Misincorporation Proton-Alkyl Exchange (MPAX): Engineering Cysteine Probes into Proteins

A method to obtain a map of protein topology is described in this unit. When combined with a high-throughput screen for detecting mutations that perturb ligand binding, this method provides a very useful tool to identify amino acid residues that contribute to protein-protein interfaces. This approach makes use of misincorporation proton-alkyl exchange (MPAX) to determine the topology of individual amino acid residues within a folded protein. Cysteine residues are incorporated at random and at a low frequency when the protein is expressed in *E. coli* cells harboring specific mutant cysteine tRNAs. These "misincorporator tRNAs" introduce cysteine in place of a given amino acid. The resulting misincorporated peptides contain one cysteine residue on average per protein molecule, in place of a specific amino acid. The chemical reactivity of the cysteine to an alkylating reagent is used in combination with 2-nitro-5-thiocyanobenzoic acid–driven chemical cleavage of the protein backbone to measure the relative burial of that residue in the folded protein structure. A surface map is thereby generated, which reveals the identity of surface-localized hydrophobic residues that are likely to contribute to hydrophobic protein-protein interfaces. Mutant protein pools generated with distinct misincorporator tRNAs can also be screened simultaneously in a single affinity chromatography step to reveal differences in ligand binding affinity.

In this unit, the use of MPAX technology to misincorporate cysteine residues (see Basic Protocol 1) and chemical cleavage of the protein backbone at the misincorporation site (see Basic Protocol 2) are described. Protocols to determine the solvent accessibility of misincorporated cysteines in the context of native versus unfolded proteins are then presented (see Basic Protocols 3 and 4). Basic Protocol 5 describes how to analyze MPAX data to enable prediction of binding interfaces. In addition, the combination of MPAX with affinity chromatography to provide a high-throughput method to select for affinity-altered mutant proteins in vitro (see Basic Protocol 6) is described. Note that this protocol is independent of Basic Protocols 3 through 5.

These methods require high-resolution separation of small peptides by gel electrophoresis (see Support Protocol 1). Finally, this unit describes the assembly of a synthetic gene that can be used to achieve maximal cysteine misincorporation (see Support Protocol 2 and 3).

STRATEGIC PLANNING

Naturally occurring cysteines

To simplify the biochemical readout of cysteine misincorporation, cysteine codons in the gene of interest may be replaced, for instance with alanine codons, by conventional site-directed mutagenesis (see Critical Parameters for further discussion). This is helpful because naturally occurring cysteine residues that have been modified by 2-nitro 5-thiocyanobenzoic acid are cleaved with 84% efficiency (Silverman and Harbury, 2002a). This results in a major cleavage product that may occlude the signal from any misincorporated cysteine that is nearby in the protein sequence (see Table 1 in Silverman and Harbury, 2002b). In addition, signal from misincorporated cysteines that are distal to both the protein kinase A (PKA) tag and the naturally occurring cysteine only arise

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from peptides that remain uncleaved at the naturally occurring cysteine (and still contain a PKA tag).

Protein kinase A labeling motif

Peptides containing misincorporated cysteines are detected by end-radiolabeling. For this purpose, a protein kinase A recognition sequence is introduced at one end of the polypeptide. Thus, the gene of interest should be prepared to include this motif (encoding amino acids N_{ter}-GRRASIY-C_{ter}).

Gene assembly

Cysteine misincorporation is enabled by co-expression of a gene of interest together with specific cysteine misincorporator tRNAs in *E. coli*. Misincorporator tRNAs recognize a subset of naturally occurring triplets that encode a given amino acid. Maximal misincorporation is possible when a synthetic gene is utilized, in which the codons are selected for optimal recognition by the tRNAs employed. Support Protocol 2 can be followed to assemble a gene containing exclusively amino acid triplet codons that match the specific misincorporator tRNAs employed (see Table 26.1.2). Support Protocol 2 is not essential but will provide maximal cysteine misincorporation.

