
Protein Aggregation Mediated by Cysteine Oxidation During the Stacking Phase of Discontinuous Buffer SDS-PAGE

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ABSTRACT

The resolution of complex protein mixtures by discontinuous buffer SDS-PAGE is accomplished by their concentration into thin bands in the stacking gel, followed by their separation during migration through the resolving gel. Recombinant human interferon-inducible protein-10 (IP-10), a 10-kDa C-X-C chemokine with four cysteines, aggregated during the stacking phase of SDS-PAGE and generated a band with an apparent molecular mass of 18 kDa. This aggregation depended on the presence of reduced sulfhydryl residues on IP-10, on the amount of loaded protein, and on the concentration of the ammonium persulfate used to polymerize the stacking gel. The aggregation of IP-10 could be prevented by reduction of its sulfhydryls with dithiothreitol followed by irreversible blockade with iodoacetamide. These methods may be useful in the prevention of aggregation of sulfhydryl-containing proteins during SDS-PAGE, especially when large quantities are analyzed to assess their purity.

INTRODUCTION

The pioneering work of Laemmli (11) has made discontinuous buffer SDS-PAGE indispensable for protein analysis. The superior resolution of discontinuous buffer SDS-PAGE depends on the concentration of proteins in sharp bands away from the bulk of the SDS in the stacking gel, followed by their resolution during migration through the separating gel. The introduction of a discontinuous buffer system with Tricine (18) has further extended the range of resolution SDS-PAGE, by improving the separation of proteins smaller than 20 kDa.
We have extensively used the Tricine system for studying the biosynthesis and purification of human interferon-inducible protein-10 (IP-10), a 10-kDa chemokine with four conserved cysteines (15). The chemokine family was so named because of the chemotactic and activating properties of its members towards neutrophils, monocytes, and lymphocytes (1,14). The chemokines have variable sequence homology to one another, and most contain four cysteine residues. Since the determination of molecular purity of the various IP-10 preparations was essential for our studies of its biological activity, all batches of IP-10 were extensively analyzed by SDS-PAGE. To exclude the presence of minor contaminant, we loaded several micrograms of purified protein in each lane of SDS-PAGE. We noted the appearance of a band with an approximate molecular mass of 18 kDa, which co-purified with IP-10 and was detected in bacterial lysates, cell-culture supernatants, and fractions of intermediate purity. This band was also erratically detected in highly purified IP-10 preparations, which had eluted as a single peak from reverse-phase high-performance liquid chromatography and which were greater than 99% pure by amino terminal sequencing. The ratio of IP-10 (migrating as a 10-kDa band) to this apparent contaminant also varied widely when the same highly purified IP-10 preparation was analyzed on different occasions. This suggested that the 18-kDa band was an artifact that was generated either during sample preparation or SDS-PAGE.

We demonstrate that the 18-kDa band is generated by cross-linking of the cysteine residues on IP-10 during the stacking phase of SDS-PAGE. We also describe simple techniques for the prevention of this artifact.

MATeRIALS AND METHODS

Acrylamide, bisacrylamide, SDS, and Tris were purchased from Roche Molecular Biochemicals (Indianapolis, IN, USA). Ammonium persulfate and β-mercaptoethanol were purchased from Sigma (St. Louis, MO, USA). Tricine, Coomassie® Blue, TEMED, bromophenol blue, low molecular weight marker proteins, and dithiothreitol (DTT) were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Iodoacetamide (IAA) and S-methylmethane-thiosulfonate (MMTS) were purchased from Fluka Chemical (Milwaukee, WI, USA).

Recombinant human IP-10 was prepared as previously described (15) and was stored frozen in small aliquots at -70°C. The chemical modification of cysteine residues in purified IP-10 was accomplished by dialysis against 50 mM Tris-HCl, pH 8.0, reduction with 20 mM DTT at room temperature for 1 h, addition of IAA or MMTS to a final concentration of 100 mM, and incubation for 1 h at room temperature. Modified IP-10 was dialyzed extensively against 140 mM NaCl, 50 mM sodium phosphate, pH 7.2, at 4°C, and was subsequently stored frozen in small aliquots at -70°C.

