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Electrochemical investigations of sarcoma-related protein kinase inhibition

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ABSTRACT

An electrochemical biosensor was developed for the determination of sarcoma (Src)-related protein kinase-catalyzed phosphorylation reactions in the presence of adenosine 5′-γ-ferrocenoyl triphosphate (Fc-ATP). The sensing platform is based on a highly specific amino acid sequence Glu-Gly-Ile-Tyr-Asp-Val-Pro (EGIYDVP), to which a Fc-PO_2 moiety can be transferred from Fc-ATP by the action of the Src kinase. The enzyme kinetics and kinase inhibition were investigated by square wave voltammetry (SWV). The kinetic parameters K_m and V_{max} were determined for Src protein kinase with respect to Fc-ATP co-substrate and were found to be 200 μM and 115 μA cm^{-2} min, for phosphorylation of the EGIYDVP peptide substrate. Furthermore, the Src-catalyzed phosphorylation of Tyr was investigated in the presence of the small molecule inhibitors PP1, PP2, SU6656, and roscovitine. PP3 does not inhibit Src activity and was used as a control. The percent inhibition at half concentration, IC_{50}, values were determined for all inhibitors under the study and were estimated to be in the 5–30 nM range. The electrochemical study suggests that the increase in inhibition efficiency was in the order PP3 < SU6656 < roscovitine < PP2 < PP1.

1. Introduction

Hundreds of protein kinases catalyze phosphorylation reactions within the cell and correspondingly regulate cell cycle and function. Over-expressed protein kinases are commonly associated with the development of diseases, including cancer, and are therefore viable targets in cancer therapy [1]. A strong correlation between cancer progression and protein kinase activity has stimulated development of kinase inhibitors, many of which have entered clinical trials, such as Gleevec®, gefitinib, desatinib and imatanib [2–5]. Despite the library of protein kinase inhibitors that are commercially available, the interest in screening and developing highly selective inhibitors is ongoing [6]. This, in turn, has created a need for analytical techniques [7] capable of probing kinase activity and inhibitor screening which include the use of radioactive [8,9] and fluorescence labels [10–19], mass spectrometry [20–22], enzyme-linked immunosorbent assay (ELISA) [23–26], surface plasmon resonance [27,28], quartz crystal microbalance [29,30], and, more recently, electrochemical [31–33] methods.

Here we report a sensitive electrochemical peptide-based biosensor for the detection of the activity of sarcoma-related protein kinase (Src) and an study of Src inhibition using a number of commercially available inhibitors. Briefly, Src is an over-expressed non-receptor protein and belongs to the Src family tyrosine kinases (SFKs). Protein tyrosine kinases (PTKs) phosphorylate the target tyrosine (Tyr) residues of proteins via transfer of a phosphate group from adenosine triphosphate to the phenolic hydroxyl group on Tyr [34]. In some cases, the Src-catalyzed phosphorylations result in the activation of an oncogenic signal transduction pathway [35,36]. For example, Src over-expression is associated with HIV disease and certain cancers, such as ovarian, breast, colon and prostate cancers [37]. The rationale for targeting Src in human disease stems from the degree of Src hyperactivity which correlates with the disease progression. Because Src activity is a good predictor of the carcinogenesis pathway, the inhibition of this protein kinase is a major target in cancer therapy. This study explores the changes in the Src kinase protein activity profile in the presence of commercially available inhibitors by electrochemical means. The Src-catalyzed phosphorylation detection was based on the use of a peptide-modified gold electrode and an adenosine 5′-γ-ferrocenoyl triphosphate (Fc-ATP) conjugate as a co-substrate. The kinase domain inhibitors under study were pyrimidine-based small molecules, pyrazolopyrimidines 4-amino-5-(4-methylphenyl)-7-(tert-butyl)parazolo [3,4-d] pyrimidine (PP1) and 4-amino-5-(4-chlorophenyl)-7-(tert-butyl)pyrazolo [3,4-d] pyridine (PP2) [38], which are the standard inhibitors for kinases belonging to the Src family of protein. As a negative control 4-amino-5-(4-phenyl)-7-(tert-butyl)pyrazolo [3,4-d] pyridine (PP3) was used which does not inhibit Src activity. Additionally, we tested the inhibitor of Src protein kinase, 2-oxo-3-(4,5,6,7-tetrahydro-1-*H*-indol-2-ylmethylene)-2,3-dihydro-1*H*-indole-5-sulfonic acid dimethylamide (SU6656) [39]. For comparison purpose, the non-specific inhibitor roscovitine [40], a well-known inhibitor of serine protein kinases, such as cyclin-dependent kinase, was investigated.

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Herein, the electrochemical peptide-based biosensor was used for the first time to extract new information regarding the activity of Src protein kinase, Src-catalyzed phosphorylation reactions and the inhibition efficacy of four small molecule inhibitors, including their IC_{50} values.

2. Experimental

2.1. General experimental conditions

All reagents and solvents were used without further purification unless otherwise specified. Src kinase was purchased from Cell Signaling and the substrate peptide EGIYDVP, seven amino acid long sequence, was purchased from BioBasic Inc. The peptide was used as received without further chemical modification. In addition to the N-terminal NH_2 group which was used for surface binding, the peptide sequence does not contain any other primary amine groups, which greatly simplifies the surface attachment. Compounds PP1, PP2, PP3 and SU6656 were received from Canadian Blood Services, while roscovitine was purchased from Sigma–Aldrich. All organic solvents were freshly distilled and the experiments in aqueous conditions were prepared using ultra-pure water (18.3 MΩ cm) from Millipore (Milli Q system). Adenosine 5′-[γ-ferrocene] triphosphate (Fc-ATP) was synthesized according to the procedure published elsewhere [32].

