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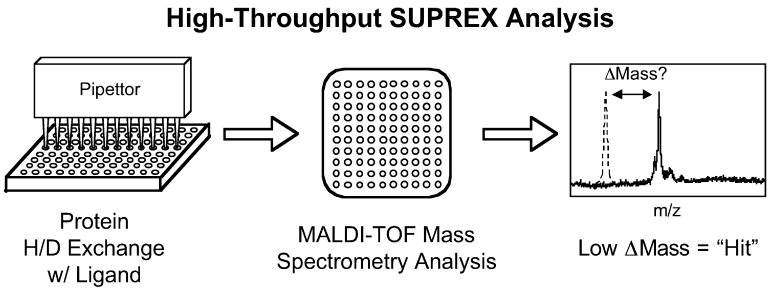
J. Comb. Chem., **2004**, 6 (2), 262-269• DOI: 10.1021/cc034051e • Publication Date (Web): 22 January 2004



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High-Throughput Screening Assay for the Tunable Selection of Protein Ligands

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Received October 1, 2003

Here, we describe a new protein−ligand binding assay that is amenable to high-throughput screening applications. The assay involves the use of SUPREX (stability of unpurified proteins from rates of H/D exchange), a new H/D exchange and mass spectrometry-based technique we recently developed for the quantitative analysis of protein−ligand binding interactions. As part of this work, we describe a new high-throughput SUPREX protocol, and we demonstrate that this protocol can be used to efficiently screen peptide ligands in a model combinatorial library for binding to a model protein system, the S-protein. The high-throughput SUPREX protocol developed here is generally applicable to a wide variety of protein ligands, including DNA, small molecules, metals, and other proteins. On the basis of the results of the model study in this work, one person with access to one MALDI mass spectrometer should be able to screen ∼10 000 compounds per 24-h period using the protocol described here. With full automation and the use of a commercially available MALDI mass spectrometer optimized for high-throughput analyses, we estimate that the SUPREX-based assay described here could be used to screen on the order of 100 000 ligands per day.

Introduction

Currently, the detection and quantification of protein−ligand binding affinities in high-throughput screening (HTS) assays is most often accomplished using spectroscopic methods. More recently, the speed, sensitivity, and generality of modern mass spectrometric methods have also been exploited in a growing number of HTS approaches for protein−ligand binding.[1−4] Spectroscopic methods have the disadvantage that they often require the introduction of a spectroscopic label into the covalent structure of the protein, ligand, or both. In addition, once a spectroscopic assay is developed for the detection of a specific protein−ligand system, it can be difficult to adapt it to another system involving a different protein or a different type of ligand. Many of the mass spectrometry-based HTS approaches used to date often involve different chromatographic techniques (e.g., affinity chromatography, size-exclusion chromatography, or affinity capillary electrophoresis). The chromatographic separation in such mass spectrometry-based HTS approaches can be time-consuming, and it can be problematic because the selection is not performed directly in solution (i.e., the ligand or protein is often covalently attached to the chromatographic support). More recently, mass spectrometric techniques for the gas-phase detection of protein−ligand complexes have been employed for HTS. The techniques must be highly optimized to ensure that the complex does not dissociate during the ionization process, and questions frequently arise about the relevance of gas-phase behavior to solution-phase binding affinity.[5−6]

Recently, we developed a new mass spectrometry-based method for the quantitative analysis of protein−ligand binding interactions in solution.[7−10] The method relies on a technique termed SUPREX to measure the increase in a protein's thermodynamic stability upon ligand binding (i.e., binding free energies).[7−14] We have shown that SUPREX-derived binding free energies can be used to determine solution-phase dissociation constants (K_d values) of protein−ligand systems with reasonable accuracy and good precision.[7−10] Several inherent advantages of SUPREX make it especially well-suited for use in HTS assays for the combinatorial analysis of protein−ligand binding properties. In particular, SUPREX is amenable to the analysis of protein−ligand systems involving a variety of different ligand classes (i.e., small molecules, peptides, oligonucleotides, and other proteins). It can be used to analyze complexes with a wide range of K_d values (i.e., K_d values from high micromolar to subnanomolar have been measured by SUPREX). Moreover, only picomole quantities of protein are required for analysis, and the protocol is amenable to automation and high-throughput analyses.

Here, we describe the use of SUPREX as a high-throughput, screening tool for the detection of protein−ligand binding in solution. In this proof-of-principle study, we demonstrate that SUPREX can be used to screen model combinatorial libraries of peptide ligands for binding to a small model protein, the S-protein. As part of this work, we describe a generic high-throughput SUPREX protocol for the direct screening of potential protein ligands from one-bead, one compound libraries.

Results and Discussion

General Strategy. The HTS protocol developed in this work is outlined in Scheme 1. The protocol involves the preparation of one-bead, one-compound libraries and the

