Electrochemical Reduction of Disulfide-Containing Proteins for Hydrogen/Deuterium Exchange Monitored by Mass Spectrometry

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ABSTRACT: Characterization of disulfide bond-containing proteins by hydrogen/deuterium exchange monitored by mass spectrometry (HDX-MS) requires reduction of the disulfide bonds under acidic and cold conditions, where the amide hydrogen exchange reaction is quenched (pH 2.5, 0 °C). The reduction typically requires a high concentration (>200 mM) of the chemical reducing agent Tris(2-carboxyethyl)phosphine (TCEP) as its reduction rate constant is decreased at low pH and temperature. Serious adverse effects on chromatographic and mass spectrometric performances have been reported when using high concentrations of TCEP. In the present study, we explore the feasibility of using electrochemical reduction as a substitute for TCEP in HDX-MS analyses. Our results demonstrate that efficient disulfide bond reduction is readily achieved by implementing an electrochemical cell into the HDX-MS workflow. We also identify some challenges in using electrochemical reduction in HDX-MS analyses and provide possible conditions to attenuate these limitations. For example, high salt concentrations hamper disulfide bond reduction, necessitating additional dilution of the sample with aqueous acidic solution at quench conditions.

Proteins are dynamic molecules which exhibit conformational flexibility/adaptability to function properly.¹ Hydrogen/deuterium exchange monitored by mass spectrometry (HDX-MS) is increasingly being used to characterize the dynamic properties of proteins.⁴⁻⁸ When a protein is incubated in D₂O, the backbone amide hydrogen/deuterium exchange kinetics directly reflect the conformational dynamics of the polypeptide backbone. Amide hydrogens which are engaged in stable hydrogen bonds are protected against exchange with the solvent. When hydrogen bonds break as a result of structural fluctuations or local/global unfolding motions, the amide hydrogens become exchange-competent through the direct exposure to the solvent.⁹,¹₀

Extracellular proteins are generally rich in disulfide bonds. Incomplete reduction of disulfide bonds generally hampers HDX-MS analysis, as proteins are often partially or fully resistant to enzymatic digestion when their disulfide bonds are intact. While some proteins are readily digested with intact disulfide bonds, peptides linked by disulfide bonds are rarely identified unless the bonds are reduced. For these reasons, protein regions with multiple disulfide bonds generally yield lower sequence coverage and thus fewer identifiable peptides in HDX-MS experiments.

For HDX-MS analyses, disulfide bond reduction represents a particular challenge, as cleavage has to be performed under cold and acidic conditions (i.e., at HDX quench conditions). Additionally, the reduction must be carried out as quickly as possible (within a few minutes) to minimize artifactual deuterium loss or gain in the backbone amides, caused by slow exchange at quench conditions. Unfortunately, the disulfide bond reduction rate of the commonly used reducing agent TCEP,¹¹ Tris(2-carboxyethyl)phosphine, is also slowed down by orders of magnitude at acidic quench conditions.¹² To mitigate the reduced reactivity at low pH, high concentrations of TCEP (>200 mM) are typically employed.¹³,¹⁴ However, we and others in the field¹⁵ have observed that high concentrations of TCEP have serious adverse effects on the chromatographic and mass spectrometric analysis. Significant amounts of TCEP are retained on reversed-phase chromatographic columns, resulting in ion chromatograms which are dominated by intense TCEP clusters.¹⁵ These abundant cluster ions interfere with peptide identification by suppression of ionization, by yielding overlapping ions, and by reducing the sensitivity in the mass spectrometer due to a high level of chemical noise. This interference can be alleviated by decreasing the amount of TCEP,¹⁵ although this solution inevitably slows the rate of reduction. Although methylesterification of TCEP increases its reactivity somewhat at low pH,¹² such derivatives also have increased hydrophobicity and lowered water solubility.

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complicating their removal by reversed-phase chromatography and limiting the concentration they can be used in.

