Biochemistry and Biophysics Reports 1 (2015) 1–7

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

Study into the kinetic properties and surface attachment of a thermostable adenylate kinase

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ARTICLE INFO

Article history:
Received 4 February 2015
Received in revised form
20 March 2015
Accepted 23 March 2015
Available online 31 March 2015

Keywords:
Thermostable adenylate kinase
Kinetic parameters
Plasma deposition
Cleaning efficacy
Enzyme inhibition

ABSTRACT

A thermostable adenylate kinase (tAK) has been used as model protein contaminant on surfaces, so used because residual protein after high temperature wash steps can be detected at extremely low concentrations. This gives the potential for accurate, quantitative measurement of the effectiveness of different wash processes in removing protein contamination. Current methods utilise non-covalent (physisorbtion) of tAK to surfaces, but this can be relatively easily removed. In this study, the covalent binding of tAK to surfaces was studied to provide an alternative model for surface contamination. Kinetic analysis showed that the efficiency of the enzyme expressed as the catalytic rate over the Michaelis constant (k_{cat}/K_M) increased from **8.45** ± 3.04 $mM^{-1} s^{-1}$ in solution to **32.23** ± 3.20 or **24.46** ± 4.41 $mM^{-1} s^{-1}$ when the enzyme was immobilised onto polypropylene or plasma activated polypropylene respectively. Maleic anhydride plasma activated polypropylene showed potential to provide a more robust challenge for washing processes as it retained significantly higher amounts of tAK enzyme than polypropylene in simple washing experiments. Inhibition of the coupled enzyme (luciferase/luciferin) system used for the detection of adenylate kinase activity, was observed for a secondary product of the reaction. This needs to be taken into consideration when using the assay to estimate cleaning efficacy.

1. Introduction

Efficient modelling of protein attachment to surfaces has generated wide interest within the scientific community over the years, largely owed to the significant number of implications this phenomenon is responsible for. Perhaps one of the most clinically relevant implications is the residual protein contamination of surgical equipment post cleaning and disinfection. Equipment such as endoscopes and other reusable tools are subject to vigorous disinfection cycles within the clinic, however there are certain biological species which are resistant to such cleaning processes. An example of which are prions, protease resistant, transmissible glycoproteins responsible for a range of transmissible spongiform encephalopathies including Creutzfeldt-Jacob disease (CJD) [1]. Therefore evaluation of proteinaceous surface contamination has become paramount in preventing the spread of disease within the healthcare setting. This has led to specific review and revision of processes for cleaning surgical instruments in the health service in England [2]. A current clinical model aimed at quantifying residual protein presence post disinfection utilises a thermostable adenylate kinase (tAK) enzyme, so used due to the resilient nature of the enzyme drawing potential comparison to equally resilient species such as prions.

The enzyme adenylate kinase (AK) is a phosphotransferase enzyme which catalyses the reversible formation of adenosine triphosphate (ATP) and adenosine monophosphate (AMP) from adenosine diphosphate (ADP), as shown in Scheme 1.

The isolation and purification of a thermostable AK (tAK) from the archaebacterium *Sulfolobus acidocaldarius* in 1993 [3], paved the way for the significant development of proteinaceous surface contamination detection within the healthcare setting. The archaebacterium from which tAK is isolated is an extremophile found growing in volcanic springs in temperatures between 75 and 80 °C. The corresponding tAK isolated from this thermophile demonstrates a temperature optimum of around 90 °C and is stable to very low pH [4]. The novelty of tAK lies with its ability to withstand extreme conditions, tAK is currently in use in the clinical setting (WASHtAK [5]) as a model for highly surface adherent species commonly associated with residual surface contamination. The technology behind this device is detailed in a previous paper [6], which highlights that protein removal is modelled by tAK as an indicator of cleaning efficiency. Briefly,

Abbreviations: AK, Adenylate Kinase; tAK, Thermostable Adenylate Kinase; ATP, Adenosine Triphosphate; ADP, Adenosine Diphosphate; AMP, Adenosine Monophosphate; CJD, Creutzfeldt –Jacob Disease; RLU, Relative Light Units

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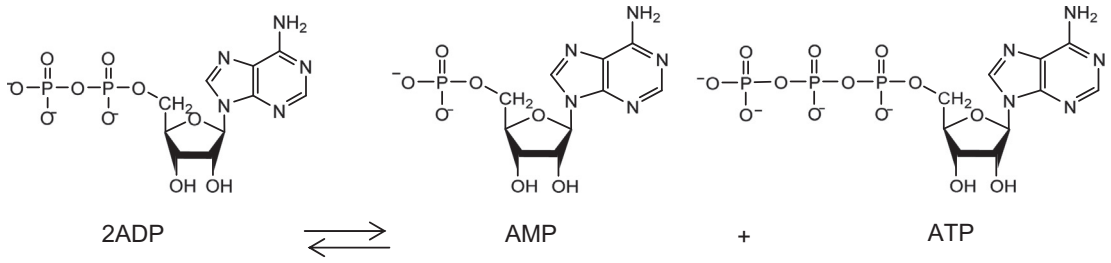
http://dx.doi.org/10.1016/j.bbrep.2015.03.011