BASIC PROTOCOL 1

INTRODUCTION OF CYSTEINE RESIDUES BY TRANSLATIONAL MISINCORPORATION

This protocol describes the replacement of a specific amino acid within a polypeptide with cysteine, using translational misincorporation in *E. coli*. The process requires a mutant cysteine tRNA bearing an anticodon triplet that matches the amino acid to be replaced (see Table 26.1.2). In the example shown in Figure 26.1.1, the cysteine tRNA anticodon has been mutated to that of valine (GAC). Simultaneous expression of this mutant tRNA and a gene of interest results in the introduction of cysteine at a random valine position in the growing polypeptide chain.

This technique makes use of vectors (pMPAX) encoding specific mutant *E. coli* cysteine transfer RNAs and cysteinyl tRNA synthetase (Silverman and Harbury, 2002a). This protocol describes how to co-express a gene of interest together with a pMPAX vector to yield an ensemble of peptides containing cysteine misincorporated at different positions. The protein can then be purified according to individual protocols.

Materials

pMPAX vectors (Harbury laboratory, Stanford University; harbury@stanford.edu)
Kanamycin-resistance plasmid (*pET28a*, Novagen; *pET28a pH6_PKA* and *pET28a PKA_pH6*, Harbury laboratory, Stanford University)
BL21(DE3) *E. coli* cells (Novagen)
LB plates (*APPENDIX 4A*) containing 30 μg/ml kanamycin and 100 μg/ml carbenicillin
LB medium (EMD Biosciences) containing 30 μg/ml kanamycin and 100 μg/ml carbenicillin
1× PBS (see recipe)
M63 medium containing 30 μg/ml kanamycin and 100 μg/ml carbenicillin (see recipe)
1 M isopropyl-β-D-thiogalactopyranoside (IPTG)
Incubator and shaker (37°C and 30°C)
Culture flasks, sterile
Additional reagents and equipment for electroporation (*UNIT 5.10*)

