Solution Behavior of IFN-β-1a: An Empirical Phase Diagram Based Approach

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ABSTRACT: An empirical phase diagram approach has been developed as a practical tool to aid macromolecular preformulation/formulation studies. This method employs an eigenvector based procedure to visualize and interpret complex data sets. Human Inteferon-β-1a, an important therapeutic protein, was used to further develop the method and test its utility. The protein was characterized in solution as a function of pH (2–8), temperature (10°C–85°C) and ionic strength ($I = 0.1$ and 1.0) using intrinsic and ANS fluorescence, Far-UV circular dichroism (Far-UV CD), Fourier Transform Infrared spectroscopy (FTIR) and derivative UV absorbance spectroscopies, as well as differential scanning calorimetry (DSC) to supplement spectroscopic thermal stability studies. Derivative UV absorbance data were initially used to construct a pH-temperature phase diagram at each ionic strength. Three distinctive phases at $I = 0.1$ and two major phases at $I = 1.0$ were identified corresponding to different conformation/aggregation states of the protein. For the first time, heterogeneous data sets (i.e., data from different techniques) including Far-UV CD, fluorescence and UV absorbance results were used to generate empirical phase diagrams. Results from different data sets are compared; precautions in applying the method and its overall utility are discussed. J Pharm Sci 94:1893–1911, 2005

Keywords: circular dichoism; fluorescence spectroscopy; FTIR; calorimetry (DSC); UV/Vis spectroscopy; protein formulation; protein aggregation; stability; empirical phase diagram

INTRODUCTION

A major challenge in protein drug development is the creation of a marketable formulation, which manifests sufficient chemical, physical, and biological stability to produce a shelf life of 24–36 months. Achieving this goal can be quite difficult due to the complexity of protein molecules, which includes their large size and their multiple levels of structure (i.e., secondary, tertiary, and quaternary) as well as their intrinsic susceptibility to a variety of degradation procedures. Traditionally, protein formulation studies have been carried out in a semi-empirical manner. For example, a limited number of methods such as HPLC and DSC are employed in an accelerated stability format under a small number of solution conditions. A number of excipients are then added to counter any observed instability. The extent of such studies is often less than ideal due to the aggressive nature of modern drug developmental timelines. The result is often suboptimal formulations, although this approach has, obviously, led to many commericially successful products. In some cases, however, this minimal formulation strategy has not been successful

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and led to product failures during development. Thus, it would seem that more intensive studies are desirable, especially if unexpected problems occur in later development stages with no detailed structural information available.

Recently, there have been attempts to develop more rapid, rational approaches to protein formulation. A wider variety of analytical methods are first employed to fully examine different aspects of a protein's structure under a range of solution conditions. The totality of such information could then provide a rigorous basis for further formulation development. The large amount of data generated, however, can be difficult to interpret due to their diversity and complexity. Thus, novel data analysis methods become necessary to find patterns among the data sets and correlate them to defined physical states of target proteins. An empirical phase diagram approach developed in our laboratory has been employed to this end. Data obtained with several techniques can empirically be combined and processed through a series of mathematical procedures to construct an intuitive visual picture, which facilitates visualization and interpretation of complex data sets. This method was previously applied to the structural characterization of Bovine Granulocyte Colony Stimulating Factor (bGCSF), using just the multiple peaks of 2nd derivative UV absorption spectral data to construct such an empirical phase diagram.[1,2]

To further develop this phase diagram method and test its overall utility as a practical tool for use in preformulation/formulation applications, we now apply the method to human Interferon-β-1a (AVONEX®, IFN-β-1a), an approved protein therapeutic for treatment of multiple sclerosis.[3] IFNs are believed to participate in various cellular pathways involving antiviral, antiproliferative, antiinfective and immunomodulating activities.[4,5] IFN-β is a 166 amino acid glycoprotein with a 4-helix bundle domain as its main structure component.[6] In this work, IFN-β is characterized as a function of pH (2–8), temperature (10°C–85°C) and ionic strength ($I = 0.1$ and 1.0). Far-UV CD and FTIR are used to evaluate the secondary structure of IFN-β. Tertiary structure is analyzed by intrinsic fluorescence, derivative UV absorption spectroscopy and ANS fluorescence. DSC measurements are employed to supplement spectroscopic thermal stability studies. Fourth derivative UV absorbance data are initially used to construct two empirical phase diagrams in a two dimensional space of pH and temperature at each ionic strength as described previously for bGCSF. Three distinctive phases are observed at $I = 0.1$ and two primary phases are seen at $I = 1.0$. To explore more subtle structural transitions not detected by the absorbance data, CD, intrinsic and ANS fluorescence data are for the first time used to construct phase diagrams. Complete data sets including data from all four techniques are also used to construct another two phase diagram. Results from different data sources are compared with each other and the unity of the phase diagram approach is considered in terms of the applicability of the various data sets.

EXPERIMENTAL

Materials

Recombinant human interferon-β-1a of greater than or equal to 98% purity was provided by Biogen Idec (Cambridge, MA). 8-anilino-1-naphthalene-sulfonic acid (ANS) was obtained from Acros Oganics (Morris Plains, NJ). All other chemicals were purchased from Fisher Scientific (Pittsburgh, PA).

Protein preparation

IFN-β was frozen and stored at −70°C in pH 7.2, 200 mM NaCl and 100 mM sodium phosphate buffer at a concentration of 0.27 mg/ml. A Millipore Ultrafiltration system was used to make concentrated stock solutions greater than 1 mg/ml. Most biophysical measurements were performed at seven pH values (pH 2–8, at one unit intervals) and two ionic strength conditions ($I = 0.1$, 10 mM phosphate and 10 mM citrate; $I = 1.0$, 100 mM phosphate and 100 mM citrate; NaCl was used to obtain the final desired ionic strength). Protein solutions under specific pH and ionic strength conditions were prepared by dialyzing concentrated stock solutions against the corresponding buffers for at least 16 h. The dialyzed solutions were diluted to desired concentrations and the final pH was verified. At $I = 1.0$, pH 2–4, precipitation of IFN-β occurred during dialysis. Therefore, the protein concentration was not high enough to obtain high resolution DSC and FTIR results. Trace quantities of aggregated protein (formed during freeze-thaw or ultrafiltration procedures) were removed by filtering through a 0.45 μm low protein-binding filter (Millipore, Bedford, MA) or by centrifugation at 10000*g* for 2 min at 4°C before concentration