³State Key Laboratory of Chemo/Biosensing and Chemometrics, Hunan University, Changsha, China

ABSTRACT

Protein kinase A was detected by quantifying the amount of ATP used after a protein kinase reaction. The ATP assay was performed using the T4 DNA ligase and a molecular beacon (MB). In the presence of ATP, DNA ligase catalyzed the ligation of short DNA. The ligation product then hybridized to MB, resulting in a fluorescence enhancement of the MB. This assay was capable of determining protein kinase A in the range of 12.5–150 nM, with a detection limit of 1.25 nM. Furthermore, this assay could also be used to investigate the effect of genistein on protein kinase A. It was a universal, non-radioisotopic, and homogeneous method for assaying protein kinase A.

INTRODUCTION

Protein phosphorylation, catalyzed by protein kinases, is a post-translational modification of proteins. It plays a pivotal role in regulating signal transduction, cellular proliferation, hormone secretion, cell differentiation, gene expression, and apoptosis.¹-⁴ During phosphorylation, protein kinases catalyze the addition of γ-phosphoryl from adenosine-5'-triphosphate (ATP) to a free hydroxyl group of serine, threonine, or tyrosine in a peptide or protein substrate.⁵,⁶ It has been demonstrated that aberrant protein kinase activity and abnormal protein phosphorylation are closely associated with various human diseases, including various forms of cancer, diabetes, Alzheimer disease, restenosis, immune deficiencies, endocrinological disorders, and cardiovascular diseases.⁷-¹² It is estimated that 25% of drug development efforts is focused on protein kinase inhibitors.¹³ Therefore, developing sensitive and specific detection methods for detecting protein kinases and their potential inhibitors is important.

Conventional techniques for detecting protein kinase activity include immunoassays using phosphate-specific antibodies labeled with fluorophores,¹⁴,¹⁵ radio-isotope assays using radioactive ³²P as phosphate group donors of ATP,¹⁶ and electrochemical methods using ferrocene- or thiol-labeled ATP.¹⁷ Although these methods can effectively detect protein kinase activity, they require intricate molecule labeling procedures and radioisotopes, which might increase the cost and time consumed for detection. Moreover, the use of radioisotopes poses a threat to human health. Therefore, there is considerable interest in developing simple, sensitive, and cost-effective methods for monitoring protein kinase activity.

After they were first reported in 1996, molecular beacons (MBs), a class of molecular probes, have been widely used in chemistry and in bioanalytical and biomedical sciences for biomolecular recognition, due to their high sensitivity and excellent specificity.¹⁸,¹⁹ MBs are probes which contain a stem-loop structure, a fluorophore and a quencher at their 5' and 3' ends, whose fluorescence is restored when they bind to a target nucleic acid sequence.¹⁸ Recently, we had developed a novel technique that used DNA ligase and T4 polynucleotide kinase in real-time, by combining the advantages of MBs and DNA ligases.²⁰,²¹ In addition, a novel method for detecting biologic small molecules such as ATP and nicotinamide adenine dinucleotide (NAD) has been developed.²²,²³ In this study, we developed a simple, fast, and sensitive assay for detecting protein kinase activity using MBs, based on DNA ligation.
Material and methods

Reagents

The MB: 5’-(TAMRA)-CCTCTCGTGCCTTGTACTTCCGTCAGAGA GG-(DABCYL)-3’. Its 5’-terminal was labeled with a fluorescent group, tetramethyl rhodamine (TAMRA). DNA fragment, N1: 5’-p-TACAAGACAC-3’ (complementary to the 10 bases at the 5’ end of the MB loop for 5’-phosphorylation) and fragment N2: 5’-GACGGGAAG-3’ (complementary to the 9 bases at the 5’ end of the MB loop). All sequences were synthesized by Takara Biological Engineering Co., Ltd. Other reagents used included dithiothreitol DTT (BBI), T4 DNA ligase (Takara), BSA (Bovogen Biologicals Pty Ltd), protein kinase A (protein kinase A from bovine heart, P5511, Sigma), cAMP (adenosine 3’-5’-cyclic monophosphate sodium salt monohydrate, 6885, Sigma), ATP (Amresco), kemptide (kemptide acetate salt, K1127, Sigma), genistein (genistein, G6649, Sigma). Deionized water was obtained from a Nanopure InfinityTM ultrapure water system (Barnstead/thermolyne Corp, Dubuque, IA).

