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Unfolding of IgG domains detected by non-reducing SDS-PAGE

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ABSTRACT

Monoclonal antibodies are very important in modern therapeutics and constitute a substantial percentage of newly approved drugs. Every therapeutic monoclonal antibody must be analyzed for structural and functional integrity, and all protein heterogeneities need to be identified and quantified. The conformational stabilities of the monoclonal antibodies are also important for antibody storage and handling stabilities. One of the first and simplest of the structural analysis techniques utilized is SDS-PAGE, which can be performed both with and without prior reduction to break disulfide bonds. This permits sizing of both heavy and light chains in the reduced condition, and sizing of the intact antibody and any disulfide aggregates in the non-reduced condition. Analyzing our human anti-cocaine monoclonal antibody, we noted unexpectedly larger apparent molecular weights and apparent protein size heterogeneities using non-reducing SDS-PAGE. These apparent molecular weight heterogeneities are not consistent with other analysis techniques. Heterogeneities were noted using several heating and pre-electrophoretic sample preparation protocols, and are modified by the inclusion of small concentrations of certain alcohols such as propanol and butanol. All of these unexpected results were also observed for a commercial human IgG_1 antibody, suggesting that these observations are applicable to IgGs in general. Thus, careful attention must be paid to the interpretation of non-reducing SDS-PAGE results for IgGs. It is hypothesized that differential thermal unfolding of the Fab, CH2 and CH3 domains of the IgGs in SDS give rise to the stable, discrete bands observed using different heating protocols prior to non-reducing SDS-PAGE.

1. Introduction

Monoclonal antibodies (mAbs) are important therapeutic agents, and both monoclonal and polyclonal antibodies are widely used in basic and translational research. However, it has been reported that a large percentage of commercially available antibodies for basic research are unable to accurately and selectively recognize their intended targets [1,2], and there are substantial ongoing efforts to systematically validate antibodies for their intended usages and targets. There are very well-developed protocols and methodologies used to characterize protein therapeutics, e.g., monoclonal antibodies, as well as fragments and derivatives of monoclonal and polyclonal antibodies. Our laboratory has developed a humanized mAb (h2E2) that has high affinity for cocaine and selectivity for cocaine over its inactive metabolites [3], which is intended for treatment of cocaine addiction. This recombinant mAb protein can be produced in gram quantities in Chinese hamster ovary (CHO) cells [4].

SDS-PAGE is one of the simplest, least expensive, and most commonly used techniques to analyze antibodies for purity. Reduced samples of the IgG class of antibodies give rise to glycosylated heavy chains of approximately 50 kDa and light chains of approximately 25 kDa on SDS-PAGE. When analyzed without reduction of disulfide bonds, these antibodies should give rise to a single band on SDS-PAGE, i.e., the intact antibody consisting of 2 heavy and 2 light chains, with a combined size of approximately 150 kDa.

In this work, we note apparent overestimated molecular weights and size heterogeneities for a recombinant humanized monoclonal anti-cocaine mAb (h2E2), as evidenced by multiple bands on non-reducing gels, some of which migrate with artificially low electrophoretic mobilities (high apparent molecular weights). These anomalies are dependent on the sample pre-electrophoretic heating temperature and duration. In addition, we noted that under some conditions, both the apparent band heterogeneity and the

Abbreviations: mAb, monoclonal antibody; h2E2, humanized anti-cocaine monoclonal antibody; CHO, Chinese Hamster Ovary.

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anomalously high molecular weights observed are modulated by the inclusion of a small concentration of certain short chain alcohols (e.g., propanol and butanol) in the sample buffer. These unexpected effects of heating and the addition of alcohols were similar for both the humanized h2E2 monoclonal antibody and a commercial human IgG_1 polyclonal antibody, suggesting that these properties may be shared by many polyclonal and monoclonal antibodies in use for both research and clinical applications.

It is well known that, in the absence of SDS, IgGs have structural domains that unfold relatively independently, and exhibit differential thermal stabilities, as measured by the multiple transitions and melting temperatures observed for the native proteins in non-denaturing buffers, as assessed by differential scanning calorimetry (DSC [5–7]). In this study, we hypothesize that the observed changes in electrophoretic mobilities and apparent size heterogeneities of these IgG antibodies in non-reducing SDS-PAGE are due to differential unfolding of the Fab, CH2, and CH3 domains of the immunoglobulins in the SDS sample buffer prior to non-reducing electrophoresis. Using such analyses could add to the existing therapeutic antibody characterization techniques, as well as possibly represent a simple means to analyze the effects of mutations on the stability of IgG protein domains in therapeutic antibodies.

2. Materials and methods

2.1. Materials

The generation and production of the h2E2 anti-cocaine monoclonal antibody was previously described [8]. The recombinant h2E2 mAb was purified by protein A affinity chromatography and used as supplied by the manufacturer, Catalent. The purity, structure, and function of the resultant mAb protein has been well characterized in our laboratory [4,9–11]. The human polyclonal IgG_1 protein used for comparative purposes was purchased from Sigma-Aldrich (catalogue #I5029, batch # SLBV3436, reported to be 96% pure by capillary electrophoresis, 1.06 mg/ml as supplied). Acrylamide, bisacrylamide, and all reagents used to pour, run, and stain SDS-PAGE gels were from BioRad. HPLC grade methanol, ethanol, 1-propanol, 2-propanol (isopropanol) and 1-butanol (butanol) were from Fisher. Pre-stained SDS-PAGE molecular weight standards (10–240 kDa) were purchased from SMOBIO Technology, Inc., catalogue # PM1700.

