Real-time Kinetics of High-mobility Group Box 1 (HMGB1) Oxidation in Extracellular Fluids Studied by in Situ Protein NMR Spectroscopy

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Background: Redox of extracellular HMGB1 protein plays an important role in inflammation.

Results: The half-life of all-thiol HMGB1 was ~17 min in serum and saliva and significantly longer in cancer cell culture medium and was modulated by exogenous ligands (e.g. heparin).

Conclusion: The extracellular environment dictates HMGB1 oxidation kinetics.

Significance: Our approach permits investigating protein oxidation in situ.

Some extracellular proteins are initially secreted in reduced forms via a non-canonical pathway bypassing the endoplasmic reticulum and become oxidized in the extracellular space. One such protein is HMGB1 (high-mobility group box 1). Extracellular HMGB1 has different redox states that play distinct roles in inflammation. Using a unique NMR-based approach, we have investigated the kinetics of HMGB1 oxidation and the half-lives of all-thiol and disulfide HMGB1 species in serum, saliva, and cell culture medium. In this approach, salt-free lyophilized 15N-labeled all-thiol HMGB1 was dissolved in actual extracellular fluids, and the oxidation and clearance kinetics were monitored in situ by recording a series of heteronuclear 1H-15N correlation spectra. We found that the half-life depends significantly on the extracellular environment. For example, the half-life of all-thiol HMGB1 ranged from ~17 min (in human serum and saliva) to 3 h (in prostate cancer cell culture medium). Furthermore, the binding of ligands (glycyrrhizin and heparin) to HMGB1 significantly modulated the oxidation kinetics. Thus, the balance between the roles of all-thiol and disulfide HMGB1 proteins depends significantly on the extracellular environment and can also be artificially modulated by ligands. This is important because extracellular HMGB1 has been suggested as a therapeutic target for inflammatory diseases and cancer. Our work demonstrates that the in situ protein NMR approach is powerful for investigating the behavior of proteins in actual extracellular fluids containing an enormous number of different molecules.

Disulfide bonds between cysteine residues are very common in extracellular proteins. In the canonical pathway, disulfide bonds are formed via oxidation in the endoplasmic reticulum before secretion to the extracellular space (1, 2). However, there are many proteins that bypass the endoplasmic reticulum and have no disulfide bonds when released (3–5). Such proteins eventually become oxidized due to the oxidative nature of the extracellular environment. Various studies have shown that the functional roles of their reduced and oxidized forms are distinctly different (reviewed in Ref. 5). Therefore, the kinetics of conversion to the oxidized state must play an important role in dictating the functions of the two forms. Rapid disulfide bond formation (seconds to minutes) would suggest that the role for the reduced form is transient and that any function of the reduced form must be rapid and immediate. On the other hand, slow oxidation would suggest that the reduced form is the functionally relevant form, that disulfide formation regulates function, and/or that the function of the oxidized form, if important, emerges over longer time scales of hours. Therefore, the kinetics of the oxidation should be an important determinant of the balance between roles of reduced and oxidized states. This balance could be shifted in some diseases, including cancer (6, 7), where extracellular redox status is significantly modulated. Currently, there is a lack of knowledge of the kinetics of protein oxidation in the extracellular space. Analyzing the compositions of fluids is not enough to predict oxidation kinetics because a large number of chemical factors affect disulfide bond formation (8–12).