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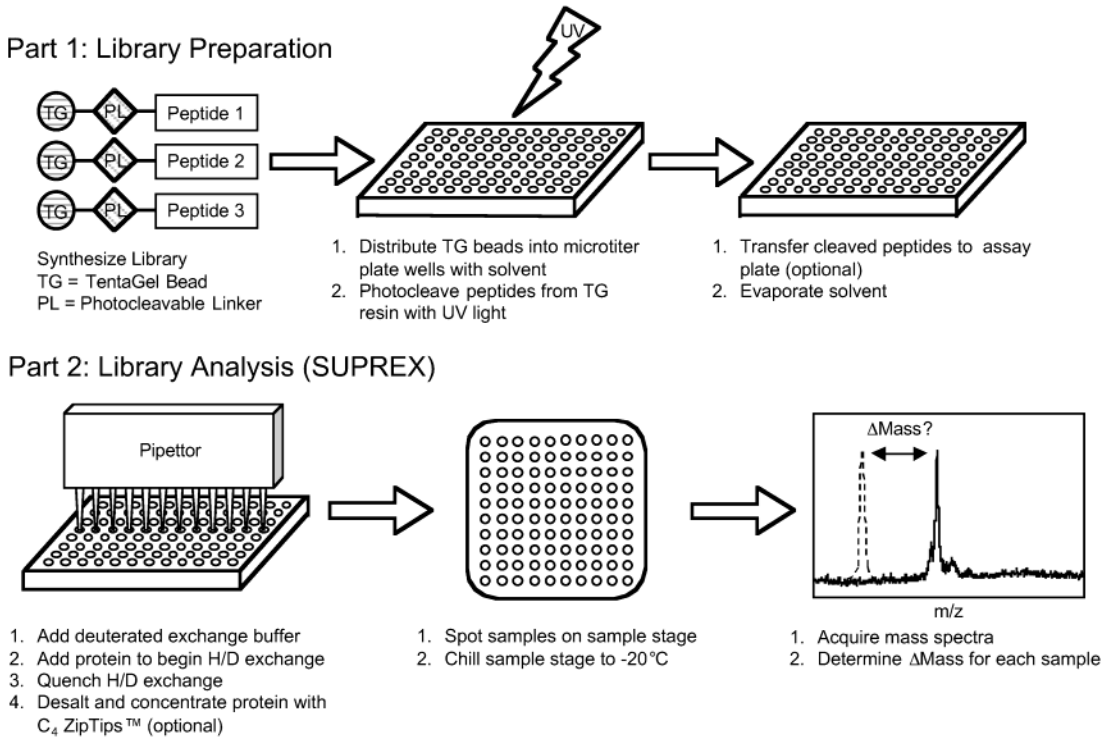
10.1021/cc034051e CCC: $27.50
Published on Web 01/22/2004

Scheme 1. High-Throughput Screening Protocol



subsequent distribution of each library member into the wells of a microtiter plate. Ultimately, the target protein is added to each well, and a single-point SUPREX analysis is performed on protein−ligand complexes in each microtiter plate well. The single-point SUPREX analysis is based on the principle that a protein's thermodynamic stability is increased upon ligand binding and that this increase in a protein's thermodynamic stability results in a shift of the protein's SUPREX curve to a higher urea concentration.[7−10] A higher concentration of urea is required to chemically denature the protein when it is complexed with ligand.

The effect that ligand binding has on a protein's SUPREX curve can be seen in Figure 1, which shows several theoretical SUPREX curves for the model protein system used in this study, S-protein (S-Pro). The theoretical SUPREX curves in Figure 1 were generated using eqs 1, 2, and 3 and using previously established thermodynamic parameters (i.e., ΔG_f, m, and K_d values) for the S-Pro system.[9] The same free ligand concentration (5 μM) and exchange time (30 min) were also used to generate each of the theoretical curves in Figure 1. Note that the SUPREX curve transition midpoints for the S-Pro−peptide complexes shifted to higher denaturant concentrations, as compared to the SUPREX curve transition for the S-Pro alone. The tighter binding peptide ligands produced larger shifts.

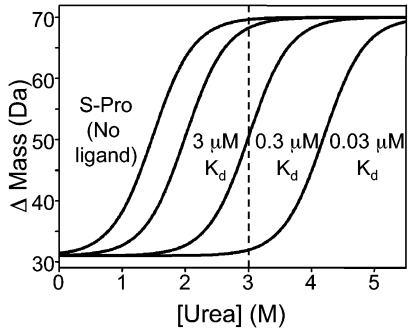


Figure 1. Theoretical SUPREX curves for the S-Pro system. SUPREX curves for S-Pro in the absence of ligand, for S-Pro in the presence of a 3 μM binder, for S-Pro in the presence of a 0.3 μM binder, and for S-Pro in the presence of a 0.03 μM binder are shown from left to right. The dotted line denotes the transition midpoint ($C^{1/2}_{SUPREX}$) of the S-Pro curve generated in the presence of the 0.3 μM binder (3.0 M urea).

The experimental determination of ΔG_f and K_d values by SUPREX requires that the transition midpoint of a protein's SUPREX curve ($C^{1/2}_{SUPREX}$) be evaluated. The determination of $C^{1/2}_{SUPREX}$ values in SUPREX experiments typically requires that at least 10 data points (i.e., Δmass measurements at a minimum of 10 different denaturant concentrations) be recorded. In theory, potential ligands in a combinatorial library could be analyzed by SUPREX for protein binding by evaluating a $C^{1/2}_{SUPREX}$ value for the protein in the presence of each ligand. Although this would ultimately permit the quantitative analysis of each ligand's binding affinity (i.e., a K_d value determination for each protein−ligand complex), it would be relatively time-consuming and require large amounts of each ligand in the library. One way to reduce both the analysis time and the required amount of each ligand is to perform a single-point SUPREX analysis.

