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Pulse Proteolysis in Protein-Ligand Interactions

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Thermodynamic changes that occur when a protein and ligand bind may be used to better understand the nature and function of protein-ligand interactions. By establishing a method to detect thermodynamic changes, identifying proteins and the ligands in which they interact are valuable in understanding the proteins involved in cellular functions and pharmacological mechanisms. The abilities of these recent techniques have the capacity to reveal a better understanding of protein-ligand interactions in biochemical pathways, pharmaceuticals, and genetic disease. Traditional methods that investigate protein stability such as circular dichroism and fluorospectrometry often require more biophysical instrumentation and a larger amount of proteins. However, pulse proteolysis later coupled with 2D gel electrophoresis provides an acceptable method for determining protein ligand interactions that minimizes the use of instruments and required samples. Later research demonstrates that the use of chromatography and SDS PAGE rather than 2D gel electrophoresis simplifies the process while still providing accurate experimental results.

Introduction

Understanding and being able to detect the interactions between proteins and other molecules has many biological implications. Protein stability and ligand binding is an important part of the nature of protein structure and folding. Many cellular systems depend on protein ligand binding to initiate and maintain biological functions. By developing a thorough understanding of proteins stability and their ligand interactions, this scientific knowledge will have great implications for drug research and chemical genetics.

When a ligand binds to a protein, changes occur in thermodynamic properties such as the stability of the protein and change in unfolding rates. Detection of thermodynamic changes can be utilized to identify proteins that interact with other biological molecules. This review serves to identify the research and techniques that are currently being used and recently developed to detect the thermodynamic changes in protein-ligand interactions. Pulse proteolysis coupled with simple analysis techniques provides a process to discover protein stability and protein-ligand interactions. The issue presented in this manuscript is the effectiveness of the technique, protein proteolysis, in identifying protein interactions.

Recent Progress

Park & Marqusee[1] reported developing a new method, pulse proteolysis, for monitoring the stability of proteins after binding to a ligand. Pulse proteolysis only requires simple biophysical instrumentation for a small sample of proteins when compared to the more traditional methods that require more instrumentation and a larger amount of pure proteins. Pulse proteolysis directly measures the fraction of folded protein (f_{fold}) by the application of a pulse that selectively digests unfolded proteins. By determining f_{fold} directly, the global stability from urea denaturation is able to be more simply calculated than from traditional methods. The accuracy of this new technique is lies within the experimental data. Global stability values obtained from pulse proteolysis agree with the results of more traditional methods.

The experimental approach used by the researchers to determine the fraction of folded protein tested recombinant *Escheria coli* ribonuclease HI (RNase H*) and its variants 153A and 153D. These proteins were placed varying concentrations of urea in the presence and absence of thermolysin. To determine f_{fold}, the fraction of folded proteins, the amount of intact RNase H* was measured by SDS-PAGE at varying concentrations of urea by after applying a pulse that selectively digests unfolded proteins was applied. A long pulse proteolysis

digests unfolded proteins causing the fraction of folded protein to be unreliable; thus, a one minute pulse is a reasonable length to avoid producing unreliable results.

After performing experiments on RNase H* variants in urea and changing the length of the pulse (which does not substantially change C_m), the concentration of urea at which half of the protein is unfolded (C_m) was determined to be 5.10 $\pm$ 0.05 M. To quantitatively determine the global stability of a protein, $\Delta G_{unf}°$ was calculated by directly multiplying the C_m value with the denaturant dependence of $\Delta G_{unf}°$, or the *m*-value. *m*-values were taken from statistical data based on the size of a protein. The experimentally obtained $\Delta G_{unf}°$ values of RNase H* and its variants in this experiment were compared those obtained by circular dichroism, and were found to be consistent.

After quantitatively determining protein stability and establishing validity in the new technique, maltose binding protein (MBP) in the presence and absence of maltose was used to establish the ability of pulse proteolysis to monitor ligand binding. MBP was digested without maltose in 2.5 M urea containing 0.2 mg/ml thermolysin to determine the C_m which was found to be 2.78 $\pm$ 0.01 M; however, when MBP was incubated with maltose the C_m increased to 3.59 $\pm$ 0.07 M. After estimating the m-value to be –4.8 kcal/mol·M, the $\Delta G_{unf}°$ values were found to be13.3 and 17.2 kcal/mol without and with maltose.