In this work, we studied the oxidation of HMGB1 (high-mobility group box 1) protein in extracellular fluids. This 25-kDa protein, which is normally located in nuclei, is passively released during cell injury and necrosis and actively secreted by some cell types via a non-canonical pathway that bypasses the endoplasmic reticulum (13, 14). Extracellular HMGB1 acts as a danger signal and inflammatory mediator via binding to cell surface receptors such as RAGE, TLR4 (Toll-like receptor 4), and CXCR4 (13–16). Extracellular HMGB1/receptor interactions promote inflammation and angiogenesis via activation of NF-κB (17, 18) and tumor proliferation via activation of p44/p42, p38, and SAPK/INK MAPKs (19, 20). When released into...
the extracellular space, HMGB1 is initially in the reduced state ("all-thiol HMGB1") (see Fig. 1A) but becomes oxidized due to the oxidative environment (5, 15, 21). HMGB1 has three cysteine residues. Cys-23 and Cys-45 in the A-domain are located in close proximity (see Fig. 1A). With a standard redox potential of $-237\text{ mV}$ (21), these two cysteine residues can easily form a disulfide bond under relatively mild oxidative conditions ("disulfide HMGB1"). Cys-106 in the B-domain remains in the reduced state but can be sulfonated in the extracellular space when exposed to a large amount of reactive oxygen species from activated leukocytes (22). The three different redox states (i.e. all-thiol, disulfide, and sulfonated) of extracellular HMGB1 play distinct roles in inflammation (reviewed in Refs. 16 and 23). All-thiol HMGB1, but not disulfide HMGB1, is able to form a complex with CXCL12 for signaling via the CXCR4 receptor and exhibits chemoattractant activity (24). Only disulfide HMGB1 can interact with TLR4 and exhibits cytokine-inducible expression was induced with 0.4 mM isopropyl $\beta$-d-thiogalactopyranoside, and the $E.\ col i$ culture was continued at 18 °C for 16 h. Harvested cells were suspended in buffer containing 20 mM sodium phosphate (pH 6.0), 1 mM EDTA, 100 mM NaCl, 2 mM DTT, and 5% glycerol and disrupted at 4 °C by sonication. The supernatant of the cell lysate was loaded onto an SP cation exchange column equilibrated with 20 mM sodium phosphate (pH 6.0) and 100 mM NaCl and eluted with a gradient of 100–2000 mM NaCl. Fractions containing HMGB1 were concentrated and passed through an S-100 column equilibrated with buffer containing 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 200 mM NaCl. After size exclusion chromatography, HMGB1 was further purified by RESOURCE Q anion exchange chromatography with a gradient of 0–1500 mM NaCl in 50 mM Tris-HCl (pH 7.5). No reducing regent was used in the column chromatography procedures except in the sonication buffer. Complete disulfide bond formation in HMGB1 was confirmed by NMR (21).

Preparation of Salt-free Powders of $^{15}\text{N}$-Labeled All-thiol HMGB1—To break the disulfide bond, 5 mM DTT was added to a solution of $^{15}\text{N}$-labeled disulfide HMGB1, and the mixture was kept overnight at 4 °C. Using an Amicon Ultra-15 unit, the solvent was exchanged with water containing 1 mM $\beta$-mercaptoethanol. Because of the lower solubility of HMGB1 in the absence of salt, the process of the solvent exchange was carried out at protein concentrations lower than 200 $\mu$m. The final solution was divided into aliquots (each containing 68 nmol of HMGB1), frozen at $-80\text{ °C}$, and lyophilized. $\beta$-Mercaptoethanol was removed during the lyophilization process, and all-thiol HMGB1 remained as a powder. The obtained powder was stored at $-20\text{ °C}$ until used.

Preparation of Salt-free Powders of $^{15}\text{N}$-Labeled Disulfide HMGB1—Salt-free powders of $^{15}\text{N}$-labeled disulfide HMGB1 were prepared in the same way as for all-thiol HMGB1 except that the samples were not treated with either DTT or $\beta$-mercaptoethanol. Disulfide HMGB1 in water was lyophilized as described above.

In Situ NMR Experiments to Measure the Half-lives of All-thiol and Disulfide HMGB1 Species in Extracellular Fluids—For the kinetic measurements, the salt-free powder of $^{15}\text{N}$-labeled all-thiol HMGB1 was dissolved in 500 $\mu$l of extracellular fluid (i.e. serum, saliva, or cell culture medium) plus 1% D$_2$O
The solution was immediately sealed in a 5-mm NMR tube with ambient oxygen pressure, and a series of $^1$H-$^{15}$N correlation spectra were recorded at 37 °C using Bruker AVANCE III spectrometers equipped with cryogenic probes ($^1$H frequencies, 800 and 600 MHz). In all experiments except those involving the cell culture medium, band-selective optimized flip-angle short-transient heteronuclear multiple quantum coherence (SOFAST-HMQC) spectra (28, 29) were recorded with a recycle delay of 0.1 s. For the cell culture medium, in which HMGB1 oxidation is relatively slow, the kinetics were measured with a series of sensitivity-enhanced $^1$H-$^{15}$N transverse relaxation optimized spectroscopy spectra (30, 31) recorded with a recycle delay of 0.8 or 1.0 s. Values of pH for the NMR samples were confirmed to be 7.4. Kinetic rate constants were determined via nonlinear least-squares fitting with MATLAB software (MathWorks). Other details of the calculations are given in the figure legends.