In the single-point SUPREX analysis, a Δmass measurement is recorded at a single denaturant concentration in the SUPREX experiment. If the denaturant concentration is appropriately chosen, the magnitude of the resulting Δmass measurement can be used to evaluate the binding properties of a given ligand. This is illustrated with the theoretical data in Figure 1. The intersection of the dotted line and the SUPREX curves in Figure 1 reveals the expected Δmass

Table 1. Composition of Model Peptide Libraries

peptide no.	peptide sequence[a]	% of beads in model libraries[b]			
		A	B	C	D
1	ac-YETAAAKFERPHVDSG-NH_2	19	96		
2	KETAAAKFERQHADSG-NH_2	17		96	
3	ac-YETAAPKFERQHVDSG-NH_2	13			96
4	KETAAAKFERQHXDSG-NH_2	28			
5	ac-YETAAAKFERQHVDSGBG-NH_2	23	4	4	4
6	ac-YETAAAKFERQHVDS-NH_2				

[a] Abbreviations: ac- is an N-terminal acetylation, $-NH_2$ is a C-terminal amidation, X is norleucine, and B is β-alanine. [b] Based on the dry weight of the peptide-containing resin beads.

values in a hypothetical "one-point" SUPREX analysis performed at 3 M urea on the S-Pro when it is complexed with three different peptide ligands. Under the conditions of this hypothetical experiment, a Δmass value of 70 is expected for the S-Pro in the absence of ligand, and Δmass values 68, 50, and 32 Da are expected for the S-protein-peptide complexes with K_d values of 3, 0.3, and 0.03 μM, respectively.

In the hypothetical single-point SUPREX experiment described above, the only K_d value that can be determined accurately is the 0.3 μM K_d value. It is only for the S-Pro−peptide complex with this K_d value that the chosen [urea] corresponds to the $C^{1/2}_{SUPREX}$ value of the theoretical SUPREX curve. However, information about the relative K_d values of the other two S-Pro−peptide complexes can be ascertained from the Δmass values. Because the Δmass measured for the tight binding complex, 32 Da, is in the pretransition region of the SUPREX curve expected for this complex, it can be concluded that the curve's transition midpoint is shifted to a higher [urea]. Therefore, the K_d value for the S-Pro−peptide complex must be <0.3 μM. Similarly, because the expected Δmass for the weak binding complex, 68 Da, is in the posttransition baseline of the SUPREX curve expected for this complex, it can be concluded that the curve's transition midpoint is shifted to a lower [urea]. Therefore, the K_d value for this complex must be >0.3 μM.

We also note that the [urea] at which single-point SUPREX analyses are performed can be easily changed to alter the maximum and minimum K_d values expected for selected protein−ligand complexes. For example, if the [urea] used in the hypothetical single-point SUPREX experiment described above were changed from 3 to 2 M, it would be possible to select peptide ligands with K_d values <3 μM instead of 0.3 μM.

D/H Back-Exchange Correction. We found that it was important to apply a back-exchange correction to the Δmass values generated in our high-throughput single-point SUPREX analyses. Such a correction was necessary because ~25 min was required to deposit samples on the MALDI sample stage. This meant that the first samples deposited on the MALDI sample stage were typically exposed to ambient air ~25 min longer than the last samples deposited on the MALDI sample stages. During this 25-min time period, we found that a significant number of deuterons were back-exchanged with protons (see filled circles in Figure 2).

The data in Figure 2 were used in eq 4 to evaluate the back-exchange rate, k, under the quench conditions of our experiment (i.e., pH 2.5 and $T = 0$ °C). The back-exchange

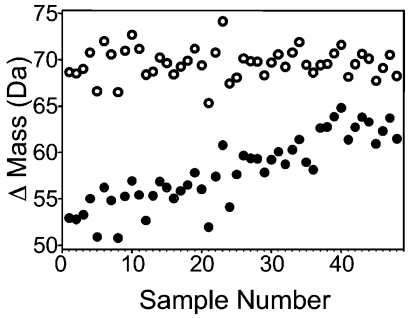


Figure 2. Raw Δmass data, filled circles, must be corrected for D to H back-exchange that occurs during the sample preparation protocol. The raw data were corrected using eqs 5 and 6, and the corrected data are displayed as open circles. The observed scatter of the corrected data is consistent with the expected mass accuracy of our MALDI-TOF instrument (~150 ppm at 11.5 kDa).

rate that we determined was 4.6×10^{-4} s^{-1}. This rate is in reasonably good agreement with the rate estimated for S-Pro under the same conditions using the program SPHERE (i.e., 1.9×10^{-4} s^{-1}).[15−17] With the back-exchange rate in our experiments established, eq 5 was used to correct the measured Δmass values in Figure 2 (see the open circles) and generate $\Delta mass_{corr}$ values. The random scatter of the corrected $\Delta mass_{corr}$ values in Figure 2 (open circles) around ~70 Da indicates that the applied correction was sufficient to account for all of the back-exchange observed in our experiments. Other studies have noted that back-exchange during mass spectral acquisition can be significant;[18,19] however, chilling the sample stage to −20 °C prior to analysis effectively eliminates this source of back-exchange during the timecourse of our experiment.

We note that the back-exchange correction described above is not typically required in conventional SUPREX analyses. This is because the ~10 samples required to generate a conventional SUPREX curve can be applied to the MALDI sample stage at approximately the same time, the samples can be dried at the same time, and the samples can be easily analyzed in less than ~10 min after their introduction into the MALDI instrument. Therefore, the number of protein deuterons exchanged for protons in the back-exchange reaction is constant and relatively small compared to the amplitude of a typical SUPREX curve.

