RESEARCH ARTICLE

Protein Covalent Dimer Formation Induced by Reversed-Phase HPLC Conditions

XIAN HUANG, JAMES BARNARD, THOMAS M. SPITZNAGEL, RAJESH KRISHNAMURTHY

Human Genome Sciences, A GlaxoSmithKline Company, Rockville, Maryland

Received 9 September 2012; revised 15 November 2012; accepted 5 December 2012

Published online in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jps.23431

ABSTRACT: Reversed-phase high-performance liquid chromatography (RP-HPLC), which is routinely used to detect and quantitate levels of protein oxidation, was used to analyze a free cysteine-containing protein. However, the RP-HPLC method appeared to induce dimerization of the oxidized protein. The purpose of this study was to understand the role of RP-HPLC conditions in inducing protein dimerization. Samples were also analyzed by orthogonal size-based analytical methods such as size-exclusion high-performance liquid chromatography and sodium dodecyl sulfate polyacrylamide gel electrophoresis. These methods indicated the presence of dimer and confirmed that the acidic solvent conditions induced the dimer formation of the oxidized protein. Furthermore, the dimerization was observed only when the protein was mildly oxidized and not when the protein was severely oxidized or in its native form. The sulfenic acid form of cysteine is a likely precursor to the disulfide formation. The amount of dimers increased with increasing concentration of trifluoroacetic acid (TFA) or formic acid is in the range of 0%–0.3%. The effect of the organic solvent was less than the effect of TFA/formic acid on dimer formation. Given that RP-HPLC is typically run with low-pH mobile phase containing an ion-pairing acid for improved resolution, its potential for inducing artifacts needs to be taken into consideration during method development. © 2012 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci

Keywords: HPLC; reversed-phase; protein aggregation (dimerization); oxidation; liquid chromatography; analytical biochemistry; cysteine oxidation; cysteine sulfenic acid

INTRODUCTION

In the development of well-characterized protein or polypeptide therapeutics, reversed-phase high-performance liquid chromatography (RP-HPLC) serves as an important analytical tool for formulation studies and stability indication.[1,2] For example, RP-HPLC has been routinely used to detect and quantitate the amount of oxidized protein at release and upon storage.[3,4] RP-HPLC methods typically use silica-based alkyl stationary phases and organo-aqueous mobile phases containing trifluoroacetic acid (TFA) for gradient elution. To demonstrate or verify the capabilities of a RP-HPLC method, proteins are often oxidized using an oxidant such as hydrogen peroxide, *t*-butylperoxide, or *t*-butyl hydroperoxide prior to the sample injection.[5–9] Analysis of these samples provides an indication of the number of oxidized forms, the ability of the method to resolve these species, rate of oxidation, and extent of oxidation. The site of oxidation is usually identified by analyzing fractions collected from the chromatographic profile followed by enzymatic digestion and peptide mapping.[10,11]

Methionine residues are often the most susceptible to oxidation.[5,8,12–20] The methionine-oxidized protein is separated from its native form presenting a peak that elutes before the main peak in a typical RP-HPLC chromatogram because of the reduction of protein-surface hydrophobicity.[21] On the contrary, if a protein contains a free (unpaired) cysteine (not cystine), the protein oxidation should involve cysteine oxidation because a cysteine thiol is more susceptible to oxidation than the methionine residue.[16,22–29]

Correspondence to: Rajesh Krishnamurthy (Telephone: +301-990-4808; Fax: +301-990-4801; E-mail: rkrishnamurthy@zyngenia.com)

Xian Huang's present address is CMC Biologics, Inc., Bothell, Washington 98021.

James Barnard's present address is Department of Pharmaceutical Sciences, University of Colorado, Aurora, Colorado 80045.

Rajesh Krishnamurthy's present address is Zyngenia, Inc., Gaithersburg, Maryland 20878.

Journal of Pharmaceutical Sciences

The recombinant protein we utilized in this work was albinterferon α-2b, which contained a free cysteine (Cys34) and 11 methionines.[30–32] In our typical RP-HPLC chromatogram of oxidized protein, we observed not only the minor front peak representing the methionine-oxidized form, but also a small back peak, which was identified as a covalent dimer form. Surprisingly, the dimerization was not found in the oxidized sample prior to RP-HPLC, but induced by the RP-HPLC method. The purpose of this study is to confirm and understand the role of RP-HPLC conditions that induced dimerization as well as the possible mechanism of dimer formation.