NMR Resonance Assignment—Because the spectra of HMGB1 samples dissolved in extracellular fluids and in buffers are very similar, resonances of HMGB1 in extracellular fluids were assigned using the NMR spectra recorded for HMGB1 in a buffer. $^1$H/$^{13}$C/$^{15}$N resonances for oxidized and reduced HMGB1 proteins were assigned with three-dimensional HNCA, HN(CO)CA, HNCO, HN(CA)CO, HNCACB, CBCA(CO)NH, C(CO)NH, $^{15}$N-edited NOESY-heteronuclear single quantum coherence; and $^{1}$F-$^{15}$N-$^{15}$N-edited HMQC-NOESY-heteronuclear single quantum coherence spectra (32) recorded at 25 °C on 0.7 mM $^{13}$C/$^{15}$N-labeled proteins in buffer containing 20 mM Tris-HCl (pH 7.5), 120 mM NaCl, and 5% D$_2$O. For all-thiol HMGB1 in the buffer, 5 mM DTT was also added, and the NMR tube was sealed in the presence of argon gas. Resonance assignment was also aided by previous assignment data for HMGB1 under different conditions (21, 33–35). NMR data were processed and analyzed using the NMRPipe (36) and NMRView (37) programs.

In this study, we use the residue numbering scheme from the initial methionine in the gene (i.e. Met-1–Gly-2–Lys-3 . . . ) because this has become the accepted norm, although the numbering scheme from the actual N-terminal glycine (i.e. Gly-1–Lys-2 . . . ) of HMGB1 has been used in many previous articles (including ours).

RESULTS

NMR of All-thiol and Disulfide HMGB1 Proteins Dissolved in Serum—We prepared salt-free lyophilized powders of $^{15}$N-labeled all-thiol HMGB1 and HMGB1 proteins and dissolved them in human serum to characterize the actual redox kinetics of extracellular HMGB1 by NMR. Fig. 1C shows NMR spectra recorded at 37 °C for all-thiol HMGB1 and disulfide HMGB1 in human serum. The spectra were similar to spectra recorded for all-thiol HMGB1 and disulfide HMGB1 in buffer containing 20 mM HEPES-NaOH (pH 7.4), 120 mM NaCl, and 5% D$_2$O (Fig. 1D). This suggests that the HMGB1 structures in serum and in

FIGURE 1. A, HMGB1 and its three cysteine residues. Cys-23 and Cys-45 form a disulfide bond under oxidative conditions. B, experimental scheme for the in situ protein NMR approach to investigate the behavior of HMGB1 in actual extracellular fluids. C, $^{1}$H-$^{15}$N SOFAST-HMQC spectra recorded with eight scans at 37 °C for all-thiol HMGB1 (red) and disulfide HMGB1 (blue) immediately after dissolving in human serum. D, $^{1}$H-$^{15}$N SOFAST-HMQC spectra of all-thiol HMGB1 (red) and disulfide HMGB1 (blue) immediately after dissolving in 20 mM HEPES-NaOH (pH 7.4), 120 mM NaCl, and 5% D$_2$O. 135 $^{15}$N-labeled HMGB1 was used for both the serum and buffer samples. Line shapes of NMR signals from HMGB1 in serum were substantially broader than those in buffer presumably due to the molecular crowding environment of the serum. E, SDS-PAGE of the serum samples used for the NMR experiments. 3 μl of serum before and after dissolving 135 μM $^{15}$N-labeled HMGB1 was loaded. The arrow indicates the position of the HMGB1 band. The strongest bands are albumin. F, changes in the $^{1}$H-$^{15}$N SOFAST-HMQC spectra recorded at 37 °C for all-thiol HMGB1 in human serum. Due to oxidation, signals from all-thiol HMGB1 became weaker, whereas those from disulfide HMGB1 became stronger.

2 The abbreviation used is: SOFAST-HMQC, band-selective optimized flip-angle short-transient heteronuclear multiple quantum coherence.
buffer are identical, although serum is a molecular crowding environment with numerous proteins as shown by the SDS-PAGE data (Fig. 1E). Our NMR data also indicate that the proteins from the salt-free lyophilized powders were properly folded when dissolved in extracellular fluids. As demonstrated in our previous study on the A-domain of HMGB1 (21), residues in close proximity to Cys-23 or Cys-45 exhibited large 1H/15N chemical shift differences between all-thiol HMGB1 and disulfide HMGB1. Due to rapid oxidation, however, the spectra recorded for the sample of all-thiol HMGB1 in serum gradually changed and finally became identical to the spectra of disulfide HMGB1 (Fig. 1F). We used this change in NMR spectra to investigate the oxidation kinetics of HMGB1 in serum.

