A new method is described for isolating and identifying proteins participating in protein-protein interactions in a complex mixture. The method uses a cyanogen bromide-activated Sepharose matrix to isolate proteins that are non-covalently bound to other proteins. Because the proteins are accessible to chemical manipulation, mass spectrometric identification of the proteins can yield information on specific classes of interacting proteins, such as calcium-dependent or substrate-dependent protein interactions. This permits selection of a subpopulation of proteins from a complex mixture on the basis of specified interaction criteria. The new method has the advantage of screening the entire proteome simultaneously, unlike the two-hybrid system or phage display, which can only detect proteins binding to a single bait protein at a time. The method was tested by selecting rat brain extract for proteins exhibiting calcium-dependent protein interactions. Of 12 proteins identified by mass spectrometry, eight were either known calcium-binding proteins or proteins with known calcium-dependent protein interactions, indicating that the method is capable of enriching a subpopulation of proteins from a complex mixture on the basis of a specific class of protein interactions. Because only naturally occurring interactions of proteins in their native state are observed, this method will have wide applicability to studies of protein interactions in tissue samples and autopsy specimens, for screening for perturbations of protein-protein interactions by signaling molecules, pharmacological agents or toxins, and screening for differences between cancerous and untransformed cells. Molecular & Cellular Proteomics 1:253–259, 2002.

Protein-protein interactions underlie a vast number of physiological processes. Cellular processes such as neuronal signaling, cell development, and growth all depend on a complex network of protein-protein and protein-small molecule interactions in the cell. These interactions may be categorized as constitutive interactions, such as between subunits of hemoglobin, and signal-dependent interactions, such as those between the subunits of cAMP-dependent protein kinase or the subunits of GTP-binding proteins. The complexity of the task of investigating these interactions is evident from the potential number of protein interactions; comprehensively screening binary interactions among 15,000 proteins would require testing $2.25 \times 10^6$ pairwise combinations of proteins. This complexity means conventional biochemical methods are of limited use. Despite intensive research, there is still no satisfactory method for systematically studying protein interactions in mammalian cells or other complex mixtures of proteins.

A number of techniques have been used to study individual protein-protein interactions, including protein cross-linking (1–3), green fluorescent protein (4, 5), phage display (6, 7), the two-hybrid system (8), protein arrays (9), fiber optic evanescent wave sensors (10, 11), chromatographic techniques (12), and fluorescence resonance energy transfer (13, 14). However, these methods are generally only useful for screening one bait protein at a time.

With the aid of high throughput robotics, the two-hybrid system has also been adapted for proteomic screening. In these experiments (15, 16), a Gal4 library of 6000 yeast proteins was partitioned into separate wells of 96-well plates, and each well was screened against an activation domain library, yielding as many as 4,549 possible interactions among yeast proteins.

Unfortunately, this technique is infeasible for screening mammalian proteins, because it would result in a large proportion of nonspecific interactions. Mammalian proteins are normally expressed in a variety of compartmentalized subcellular organelles and in specific cell types and are extensively post-translationally modified in a tissue-specific manner. There is no technology available for performing a two-hybrid analysis on a tissue sample. Thus, natural protein interactions associated with physiological processes such as learning or Alzheimer’s disease, or interactions resulting from signaling, cannot be studied with this technique.

Here, a new method is described that allows screening of both constitutive and signal-mediated protein-protein interactions. The new method has several advantages. 1) It is a protein-based technique and does not require cloning but uses the native proteins in their native, folded states, which are properly post-translationally modified. 2) The interactions are accessible to chemical manipulation, permitting interesting subpopulations of protein-protein interactions, such as calcium-dependent protein-protein interactions, to be easily
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studied. 3) Perturbations of protein-protein interactions by pharmacological agents or toxins, or differences between cancerous and untransformed cells, can also be screened. 4) If an appropriate control group is used, only naturally occurring protein interactions are observed. Non-physiological interactions are eliminated from analysis, because non-physiological interactions are identical in both groups. This method was tested by investigating calcium-dependent protein interactions.