However, it was found the MBP-maltose complex demonstrated slow unfolding which creates uncertainty in the C_m value. To address this uncertainty, MBP was incubated in 4.5 M of urea with and without maltose ligand, rather than the original 2.5 M. After one hour, MBP was fully digested while MBP with ligand again demonstrated slow unfolding. These final experimental results suggest that pulse proteolysis is an acceptable method to screen ligand binding.

An alternate method to investigating interactions between proteins and small molecules such as substrates or metabolites employ the use of biophysical assays and genetic arrays. However, these approaches are labor intensive and costly, often resulting in a low-throughput. Liu, Kihara, & Park[2] developed a target identification method that combined several different approaches to understand interactions between proteins and small molecules. When a protein forms a complex with a ligand, energetic properties of a protein change as a result of this formation. Thus, energetic-based target identification exploits these energy changes during binding to interactions between proteins.

To identify ATP binding proteins in *Escherichia coli*, Liu, Kihara, & Park[2] combined pulse proteolysis with two-dimensional (2D) gel-electrophoresis. To begin their experiment, an *E. coli* lysate was incubated for 2 hours in a buffer containing 3.0 M urea and 1.0 mM of adenosine 5'-[γ-thio]triphosphate (ATPγS). A control group without ATPγS was also incubated in 3.0 M urea for 2 hours. Once incubated, 0.2 mg/ml thermolysin was used to treat the resulting proteins for 1 minute before being analyzed by 2D gel electrophoresis.

Although each experimental group appeared to be the same, there were small differences in the intensities of spots on the gel. Twelve spots were chosen for protein identification and in-gel digestion was used to determine the identities of the 12 proteins in the higher intensity locations followed by matrix-assisted laser desorption/ionization (MALDI) tandom time-of-flight (TOF) mass spectroscopy. The identities of the proteins were entered into a genomics database to identify each proteins function. Out of the 12 proteins, there were 3 proteins that were not annotated by the database to be ATP binding while the remaining proteins were annotated ATP binding.

Three more experiments were performed by Liu, Kihara, & Park[2] on the three proteins annotated without ATP binding. These proteins include glyceradehyde-3-phosphate dehydrogenase (GADPH), dihydrolipoamide dehydrogenase (E3), and periplasmic-binding protein (mlaC). Experiments were performed separately on each protein utilizing pulse proteolysis to monitor the proteins unfolding in concentrated urea. These experiments were conducted to investigate the effect of ATP on the thermodynamic stability and individual unfolding kinetics. The results confirm that ATP does have an effect on the energetic properties of proteins.

Recent research on pulse proteolysis[1,2] demonstrates that pulse proteolysis coupled with the use of energetics is an effective tool to understand and identify protein-ligand interactions by monitoring the protein stability after ligand binding. However, Chang, Schlebach, VerHeul, & Park[3] have recently created a simpler approach to the formerly discovered energetic based methods. The newly discovered approach utilizes chromatography to fraction out proteins, application of pulse proteolysis, and analysis with SDS PAGE rather than 2D gel electrophoresis. Chang et al.[3] suggests that SDS is a simpler, more quantitative method than 2D gel electrophoresis and is more easily reproduced.

The *Escherichia coli* proteome was fractioned by Chang et al.[3] using anion exchange chromatography into fifteen 1.5 ml fractions. Each of the fifteen fractions, except for two, contain about 30 to 50 proteins and thus are capable of being monitored for ATP binding by pulse proteolysis and SDS PAGE. Each fraction was incubated with and without 1 mM of ATPγS in 0 to 4.0 M urea. After each fraction was incubated overnight, pulse proteolysis was applied to digest unfolded proteins. Once the reactions of each fraction were complete, SDS PAGE was used to analyze the stabilization of the target protein.

The proteins in the bands of the SDS that have more than a 50% change in their band intensity were identified by in-gel digestion and matrix-assisted laser desorption/ionization (MALDI) tandem time-of-flight (TOF) mass spectroscopy. The result of band analysis and protein identification revealed that there were 30 ATP-binding proteins. When these results were compared with EcoCyc genomics database and previous identification using 2D gel electrophoresis, the results were similar. The new technique found that 21 of the 30 proteins are currently known to interact with ATP while the other 9 have had not yet been discovered to have had interactions with ATP.