Fluorescence measurement

All fluorescence measurements were performed with a F2500 (Hitachi, Japan) fluorescence spectrophotometer, equipped with an aqueous thermostat (Amer sham), with excitation at 521 nm and emission at 578 nm. TAMRA, labeled at the 5’ end of the MB, was used as the fluorophore. To ensure high sensitivity and sufficiently high activity of protein kinases, the assay was performed at 37 °C. The excitation and emission slits were 2.5 nm and 5 nm in diameter, respectively. The initial rate of enhancement of the sample fluorescence was defined as the change in fluorescence intensity per unit time. It was calculated from the average change in fluorescence intensity within 40 s of the beginning of the reaction.

Investigation of feasibility

Solution 1 comprised 250 nM MB, 250 nM each N1 and N2, 50 mM Tris-HCl (pH 7.0), 7.5 mM MgCl2, and 0.05% BSA. Six samples were prepared: solution A was composed of solution 1 and 100 ATP nM; solution B was composed of solution 1, 100 nM ATP, and 1 µM cAMP; solution C was composed of solution 1, 100 nM ATP, 1 µM cAMP, and 1 µM kemptide; solution D was composed of solution 1, 100 nM ATP, 1 µM cAMP, and 50 nM protein kinase A; solution E was composed of solution 1, 100 nM ATP, 1 µM cAMP, 1 µM kemptide, and 50 nM protein kinase A. Solutions A, B, and C were added to 0.56 U T4 DNA ligase after the sample was stabilized; solutions D and E were added to 0.56 U T4 DNA ligase after the addition of protein kinase A. Solution F was similar to E, but after the addition of protein kinase A, it was incubated for 5 min and 0.56 U T4 DNA ligase was then added. The total volume of the 6 samples was 80 µL. To observe the changes in fluorescence intensity, the initial rate of fluorescence enhancement was recorded. The fluorescence enhancement rate is defined as a change in fluorescence intensity per unit time, and calculated according to the average of the change in fluorescence intensity within 40 seconds from the beginning of the reaction.

Establishment of ATP standard curve

After the fluorescence intensity of the solution 1 was stabilized, 0.56 U T4 DNA ligase was added using the micro sampler. When the fluorescence intensity of the solution was again stable, different trace amounts of ATP were added into the injector, and the final concentrations were measured at 10, 25, 50, 100, 150, and 200 nM. These measurements were compared with the standard ATP curve, and the concentrations were obtained according to the literature. The initial reaction rates were then calculated.

Optimization of detection conditions

To obtain the optimal results, the experimental conditions, including the concentration of Mg2+ and the reaction temperature were evaluated. The Mg2+ concentration range was 5 to 25 mM, and the reaction temperature was 25 to 40 °C.

Detection of protein kinase A activity and establishment of standard curve

Solution 2 comprised 250 nM MB, 250 nM each N1 and N2, 50 mM Tris-HCl (pH 7.0), 7.5 mM MgCl2, 0.05% BSA, 200 nM ATP, 1 µM cAMP, and 1 µM kemptide, The total volume, 80 µL, was placed in a quartz colorimetric dish, which was placed in the F-2500 fluorescence spectrophotometer. After the fluorescence intensity was stabilized, protein kinase A was
added. T4 DNA ligase (0.56 U) was then added, and fluorescence curves were recorded simultaneously.