EXPERIMENTAL PROCEDURES

Titration of CNBr-Sepharose—Cyanogen bromide activated Sepharose 4B (Amersham Biosciences) was rehydrated and washed three times with water before use. CNBr-Sepharose was titrated with rat brain extract by incubating a fixed quantity of extract at room temperature with varying amounts of CNBr-Sepharose. After 1 h, samples were centrifuged, and the unbound protein was measured using a dye-binding assay (17), and the quantity of CNBr-Sepharose to reduce the protein concentration by 50% was calculated.

Isolation of Interacting Proteins—One rat brain was homogenized by sonication in 10 mM NaHCO₃ pH 7.7, containing 5% CHAPS, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM CaCl₂, and centrifuged at 100,000 × g for 20 min. A quantity of rehydrated CNBr-Sepharose sufficient to bind 50% of the protein was added, and the sample was shaken at room temperature for 1 h. Tris acetate was then added to 0.1 M to block unreacted CNBr, and incubation was continued for another 30 min. The mixture was transferred to a small chromatography column and washed extensively with 100 mM Tris acetate containing 1 mM CaCl₂. When the A₂₈₀ of the eluate reached zero, the proteins retained by calcium-dependent interactions were eluted with 25 mM EGTA, desalted, and concentrated in a Centricon-3 ultrafiltration device, mixed 1:1 with IEF sample buffer (8.5 M urea, 2 M thiourea, another 30 min. The mixture was transferred to a column and washed with 100 mM Tris-HCl containing 1 mM CaCl₂, desalted, and concentrated in a Centricon-3 ultrafiltration device, mixed 1:1 with IEF sample buffer (8.5 M urea, 2 M thiourea, another 30 min. The mixture was transferred to a column and washed with 100 mM Tris-HCl containing 1 mM CaCl₂, desalted, and concentrated in a Centricon-3 ultrafiltration device, mixed 1:1 with IEF sample buffer (8.5 M urea, 2 M thiourea, another 30 min. The mixture was transferred to a column and washed with 100 mM Tris-HCl containing 1 mM CaCl₂, desalted, and concentrated in a Centricon-3 ultrafiltration device, mixed 1:1 with IEF sample buffer (8.5 M urea, 2 M thiourea, another 30 min. The mixture was transferred to a column and washed with 100 mM Tris-HCl containing 1 mM CaCl₂, desalted, and concentrated in a Centricon-3 ultrafiltration device, mixed 1:1 with IEF sample buffer (8.5 M urea, 2 M thiourea, another 30 min. The mixture was transferred to a column and washed with 100 mM Tris-HCl containing 1 mM CaCl₂, desalted, and concentrated in a Centricon-3 ultrafiltration device, mixed 1:1 with IEF sample buffer (8.5 M urea, 2 M thiourea, another 30 min. The mixture was transferred to a column and washed with 100 mM Tris-HCl containing 1 mM CaCl₂, desalted, and concentrated in a Centricon-3 ultrafiltration device, mixed 1:1 with IEF sample buffer (8.5 M urea, 2 M thiourea, another 30 min. The mixture was transferred to a column and washed with 100 mM Tris-HCl containing 1 mM CaCl₂, desalted, and concentrated in a Centricon-3 ultrafiltration device, mixed 1:1 with IEF sample buffer (8.5 M urea, 2 M thiourea, another 30 min. The mixture was transferred to a column and washed with 100 mM Tris-HCl containing 1 mM CaCl₂, desalted, and concentrated in a Centricon-3 ultrafiltration device, mixed 1:1 with IEF sample buffer (8.5 M urea, 2 M thiourea, another 30 min. The mixture was transferred to a column and washed with 100 mM Tris-HCl containing 1 mM CaCl₂, desalted, and concentrated in a Centricon-3 ultrafiltration device, separated by electrophoresis in a 4–20% SDS-polyacrylamide gel, and blotted onto nitrocellulose membranes.