In view of the shortcomings mentioned above, an alternative, and preferably more efficient, method for disulfide reduction is highly desirable for HDX-MS experiments. Here, we explore an alternative approach, implementing an electrochemical cell into the HDX-MS workflow to achieve efficient online electrochemical reduction of disulfide-containing proteins. Reduction is achieved using a titanium-based working electrode which is specifically designed for reduction of disulfide bonds. The electrode acts through a combination of cathodic and anodic square wave voltage pulses generated by a potentiostat. The anodic pulse creates an oxide layer on the metal surface of the working electrode, the thickness of which is decisive for the disulfide reduction efficiency during the cathodic pulse. Our studies clearly demonstrate that online electrochemical reduction can be implemented successfully into the HDX-MS workflow.

**EXPERIMENTAL SECTION**

**Electrochemical Reduction.** A µ-PrepCell thin-layer electrochemical reactor cell (Antec, Zoeterwoude, NL) was equipped with a 12 × 30 mm × 1 mm titanium-based working electrode (also Antec). A detailed introduction to the electrode can be found in the work of Kraj et al. A single 50 μm spacer was used, resulting in an internal volume of approximately 11 μL. The cross section of the electrochemical cell is shown in Figure 1A. A solvent inlet and outlet allows a thin layer (0.15 mm) of solvent to flow over the surface of the working electrode. Reduction of disulfide bonds occurs when a square-wave negative potential pulse is applied. The cell was operated in a standard three electrode configuration, using a working electrode (WE), a titanium counter electrode (AUX), and a Pd/H₂ reference electrode (REF). The cell was powered by a ROXY potentiostat (Antec) operating in pulse mode, delivering a reducing square-wave negative potential pulse (E1) for 2000 ms, followed by an oxidizing square-wave positive potential pulse (E2) for 1000 ms. The E2 voltage was set to one-third of the absolute value of the E1 voltage (see Results and Discussion for information on absolute values). For direct infusion experiments an aqueous 10 μM insulin solution, made using degassed 10% (ν/ν) acetonitrile and 1% (ν/ν) formic acid, was infused through the cell and directly into the electrospray ion source using a glass syringe mounted in a syringe pump. When used in conjunction with the Waters HDX unit, the electrochemical reduction cell was located in the flow path between the sample loop and trap column, physically close to the pepsin column compartment, as shown in Figure 1B.

**Liquid Chromatography and Mass Spectrometry.** Samples were injected into a Waters HDX unit (Waters, Milford, MA, USA) which traps and desalts samples, while providing cooling for chromatographic solvents, the sample loop, switching valves, the reversed-phase trap, and the analytical column. All were cooled to 0 °C. Samples were flushed from the cooled sample loop, through the electrochemical reduction cell, and onto the reversed-phase trap using 1% formic acid flowing at 50 μL/min. For local exchange experiments, proteins were digested online using an Upchurch guard column (1.0 × 20 mm, IDEX, Oak Harbor, WA, USA) packed in-house with agarose-immobilized pepsin (Thermo Scientific Pierce, Rockford, IL). The pepsin column was mounted in the desalting flow path, immediately after the electrochemical reduction cell (Figure 1B), in a compartment cooled to 10 °C. The lid to the compartment housing the pepsin column was replaced by a block of polystyrene foam which provided temperature insulation. Mass spectrometric analyses were carried out using a Waters Synapt G1 instrument. Further details on the setup are available in the Supporting Information.

**RESULTS AND DISCUSSION**

Electrochemical reduction of disulfide bonds coupled with online mass spectrometric analysis is traditionally obtained by mercury–gold amalgam and boron-doped diamond. The cross section of the electrochemical cell is shown in Figure 1A. A solvent inlet and outlet allows a thin layer (0.15 mm) of solvent to flow over the surface of the working electrode. Reduction of disulfide bonds occurs when a square-wave negative potential pulse is applied. The cell was operated in a standard three electrode configuration, using a working electrode (WE), a titanium counter electrode (AUX), and a Pd/H₂ reference electrode (REF). The cell was powered by a ROXY potentiostat (Antec) operating in pulse mode, delivering a reducing square-wave negative potential pulse (E1) for 2000 ms, followed by an oxidizing square-wave positive potential pulse (E2) for 1000 ms. The E2 voltage was set to one-third of the absolute value of the E1 voltage (see Results and Discussion for information on absolute values). For direct infusion experiments an aqueous 10 μM insulin solution, made using degassed 10% (ν/ν) acetonitrile and 1% (ν/ν) formic acid, was infused through the cell and directly into the electrospray ion source using a glass syringe mounted in a syringe pump. When used in conjunction with the Waters HDX unit, the electrochemical reduction cell was located in the flow path between the sample loop and trap column, physically close to the pepsin column compartment, as shown in Figure 1B.