SDS-PAGE with Tricine was performed as previously described (15) using stacking gels containing 6% polyacrylamide and 750 mM Tris-HCl, pH 8.45, and separating gels consisting of 10%–20% polyacrylamide gradients in 1.0 M Tris-HCl, pH 8.45. We used 200 mM Tris-HCl, pH 8.9, in the anode reservoir, 100 mM Tris and 100 mM Tricine in the cathode reservoir, and 0.1% SDS throughout the system (18). Ammonium persulfate was prepared as a 10% (w/v) stock solution, was stored in small aliquots at -20°C, and was thawed only once for use before being discarded. The standard ammonium persulfate concentration of 0.05% was used in the stacking and separating gels, except when 0.1% or 0% ammonium persulfate was used in the stacking gel, as indicated in the figure legends. To stack the proteins without any exposure to ammonium persulfate before entrance into the separating gel, we used stacking gel with 2% agarose instead of acrylamide.

Samples were routinely solubilized by suspending in sample buffer at a final concentration of 17 mM Tris-HCl, pH 6.8, 4% (w/v) SDS, 2% (v/v) glycerol, 2% (v/v) β-mercaptoethanol, 0.002% (w/v) bromophenol blue, and 0.02% (w/v) pyronin-Y. They were then heated in a boiling water bath for 5 min. Solubilization without a reducing agent was accomplished by suspension in the same sample buffer without β-mercaptoethanol and then heating in a boiling water bath for 5 min. The samples were electrophoresed overnight at a constant current of 30 mA in a vertical electrophoresis apparatus (Bethesda Research Laboratories, Rockville, MD, USA). Western blotting of IP-10 was performed with methodology and antisera previously described (15).

To determine whether the cysteines could be successfully blocked while protein was suspended in sample solubilization buffer, we changed the β-mercaptoethanol to 20 mM DTT. After suspending the specimen in this buffer and heating in a boiling water bath for 5 min, IAA was added to a final concentration of 100 mM to block sulfhydryls and destroy excess DTT. After incubation for 20 min at 37°C, specimens were analyzed by Tricine-SDS-PAGE as previously described.

After electrophoresis, the gels were stained either with Coomassie brilliant blue (16) or with the Rapid-Ag Silver Stain Kit (ICN Biomedicals, Costa Mesa, CA, USA). To this effect, gels were washed twice for 15 min with 250 mL 10% (w/v) trichloroacetic acid, transferred to a clean glass dish, washed twice for 15 min each with double-distilled water, and then stained with silver as previously described (10). Color development was stopped by soaking the gels in clean glass containers with 0.1% (v/v) glacial acetic acid, 20% (v/v) methanol, and 2% (v/v) glycerol, followed by photography and by air drying at room temperature overnight as previously described (15).

RESULTS

All experiments were performed with highly purified IP-10, which had eluted as a single peak from reverse-phase high-performance liquid chromatography and was indistinguishable from naturally secreted IP-10 (15). Purified recombinant IP-10 was solubilized at a final concentration of 200 μg/mL, and increasing amounts were subsequently analyzed in Tricine SDS-PAGE containing 0.05% ammonium persulfate in both the stacking and the separating gels. When 0.5 or 1 μg of
the same preparation were loaded, there was no detectable 18-kDa band (Figure 1A). However, when 6 or 10 µg IP-10 were loaded in each lane, 30%–50% of the material migrated as an 18-kDa band. These results suggested that the 18-kDa band might represent an impurity comprising 30%–50% of the total protein. However, the intensity of the 18-kDa band was such that if 30%–50% of the total protein in the lanes with 6 or 10 µg IP-10 was a contaminant, it should have been easily detected in the lane containing 1 µg protein (Figure 1A).

Further experiments demonstrated that both the 10- and 18-kDa bands were recognized with an antiserum against IP-10 in western blotting (results not shown). This excluded the possibility that the 18-kDa band simply represented an impurity. However, it would still be possible for the 18-kDa band to represent a heterodimer between IP-10 and a contaminating 8-kDa peptide. This was also unlikely because amino acid analysis and limited amino terminal sequencing confirmed greater than 99% purity for the IP-10 preparations used in these experiments.