Western Blot Analysis—Samples were analyzed by electrophoresis on a 4–20% acrylamide gradient SDS gel, followed by blotting onto nitrocellulose, probed with antibody, and visualized with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Mass Spectrometry—Stained protein spots were excised from the two-dimensional gel and digested with trypsin, using the in-gel method described by the Association of Biomolecular Resource Facilities (18). Digestion with trypsin was carried out overnight at 37 °C, and peptides were extracted from the gel into 5% formic acid:acetonitrile (1:1) and a second extraction into 5% formic acid:acetonitrile (5:95). The extracts were pooled, the volume was reduced by vacuum centrifugation, and the final volume was brought up to 10 μl with 0.1% trifluoroacetic acid. Contaminating salts and particulates were removed by binding the peptides to a C18-ZipTip (Millipore, Bedford, MA), washing with 0.1% trifluoroacetic acid, and elution into 10 μl of 0.1% trifluoroacetic acid:acetonitrile (1:1). The peptides from the tryptic digests were analyzed by LC-MS/MS. Liquid chromatography was performed using a Michrom Magic high pressure liquid chromatography system with a constant pressure splitter to reduce the flow rate through the column to 400 nl/min. Peptides were separated by reversed phase chromatography, using Vydac C18, 5-μm particle, 300-Å pore packing. A column of ~5 cm was packed into a 75-μm inner diameter fused silica capillary (PicoFrit; New Objective, Cambridge, MA). Peptides were separated using a linear gradient from 2–85% (Buffer A, 5% acetonitrile in water with 0.5% acetic acid and 0.005% trifluoroacetic acid; Buffer B, 80% acetonitrile, 10% N-propanol, 10% water, with 0.5% acetic acid, 0.005% trifluoroacetic acid). The LC effluent was electrosprayed directly into the sampling orifice of an LCO DECA spectrometer (Thermo Finnigan, San Jose, CA) using an adaptation of the microscale electrospray interface (19). The LCQ DECA was operated to collect MS/MS spectra in a data-dependent manner, with up to four of the most intense ions that exceeded a pre-set threshold being subjected to fragmentation and analysis. The MS/MS data generated was analyzed, and matches to protein sequences in the NCBI non-redundant database (mammalian subset) were determined using both SEQUEST (20) and MASCOT (21) programs.

Sequence identification was based on the Mowse score (10 × log(P), where P is the probability that the observed match found by the Mascot software is a random event). Protein scores greater than 60 were significant at p < 0.05. In each case, the predicted M, and pl of the identification matched the observed M, and pl values within ± 5%.

Computer Analysis—Image quantitation, spot alignment, and molecular weight estimation were done using the image analysis program tnmage (entropy.bmi-jhu.org).

RESULTS

Initial attempts to identify protein-protein interactions using bifunctional cross-linking reagents resulted in complex mixtures of often insoluble aggregates containing multiple proteins, presumably caused by the small molecular size of the cross-linking reagent, which allowed it to bind to both interacting partners at multiple locations. This problem was eliminated when CNBr-activated Sepharose was used instead of a cross-linking reagent.

Binding of free NH₃ groups to CNBr-activated Sepharose could produce six possible categories of outcomes, depending on whether one or both components of the interacting pair of proteins A and B bind to the Sepharose particle (Fig. 1, top). The categories are as follows.

1. Protein A bound to both particles and Protein B free.
2. Protein A bound to a particle and Protein B free.
3. Protein A and protein B bound to different particles.
4. Protein A bound to one particle and protein B bound to both particles.
5. Protein A and B both bound to both particles.
6. Protein A and B both bound to one particle.

Because of the large size of the Sepharose particle, out-

1 The abbreviations used are: CHAPS, 3-[3-cholamidopropyl]ldimi-
methylammonio]-1-propanesulfonic acid; LC-MS/MS, liquid chromato-
graphy-tandem mass spectrometry.
comes in which the protein complex is bound to different particles (1, 3, 4, and 5 in Fig. 1, top) are eliminated, because mechanical stress overcomes the chemical bond. Outcomes in which both partners are bound to the same particle (6) should be relatively rare because of the sparsity of activated groups on the Sephadex particle. This leaves only outcome 2, in which one protein is bound covalently to a particle and the other is non-covalently associated with the bound protein.

To select non-covalently interacting proteins, the protein mixture is reacted with cyanogen bromide-activated Sepharose in such a way that 50% of the proteins are bound covalently. The remaining proteins are either washed away or retained on the Sepharose by interacting non-covalently with the covalently bound proteins. After washing in appropriate buffer, the principal components would be proteins bound at a single site (outcome 2), in which one partner is attached covalently to a particle and its non-covalently attached interacting partner is retained by virtue of its affinity to the bound protein. The non-covalently attached protein is then eluted by washing in 8 M urea (Fig. 1, bottom) and applied to a two-dimensional polyacrylamide gel. Alternatively, the elution buffer can be modified to examine specific types of protein-protein interactions, such as substrate-dependent or calcium-dependent interactions.