Model Library Screening. Four model peptide libraries (see Table 1) were constructed to assess the utility of the HTS assay outlined in Scheme 1. Model library A consisted of roughly equal numbers of beads containing peptides 1−5. It was used to evaluate the ability of our HTS assay to select ligands with varying protein binding affinities. Model

Table 2. Summary of HTS Results from Model Library A Using a One-Bead-Per-Well Format

peptide no.	SUPREX K_d (μM)[a]	2.0 M urea selection[b] ($K_d \leq \sim 3\ \mu$M)	3.0 M urea selection[b] ($K_d \leq \sim 0.3\ \mu$M)	3.5 M urea selection[b] ($K_d \leq \sim 0.1\ \mu$M)
1	>1000	0 (17)	0 (18)	0 (16)
2	250	0 (12)	0 (13)	0 (12)
3	9.7	0 (21)	0 (7)	0 (13)
4	0.40	17 (17)	15 (18)	0 (25)
5	0.034	21 (21)	26 (26)	15 (20)

[a] K_d values were determined in ref 9 for a series of peptides that were nearly identical to the peptides analyzed in this work. On the basis of results presented in refs 31−33, the small differences between the peptide sequences in this work and those in ref 9 are not expected to significantly alter the K_d values. [b] Results are reported as *X* (*Y*) where *X* is the number of wells containing the given peptide that resulted in an assay hit, and *Y* is the total number of wells analyzed that contained the given peptide.

libraries B, C, and D each contained a small number of peptide 5-containing beads and a large number of beads containing either peptides 1, 2, or 3 (respectively). Libraries B, C, and D were designed to evaluate whether tight binding ligands (i.e., peptide 5) could be detected in libraries containing a large number of relatively weak binding ligands.

Initially, peptides from model library A were screened for S-Pro binding according to Scheme 1. A total of three 96-well microtiter plates containing random peptides from library A were analyzed in single-point SUPREX experiments that employed a deuterated exchange buffer containing either 2.0, 3.0, or 3.5 M urea. These urea concentrations correspond to the $C^{1/2}_{\mathrm{SUPREX}}$ values predicted by eq 2 for S-Pro−peptide complexes with K_d values of 3, 0.3, and 0.1 μM, respectively. In these initial experiments, the peptide material in each well came from a single resin bead. Representative $\Delta\mathrm{mass}_{\mathrm{corr}}$ data that we collected in these experiments are summarized in Figure 3. The single-point SUPREX experiments in Figure 3A, B, and C were designed to select for peptide ligands binding to the S-Pro with K_d values $\leq \sim 3$, 0.3, and 0.1 μM, respectively. The hits in these experiments were defined as all microtiter plate wells in which the $\Delta\mathrm{mass}_{\mathrm{corr}}$ values differed from the average $\Delta\mathrm{mass}_{\mathrm{corr}}$ values of the negative controls (see the Experimental Section) by more than 2.5 SD. Ultimately, the identity of the hits and nonhits in each microtiter plate were determined by recording the molecular weight of the peptide in each well using MALDI-TOF-MS.

The hits and non-hits from our experiments are summarized in Table 2. No false positives were detected in our experiments. However, several false negatives were detected in the single-point SUPREX experiments at 3.0 and 3.5 M urea. As expected, the peptides missed in our selections were the peptides with K_d values close to (i.e., within 3-fold of) the K_d value for which the selection was designed. In cases in which the K_d value of the ligand is close to the K_d value of the selection, the observed Δmass value can be very sensitive to the ligand concentration in the assay. The K_d values of the selections in this work were determined assuming a total ligand concentration of 6 μM in each assay. This was the total ligand concentration expected in each assay on the basis of our measured photocleavage yields (see the Experimental Section).

In our assay, false negatives can be produced in wells where the total ligand concentration is less than the expected 6 μM. The few false negatives that we observed in our experiments are likely due to low peptide concentrations.

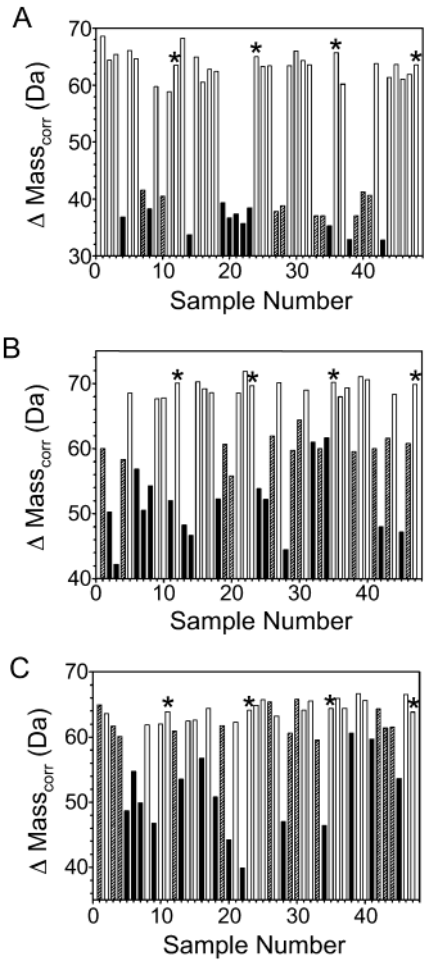


Figure 3. Typical screening results obtained from the analysis of model library A. Results from single-point SUPREX analyses at 2.0, 3.0, and 3.5 M urea are shown in A, B, and C, respectively. Open bars topped by an asterisk represent the negative controls (S-Pro in the absence of peptide); all other open bars represent microtiter plate wells that contained peptides 1, 2, or 3. Gray bars represent wells that contained peptide 4, and black bars represent wells that contained peptide 5. Peptide identities were determined by MALDI-TOF-MS mass measurements only after the screening analysis was performed.

