An Improved Methodology for Multidimensional High-Throughput Preformulation Characterization of Protein Conformational Stability

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ABSTRACT: The empirical phase diagram (EPD) technique is a vector-based multidimensional analysis method for summarizing large data sets from a variety of biophysical techniques. It can be used to provide comprehensive preformulation characterization of a macromolecule's higher-order structural integrity and conformational stability. In its most common mode, it represents a type of stimulus–response diagram using environmental variables such as temperature, pH, and ionic strength as the stimulus, with alterations in macromolecular structure being the response. Until now, EPD analysis has not been available in a high-throughput mode because of the large number of experimental techniques and environmental stressor/stabilizer variables typically employed. A new instrument has been developed that combines circular dichroism, ultraviolet absorbance, fluorescence spectroscopy, and light scattering in a single unit with a six-position, temperature-controlled cuvette turret. Using this multifunctional instrument and a new software system, we have generated EPDs for four model proteins. Results confirm the reproducibility of the apparent phase boundaries and protein behavior within the boundaries. This new approach permits two EPDs to be generated per day using only 0.5 mg of protein per EPD. Thus, the new methodology generates reproducible EPDs in high-throughput mode and represents the next step in making such determinations more routine. © 2012 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 101:2017–2024, 2012
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INTRODUCTION

An empirical phase diagram (EPD) is a data visualization tool used to assist in comparing alterations in macromolecular states due to environmental stress. The technique consists of applying principal component analysis to a series of multidimensional biophysical measurements to find an optimal lower-dimensional representation of these large data sets and then using this representation to construct a color diagram in which multidimensional differences in measurements are easily discernible. This approach may be thought of as intermediate between that of high-resolution techniques such as X-ray crystallography and nuclear magnetic resonance, and individual biophysical analyses such as circular dichroism (CD), fluorescence spectroscopy, or static light scattering. The information content of EPDs is relatively high because of the use of stress variables to create what is effectively a stress–response diagram. Applications of EPDs have included the selection of excipient screening conditions and the determination of stabilizing solution conditions, as well as the comparison of proteins (e.g., mutants). The EPD technique has been used to assist in characterizing, stabilizing, and formulating proteins, viruses, virus-like

Additional Supporting Information may be found in the online version of this article. Supporting Information

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particles, and whole bacterial cells as pharmaceutical dosage forms for use as therapeutic drugs and vaccines.[1,2]

The time and effort required to generate an EPD for individual proteins have, however, somewhat limited the method's general applicability. It typically takes several days or up to 1–2 weeks to collect individual data sets with multiple biophysical techniques, process the large combined data set, and generate an EPD. Reducing the total time to a day or less would allow EPDs to be more routinely used during formulation development as a tool for excipient screening and to enable more reliable comparisons of the stability of higher-order macromolecular states.

Multimode instruments have recently become available that could significantly reduce the time to collect the experimental data used to generate an EPD. The Olis MultiScan (also referred to as "the protein machine"; Olis, Bogart, GA) is a cuvette-based spectrophotometer that measures CD, ultraviolet (UV) absorbance, fluorescence, turbidity, and light scattering with high-level photometric and wavelength accuracy and repeatability. In contrast to most plate readers, this instrument measures full spectra, in addition to incorporating a temperature-controlled sample chamber capable of performing temperature studies from 0°C to 100°C.

To automate data analysis, we have developed a software tool called the Declarative Array Transformer (DART; manuscript in preparation). In essence, this tool allows one to perform general mathematical, graphical, and file operations on data arrays without concern over the propagation and use of array axis information. DART is used to write data processing scripts in a declarative programing style, meaning that control flow operations such as looping and if/then statements are not necessary for most tasks. Scripts written in DART are self-explanatory and easily modified by nonexpert programers. Additionally, reproducibility of experiments and traceability of data are increased by saving data processing scripts along with their output data.

In this manuscript, we describe for the first time the Olis MultiScan and its use to generate EPDs for four model proteins, including aldolase, bovine serum albumin (BSA), α-chymotrypsin, and lysozyme. These proteins cover a range of molecular weights (∼14–160 kDa), secondary structures (∼10%–67% alpha-helical and ∼10%–49% beta-sheet), and thermal stabilities (transition temperatures from ∼44°C to 74°C).[3–8] These model proteins were characterized over a grid of environmental conditions consisting of solution pH values from 3 to 8 and temperatures from 10°C to 85°C. The characterization of each protein was performed over a 12-h period. At each combination of temperature and pH, the following biophysical measurements were taken: CD at 217, 222, and 235 nm; absorbance from 238 to 343 nm [including optical density (OD) measurements from 320 to 340 nm]; and intrinsic tryptophan (Trp) fluorescence between 255 and 420 nm with 295 nm excitation. DART was then used to import and regularize the data, filter it, and generate EPDs. The resulting EPDs have been interpreted in light of the original raw data, and the phase boundaries and protein behavior were found to be reproducible and similar to those obtained by independent measurements using separate instruments.

MATERIALS AND METHODS

Materials

Albumin (from bovine serum), aldolase (from rabbit muscle), α-chymotrypsin (from bovine pancreas), and lysozyme (from chicken egg white) were obtained in the form of lyophilized powder from Sigma Life Sciences (St. Louis, Missouri). All chemicals were of reagent grade and purchased from Fisher Scientific (Pittsburg, Pennsylvania).

Citrate–phosphate buffer was prepared at 20 mM at pH 3, 4, 5, 6, 7, and 8 from citric acid anhydrous and sodium phosphate dibasic anhydrous. The ionic strength of each buffer was controlled to $I = 0.15$ (dimensionless) by the addition of NaCl. For each pH, the lyophilized protein samples were dissolved into 2 mL of H_2O, and all protein solutions were dialyzed into between 1 and 2 L of citrate–phosphate buffer in Thermo Scientific Slide-a-Lyzer 0.5–3 mL, 3500 Da molecular weight cutoff dialysis cassettes (Thermo Scientific, Waltham, Massachusetts). The concentration of each sample was obtained by absorbance spectroscopy with 1 cm path length at 280 nm using an Agilent Technologies 8453 spectrophotometer (Agilent Technologies, Santa Clara, California) and known extinction coefficients for each model protein. Samples were diluted to 0.2 mg/mL. The citrate–phosphate buffers used for protein dilution and as instrument controls were filtered with a Millipore Millex 0.45-μm syringe filter (Millipore, Billerica, Massachusetts). Samples were stored at 4°C, and measurements were taken within 2 weeks of reconstituting the lyophilized protein powders with the citrate–phosphate buffers (except for lysozyme, which was used within 3 weeks).

Methods

High-Throughput Spectroscopy

High-throughput spectroscopy was performed with the Olis MultiScan (Olis) equipped with a Quantum Northwest peltier temperature controlled 6-position cuvette turret (Quantum Northwest, Inc., Liberty Lake, WA). The Olis MultiScan uses a 150 W xenon arc lamp and dual-grating rapid scanning monochromators (RSM-1000) for CD and fluorescence

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