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working electrodes. Recently, a new titanium-based electrode, nevertheless, proved superior to these original electrodes with respect to disulfide bond reduction in small proteins as nearly quantitative reduction of disulfide bonds was obtained for insulin, somatostatin, and α-lactalbumin.16 Importantly, we exploited the efficacy of this new titanium-based electrode by implementing it for online reduction of disulfide bonds in proteins at HDX quench conditions. To exemplify the versatility of this approach, we probed two inherently difficult proteins dominated by intermolecular dynamics (i.e., self-assembly of insulin hexamers) or intramolecular dynamics (multidomain flexibility of the urokinase-type plasminogen activator receptor, uPAR, containing no less than 10% cysteine ~14 disulfide bonds).

Insulin consists of two polypeptide chains, A and B, linked by two interchain disulfide bonds (Figure 2A). Chain A contains one additional intrachain disulfide bond. Reduction of the two interchain disulfide bonds in insulin is readily detected by mass spectrometry by the appearance of peaks corresponding to the individual chains. Note that chain B is more efficiently ionized by electrospray as it contains several basic residues, while chain A is devoid of any basic residues.

The urokinase-type plasminogen activator receptor (uPAR) was chosen as a more complex challenge for our electrochemical reduction approach. The urokinase-type receptor (uPAR) contains three homologous LU-domains with 14 intradomain disulfide bonds.22 Recent studies have shown that the interdomain flexibility of uPAR plays a key role in the allosteric regulation of uPAR-mediated adhesion on vitronectin by its high-affinity protease ligand uPA.22–24 We have previously applied HDX-MS and chemical reduction using TCEP to probe the loss of flexibility when introducing an artificial interdomain disulfide-bond (H47C-N259C), which creates a stabilized and constitutively active receptor and alleviates the regulatory effect caused by uPA binding.24

Disulfide Bonds Can Be Efficiently Reduced under HDX Quench Conditions, Using Electrochemical Reduction. When attempting to reduce insulin using 400 mM TCEP at HDX quench conditions, a very poor reduction yield was observed. Ions belonging to reduced chain B were very low abundant, even after 10 min of TCEP reduction, and intact insulin still accounted for more than 80% of the total ion intensity after 50 min (Figure S1, Supporting Information). In contrast, when insulin was infused through the electrochemical reduction cell at a flow rate of 50 μL/min using 10% acetonitrile and 1% formic acid, quantitative reduction of the interchain disulfide bonds in insulin was readily achievable (compare Figure 2B,D). At greater flow rates (corresponding to a lower residence time inside the cell), reduction became incomplete. This is exemplified in Figure 2C, where the presence of peaks corresponding to intact insulin becomes visible in the mass spectrum when reduction was performed at 100 μL/min. When the reduction cell was used online with a Waters HDX-unit, placed in the desalting flow path as illustrated in Figure 1B, the solvent had to be changed to 1% formic acid to be compatible with the reversed-phase trap column. Despite this solvent change, the reductive efficiency of the cell was maintained in the online setup.

Online Electrochemical Reduction and Back-Exchange. In HDX-MS setups employing online desalting, higher desalting flow rates are generally preferred to minimize desalting time. A lower desalting flow rate increases the time required for sample desalting, resulting in increased loss of deuterium label due to back-exchange with chromatographic solvents. Placing the reduction cell in the desalting flow path of our HDX setup meant that our standard desalting flow rate had to be lowered to ensure efficient reduction, from 300 μL/min to 50 μL/min. Additionally, the cell itself was expected to promote back-exchange, as it added a localized, noncooled, environment to our desalting flow path. To investigate how these parameters impacted back-exchange, equilibrium labeled insulin chain B (80% D2O, 28 backbone amides) was injected in its reduced state and its deuterium content was measured in the absence and presence of the cell at the two aforementioned flow rates. Our standard setup, without the electrochemical cell, desalting for 0.5 min at 300 μL/min yielded a deuterium content of 16.13 ± 0.14 (average ± SD, n = 3) corresponding to 28.0% back-exchange (1 − 16.13/(0.8 × 28) = 28%). Using a flow rate compatible with electrochemical reduction (50 μL/min for 3 min) resulted in somewhat lower deuterium content (14.35 ± 0.07 deuterons, 36.0% back-exchange). Placing the electrochemical cell in the flow path (with no voltages applied), further increased the level of back-exchange (13.09 ± 0.08 deuterons, 41.6% back-exchange), while applying voltages to the cell only marginally affected the deuterium content (12.94