Taken together, these results suggested that the 18-kDa band represented an aggregate of IP-10 generated either during sample solubilization or during SDS-PAGE. We explored these alternatives by solubilizing IP-10 at final concentrations of 50, 75, 150, and 290 µg/mL and by subsequently loading in each lane different volumes so that the same amount (1 or 3.8 µg) of protein was analyzed in each lane. The 18-kDa band was detected only when 3.8 µg, but not 1 µg, was loaded, irrespective of the IP-10 concentration during solubilization (data not shown). These results suggested that the 18-kDa band was not generated during sample solubilization.

Therefore, we investigated the remaining possibility, namely that aggregation occurred during SDS-PAGE. A likely location for the aggregation of IP-10 was the stacking gel, where proteins are highly concentrated into a very small volume. During this time, the highly concentrated proteins are exposed to ammonium persulfate, which is an oxidant. These conditions would favor disulfide bond formation.

To test this hypothesis, IP-10 was analyzed by SDS-PAGE with either standard (0.05%, Figure 1A) or increased concentration (0.1%, Figure 1B) of ammonium persulfate. When 6 or 10 µg

Figure 1. Aggregation of IP-10 during SDS-PAGE. Purified protein was solubilized at a final concentration of 200 µg/mL. Increasing volumes (and increasing amounts of protein) were loaded on SDS polyacrylamide gels with stacking gels containing 0.05% (A) or 0.1% (B) ammonium persulfate. Both gels had the standard concentration of 0.05% ammonium persulfate in the separating gels. The amount of loaded protein is shown in micrograms at the top of each lane. MW, molecular mass markers, whose sizes are indicated by the numbers on the side of the figure.
proteins were loaded, the formation of the 18-kDa band increased as the concentration of ammonium persulfate increased from 0.05% to 0.1%. Aggregation was not detectable with 0.5 or 1.0 μg IP-10 at either 0.05% or 0.2% of ammonium persulfate in the stacking gel.

To determine if sulphydryl oxidation was involved in the formation of the 18-kDa band, we reduced IP-10 with DTT and then blocked the sulphydryls irreversibly with IAA, or reversibly with MMTS (2,19). Subsequently, native or modified IP-10 was solubilized with or without β-mercaptoethanol and was analyzed by SDS-PAGE containing varying concentrations of ammonium persulfate in the stacking gel. When unmodified IP-10 was solubilized by heating in sample buffer without β-mercaptoethanol and analyzed in gels of varying ammonium persulfate concentrations (Figure 2, A, B, and C, right column of gels, lane NONE), we detected the 18-kDa band and larger bands. This suggests that in our recombinant IP-10 preparations the sulphydryl bridges are not formed and that heating without reducing agent causes aggregation. Unmodified IP-10 showed progressive generation of the 18-kDa band when solubilized with β-mercaptoethanol as ammonium persulfate concentration in stacking gel increased from 0% to 0.1% (Figure 2, A, B, and C, lane NONE, left column of gels). IP-10, irreversibly modified by IAA, migrated as a 10-kDa monomer, whether it was solubilized in the presence or absence of β-mercaptoethanol and subsequently analyzed through stacking gels containing 0%, 0.05%, or 0.1% ammonium persulfate (Figure 2, lane IAA). This suggests that, when sulphydryls are irreversibly blocked, aggregation is not possible. IP-10, modified by MMTS, also migrated as a monomer at 10 kDa when solubilized without β-mercaptoethanol and analyzed with stacking gels containing 0%, 0.05%, and 0.1% ammonium persulfate (Figure 2, lane MMTS, right stack of gels). These results are consistent with those derived with IAA modification and suggest that the aggregation does not occur in the absence of reduced sulphydryls. However, when reduced sulphydryls were regenerated by reduction of MMTS-treated IP-10 (Figure 2,

![Figure 2. Modification of sulphydryl prevents IP-10 aggregation. Purified IP-10 was modified with IAA or MMTS, as described in Materials and Methods, and then solubilized at a final concentration of 60 μg/ml in solubilization buffer with β-mercaptoethanol (left column of gels designated with BME) or without β-mercaptoethanol (right column of gels designated without BME). Subsequently, 3 μg protein were analyzed in gels with 0.05% (A), 0.1% (B), or 0% (C) ammonium persulfate in the stacking gel IP-10. Modification by IAA or MMTS is indicated at the top of each lane. NONE indicates native IP-10, which was solubilized and analyzed without any chemical modification. BME indicates the presence of β-mercaptoethanol in the solubilization buffer. MW, molecular mass markers, whose sizes are indicated by the numbers on the side of the figure.](image)
lane MMTS, left column of gels), the 18-kDa aggregate appeared, and its amount increased as the ammonium persulfate concentration rose to 0.1%.

