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Detecting and exploring partially unfolded states of proteins using a sensor with chaperone bound to its surface

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ABSTRACT

We have developed a sensor concept capable of discriminating environments that induce proteins to enter unfolding intermediate states. Such a sensor detects the presence of environmental stressors such as chemical agents in aqueous media, thermal stress or the presence of ionizing or non-ionizing radiation by monitoring the conformation state of a "sensor protein". In this paper, we demonstrate the concept by using surface plasmon resonance to monitor binding of thermally and chemically stressed sensor proteins to a chaperone, α-crystallin, bound to the sensor surface. Citrate synthase and insulin were used as example sensor proteins to detect the presence of thermal stress and chemical stress, respectively.

It was shown that α-crystallin retained its chaperone action after immobilization on the Biacore sensor chip. The binding of early and late unfolding intermediates of citrate synthase was discriminated using the association and dissociation behaviour of the binding. The sensor is therefore capable of assessing the severity of an environmental stress.

1. Introduction

Organisms respond to the presence of chemical and physical stressors in their environment through the heat shock response. The organism detects the presence of the stressor at the cellular level when unfolding of proteins occurs. Unfolding may trigger the heat shock response in which molecular chaperones, also known as heat shock proteins are expressed. Molecular chaperones bind to the unfolding intermediate conformation states of the stressed proteins, stabilizing them and preventing them from aggregating. The stabilized proteins are then either refolded or eliminated from the cell (Das and Surewicz, 1995; Raman et al., 1995). Recently, concepts in which the heat shock response is used as an environmental sensor have been reported. The use of the expression of the heat shock protein HSP70 to monitor environments has been reviewed by Mukhopadhyay et al. (2003). In all of the concepts so far described, a whole organism has been used as the sensing element. A wide variety of organisms have been used to detect a range of stressors. Examples of environments monitored are soil, marine environments, air and fresh water. Examples of organisms used include human epithelial cells, fresh water sponges, earthworms, fish, algae, rotifers, mollusks and centipedes. Nadeau et al. (2001) have used the heat shock response in earthworms to detect the presence of heavy metals in soils for example. The use of a whole organism as a sensor has disadvantages including the need to maintain a viable population and the need to extract the heat shock protein and quantify its expression. These requirements lead to a cumbersome sensing system with a slow response time. In this paper, we propose a sensing strategy that directly measures the response of a protein to the stressor and eliminates the step in which the chaperone is expressed. Our approach is to prepare a surface with immobilized chaperone and to monitor with surface plasmon resonance the attachment of the protein acting as the environmental sensor. This concept makes the chaperone continuously available and the presence of stressed protein is detected quickly by monitoring its binding to the chaperone. The concept relies on the α-crystallin remaining functional when it is bound to a surface. Previous work (Peterson et al., 2005) suggests this is possible.

By directly monitoring the binding between the stressed protein and the chaperone, a new capability of discriminating between conformation states of a protein is added. This offers an important advantage over methods detecting the mere presence of stressors. A sensor based on discrimination of conformation states could monitor an environment for the presence of aggressive chemical or physical agents as well as for the presence of mild agents that induce only tertiary level structural changes in the sensor protein. Methods based on whole organisms may not be sensitive enough to detect changes in the level of expression of the heat shock proteins under the influence of mild stressors.

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In order to develop an environmental sensor based on chaperone binding, a convenient means is required for detecting binding of a protein to its chaperone. Chaperones in the small heat shock protein family, such as α-crystallin, bind to unfolding proteins and are believed to operate by seeking and attaching to exposed hydrophobic regions.

Citrate synthase (CS) and insulin were chosen as the sensor proteins for this work. CS was chosen because of its known sensitivity to temperature stress (Manna et al., 2001). CS is present in a wide range of organisms from bacteria and fungi to humans. It is a large (87 kDa) enzyme and is important in catalysing the formation of citrate from oxaloacetate and acetyl coenzyme A. It exists as a homo-dimer of a polypeptide chain of 48 kDa (Remington et al., 1982; Rajaraman et al., 2001). The structure of CS in the crystalline state is known and is reported in Remington et al. (1982) and Mulholland and Richards (1998).

Insulin was chosen because of its known sensitivity to chemical stress (Abgar et al., 2000). Insulin is a small protein (5.7 kDa) produced by the B-cells of the pancreas (Bucchini et al., 1989). Insulin is easily destabilized by chemical agents such as dithiothreitol (DTT) and forms large complexes (Abgar et al., 2000).

There are three steps in our proof of concept. We first demonstrate that α-crystallin retains its chaperone function while bound to a surface and secondly, we demonstrate the detection of unfolding of CS and insulin. Finally, we demonstrate the ability to discriminate between the unfolding intermediates of CS and therefore to assess the severity of the stress. To illustrate the potential of this technique as a discriminating sensor of protein conformation, we use it to probe the unfolding pathways of CS.