2.2. Preparation of the working peptide-modified gold electrode

Gold rod electrodes (99.99% purity) with surface area of 0.02 cm^2 were obtained from CHInstruments. The cleaning procedure was achieved using following steps. The gold electrodes were cleaned by polishing with slurry of 0.05 μm Al_2O_3 until a mirror finish was obtained. After 5 min of sonication in milliQ water, the gold electrodes were rinsed with water and ethanol. The electrodes were then cleaned electrochemically by cyclic voltammetry (CV) in 0.5 M H_2SO_4 between 0 and 1.2 V range. Next, the gold electrodes were incubated with 2 mM *N*-hydroxysuccinimide lipoic active ester (lipoic-NHS) ethanolic solution for 1 day at 278 K. The lipoic-NHS derivative was synthesized according to the previously reported procedure [41]. All kinase experiments were performed on the immobilized peptides on gold electrodes. The peptide immobilization was achieved as follows. For the inhibition studies, the electrodes were washed with fresh ethanol and incubated with 100 μM substrate peptide solution EGIYDVP in milliQ water for 20 h at 278 K. A surface concentration of the peptide resulting from an immobilisation with 100 μM EGIYDVP was used throughout, unless stated otherwise. For the peptide coverage experiments, the gold electrodes were incubated in a peptide solution at different working concentrations (0.5, 5, 10, 25, 50, 75, 100, 150 μM in milliQ water). Following the peptide incubation the electrodes were rinsed with milliQ water and blocked with ethanolic 100 mM ethanolamine solution (1 h) followed by ethanolic 10 mM dodecanethiol solution (20 min) for the back-filling of the remaining uncovered empty spots on the Au surface.

2.3. Preparation of the protein kinase assays for kinase-catalyzed phosphorylation reactions

Electrochemical experiments were carried out with the peptide-modified Au electrode as working electrode. The Src kinase assays were performed in the specific buffer according to the Cell Signaling protocol. The Src kinase assay buffer was based on 5 mM MOPS pH 7.5, 2.5 mM β-glycerophosphate, 1 mM EGTA, 0.4 mM EDTA, 2.5 mM $MnCl_2$ and 4 mM $MgCl_2$. The phosphorylation reaction was performed by incubating the working electrode in a total kinase reaction volume of 20 μL, based on the kinase assay buffer, in two different ways. Firstly, the control experimental conditions included the Src kinase protein (1 μg mL^{-1}) and Fc-ATP (200 μM) unless stated otherwise. Secondly, the inhibition studies were performed in the presence of Src kinase protein (1 μg mL^{-1}) and Fc-ATP (200 μM) and inhibitor under study at variable concentrations. The working inhibitor concentrations were 0, 0.2, 0.4, 0.8, 1, 2, 4, 8, 10, 20, 40, 80 and 100 nM. Inhibitors under study were PP1, PP2, SU6566 and roscovitine and they were used as solutions in dimethylsulfoxide (DMSO). The final DMSO concentration in the kinase assay was below 5% and did not affect the overall phosphorylation reaction. Initially, the inhibitor, at the tested concentration, was included in the reaction mixture with kinase assay buffer and Src protein. Following 25 min of incubation time, the phosphorylation reaction was initiated by addition of Fc-ATP (200 μM in Millipore water). After 2 h of incubation of Au electrodes in kinase assay solution at 316 K in a heating block (VWR Scientific, USA), the electrodes were washed using the kinase assay buffer prior to electrochemical measurement.

2.4. Electrochemical measurements

All square-wave voltammetry (SWV) experiments were carried out using a CHInstrument potentiostat 660B (Austin, TX). All electrochemical measurements were performed in the 0.1 sodium phosphate buffer, pH 7.4. In a typical electrochemical experimental setup a peptide-modified Au electrode was used as a working electrode, Ag/AgCl in 3 M KCl as the reference electrode, which was connected with the electrolyte via a salt bridge, and platinum wire as the counter electrode.

For SWV measurements, the potential was scanned from 0.2 to 0.6 V with a step potential of 4 mV, frequency at 15 Hz, quiet time at 2 s and a pulse amplitude of 25 mV in the same buffer. The inhibition parameters, IC_{50} values were extracted from the average current density as a function of inhibitor concentrations by using a dose response curve and a growth/sigmoidal function from OriginPro 8 software. The kinetics of the Src-catalyzed phosphorylation was evaluated using the solution Michael–Menten equation with respect to the co-substrate Fc-ATP. The parameters for the surface Src-catalyzed reaction were evaluated based in Eq. (1) as previously reported for the surface enzyme catalyzed reactions [42]:

$$V = \frac{V_{max}[E]}{K_M + [E]} \quad (1)$$

The V_0 is the steady-state current density per minute, V_{max} is the maximal current density per minute, [*E*] is the enzyme concentration and K_M is the surface Michaelis–Menten constant.

3. Results and discussion

3.1. Peptide-based biosensor for protein kinase determination

Electrochemical detection of Src protein kinase activity was based on the peptide-biosensor shown in Scheme 1. The design revolved around the immobilization of *N*-hydroxysuccinimide lipoic acid active ester (lipoic-NHS) onto Au electrodes. Subsequently, the activated lipoic-NHS was reacted with the amino terminal group of the target peptide substrate, EGIYDVP. This specific sequence was chosen due to its previous use as the appropriate substrate for other protein tyrosine kinases including Src protein kinase [43,44]. Additionally, the chosen peptide does not contain any reactive side chains, that could potentially complicate the coupling chemistry to the surface, but rather amide formation with surface bound lipoic-NHS groups goes exclusively via NH_2 group of the peptide N-terminal. Following the peptide immobilization, the peptide film was blocked and diluted with 100 mM ethanolamine and back-filled with 10 mM dodecanethiol. Next, the