Kinetics of HMGB1 Oxidation in Serum—We recorded a series of 1H/15N SOFAST-HMQC spectra immediately after dissolving 15N-labeled all-thiol HMGB1 in serum. SOFAST-HMQC allows acquisition of high-resolution 1H-15N correlation spectra in a few minutes and is therefore ideally suited for measuring real-time kinetics (28, 29). NMR signals from disulfide HMGB1 gradually became stronger and predominant, whereas signals from all-thiol HMGB1 became weaker and eventually disappeared in 50 min (Fig. 1F). This clearly reflects the oxidation process of all-thiol HMGB1 in serum. Using the SOFAST-HMQC spectra, we obtained the time course data of the fraction of oxidation (Fig. 2A). By nonlinear least-squares fitting with the experimental data, we determined the apparent pseudo-first-order rate constant ($k_{ox}$) for HMGB1 oxidation. The half-life ($t_{1/2}$) of all-thiol HMGB1, which was calculated as $k_{ox}^{-1}$ in 2, was determined to be 17 ± 1 min (Table 1). We also measured $k_{ox}$ at different concentrations of HMGB1 (Fig. 2B). $k_{ox}$ was slightly smaller at concentrations higher than 200 μM. This is probably because the overall concentration of the oxidants that oxidized HMGB1 was not high enough to make the process completely pseudo-first-order. Because the in vivo concentration of extracellular HMGB1 is lower than micromolar, HMGB1 oxidation in vivo should occur in a completely pseudo-first-order manner, and the half-life in vivo should be only slightly shorter than what we observed in situ.

Clearance of HMGB1 in Serum—Our NMR data also provide kinetic information on the clearance of HMGB1 due to protease activities in extracellular fluids. Fig. 2C shows the time course of the loss in total intensity of NMR signals whose positions (i.e. 1H/15N chemical shifts) were unaffected by the oxidation. The HMGB1 band in SDS-PAGE became weaker over hours as well, which indicates that the decrease in NMR signal intensities is caused by a decrease in the total amount of HMGB1. Apparent kinetic rate constants for HMGB1 clearance ($k_{cl}$) were determined via monoexponential fitting with the time course data shown in Fig. 2C. The half-life of disulfide HMGB1 in serum was calculated as $k_{cl}^{-1}$ in 2 to be 642 ± 49 min (Table 1). Interestingly, neither 1H-15N spectra nor SDS-PAGE showed degradation products of HMGB1. These results imply that, once cleavage occurs, HMGB1 molecules become highly susceptible to complete digestion by extracellular proteases to an amino acid level.

Kinetics of HMGB1 Oxidation and Clearance in Saliva—Salivary gland cells are known to produce extracellular HMGB1 (38). To examine whether or not the molecular behavior of

![FIGURE 2. Kinetics of oxidation and clearance of HMGB1 in human serum.](image)

A, oxidation kinetics of HMGB1 in human serum measured by in situ protein NMR. The vertical axis represents the fraction of oxidation ($p_{ox}$) measured as (1/$N_{ox}$), where $i_O$ and $i_S$ are signals intensities for all-thiol and disulfide HMGB1 proteins, respectively, and $i$ is indices for analyzed residues. Intensities were measured for NMR signals from residues that exhibit relatively strong, well isolated signals for both all-thiol and disulfide states (i.e. those from Thr-22, Lys-29, His-31, and Gly-S8 backbone amide groups and the Gln-21 side chain NH$_2$ group). Experimental $p_{ox}$ data were fitted to $1 - \exp(-k_{ox}(t-t_0))$, where $k_{ox}$ represents a pseudo-first-order rate constant for the oxidation of all-thiol HMGB1, and $t_0$ represents a time shift due to the difference between the effective times and the mid-times for recording NMR spectra. This shift was applied to the horizontal axis of the time course data shown. B, kinetic rate constants ($k_{cl}$) for HMGB1 at different concentrations (90, 135, 180, 225, and 270 μM) in human serum. C, decrease in the total intensity of NMR signals from amide groups whose 1H/15N chemical shifts were unaffected by oxidation. SOFAST-HMQC spectra recorded for 135 μM 15N-labeled HMGB1 in human serum were analyzed. The curve represents the best fit to $1 - \exp(-k_{cl}(t-t_0))$, where $k_{cl}$ is a pseudo-first-order rate constant for HMGB1 clearance. The vertical axis represents the total intensity divided by a, A.U., arbitrary unit.