2. Materials and methods

2.1. Immobilizing α-crystallin on a surface plasmon resonance chip

Bovine α-crystallin was obtained from Sigma–Aldrich, USA. 1 mg/ml bovine α-crystallin in HBS buffer (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% Surfactant P20; Biacore) was diluted in 10 mM CH_3COONa pH 4.5 to a final concentration of 100 μg/ml and immobilized on a CM5 Biacore sensor chip (Biacore AB, Uppsala, Sweden) via the standard Biacore amine coupling chemistry protocol. The CM5 chip allows four separate flow cells to be operated in the Biacore 3000 instrument used in these experiments. Coupling is achieved by activating the surface of the sensor chips with equal volumes of 50 mM *N*-hydroxysuccimide (NHS)/200 mM *N*-ethyl-*N*′-(3-diethylaminopropyl)-carboiimide (EDC) (Biacore, AB, Uppsala, Sweden) to form active sites. In the Biacore protocol the binding is believed to be between carboxyl groups on the surface and amine groups on the protein. The α-crystallin solution (100 μg/ml) is then run over the chip for 6 min at a rate of 5 μl/min, where the amine groups on α-crystalline react with the activated esters. In the final stage of immobilization, the surface is blocked by ethanol amine. The α-crystallin was immobilized on the sensor chip to give an attachment signal increase of approximately 5000 resonance units (RU). The RU is a convenient arbitrary unit related to the angle shift in the minimum in reflectance due to the surface plasmon resonance. A control surface without α-crystallin was prepared by activating and blocking as described above but omitting the flow of the α-crystallin solution.

2.2. Surface plasmon resonance sensing of binding

The sensor protein, CS or insulin was used as the analyte in HBS buffer and subjected to analysis in a Biacore 3000 surface plasmon resonance detection system. The standard operating procedure consisted of an initial flow of HBS buffer over the Biacore chip to give a baseline. The association phase, in which the binding of the analyte protein occurs, is examined by the injection and flow of the analyte protein diluted in the same HBS buffer. The dissociation phase in which the analyte protein is released is examined by flow of pure HBS buffer. The change in RU during each of these flow stages was used to quantify the attachment of the analyte protein to α-crystallin during the association phase and to examine the amount of residual binding during the dissociation phase. For every experiment using a flow cell containing immobilized α-crystallin on the chip surface, another control experiment was carried out using the same procedure except that the step for immobilization of α-crystallin was omitted. In all experiments, the analyte protein was diluted in HBS buffer and was centrifuged prior to heating in the water bath or to treatment with chemical agents. Experiments were repeated at least three times for each measurement to ensure reproducibility of results. Flow rates and total volumes injected were kept the same in comparative data sets. Appropriate values of these quantities were used to allow assessment of the attachment process in both the association and dissociation phases. The values used for CS are shown in the captions to each Fig.

2.3. Investigating the effect of temperature stress

CS was used as the sensor analyte protein for these experiments. Porcine heart CS was obtained from Sigma–Aldrich and was prepared at a concentration of 100 μg/ml in HBS buffer and heated in a water bath to 42, 54 and 63 °C for 5, 10 and 15 min. The analyte was passed over the immobilized α-crystallin and over a control surface with no α-crystallin at a flow cell temperature of 25 °C.

To determine how the sensor response depends on the temperature of its surface the effect of heating the immobilized α-crystallin on its ability to bind CS was investigated. The CS solution at a concentration of 150 μg/ml in HBS buffer was run over a Biacore sensor chip held at 25 and 37 °C. The immobilized α-crystallin on the surface of the chip is expected to be at a temperature close to that of the chip because of the small flow rates of the analyte. The CS was thermally stressed by preheating in a water bath at various temperatures ranging from 42 to 66 °C for 5 min as well as to 63.5 °C for 5 and 10 min.

CS was diluted in HBS buffers to concentrations of 50, 100 and 150 μg/ml and its binding to α-crystallin and to a blank flow cell was studied at 25 °C. The binding of the same CS concentrations was studied for samples preheated in a water bath to 43 and 67 °C for 5 min.

2.4. Investigating the effect of chemical stress

DTT obtained from Sigma–Aldrich was used as a chemical agent to induce unfolding. DTT is known to attack the disulphide bond in proteins causing unfolding with the loss of tertiary structure (Azzoni et al., 2004). For these experiments, insulin was used as the analyte protein. In order to confirm that DTT binding to α-crystallin did not produce an interfering background binding, DTT was prepared at a concentration of 20 mM. A volume of 120 μl was allowed to flow on a blank flow cell that does not contain α-crystallin and on a flow cell that contains α-crystallin