Such low peptide concentrations could have resulted from reduced peptide synthesis yields or reduced photocleavage yields on some beads. However, it is important to emphasize that these apparent bead-to-bead variations only affected the selection of peptide ligands with K_d values close to (i.e., within 2−3-fold of) the K_d value of the selection. In addition, even in these cases, our results indicate that the false negative rate in this work was < 25%.

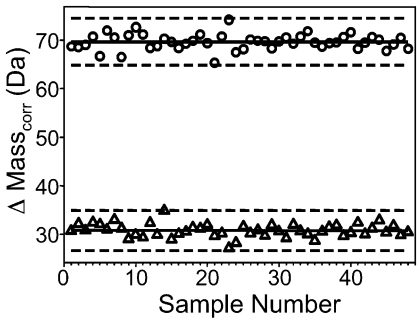


Figure 4. Screening results obtained from the analysis of a series of negative controls (open circles; S-Pro in the absence of peptide) and a series of positive controls (open triangles; S-Pro in the presence of 10 μM peptide 6). For each data set, the solid line marks the mean and the dotted lines represent plus and minus 3 SD from the mean.

The peptides from model libraries B, C, and D were also screened for S-Pro binding. The protocol used to screen each library was identical to the protocol described above for the analysis of model library A, with the exception that the peptide material in each well was derived from multiple resin beads (usually between 5 and 10 beads). This meant that peptide material from ~480 to 960 randomly chosen beads from each library was analyzed in a series of 96 single-point SUPREX experiments using 3.0 M urea. A total of 39 microtiter plate wells were identified as containing a hit in the these experiments with libraries B, C, and D. It was confirmed by MALDI-TOF-MS that peptide 5 was present in all but 2 of the 39 wells that were identified as hits; therefore, only 2 false positives appeared in our screen, and these false positives appeared only in our analysis of library D. Interestingly, each of the two wells in which the false positives appeared contained peptide material with an unexpected mass of 1911 Da. Thus, one explanation for the two false positives we detected in our screening experiment may be that this unknown peptide material actually binds to S-Pro, resulting in a complex with a K_d value of <0.3 μM.

Overall, our screening results with libraries B, C, and D suggest that the HTS strategy described here is amenable to multiplex analyses containing up to 10 unique ligands per microtiter plate well. The interaction of S-Pro with a small amount (6 μM) of a tight binding ligand was readily detected in the presence of an excess (60 μM) of weak binding ligands. The K_d differences between the strongest and weakest ligands in model libraries B, C, and D were 29 000-, 7400-, and 280-fold, respectively.

***Z′* Test.** The back-exchange corrected results from both negative and positive controls are shown in Figure 4. The single-point SUPREX data shown for the negative controls (S-Pro without ligands, open circles) and for the positive controls (S-Pro plus 10 μM peptide 6, open triangles) was obtained using an H/D exchange time of 30 min and a deuterated 3.0 M urea buffer. The negative and positive control data sets resulted in average Δmasses of 69.6 and 30.8 Da with standard deviations of 1.6 and 1.4 Da, respectively. Analysis of the results with eq 6 resulted in a Z' of 0.77. Screening techniques with a Z' value of >0.5 are generally deemed appropriate for large-scale HTS applications. Therefore, our results suggest that the single-point SUPREX strategy experiment is well-suited for large-scale HTS applications.

Throughput. In the single-point SUPREX strategy described here, a series of pipetting steps are required to initiate the H/D exchange reaction, to quench the H/D exchange reaction, to desalt the sample, and to spot the sample on the MALDI sample stage. In our work, these pipetting steps were performed manually using a 12-channel pipettor, and it took ~65 min to process the samples in 48 wells of a microtiter plate. Thus, samples from more than 1000 microtiter plate wells could be prepared by a single individual for MALDI analyses in a 24-h period. This is about one-half of the maximum throughput of the MALDI instrument used in this work. As part of this work, we have determined that single-point SUPREX experiments can be performed using as many as 10 beads/well. Therefore, it would be relatively straightforward to screen 10 000 ligands/day for protein binding using the HTS strategy described here.

We note that the speed of the single-point SUPREX experiments described here could possibly be enhanced to permit the screening of >100 000 compounds/day/mass spectrometer. We estimate that the use of a liquid-handling robot for the pipetting steps instead of manual pipetting could potentially increase the number of samples prepared per day from 10 000 to 100 000/day. This number of samples per day could easily be analyzed using a MALDI-TOF instrument equipped with a high-repetition-rate (e.g., 200 Hz) laser for high-throughput analyses.

Scope. Successful SUPREX analyses require that the protein under study exhibit so-called EX2 exchange behavior (i.e., the protein's folding rate must be greater than the intrinsic chemical exchange rate of the amide protons in the protein).[20] Such EX2 exchange behavior is also a requirement for the single-point SUPREX analyses described in this work. We note that the experimental conditions (i.e., buffer pH and temperature) employed in SUPREX analyses can often be chosen to ensure that the protein under study exhibits EX2 exchange behavior.