To test the feasibility of the method as a screening technique, we first investigated the ability to detect known calcium-dependent interactions of the method. A sample of rat brain homogenate CHAPS extract was bound to CNBr-activated Sepharose, and the interacting proteins were washed with Tris acetate buffer and eluted with EGTA. EGTA was used at one-fourth the concentration of Tris acetate, so that no change in the concentration of carboxyl or amino groups, which might elute proteins by virtue of a change in ionic strength, would occur upon the transition to EGTA. A separate sample was bound to a conventionally prepared calmodulin affinity column and eluted with EGTA for comparison. Western blot analysis showed that all four calmodulin-binding proteins tested (CaM kinase I and II, MARCKS, and protein phosphatase 2A) were detectable with the CNBr method, with results comparable with those obtained with conventional calmodulin affinity chromatography (Fig. 2). A control sample of CNBr-activated Sepharose inactivated by incubating with Tris buffer showed no calcium-dependent protein binding (left lanes).

To study the specificity of the method, a sample of rat brain extract was bound to CNBr-Sepharose as before, and the EGTA-eluted proteins were separated by two-dimensional polyacrylamide gel electrophoresis. Fig. 3A shows a Coomassie-stained gel from this experiment. The total number of measurable spots (~172) was smaller than the 300–400 spots visible when all interacting proteins were eluted with 8 M urea (Fig. 3B) and much smaller than the 1000–1200 spots visible from unselected extract (not shown). Twenty-three of the more intense spots detected on the two-dimensional gel were subjected to digestion with trypsin, and the resulting peptides were analyzed by LC-MS/MS. Matching
the LC-MS/MS data with the peptide and fragment masses from sequences in the protein database resulted in positive identification for 12 of the 23 proteins analyzed. Of the 12 identified proteins, eight are proteins known to either bind calcium or interact with other proteins in a calcium-dependent manner (Table I).

Attempts to identify the remaining 11 large spots in Fig. 3A, including the large spots at 10 kDa, pI 7.2 and 52 kDa, pI 4.7, were unsuccessful. Although numerous peptides were obtained, analysis of the mass spectrometric data did not produce a match with any protein in the database.

The most abundant protein spot on the two-dimensional gel (spot 1) was identified as the calcium-binding protein calmodulin (Table I and Fig. 3A). This spot is also detectable in crude extract but is a relatively minor component (Fig. 4). Most of the remaining identifiable spots, including ATP synthase, mitochondrial ATPase inhibitor, and heterogeneous nuclear ribonucleoprotein A2, are also either known calcium-binding proteins or proteins that interact directly with calcium-binding proteins (22–26). Peptides from S100, another calcium-binding protein that undergoes numerous calcium-dependent protein interactions (27), were also detected. Although the Mr and pI were identical with S100, because of the small number of observed peptides in the digestion, the mass spectrometric identification did not reach statistical significance.

Table I summarizes the proteins identified by mass spectrometry. With the exception of hemoglobin, citrate synthase, and carbonic anhydrase, all the proteins identified were either known calcium-binding proteins or were proteins with well characterized calcium-dependent interactions. For example, tropomyosin is associated with the well known actin-tropomyosin-myosin complex. Ca\(^{2+}\) binding to troponin enables troponin to bind tropomyosin and shift it from the binding sites of myosin on the actin proteins. Without the presence of Ca\(^{2+}\), troponin is no longer able to bind to tropomyosin, and tropomyosin again blocks the binding sites of myosin on the actin proteins. Tropomyosin also binds to the calcium-binding protein calcyclin (28). Similarly, Rho GDP dissociation inhibitor strongly binds to the low molecular weight GTP-binding protein Rho, which participates with the calcium-binding protein cadherin in reorganization of actin cytoskeleton (29). Calponin is also a substrate of Rho kinase (30).