MATERIALS AND METHODS

Materials

The recombinant protein utilized in our work was albinterferon α-2b, which is formed by genetically fusing human serum albumin to interferon α-2b. The fusion protein, which has a longer half-life compared with interferon α-2b, has a molecular weight of 88kDa with a Cys34, 19 disulfide bonds, 11 methionines and was produced in Human Genome Sciences, Inc. (Rockville, Maryland), at 5.5 mg/mL in formulation buffer (pH 7.2). The 70% (w/w) *t*-butyl peroxide and 30% (w/w) H_2O_2 were from Sigma (St. Louis, Missouri), trifluoroacetic acid (TFA) was from Fisher (Pittsburgh, Pennsylvania), and formic acid was from Fluka (Gillingham, Kent, UK). Other reagents were from J.T. Baker (Phillipsburg, NJ).

Dialysis

Two types of dialysis devices from Pierce (Rockford, Illinois) were used for the buffer/solvent exchanges: (1) Slide-A-Lyzer® dialysis cassettes, 3500 molecular weight cut-off (MWCO) for 3–12mL sample volume and (2) Slide-A-Lyzer® MINI dialysis units, 3500 MWCO for sample volume 10–100 μL.

Oxidation

Albinterferon α-2b was oxidized by the following two methods in parallel: (1) A certain amount of protein (e.g., 5 mL of 5.5 mg/mL protein in formulation buffer at pH 7.2) was treated with *t*-butyl peroxide at an initial concentration of 0.03% (w/v) at 5°C for 24 h and (2) 5 mL of the same protein solution was treated with hydrogen peroxide at an initial concentration of 0.003% (w/v) at 5°C for 1h. The choice of oxidants and concentration were based on conditions used in forced oxidation studies with other proteins. Each sample was then dialyzed with a Slide-A-Lyzer® dialysis cassette (3500 MWCO) against two 500 mL formulation buffer exchanges to replace the oxidizing buffer and to stop the forced oxidation. Oxidized samples were stored in 500μL aliquots at −80°C until use. The samples prepared by the second (hydrogen peroxide) method were used only for the oxidation pathway study.

Incubation in Acidic Solvents

To simulate the RP-HPLC mobile phase conditions and to investigate their effect on albinterferon α-2b dimerization, solvents containing 0, 20, 40, or 60% (v/v) acetonitrile (ACN) in water and 0.05, 0.1, 0.15, or 0.3% (v/v) TFA or formic acid were prepared. Each oxidized or untreated (control) sample (100μL) was dialyzed against the different solvents for 45 minutes typically at room temperature (about 25°C). Then, each sample (in an incubating solvent) was dialyzed against (back to) formulation buffer prior to size-exclusion high-performance liquid chromatography (SE-HPLC) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. For each incubated sample, the RP-HPLC was also performed to detect the dimer content.

Size-Exclusion High-Performance Liquid Chromatography

SE-HPLC was performed on Waters 2690 Alliance HPLC system (with Waters 2996 photodiode array (PDA) detector) using a Tosohaas G3000SWXL column (7.8 × 300 mm; Tosoh, Grove City, Ohio) at room temperature. Isocratic elution of the buffer containing 104 mM sodium phosphate and 100mM sodium sulfate (pH 6.7) was carried out at a flow rate of 1.0 mL/min for 20 min. Detection was performed at UV 280 nm.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Novex 4%–12% bis–*tris* precast polyacrylamide gels and relevant reagent kits from Invitrogen (Carlsbad, California) were used to perform SDS-PAGE. Unless otherwise noted, the Novex precast gel electrophoresis guide was used to design the sample preparation and SDS-PAGE experiments. Both reduced and nonreduced protein samples were prepared using the NuPAGE reagent kit. Dithiothreitol in the reagent kit was used for the disulfide reduction. Reduced samples were prepared using a 25:20:5 ratio of the NuPAGE lithium dodecyl sulfide sample buffer–test sample–NuPAGE 10× reducing agent. Nonreduced samples were prepared using a 25:20:5 ratio of NuPAGE lithium dodecyl sulfide sample buffer–test sample–water for injection. Test sample concentration was adjusted to allow for a 4 μg load at a 25 μL load volume. Both nonreduced and reduced samples were heated at 95°C for 2min. SDS-PAGE was performed at a constant voltage of 200V for 35min. The resulting gels were stained for 1h with Invitrogen Simply Blue, followed by 2h destaining in deionized water. Densitometry was performed using a