Conventional SUPREX analyses of protein−ligand complexes to derive K_d values require that the protein and the protein−ligand complex under study exhibit reversible, two-state folding properties (i.e., partially folded intermediates are not populated in the equilibrium unfolding reaction). This assumption of two-state folding behavior is important for the calculation of K_d values from changes in a protein's $C^{1/2}_{SUPREX}$ value upon ligand binding. We note that this assumption was used to generate the theoretical SUPREX curves for the S-Pro and S-Pro−peptide complexes in Figure 1. The results of our previous biophysical studies on the S-Pro system are consistent with the protein's equilibrium unfolding properties' being well-modeled by a two-state process.[9] We should note, however, that such two-state folding is not a necessary prerequisite for the HTS assay described here. The main requirement for the HTS assay described here is that there must be a measurable shift in a protein's $C^{1/2}_{SUPREX}$ value upon ligand binding. We have previously shown that such $C^{1/2}_{SUPREX}$ value shifts can be observed in the SUPREX analyses of multistate protein folding and ligand binding reactions.[10]

The work described here was focused on the selection of tight-binding peptide ligands to the S-Pro; however, we note that the single-point SUPREX assay described here is not limited to the selection of peptide ligands. One advantage to using the single-point SUPREX assay as an HTS tool is that it can be used to select a wide range of structurally diverse ligands, including small molecules, nucleic acids, peptides, and even other proteins.[7−10] There are few HTS assays that display this generality toward ligand type. The technique is also applicable to the selection of ligands with a wide range of binding affinities. In theory, there is no lower boundary to the range of ligand K_d values that can be selected for in the assay. However, the assay does require that there be an excess of ligand over protein and that the ligand concentration in the assay be greater than the K_d value being selected. In this work, the amount of material released from each bead (~60 pmol) and the assay volume (10 μL) dictated that only ligands with K_d values less than ~6 μM could be efficiently selected. However, we note that the use of larger resin beads and the use of other parallel synthesis techniques can be used to generate ligand libraries containing larger amounts of material.

Conclusions

We have demonstrated that a single-point SUPREX protocol can be used to detect peptide binding in the S-Pro system in a high-throughput fashion. We have also shown that such a single-point SUPREX protocol is amenable to the screening of one-bead, one-compound combinatorial libraries. In addition, the technique can be used in a multiplex fashion (multiple ligands per microtiter plate well). The single-point SUPREX protocol described here is a general technique with respect to the type of ligand being screened, and it can potentially be used to screen over 100 000 compounds/day.

Experimental Section

Reagents. Deuterium oxide (D_2O; 99.9% atom D), deuterium chloride (20 wt % in D_2O, 99.5% atom D), sodium deuterioxide (40 wt % in D_2O, 99.9% atom D), piperidine, diisopropyl ethylamine (DIEA), and triisopropyl silane (TIS) were purchased from Aldrich. Urea was purchased from either Mallinckrodt (ACS grade) or ICN Biomedicals (Ultrapure). Deuterated urea (urea-d_4) was prepared by repeated dissolution and lyophilization of fully protonated urea in D_2O until the calculated deuterium content was >99%. Sinapinic acid (SA) and α-cyano-4-hydroxycinnamic acid (CHCA) were obtained from either Aldrich or Sigma. Trifluoroacetic acid (TFA) was from Halocarbon, and acetonitrile (MeCN) and methanol (MeOH) were from Fisher. Dimethyl formamide (DMF) was from J. T. Baker. Bovine pancreatic ribonuclease A (RNase A), subtilisin Carlsberg, and hen egg white lysozyme were from Sigma.

General Methods and Instrumentation. MALDI mass spectra were acquired on a Voyager DE Biospectrometry Workstation (Perseptive Biosystems). Spectra were collected in the linear mode using a nitrogen laser (337 nm, 3 Hz). SUPREX samples were prepared for MALDI analysis as described below. Either SA or CHCA was used as the matrix in the MALDI analyses in this work. Positive ion mass spectra were collected in the autosampler mode using the following parameters: 25-kV acceleration voltage, 23.25−23.50-kV grid voltage, 75-V guide wire voltage, and 225-ns delay time. Each mass spectrum represents the sum of the data obtained from between 13 and 25 laser shots. Raw MALDI spectra were processed with an in-house Microsoft Excel macro that performed the following operations: a 19-point floating average smoothing of the data, a two-point mass calibration of the spectra using the protein ion signals from the internal mass calibrants, and a center of mass determination for the protein's $[M + H]^{1+}$ peak.

A Hewlett-Packard 8452A diode array UV/vis spectrophotometer was used for protein concentration determinations. S-Pro concentrations were determined using absorbance measurements at 280 nm ($\epsilon_{280} = 9800\ M^{-1}\ cm^{-1}$).[21] Urea concentrations were determined with a Bausch & Lomb refractometer as described.[22] pH measurements were performed with a Jenco 6072 pH meter equipped with a Futura calomel pH electrode from Beckman Instruments. To correct for isotope effects, the measured pH of each D_2O solution was converted to pD by adding 0.4 to the measured pH value.[23]

Protein and Peptide Samples. RNase S was prepared from RNase A by using subtilisin Carlsberg to selectively cleave the peptide bond between residues 20 and 21 of RNase A, as previously described.[24] The two major peptide fragments formed in the proteolysis reaction, S-Pro and S-Peptide, were separated as described elsewhere.[9] S-Pro was folded by dissolution of the pure, lyophilized product in a 50 mM sodium acetate buffer (pH 6.0) containing 100 mM NaCl.