**DISCUSSION**

In this experiment, a new selection method for investigating protein-protein interactions was examined, and its reliability and specificity were tested by examining calcium-dependent protein interactions. Over 30% of the total protein observed was calmodulin, a calcium-binding protein that binds to numerous other proteins in a calcium-dependent manner (31). Four other proteins (ATP synthase, two forms of ATPase inhibitor, and S100) are also known calcium-binding proteins, whereas three (tropomyosin, Rho GDP dissociation inhibitor, and heterogeneous nuclear ribonucleoprotein A2) are known to be associated intimately with calcium-binding proteins. It should be noted, however, that this method is not merely a novel way of detecting calcium-binding proteins. Only the subset of proteins that bind to some other protein in a calcium-
dependent manner would be detectable in this experiment. This will include some calcium-binding proteins, as well as their targets, such as calcium-dependent kinases and signaling proteins such as Rho and Rab, which interact with calcium-binding proteins in a calcium-dependent manner.

Three unexpected proteins, hemoglobin, carbonic anhydrase 2, and citrate synthase, were also detected. Although hemoglobin binding to other hemoglobin subunits depends on Fe²⁺ and O₂, it is not known to bind calcium; however, hemoglobin can bind to reticulocyte membranes in the presence of calcium (32), suggesting that it may be a partner for some other calcium binding membrane-bound protein. Similarly, it is possible that citrate synthase and carbonic anhydrase 2 can associate with some other as yet uncharacterized protein in the presence of calcium.

The method described here should be useful for investigating protein-protein interactions in mammalian tissues. For example, it has been suggested that Alzheimer’s disease and other neurodegenerative disorders are triggered by pathological protein-protein interactions (33, 34). Similarly, cell signaling, synaptic plasticity, learning, and development are dependent on a complex network of protein-protein interactions. This method would be useful in isolating macromolecular protein complexes as part of a proteomic screening to identify relevant protein-protein interactions for further study.

Non-physiologically relevant interactions are a significant potential problem in many studies of protein-protein interactions, including the two-hybrid system. In the present method, when a tissue sample is homogenized, in addition to protein interactions present in the original cell, nonspecific protein interactions will also occur between proteins that normally are not in contact with each other. For example, interactions between membrane and nuclear proteins or between astrocyte and neuronal proteins could occur. To prevent this from producing false interactions, it is necessary to use both a treatment and control sample and consider only differences between the two samples as representing potentially significant interactions. Because any nonspecific or non-physiological protein interactions occurring after homogenization of the cells would be identical in both the control and treatment groups, it is easily possible to distinguish normal, physiologically relevant protein-protein interactions from artifactual ones. Nonspecific interactions would be identical in both the control and treated groups, whereas changes in binding that occurred in vivo caused by the treatment would be readily detectable.

As an example of such a differential analysis, the method could be used to study protein interactions that may occur after associative learning in the rat water maze task (35, 36). This experiment uses a trained group, which swims in a tank of water containing a concealed platform, and an untrained control group, which is allowed to swim in a tank with no platform. Interacting proteins from hippocampal extracts of each group would then be isolated separately using the CNBr-Sepharose method and analyzed on two-dimensional polyacrylamide gels. Any differences in protein interactions produced by learning would be reflected as spots for which the intensity in the trained group differs from the intensity of the corresponding protein spot in the control groups. Fig. 5 demonstrates the results from such an experiment, with two proteins possibly exhibiting a learning-specific increase in protein interactions (upper center in left panel). Once these proteins are identified by mass spectrometry or other means,

### Table 1: Proteins with calcium-dependent protein interactions

| Spot | M<sub>r</sub> | pI  | % Coverage | Identification          | Category          |
|------|---------------|-----|------------|-------------------------|-------------------|
| 1    | 17,420        | 4.25| 37         | Calmodulin              | Calcium binding   |
| 2    | 5,370         | 4.93| 21         | S100 β chain            | Calcium binding   |
| 3    | 10,100        | 7.66| 63         | Hemoglobin α 1          | Calcium binding   |
| 4    | 60,220        | 5.52| 48         | ATP synthase            | Calcium binding   |
| 5    | 9,770         | 4.42| 9          | ATPase inhibitor        | Calcium binding   |
| 6    | 8,750         | 4.45| 18         | ATPase inhibitor        | Calcium binding   |
| 7    | 61,660        | 6.28| 12         | Dihydropyrimidase related | Calcium binding |
| 8    | 26,980        | 7.67| 26         | Carbonic anhydrase 2    | Calcium binding   |
| 9    | 36,470        | 8.18| 2          | Heteronuclear RNP A2   | Calmodulin binding|
| 10   | 31,330        | 5.23| 28         | Tropomyosin             | Calcyclin binding |
| 11   | 30,480        | 5.80| 41         | Rho GDI-1               | Binds Cadherin via Rho |
| 12   | 44,510        | 9.06| 6          | Citrate synthase        |                   |

Fig. 4. A comparison of calmodulin spot from two-dimensional gel after enrichment of Ca²⁺-dependent protein-protein interactions (left) or without enrichment is shown. Identical amounts of total protein (100 μg) were applied to each gel. The calmodulin spot is enriched 50-fold.