**Effects of drugs on the activity of protein kinase A**

The changes in fluorescence intensity were monitored by adding different concentrations of genistein to solution 2. The initial reaction rate was then calculated. Under the same conditions, the effect of drugs on the activity of protein kinase A was investigated.

**Results and discussion**

**Detection principle**

The principle behind the application of MBs for monitoring protein kinase A activity is schematically shown in Figure 1. As shown in Figure 1a, protein kinase A catalyzed the phosphorylation of Ser residues to the special substrates of the Kemp peptide (Leu-Arg-Arg-Ser-Leu-Gly, kemptide). During this process, cAMP was required as a secondary factor, and an equal amount of ATP was also consumed. The activity of protein kinase A could be assayed by detecting the remaining ATP in the solution after the kinase reaction. As shown in Figure 1b, the ATP assay comprised MBs, T4 DNA ligase, and 2 short oligonucleotides (oligos). In the presence of ATP, the T4 DNA ligase catalyzed the end-joining of the 2 oligos to form a longer DNA strand, which was complimentary to the whole MB loop. As a result, the hairpin structure of the MB was disrupted to form a double-stranded DNA with the ligation product. Consequently, the fluorescence was restored.

**The feasibility of protein kinase A activity detection using a MB probe**

As shown in Figure 2, column A represents the initial rate of enhancement of fluorescence for samples, in which only ATP was added; it therefore serves as a standard for other samples for the normalized treatment. Column B represents the initial rate of enhancement of fluorescence for samples, in which cAMP was added; it was observed that the reaction velocity in this case was not as fast as that in A, due to the inhibition of DNA ligase activity by cAMP. Column C was disrupted to form a double-stranded DNA with the ligation product. Consequently, the fluorescence was restored.

![Figure 1. Schematic illustration of the protein kinase A activity assay. (a) Protein kinase A consumes ATP and catalyzes the phosphorylation of kemptide. (b) ATP detection using molecular beacons (MBs), based on DNA ligation. In the presence of ATP, the T4 DNA ligase catalyzes the ligation of 2 short oligos to form a longer oligo. This ligation product then hybridizes with the MB, thus restoring its fluorescence.](image-url)

![Figure 2. Feasibility analysis for protein kinase A activity.](image-url)
represents a lack of protein kinase A, while column D represents a lack of substrate kemptide; thus, the rate of enhancement in D is not faster than that in B, i.e., ATP was not consumed. In column E, protein kinase A was not allowed to react for some time, and so, there was no ATP reduction, and the rate was similar to that of B. The solution composition of columns E and F were same; the only difference was that in E, protein kinase A reacted 5 min after the addition of ligase. It was observed that the rate of enhancement of fluorescence was significantly lower whenever ATP was partially consumed. Thus, we demonstrated that this method could be used to detect the activity of protein kinase A.

**Establishment of ATP standard curve**

The standard curve for detecting ATP should be established in its presence. Because of the inhibitory effect of cAMP on ligase activity, a relatively large concentration of the linear range was used for the detection. This could ensure that the ATP concentration in the system was always in the linear range; in addition, it also made it possible to detect low concentrations of protein kinase A. Figure 3 shows a graph where the ATP concentration is plotted against the initial velocity of fluorescence enhancement after addition of ligase. In the range of 10–200 nM, the reaction rate was proportional to the concentration of ATP, and a linear relationship was observed. Therefore, an ATP concentration of 200 nM was selected for subsequent experiments.