**TABLE 1**

| Extracellular fluids | Oxidation $t_{0.5}$ | Clearance $t_{0.5}$ |
|----------------------|---------------------|---------------------|
| Human serum          | 17 ± 1              | 642 ± 49            |
| Human saliva         | 18 ± 2              | 65 ± 6              |
| RPMI 1640 medium + 10% FBS | 25 ± 3            | 464 ± 41            |
| PC-3M cell culture medium |                |                     |
| 30% cell confluency | 27 ± 3              | 480 ± 48            |
| 60% cell confluency | 84 ± 3              | >1500’              |
| 95% cell confluency | 201 ± 6             | >1500’              |
| PC-3M + heparin     | 43 ± 2              | >1500’              |
| PC-3M + glycyrrhizin| 289 ± 9             | >1500’              |

* Determined as $k_{cl}^{-1}$ in 2. This corresponds to the half-life of all-thiol HMGB1.
* Determined as $k_{cl}^{-1}$ in 2. This corresponds to the half-life of disulfide HMGB1.
* Control for PC-3M data. No cells were cultured (see “Experimental Procedures”).
* Extracellular fluid at a cell confluency of 30%.
* Extracellular fluid at a cell confluency of 60%.
* Too slow to determine.
* Extracellular fluid at a cell confluency of 95%.
* 2.5 mM heparin octasaccharide dissolved together with HMGB1.
* 2.0 mM glycyrrhizin dissolved together with HMGB1.
HMGB1 in saliva is similar to that in serum, we also investigated oxidation and clearance of HMGB1 in saliva using the in situ protein NMR approach. Interestingly, HMGB1 clearance in saliva was found to be 10-fold faster than that in serum (Fig. 3A), whereas HMGB1 oxidation in saliva was as fast as that in serum (Fig. 3B). Thus, the function of different redox forms of extracellular HMGB1 is very likely dependent on the nature of the extracellular fluid.

**Kinetics of HMGB1 Oxidation and Clearance in Extracellular Fluids from Cell Culture**—Using the in situ protein NMR approach, we also investigated the kinetics of HMGB1 oxidation and clearance in extracellular fluids from the culture of the prostate cancer cell line PC-3M (27). Extracellular fluids at different cell confluences (0, 30, 60, and 95%) were analyzed. Interestingly, the oxidation kinetics were found to strongly depend on the cell confluence. HMGB1 oxidation was slower at a higher confluency (Fig. 4). At the highest confluency, the half-life of all-thiol HMGB1 was longer than 3 h (Table 1), suggesting a more hypoxic environment in the extracellular fluid. In such a hypoxic environment (e.g., in cancer), slow oxidation can suppress the role of disulfide HMGB1.

**Impact of Exogenous Ligands on the Oxidation of HMGB1**—Since blockade of extracellular HMGB1 signaling was shown to be effective for suppressing tumor growth and metastases (20), a large number of strategies to block HMGB1 signaling have been proposed for therapeutics of cancer and inflammatory diseases (e.g., reviewed in Refs. 14, 15, and 39–43). Compounds that directly bind to HMGB1 were discovered previously. Among them are glycyrrhizin and heparin (44–46). Using extracellular fluids from PC-3M cell culture (95% confluency), we investigated the impact of these compounds on the oxidation and degradation of HMGB1. The dissociation constant \( K_d \) for the HMGB1-glycyrrhizin complex was reported to be 156 ± 3 \( \mu M \) (45). By NMR-based titration, we determined the \( K_d \) for the HMGB1-heparin octasaccharide complex to be 259 ± 72 \( \mu M \) (Fig. 5). For these ligands, we compared the kinetics of HMGB1 oxidation for free and ligand-bound HMGB1 in PC-3M extracellular fluids. In these experiments, the concentrations of glycyrrhizin and heparin were 2.0 and 2.5 mM, respectively, at which >90% of HMGB1 should be bound. The oxidation time course data for these samples are shown in Fig. 6, and the half-lives determined from the data are shown in Table 1. Although both glycyrrhizin and heparin are inactive in terms of thiol/disulfide redox chemistry, the binding of these compounds significantly affected the oxidation of HMGB1.