![Calmodulin spot comparison](image-url)
their binding partners can be identified easily. This could provide a useful means of identifying new signaling pathways relevant to physiological processes.

Other examples of the use of this differential analysis would be testing for protein interaction differences between normal and cancer cells and testing of in vivo effects of a pharmacological agent. Any differences between the two groups cannot result from artifactual interactions occurring during sample processing but represent differences in in vivo protein interactions produced by the treatment.

When isolating in vitro interacting proteins, such as the set of proteins that undergo calcium-dependent interactions or the set whose interactions depend on the presence of some pharmacological agent, the calcium or pharmacological agent being tested would be added to the buffer at each step in sufficient concentration to ensure tight binding of all relevant proteins. During the elution step, instead of 8 M urea, the proteins would be eluted by washing the chromatography column with a buffer identical in all respects except that the pharmacological agent is omitted. The ionic strength and pH level of the two buffers should be identical to avoid eluting any proteins by virtue of a change in pH level or ion concentration. In this case, it is not strictly necessary to compare two separate samples, because the protein interactions of interest are those occurring in the test tube.

Although this method can give as much as 50-fold enrichment of interacting proteins, it is possible that some non-interacting proteins could also be detected if their affinity for Sepharose is higher than the affinity of the proteins for each other. Although protein adsorption to Sepharose is higher (37), the effects are generally small and would be eliminated by adding a control group as described above. Nonspecific binding to Sepharose was not observed in this experiment.

The data produced using this method will consist of raw information concerning proteins that may interact with some other as yet unidentified protein or proteins. Although this is valuable information in itself and reduces the problem space by several orders of magnitude, it is still necessary to validate the putative protein interactions. Once the target proteins are identified, their binding partners can be found easily using conventional techniques such as affinity chromatography (12) or two-hybrid analysis. To provide a complete understanding of the interaction, it is also necessary to confirm that the putative interaction occurs using some alternative method. Confirmation of the observed change is, of course, also necessary in other screening methods such as DNA microarrays, phage display, or the two-hybrid system. Because the present method only measures the total quantity of protein in an interacting state, validation of the interaction is also needed to determine the biochemical basis for the increased levels of interaction, which could be produced by greater affinity of the target protein, greater abundance, or even, in unusual cases, induction of an activator or reduced levels of some inhibitor of the interaction.

This technique could also be modified by adding a cross-linking step after the initial wash and substituting thiol-Sepharose for CNBr-Sepharose. This would permit the pair of interacting proteins to be separated by cleavage of the disulfide bond linking the protein to Sepharose, allowing the cross-linked protein pair to be separated and identified as a single unit.

Besides calcium-dependent protein interactions, numerous examples exist of protein-protein interactions mediated by GTP, cAMP, protein phosphorylation, enzyme substrates, or other biochemical phenomena. The present method could be used to investigate these categories of protein interactions, for example by comparing patterns produced in the presence or absence of a protein phosphatase or nucleotide phosphohydrolase.

The new method has the advantage of screening the entire proteome simultaneously, unlike other methods, which can only detect proteins binding to a single bait protein at a time. In addition, the method does not require cloning but isolates naturally occurring interactions between proteins in their native, folded state that are properly modified post-translationally. The proteins are also accessible to chemical manipulation, permitting selection of a subpopulation of proteins from a complex mixture on the basis of specified interaction criteria. The method would be useful not only for studying protein-protein interactions but also for identifying the site of action of low molecular weight compounds such as xenobiotics or pharmacological agents. Previously, determining whether a xenobiotic affected protein-protein interactions was a daunting task unless one of the target proteins was known. With the current method, the entire proteome can be screened rapidly to identify those
proteins whose interactions are affected by a molecule of interest, yielding specific targets for further investigation.

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