![Figure 3. Standard curve of ATP detection in the presence of 1 μM cAMP.](image)

**Optimization of detection conditions**

Although Mg$^{2+}$ ions are involved in the activation and catalytic reaction of many important enzymes in living organisms, high concentrations of Mg$^{2+}$ ions may inhibit the activity of enzymes. The 2 enzymes involved in the present study (protein kinase A and T4 DNA ligase) require Mg$^{2+}$ ions for their activity. However, the optimum concentrations may be different, and so, it was necessary to investigate the effect of Mg$^{2+}$ ions on the reaction with and without protein kinase A. In Figure 4a, the solid line shows the reaction rate in the presence of protein kinase A, and the dotted line represents the reaction rate without protein kinase A. In the presence of protein kinase A, the change was not obvious. Without protein kinase A, the reaction rate was highest when the concentration of Mg$^{2+}$ ions was about 10 mM. The values in the figure are normalized based on this maximum value. Figure 4b shows a histogram for the differences at each point in Figure 4a; the greater the difference, the more suitable the corresponding concentration of Mg$^{2+}$ ions is for the system. The optimum concentration of Mg$^{2+}$ ions was selected to be 10 mM.

We also investigated the influence of temperature on the reaction velocity in the absence and presence of protein kinase A. The numerical values in Figure 5a are normalized, based on the highest value. The highest points of both the bell-shaped curves (solid and dotted lines) corresponded to 37 °C. This was likely because, while the activity of protein kinase A was enhanced, the ligase activity was even stronger, and so, it could not accurately reflect the amount of ATP consumed (being consumed by ATP). Therefore, it was necessary to differentiate between the 2, to precisely evaluate the effect of temperature. Figure 5b is a histogram showing the differences at each point of Figure 5a. The biggest difference of ΔV was 37 °C, and so, it was chosen as the experimental temperature.

**Detection of protein kinase A activity**

Figure 6a shows a real time fluorescence diagram of the reaction after ATP was consumed by different concentrations of protein kinase A for 5 min. When the concentration of protein kinase A increased, the consumption of ATP also increased, thus causing a decrease in the ATP that remained in the system and in the initial rate of enhancement of fluorescence. Figure 6b shows the relationship between the
concentration of protein kinase A and the initial velocity of the ligation reaction, obtained from catalysis with different concentrations of protein kinase A. The inset of the Figure 6b showed that the reaction velocity showed a linear correlation with the protein kinase A concentration ranging from 12.5 to 150 nM. The limit of detection of the proposed strategy was estimated to be 1.25 nM, which was lower than label-free colorimetric method, and fluorescence method. Although substrate specificity differed among the kinases, they all used ATP as the phosphate donor substrate. Therefore, determining kinase activity in terms of ATP consumption, with a homogeneous format and a short reading time, could provide a universal kinase assay for high throughput screening.

**Effect of genistein on the activity of protein kinase A**

Genistein is known to be an effective inhibitor of protein kinase A, and we investigated its effect on the

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**Figure 4.** Effect of Mg$^{2+}$ ion concentration on the reaction velocity. (a) The reaction velocity in the absence (dashed line) and presence (solid line) of protein kinase A with different concentrations of Mg$^{2+}$ ions; (b) The differences in reaction velocity at different concentrations of Mg$^{2+}$ ions.

**Figure 5.** Effect of temperature on the reaction velocity. (a) The reaction velocity in the absence (dashed line) and presence (solid line) of protein kinase A at different temperatures. (b) The differences in reaction velocity at different temperatures.
activity of protein kinase A in our system. Figure 7 shows a relationship chart between the concentration of genistein and the reaction rate, normalized with a sample of a non-drug. It was observed that, when the concentration of genistein was increased, the activity of protein kinase A decreased. This decrease in activity was about 50% when 25 mg/L of genistein was used, which is similar to the previous results. Therefore, this method could be used for screening protein kinase A inhibitors.

Conclusion

In this study, we established a new method for analyzing protein kinase activity, based on ATP consumption. This new method combines ATP detection technology with MBs, based on DNA ligation. This assay could determine protein kinase A, which was used as a model, in the range of 12.5~150 nM, with a detection limit of 1.25 nM. Our method is advantageous in that it requires no isotope labeling and is completely safe. It is versatile and can be used for detecting all protein kinases without specific antibodies. In addition, it does not require the design and synthesis of any special substrates. The whole process can be completed in less than 10 min. It is expected to be used for drug screening, and could help in studying protein kinases as drug targets.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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