The fraction of known ATP binging proteins obtained by Chang et al.[3] is similar to the results obtained when 2D gel electrophoresis is used to identify protein-ligand interactions thus suggesting the reliability in use of SDS PAGE to discover protein-ligand interactions. To validate the use of this simplified approach in identifying protein-ligand interactions, phosphoglyceromutase from *E. coli* was tested. The enzyme was overexpressed, purified after cloning from *E. coli* genomic DNA, then incubated in varying concentrations of urea with and without 1.0 mM of ATP. After incubation, the enzyme sample was subjected to pulse proteolysis. The result of the proteolysis was an increase in the C_m from 1.36 M to 1.81 M. The change in C_m indicates the ATP binding of phosphoglyceromutase. The results of this experiment demonstrate the ability of this simpler approach to identify protein-ligand interactions.

Discussion

The technological development of quantitatively determining protein stability simplifies more traditional techniques that are used to monitor protein-ligand binding. The results of pulse proteolysis for RNase H* and MBP reflect results found by more traditional methods. The reflection of experimentally obtained values of global protein stability found using pulse proteolysis with those previously found using traditional techniques suggest a strong reliability of pulse proteolysis. The consistent results suggest the ability of this technique to accurately monitor protein stability and also ligand binding.

Pulse proteolysis simplifies the traditional biophysical techniques that required expensive instruments and extensive amounts of purified protein. This method simplifies traditional methods because it not only reduces the instrumentation required also allows examination of proteins in smaller amounts, even unstable proteins. The ability of pulse proteolysis to adapt to a high throughput form of ligand screening address the high throughput determination of protein stability required in protein engineering and drug discovery.[1]

Furthermore, pulse proteolysis coupled with 2D gel electrophoresis was found to be a reliable method in determining the thermodynamic properties by identifying ATP-binding properties in *Escherichia coli*. Energetics-based target identification was validated by the experimental results obtained when identifying ATP binding proteins in *E. coli* lysate. Of the 10 proteins identified has interacting with ATP, the interactions of approximately six proteins were validated with a genomic database such as EcoCyc or Gene Ontology while the remaining proteins excluding periplasmic-binding protein (mlaC) were investigated with pulse proteolysis. Pulse proteolysis confirms that these proteins are in fact ATP-binding proteins.

As a result of experimental results, the energetics based target identification using pulse proteolysis was identified to be an accurate method to identifying enzymes-substrate interactions. This method helps address some of the obstacles presented by more traditional methods. This method overcomes traditional limitations by minimizing nonspecific binding and the adverse effects of modified binding. Furthermore, energetics based target identification is able to be applied to any protein-ligand interaction. This is important in identifying interactions involving regulatory molecules, signaling molecules, inhibitors, and drugs.[2] But of even greater importance than identifying known interactions, energetics based target identification addresses the discovery of unknown interactions which is a powerful implication that has many benefits in science and medicine.

In the final study, pulse proteolysis coupled with SDS PAGE and mass spectroscopy reveal protein metabolite interactions that occur on the proteomic scale. The SDS PAGE requires less time and effort, more reproducible in experiments, easier to quantitatively compare samples than offered by 2D gel electrophoresis.[3] Furthermore, this approach allows observation of about 10% of the genes in the *E. coli* genome; however, without extensive instrumentation this coverage of the *E. coli* is reasonable.[3] The ability of this approach to detect the ATP binding ability of phosphoglyceromutase indicates the useful ness of this technique in identifying other protein-ligand interactions including those of pharmaceutical drugs. By discovery these known interactions between proteins and substrates, this approach may help recognize at the systems level unknown biochemical pathways and regulatory networks.[3]

These experimental approaches in identifying protein ligand interactions address the limitations imposed by more traditional methods. These methods often required greater biophysical instrumentation and larger amounts of purified protein samples. This new technology proposed by the researchers simplifies the instrumentation

and protein samples without sacrificing accuracy and efficiency. However, there are still improvements and questions that are not addressed. Further

The implications afforded by each of these three experimental techniques have a significant impact on our understanding of the modes of action of many molecules within our environment and bodies. At the systems level, protein interactions have immense implications which may be supportive or detrimental to the organism. Furthermore, pulse proteolysis may be a useful tool in diagnosis of genetic disease because of its ability to screen for stabilizing or destabilizing mutations. In pharmaceutical research, it is known that drugs commonly interact with several different protein targets.

A better understanding of protein binding may allow scientists to develop more highly selective molecules that improve the health of an organism in many aspects—cellular function, system homeostasis, and genetic replication. However, simple, cost-effective, and accurate techniques are continually required to address the problems that continue to evolve. However, if protein-ligand interactions are able to be accurately monitored and identified, the benefits of protein engineering and pharmaceuticals will continue to rapidly progress without bound.

References

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