![Figure 2.](image-url)
± 0.02 deuterons, 42.2% back-exchange). When using the electrochemical reduction cell online, the deuterium content was therefore determined to be 80% of the value obtained at standard operating conditions. At a later occasion, we were able to investigate the level of back-exchange when the cell was located inside the refrigerated chamber of the HDX unit, using a larger protein (human growth hormone). The level of back-exchange only decreased slightly (∼5%) when the temperature of the cell was lowered from room temperature to 0 °C. Surprisingly, the electrochemical reduction efficiency was virtually unaffected by the lower temperature when tested using insulin (data not shown). For practical reasons, however, the remaining experiments were conducted while operating the cell at room temperature (as shown in Figure 1B).

Deuteration Patterns Are Qualitatively Retained after Electrochemical Reduction. The self-association of insulin and its analogs have previously been studied by HDX-MS global exchange analyses of the intact protein or pepsin digested with intact disulfide bonds. Nakazawa et al. have investigated insulin hexamers using HDX-MS and chemical reduction with TCEP. They also observe a poor reduction efficiency when using TCEP under quench conditions (personal correspondence). Given that we were readily able to reduce insulin under HDX quench conditions using the titanium-based working electrode, we conducted an analogous experiment, analyzing insulin self-association in R-state hexamers using HDX-MS and electrochemical reduction. The B-chain in the R-state hexamer was found to be strongly protected, with only ~50% of its backbone amides being deuterated at the longest exchange time (1000 s, Figure S2, Supporting Information). Local exchange analysis identified a 6 amino acid stretch spanning B12 to B17 as the main contributor to this protection (Figure S3A, Supporting Information). Protection of this region is in good agreement with published crystal structures, where the B12–B17 residues are located in the middle of an α-helix, buried in the core of the hexamer (Figure S3B, Supporting Information). Protection of the backbone amides located at the core of the hexamers directly reflects the high kinetic stability of the R-state hexamers and is in agreement with NMR measurements of these. The data are also in excellent agreement with an H/D exchange neutron diffraction study of crystalline T-state insulin hexamers. Taken together, these findings clearly indicate that HDX-MS with online electrochemical reduction was able to reduce insulin quantitatively under quench conditions, while retaining backbone deuteration patterns which are consistent with previous observations.

We also sought to compare the observed deuteration patterns in samples subjected to chemical (TCEP) and electrochemical reduction, to ascertain whether the same qualitative patterns resulted. For this goal, we studied uPAR wt and the stabilized uPARH47C–N259C mutant. The deuterium content of the peptides resulting from online electrochemical reduction and pepsin digestion were compared to our previous measurements using TCEP (Figure 3 and Figure S5, Supporting Information). While electrochemically reduced uPAR had a lower overall deuterium content, both reduction methods resulted in the same qualitative deuteration patterns for uPAR wt and uPARH47C–N259C. For example, the pronounced stabilizing effect in uPAR domain 1 caused by the artificial disulfide-bond is readily apparent by both approaches (indicated by the arrow in Figure 3). These observations further highlight that electrochemical reduction is indeed a feasible alternative to chemical reduction by TCEP, also in the case of challenging systems with disulfide stabilized structures. It is important to note that these data should not be taken as a reporter for back-exchange induced by electrochemical reduction, compared to TCEP-reduction, as samples were prepared and reduced differently (see Figure S5 legend, Supporting Information, for further explanation).