We attempted to irreversibly modify the sulfhydryls when IP-10 was suspended in SDS-PAGE sample buffer to simplify the analytical procedures and minimize the loss of protein during dialysis. However, large amounts of the 18-kDa band were observed with this approach (data not shown), suggesting that a blockade of sulfhydryls was not efficient in sample buffer. We suspect that the generation of hydrogen ions (estimated at 40 mM for a complete reaction of 20 mM DTT by 100 mM IAA) greatly exceeded the available buffering capacity of the solution (total concentration of Tris was 17 mM). At the ensuing acidic pH, sulfhydryl modification by IAA is expected to be ineffective. Therefore, we suggest that reductions of sulfhydryls, and their subsequent blockade by IAA, should be performed in well-buffered solutions to maintain the necessary slightly alkaline pH. Proteins should then be dialyzed, quantitated, solubilized in sample buffer, and analyzed by SDS-PAGE.

DISCUSSION

We demonstrated that IP-10 aggregates during stacking of proteins in SDS-PAGE. This aggregation is dependent on reduced sulfhydryls, on the amount of loaded protein, and on the concentration of ammonium persulfate in the stacking gel and can be prevented by irreversible blockade of sulfhydryls by IAA.

These results suggest that solubilization of proteins in SDS in the presence of reducing agents does not always protect sulfhydryls from subsequent oxidation during the stacking phase of SDS-PAGE. Presumably, the concentration of IP-10 in a thin band away from the bulk of the SDS and β-mercaptoethanol in the stacking gel facilitates the formation of disulfide bonds, provided that a critical protein concentration is exceeded and that sufficient oxidant concentration is present. The detection of aggregation when IP-10 was analyzed with SDS-PAGE having stacking gels of agarose, without any ammonium persulfate, suggests that there is enough ambient oxygen to catalyze the oxidation of sulfhydryls.

Artifactual protein aggregation has been previously reported during SDS-PAGE. Reversible aggregation of the sialoglycoproteins of the murine and human erythrocyte ghosts in both continuous and discontinuous buffer SDS-PAGE was observed after boiling in a sample buffer at high protein concentrations and was presumably dependent on methionine sulfoxide formation.
These sialoglycoprotein multimers could be dissociated by heating the samples at lower protein concentrations (6–8). Since IP-10 aggregation was independent of protein concentration during solubilization in sample buffer, this was not the mechanism in our case.

Oxidation of cysteines and formation of intramolecular disulfide bonds has also been detected during SDS-PAGE of the rat glucocorticoid receptors, where it generated diffuse bands and minor molecular weight shifts (13). Similarly, oxidation of cysteines mediated by persulfate has been reported during isoelectric focusing of human globin chains, where it caused artificial band heterogeneity (3–5). Cysteine oxidation and formation of intramolecular disulfide bonds is also responsible for the doublets generated by some proteins in Laemmli or Tricine SDS-PAGE (12,18). This artifact could also be prevented by reduction and carboxamidation with iodoacetamide. Free cysteines can also be irreversibly alkylated by untreated free acrylamide present in the SDS gels (9).

However, protein aggregation caused by disulfide bond formation during stacking of discontinuous SDS-PAGE has not been previously reported, to our knowledge. Significant aggregation was observed even after complete elimination of ammonium persulfate (by using agarose in the stacking gel). This suggested that there was enough ambient oxidant around to catalyze the aggregation of IP-10.

The methodology reported here may help detect and prevent aggregation of other chemokinases and possibly of other sulfhydryl-containing proteins. It may also be useful when purity is being assessed by analysis of large amounts of protein.

REFERENCES


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