The binding of heparin was found to promote the oxidation by 4-fold. It is well known that HMGB1 undergoes conformational equilibrium between the open and closed states via intramolecular interactions between the C-terminal acidic tail and A/B-domains (34, 35). Because heparin is also highly negatively charged, HMGB1/heparin interactions may weaken intramolecular interactions between the A-domain and the acidic tail and increase the exposure of the pair of the Cys-23/Cys-45 thiol groups, thereby enhancing their oxidation. On the contrary, glycyrrhizin binding to HMGB1 was found to slow HMGB1 oxidation by −1.4-fold (Fig. 6). According to the NMR-based structure model of the HMGB1-glycyrrhizin complex of
Kinetics of HMGB1 Oxidation in Extracellular Fluids

Mollica et al. (45), glycyrrhizin bound to HMGB1 covers the regions of the Cys-23 and Cys-45 side chains, and so glycyrrhizin may block oxidant accessibility to the Cys-23/Cys-45 thiol groups. A computational study suggested that the HMGB1/glycyrrhizin interactions could be non-directional and dynamic (44), which may weaken the protection of the thiol pair from oxidants. Our present data suggest that non-redox-active ligands can regulate HMGB1 oxidation.

DISCUSSION

Relevance to Inflammation—All-thiol HMGB1 is considered to recruit leukocytes in inflammation (16, 23, 24). Venereau et al. (24) found that this role is associated with the binding of the all-thiol HMGB1-CXCL12 heterodimer to CXCR4 on leukocytes. In this study, we have shown that the half-life of all-thiol HMGB1 is only ~17 min in serum. Our data suggest that the half-life can become even shorter via interactions with heparan sulfate, which is structurally analogous to heparin and highly abundant on the cell surface. To recruit leukocytes, the binding of the all-thiol HMGB1-CXCL12 heterodimer to CXCR4 should occur rapidly before the HMGB1 molecule is oxidized. Heterodimer formation with CXCL12 may protect HMGB1 from oxidants. This is possible, as we found that the binding of ligands significantly modulates the kinetics of HMGB1 oxidation. Because of the short half-life of all-thiol HMGB1, most of the extracellular HMGB1 molecules at the injury site should be disulfide HMGB1 when leukocytes arrive as a result of the chemotactant activity of all-thiol HMGB1. Disulfide HMGB1, but not all-thiol HMGB1, is capable of activating the leukocytes and triggering the release of proinflammatory cytokines/chemokines (16, 23, 24, 26). In this sense, the short half-life of all-thiol HMGB1 in serum seems advantageous for the timely switching of HMGB1 function in inflammation.

Variations in the Half-lives of HMGB1 in Different Extracellular Fluids—We found large variations in the half-lives of HMGB1 in different extracellular fluids. For example, the half-life of all-thiol HMGB1 in PC-3M cell culture medium (95% confluency) was as long as ~3 h, whereas those in serum and saliva were ~17 min. Because all experiments were conducted under ambient oxygen pressure, the kinetic variation in HMGB1 oxidation can be largely due to different levels of redox factors intrinsically present in individual fluids. Our data also indicate that the half-lives of disulfide HMGB1 in serum and saliva differ by 10-fold, most likely due to different degrees of protease activity. These data suggest that the balance between the roles of all-thiol HMGB1 and disulfide HMGB1 depends significantly on the extracellular environment.

Potential Application of in Situ Protein NMR—This work has demonstrated that in situ protein NMR can provide important insights into the molecular behavior of a particular protein in actual extracellular fluids. The NMR data for 15N-labeled HMGB1 in human serum provided information specific to the behavior of HMGB1, although serum is a molecular crowding fluid with an enormous number of different proteins (60–80 mg/ml) and metabolites. Requiring only 0.50 ml of fluid, in situ protein NMR can potentially be used to investigate the molecular behaviors of extracellular proteins in various clinical specimens (e.g. serum, saliva, mucus, etc.). This approach is in contrast to NMR metabolomics, which focuses exclusively on the metabolite compositions of clinical specimens. Because of the orthogonal information they can provide, the combined use of NMR metabolomics and in situ protein NMR might allow more robust detection of abnormalities associated with diseases.

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Kinetics of HMGB1 Oxidation in Extracellular Fluids

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