The Limitations of Online Electrochemical Reduction. Although the above-mentioned examples show that electrochemical reduction is compatible with HDX-MS, we observed that certain buffer conditions had an adverse impact on the electrochemical reduction efficiency. For example, the reduction efficiency of uPAR was greatly reduced when the electrochemical reduction was carried out in the presence of PBS (acidified with 1% formic acid). This negative effect was substantially lessened when PBS was diluted 10- or 50-fold with 1% formic acid, with greater dilutions offering increased disulfide bond reduction (Figure S6, Supporting Information). This indicates that relatively high salt concentrations in labeled samples are detrimental to the disulfide reduction. A similar phenomenon was encountered when attempting to reduce insulin in the presence of 300 mM ammonium acetate acidified with 1% formic acid (data not shown). Lowering the buffer concentration by a 400-fold dilution with 1% formic acid restored the electrochemical reduction efficiency. For some samples, a high dilution factor may not be a feasible solution, e.g., when the protein concentration in the labeling solution is low. In such cases, online size-exclusion columns could be used to separate low molecular weight buffer components and salts from the proteins prior to reduction. It is worth noting that the negative impact of salts on the efficiency of the electrochemical...
reduction only reflects our experiences with the ammonium acetate and PBS buffers used in this study.

Throughout our experiments, we discovered that it was essential to adjust the E1 and E2 voltages applied to the cell. A high negative E1 voltage (e.g., $-3.0\, \text{V}$) gave efficient reduction but could also result in the electrolysis of water. In our experience, setting the E1 voltage approximately 50 mV above the onset voltage for electrolysis yielded efficient reduction, while avoiding the formation of gas bubbles from electrolysis of water. The E1 voltage was adjusted a few times during our experiments, indicating that the surface of the working electrode changed its reactivity slightly with time. This was most likely due to the active oxide-layer covering the electrode shrinking or increasing in thickness if the E1 (reducing) and E2 (oxidizing) voltages were not balanced properly. In our experience, quite stable operation could be achieved by adjusting the E2 voltage to one-third of the absolute value of the E1 voltage (when applying E1 for 2000 ms and E2 for 1000 ms). We only tested this 2000 ms/1000 ms pulse sequence during our experiments, but it is possible that the pulse sequence could be further optimized to better accommodate online reduction.

According to the manufacturer’s specifications, the maximum pressure limit for the current electrochemical $\mu$-PrepCell is 50 bar. This puts some restrictions on the maximum flow rate of the desalting solvent combined with trap column diameter and particle size. In our setup, we were able to keep the pressure below 45 bar when running at 50 $\mu\text{L/min}$. 

**CONCLUSION**

Our results show that the cell is capable of reducing disulfide bonds very efficiently under HDX quench conditions, while only increasing the level of back-exchange slightly. Importantly, the reduction process itself did not perturb the deuteration patterns in a qualitative manner. On the basis of these findings, we believe that online electrochemical reduction using the titanium-based working electrode is a viable alternative to chemical reduction methods for HDX-MS analyses. With further improvements, such as the discovery of buffer systems which are more compatible with electrochemical reduction, electrodes or pulse sequences which provide more efficient reduction at greater solvent flows, and cells with higher pressure tolerance, electrochemical reduction could become an attractive permanent solution for disulfide bond reduction in HDX-MS workflows. Lastly, we believe top-down fragmentation HDX-MS analyses of disulfide-containing proteins could benefit greatly from this approach.

**ASSOCIATED CONTENT**

Supporting Information

Experimental sections describing the materials used, sample preparation, extended specifics of the LC and MS setups, employed data analysis software, and details regarding the back-exchange measurements made using Human Growth Hormone. Chemical reduction of insulin using TCEP under quench conditions (Figure S1), mass spectra depicting deuterium uptake in intact chain A and B of insulin (Figure S2), HDX-MS analysis of peptic peptides derived from insulin forming R-state hexamers (Figures S3 and S4), deuterium uptake in peptides derived from uPAR wt and H47C-N259C, comparing chemical and electrochemical reduction (Figure S5), and a comparison of the electrochemical reduction efficiency on uPAR at decreasing concentrations of PBS (Figure S6). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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