The six peptides used in this study are summarized in Table 1. Peptides 1−5 were prepared on 130-μm TentaGel S NH_2 resin (Rapp Polymere) using manual SPPS protocols for fluorenylmethoxycarbonyl (Fmoc)-based peptide synthesis.[25] Prior to the assembly of each peptide on the TentaGel resin, a photolinker, 4-{4-[1-(fmoc-amino)ethyl]-2-methoxy-5-nitrophenoxy}butanoic acid (Novabiochem), was reacted with the resin for 24 h in the dark. In this reaction, 500 mg of the resin was combined with 0.34 mmol of the linker that was preactivated in 5 mL of DMF containing 0.30 mmol of HBTU and 0.81 mmol of DIEA.

Peptides 1−5 were assembled on the photolinker-containing resin in stepwise fashion under low-light conditions. Note that the five peptides in this work were prepared in parallel syntheses. After peptides 1−5 were assembled, the peptide containing resin from each synthesis was dried under vacuum, and side chain deprotection of the resin-bound peptides was accomplished by the addition of 95/2.5/2.5 TFA/H_2O/TIS (v/v/v). After 3 h, the peptide resin from each synthesis was flow-washed with DMF, MeOH, H_2O, MeOH, and DMF (in that order) before each batch of resin was stored in DMF at 4 °C in the dark until needed. After photolysis (see below), the identity of each peptide was confirmed by a MALDI-TOF-MS measurement of its mass.

Resin beads containing peptides 1−5 were combined in various ratios to generate the model peptide libraries in this

work (see Table 1). The resin beads in the resulting libraries were then manually dispensed into 384 well microtiter plates, and the peptides were liberated from their solid supports during a 3-h irradiation with 366-nm light from a hand-held UVGL-55 ultraviolet lamp (Ultraviolet Products) in the presence of ~20 μL of a 200 mM ammonium acetate solution containing 20% ethanol (the uncorrected pH was ~7). The UV lamp was positioned ~4 cm above the 384-well plate. The irradiated solutions were transferred into 96-well microtiter plates, and the solvent was evaporated at 50 °C overnight. One out of every 12 wells in each 96-well microtiter plate contained just 20 μL of the buffer (200 mM ammonium acetate, 20% ethanol, pH 7) and not peptide. These wells were used as the negative controls in the single-point SUPREX experiments described below.

Peptide 6 was synthesized in our laboratory using standard methods for manual solid-phase peptide synthesis (SPPS) and in situ neutralization protocols for *tert*-butoxycarbonyl (Boc) chemistry as described elsewhere.[26] Peptide 6 was purified by RP-HPLC.

Photocleavage Yield. The amount of peptide material liberated from an individual resin bead upon photolysis was estimated using two different methods. In one method, a Gly residue was coupled to the photolinker resin, the succinimidyl ester of carboxyfluorescein (Molecular Probes) was coupled to the Gly residue (Gly-Fluor beads), and the resin was dried under vacuum. Several ~1-mg portions of the dried Gly-Fluor beads were exactly weighed out in 0.5-mL Eppendorf tubes. The resin in each tube was allowed to swell in 40 μL of a 20 mM sodium phosphate buffer (pH 6.0) before it was irradiated from above (tube caps open) with 366-nm light from the UVGL-55 lamp for various lengths of time. After the irradiation, 40 μL of DMF was added to each tube, and the tubes were sonicated at 50 °C for 30 min. An aliquot of this solution was diluted ~30-fold into a 50 mM potassium phosphate buffer (pH 9.0), and the absorbance of the resulting solution at 495 nm was used to calculate the amount of fluorescein released ($\epsilon_{495} = 7.52 \times 10^4$ $M^{-1}cm^{-1}$).[27]

The second method employed to quantify the amount of material liberated from an individual resin bead upon photolysis involved subjecting resin beads containing peptide 5 to the same photolysis procedure described above for the Gly-Fluor beads, and then performing a ninhydrin test on the solution to determine the amount of peptide that was liberated into the solution.[28] A ninhydrin test was also performed on the peptide 5-containing resin beads prior to photolysis as a control to establish the amount of peptide 5 initially on the resin.

SUPREX Sample Preparation and Data Collection. All pipetting steps in the high-throughput analyses described here were performed manually with a 12-channel pipettor. Initially, 9 μL of a deuterated exchange buffer was added to the peptide-containing wells of the microtiter plates prepared above. The deuterated buffers in this work contained 50 mM sodium acetate and 100 mM NaCl at a pD of 6.0; the urea-d_4 concentration was either 2.2, 3.3, or 3.9 M. The microtiter plate was placed in a 10 °C water bath and allowed to equilibrate for at least 20 min. The H/D exchange reaction of the S-Pro was initiated by the addition of a 1-μL aliquot of a fully protonated 10 μM S-Pro solution to each well in the microtiter plate. Note that the final concentration of urea in the buffers after the addition of protein was 2.0, 3.0, or 3.5 M. The 12-channel pipettor used in this work permitted the transfer of S-Pro to the microtiter plates in a row-by-row fashion. The addition of S-Pro to the first four rows of the 96-well microtiter plate was staggered by 8.5 min to allow ample time for subsequent sample manipulations. After 30 min of H/D exchange, 20 μL of ice-cold 0.5% TFA was added to each well in a row-by-row fashion in order to quench each exchange reaction. We note that the manual pipetting steps employed in this work permitted the preparation of only 48 samples (i.e., one-half of a 96-well microtiter plate) at a time.