2.2. Methods

The 7% SDS-PAGE gels were poured, run, and stained with Coomassie Blue, basically as described by Laemmli [12]. Antibody samples were usually diluted to a final protein concentration of 0.2 mg/mL in non-reducing sample buffers (containing 5% SDS, and usually containing 25% glycerol to provide the needed density for loading, but sometimes replacing the glycerol with 8 M urea). Identical results were obtained loading 2 μg IgG per well, after pre-electrophoretic heating of 0.1–1.0 mg/ml IgG concentrations in SDS sample buffer, indicating that the results are protein concentration-independent and not due to overloading of the gels (data not shown). Some samples also contained low concentrations (1–10%) of various short chain alcohols (methanol, ethanol, 1- and 2-propanol, or 1-butanol). After incubation at various temperatures for varying times (boiled (100 °C) samples were always incubated in boiling water for 5 min) prior to electrophoresis, typically 10 μL (2 μg) of each sample was loaded into individual wells of 15 well, 1.5 mm thick 7% acrylamide gels. Experiments were designed so that comparisons of conditions and effects on electrophoretic mobility were done on the same gel. All gels were used at least 24 h after they were poured (polymerized), and all gel sample wells were washed 3 times with Laemmli running buffer, prior to loading samples, to eliminate possible artefacts due to reaction of the protein samples with any residual unpolymerized acrylamide or residual free radicals. Electrophoresis was performed until the bromophenol blue tracking dye reached the bottom of the gel. Pre-stained molecular weight standards (10 μL) were also run on each gel. Gels were stained at room temperature for 30–45 min with Coomassie blue, and destained for 4–20 h prior to photography. Each panel in each figure in this study depicts a single SDS-PAGE gel, to enable direct comparisons of bands. The vertical dashed lines in some figures were overlaid on the gel image to aid in alignment of lanes between panels, as well as to facilitate reader understanding and interpretation of the gels, and do not represent lanes from different gels that were aligned following cutting and pasting from different photographic images.

3. Results

We previously noted that when different laboratory personnel analyzed the h2E2 anti-cocaine mAb under development in our laboratory as a potential therapeutic for cocaine addiction, varying molecular weights were obtained using non-reducing SDS-PAGE. Specifically, larger than expected molecular weight(s) and size heterogeneity were often observed, which were not consistent with other analyses done on the same mAb. Therefore, a systematic analysis of the pre-electrophoresis heating and treatment of the mAb sample was initiated. Possible sources of artefacts due to reaction of the sample with incompletely polymerized acrylamide remaining in the gel and the gel wells and heating-induced inter-antibody disulfide formation were eliminated by using fully polymerized "aged" gels, by washing the sample gel wells with running buffer prior to sample loading, and by including a sulfhydryl-reactive alkylating agent (*N*-ethylmaleimide) in the non-reducing sample buffer. None of these treatments eliminated the size or size heterogeneity artefacts. We then evaluated various heating protocols and the use of water soluble and miscible short chain alcohols to determine if they were able to eliminate the presumed non-covalent aggregates or the possible incomplete denaturation in SDS, which might explain the unexpected results.

Fewer apparent size heterogeneities were evident when not heating (22 °C) or only gently heating (37 °C) the samples. Also shown in Fig. 1 is the effect of pre-incubation for 10 min with 5% (vol/vol) of five short chain alcohols on the electrophoretic banding patterns of both h2E2 mAb and an IgG_1 polyclonal antibody. As is evident from Fig. 1, there are few anomalies seen with either the mAb (top Panel A) or the IgG1 (bottom Panel B) without heating (22 °C). However, at 37 °C a doublet is seen for the mAb, and multiple bands are noted for the polyclonal IgG_1 without any added alcohol. Addition of some alcohols (1- and 2-propanol, as well as 1-butanol) eliminated this apparent size heterogeneity, and also reduced the apparent molecular weights. The actual molecular weight of the h2E2 mAb used in this study has been accurately determined by mass spectral analysis of the Fab and Fc fragments [7]: the total Mw = 145,036 = (45,868 X 2) (due to 2 Fab fragments) + 53,300 (due to the Fc fragment, having the most abundant (G_{0F}) glycoform). In addition, mass spectral analysis of the intact mAb was performed, demonstrating a mass spectral peak from just above 145 kDa to just below 146 kDa, with a maximum at 145,412 Da (unpublished results). Thus, the small degree (approximately 1 kDa) of actual size heterogeneities revealed by mass spectral analyses are not consistent with the large changes in electrophoretic mobilities (apparent molecular weights) for this mAb, which are observed on non-reducing SDS-PAGE (e.g., Figs. 2 and 3).

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