Scheme 1. tAK catalysed ATP formation.

in this system tAK is physisorbed to a polypropylene strip and is placed into a surgical equipment washer to undergo the standard disinfection cycle that reusable surgical equipment is subject to. Post wash cycle the polypropylene strip is removed and the residual tAK is exposed to its substrate (ADP) which is then converted to ATP. Subsequently the ATP concentration is measured via a well-known coupled enzymatic reaction involving a standard preparation of luciferase and its substrate luciferin. This assay has been extensively optimised over the years in order to accurately determine ATP concentration as a function of light output [7]. Ultimately the system relies on the excitation of luciferin to a higher energy level using ATP, the decay from this excited state to the ground state then leads to the generation of light in the form of bioluminescence. The full mechanism of bioluminescence emission from the luciferase/luciferin assay has been published elsewhere [8].

Previous studies on AK isolated from *Sulfolobus acidocaldarius* (in conjunction with the luciferase method of quantification) have been largely studied at high temperatures close to the optimum. One such study conducted at 70 °C [3] reported a K_M value of 0.7 mM with ADP as the substrate in the presence of 5 mM MgCl. The study also reported a k_{cat}/K_M (catalytic capacity) value of $2.8 \times 10^5\ M^{-1}\ s^{-1}$ based on a calculated theoretical V_{max} value. The kinetic properties of this specific AK at 25 °C, which would be the case were it used for daily monitoring of cleaning efficacy, are absent in the literature both in solution and bound to surfaces. As such there is no direct comparison or evaluation of the effect on activity when the enzyme is restricted to a solid support, such as it is in its current clinical application.

There are a number of ways in which proteins can bind to surfaces, both the prevention and promotion of such a phenomenon has been vastly studied highlighting the effects of protein conformation/orientation, hydrophobicity/hydrophilicity, electrostatic nature of the support, binding temperature/pH and the structural stability of the protein in question [9,10]. The prevention of protein attachment to surfaces is of particular importance when considering the anti-fouling properties of a range of materials including, but not limited to biosensors, surgical equipment/medical devices, food containment and industrial equipment, the significance of which has been reviewed elsewhere [11].

Plasma activated materials have generated considerable interest in terms of functionalising solid supports to promote protein attachment to surfaces, and this may be useful if one wishes to accurately model the removal of tightly adhered proteins, including prions, The highly energised state of plasma has the potential to modify the outermost layer of a polymer surface by deposition of various functional groups. The successfully deposited functional groups (which can vary from amines to carboxyl groups depending on the monomer used) can then go on to react with a variety of species which would otherwise remain unreactive towards the inert polymer support. Not only does plasma deposition provide a predominantly solvent free alternative to wet chemistry, which in turn reduces the amount of chemical waste, it is also a largely 'controllable' process, meaning surface modification can be achieved homogeneously with the final composition tailored towards the intended application [12]. Numerous biomolecules have been successfully anchored onto polymer supports following plasma activation of the surface, many of which have been reviewed elsewhere [13]. Plasma activation of surfaces to improve coating has been used on a large scale in the automotive industry, and this suggests that such processes should be scaleable if required for high volume applications.

One of the main concerns when considering the possibility of utilising surface bound proteins is the apparent loss of activity when restricting biologically active species to a solid support. Random attachment (disregarding the orientation of the protein) can often result in the modification of critical resides within the protein structure which can directly affect the overall stability of the complex. This in turn can have repercussions in terms of accessibility of the protein in question, often as a result of steric hindrance affecting access to particular biologically active sites within the protein structure. One must also consider the impact of utilising potentially charged functional groups (such as those commonly found in lysine and aspartic acid residues), the masking or complete removal of such a charge can have detrimental effects in terms of protein stability [14]. An advantage of using a thermostable adenylate kinase which is largely resistant to extreme pH is the resilient nature of the protein itself, tAK is essentially a stable protein reducing its capacity to denature when anchored to a solid support, thus increasing the ways in which immobilisation can be carried out.

To date there have been no studies carried out evaluating the kinetic behaviour of tAK and the subsequent coupled luciferase system when considering surface binding. This report aims to detail the catalytic activity and surface binding properties of tAK when immobilised onto a solid support with the intention of potentially providing a more robust challenge for use in cleaning process validation and modelling.

2. Materials and methods

2.1. Materials

tAK was obtained from the Technology Development Group, Public Heath England (PHE – Salisbury UK). Its isolation, expression in *Escherichia coli* and purification has been detailed previously [6,15]. Mucin from porcine stomach was purchased from Sigma-Aldrich (Poole, Dorset, UK). ATP Reagent (luciferase/luciferin/divalent metal ions and stabilisers), Diluent C (reconstitution solution for ATP Reagent), tris-EDTA buffer, ADP and ATP standard solutions were all purchased from BioThema (Sweden).

AMP, ethanol, maleic anhydride and preformed phosphate buffered saline (PBS) tablets (pH 7.4) were purchased from Sigma-Aldrich (Poole, Dorset, UK).