The S-Pro samples in each microtiter plate well were desalted and concentrated using C_4 ZipTips (Millipore) as described elsewhere.[11] The S-Pro was eluted from the ZipTip into the wells of an ice-cold microtiter plate using 7 μL of an ice-cold solution of H_2O/MeCN/TFA (28/72/0.1, v/v/v). Ultimately, a 2-μL aliquot of the ZipTip eluent was spotted on an ice-cold MALDI sample stage containing a series of previously dried 2-μL spots from an internal standard containing matrix solution (i.e., a saturated solution of SA in 55/45/0.1 H_2O/MeCN/TFA containing lysozyme as an internal calibrant). Solvent evaporation was assisted with the gentle flow of air from a small (6-in.-diameter) tabletop fan. The fan also prevented condensation from forming on the top of the chilled MALDI sample stage, although we note a small amount of condensation did form on the underside of the sample stage. After the spots were dry (~5 min), the underside of the MALDI sample stage was wiped dry, sealed inside a ZipLock bag containing a drying agent (DrieRite), and placed in a −20 °C freezer for at least 1 h.

Chilling the MALDI sample stage prior to analysis was critical in the experiments described here because it eliminated the back-exchange of deuterons to protons that can occur in the MALDI source chamber during the acquisition of mass spectra.[18,19] In addition, chilling the MALDI plate also allowed mass spectral analyses to be performed at a future time without any significant back-exchange. MALDI samples prepared as described above could be stored at −20 °C for at least 24 h with no detectable back-exchange.

Theoretical SUPREX Curves. The theoretical SUPREX curves in this work were generated using eqs 1, 2, and 3 (below) and using the data from ref 9.

$$\Delta\text{mass} = \Delta M_0 + \frac{a}{1 + e^{-([\text{denaturant}] - C_{\text{SUPREX}}1/2/b)}} \qquad (1)$$

In eq 1, Δmass is the difference between the measured protein mass and the fully protonated protein mass, ΔM_0 is the change in mass measured before the globally protected hydrogens in the protein exchanged with deuterons (31 Da); a is the amplitude of the curve (39 Da); [denaturant] is the molar denaturant concentration; $C^{1/2}{}_{\text{SUPREX}}$ is the [denaturant] at the transition midpoint of the curve, and b is a parameter that describes the steepness of the transition (0.325 M).

$$RT\left[\ln\frac{\left(\frac{\langle k_{int}\rangle t}{0.693}-1\right)}{\left(\frac{n^n}{2^{n-1}}[P]^{n-1}\right)}\right] = -mC^{1/2}_{SUPREX} - \Delta G_f \qquad (2)$$

In eq 2, which is derived in ref 9, R is the gas constant, T is the temperature in Kelvin (K was 283 in all the experiments described here), $\langle k_{int}\rangle$ is the average intrinsic exchange rate of an amide proton which can be estimated for a given set of experimental conditions (i.e., temperature and pH) and for a given amino acid sequence on the basis of model dipeptide data (a $\langle k_{int}\rangle$ value of 0.234 s^{-1} was used for all calculations in this work on the S-Pro), t is the H/D exchange time (t was1800 s in all the experiments desribed here), n is the number of subunits in the protein ($n = 1$ for the S-Prot system), [P] is the protein concentration expressed in n-mer equivalents, m is physically related to the change in solvent accessible surface area upon unfolding and is defined as $\delta\Delta G_f/\delta$[denaturant], and ΔG_f is the free energy of folding in the absence of denaturant. We note that eq 2 is valid only for proteins that exhibit reversible, two-state equilibrium unfolding behavior, for cases in which the product $\langle k_{int}\rangle t$ is >0.693, and for finite values of t such that $C^{1/2}{}_{SUPREX}$ values are ≥ 0 M denaturant.

$$K_d = [L]/(e^{-\Delta\Delta G_f/nRT} - 1) \qquad (3)$$

In eq 3, [L] is the concentration of free ligand (5 μM), n is the number of independent binding sites (1), and $\Delta\Delta G_f$ is the change in folding free energy upon peptide binding.[29]

Back-Exchange Correction. Equation 4 was used to determine the back-exchange rate in this work.[18]

$$\Delta mass_{meas} = B_1 + B_2 e^{-kt_{meas}} \qquad (4)$$

In eq 4, $\Delta mass_{meas}$ is the mass difference between the measured protein mass and the fully protonated protein mass, B_1 represents the deuterons that are not susceptible to back-exchange, B_2 represents the deuterons that are susceptible to back-exchange, k is the rate constant for back-exchange that occurs while the MALDI sample stage is exposed to ambient air, and t_{meas} is the amount of time samples remain exposed to ambient air. After B_1, B_2, and k are determined, the results from any experiment can be corrected using eq 5.[18]

$$\Delta mass_{corr} = B_2(e^{-kt_{corr}} - e^{-kt_{meas}}) + \Delta mass_{meas} \qquad (5)$$

In eq 5, B_2, k, t_{meas}, and $\Delta mass_{meas}$ are the same as in eq 4; $\Delta mass_{corr}$ is the corrected Δmass; and t_{corr} is the common time to which all the results are corrected ($t_{corr} = 0$ in this work).

***Z′* Test.** Equation 6 was used to help validate our SUPREX-based HTS protocol.[30]

$$Z' = 1 - (3\sigma_{c+} + 3\sigma_{c-})/(\mu_{c+} - \mu_{c-}) \qquad (6)$$

In eq 6, σ_{c+} is the standard deviation of the positive control (protein plus ligand), σ_{c^-} is the standard deviation of the negative control (protein without ligand), μ_{c^+} is the mean of the positive control, and μ_{c^-} is the mean of the negative control.

Acknowledgment. Financial support for this work was from a PECASE Award (NSF-CHE-00-94224) to M.C.F.

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CC034051E