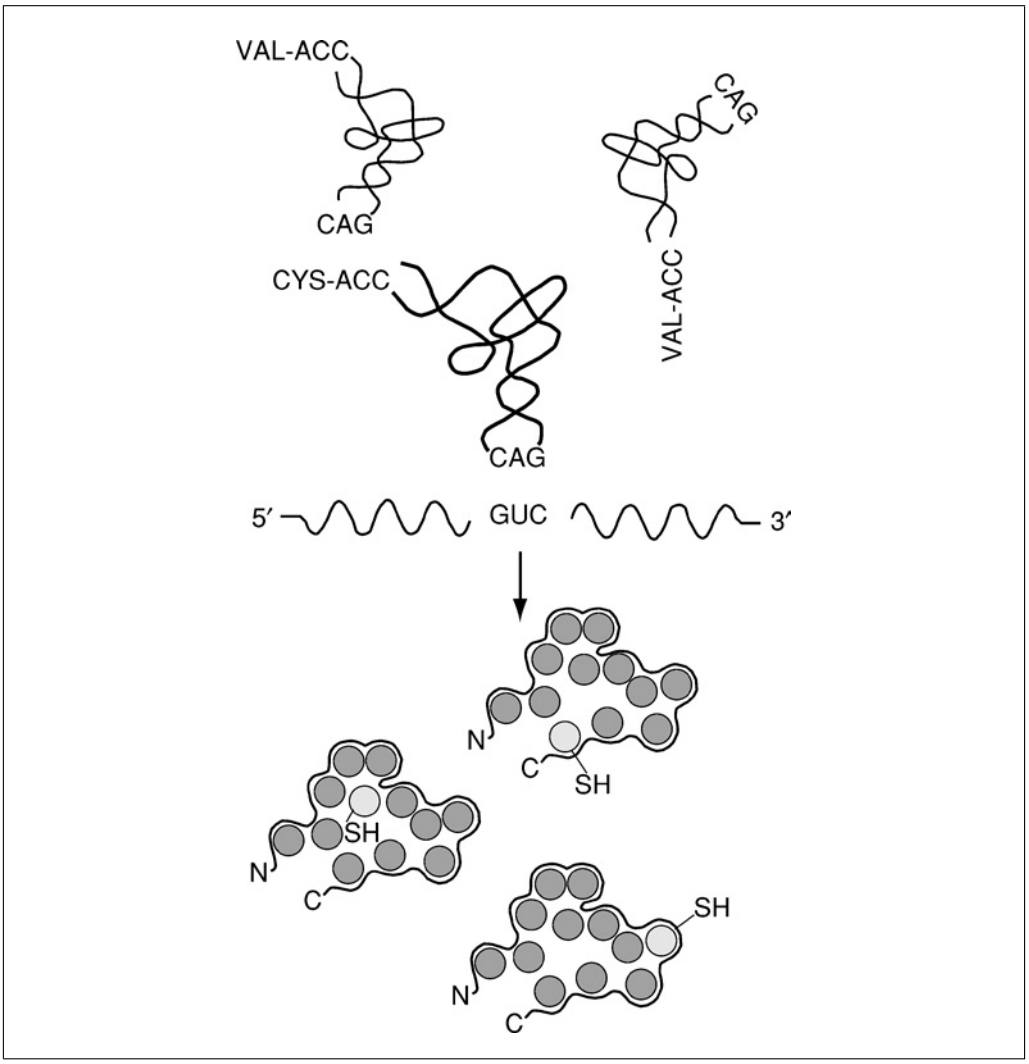

Figure 26.1.1 Translational misincorporation of cysteine residues at valine positions by use of a mutant cysteine transfer RNA.

1. Mix ~1 μg each of pMPAX vector and a kanamycin-resistance plasmid containing the gene of interest under the control of a T7 promoter.

   Note that the gene of interest is fused to a sequence encoding a protein kinase A recognition motif.

   Use sterile technique through step 7.

2. Electroporate (*UNIT 5.10*) the DNA mixture from step 1 into BL21(DE3) *E. coli* cells.

   Electroporation is the method of choice for transforming E. coli because this method yields a high number of double transformants. It is recommended to transform E. coli cells with the gene of interest in the absence of pMPAX and grow on kanamycin plates and medium as a negative control.

3. Spread 50 μl of transformed cells onto LB plates containing 30 μg/ml kanamycin and 100 μg/ml carbenicillin. Incubate overnight at 37°C.

4. Transfer a single colony into 50 ml LB containing 30 μg/ml kanamycin and 100 μg/ml carbenicillin at the end of the day. Shake overnight at 37°C.

5. Wash cells with 1× PBS, centrifuge 15 min at 5000 × *g*, 4°C, decant supernatant, and resuspend in 50 ml of 1× PBS.

6. Inoculate cells 1:50 into M63 medium containing 30 μg/ml kanamycin and 100 μg/ml carbenicillin. Shake at 37°C until cells reach an OD_{600} of ~0.5 (4 to 6 hr).

   An initial expression volume of 100 to 500 ml is recommended. This can subsequently be scaled up depending on the amounts of protein required.

7. Add IPTG to a final concentration of 1 mM. Shake for 3 hr at 30°C or according to a protein-specific optimized protocol.

8. Centrifuge cells 15 min at 5000 × *g*, 4°C. Discard supernatant. Lyse cells and purify the protein of interest by an appropriate protocol (see *UNITS 6.1, 6.2, 6.3, 6.6, 6.7 & 8.1*).

   Non-lysed bacterial pellets can be frozen in liquid nitrogen and stored for up to 6 months at −80°C.

BASIC PROTOCOL 2

CHEMICAL CLEAVAGE AT CYSTEINE

MPAX takes advantage of the fact that cysteine residues within a polypeptide chain can effect polypeptide cleavage upon cyanylation with 2-nitro 5-thiocyanobenzoic acid (NTCB) followed by incubation in ammonia (Fig. 26.1.2; Jacobson et al., 1973; Wu and Watson, 1998; Silverman and Harbury, 2002a; Burguete et al., 2004). In this protocol, proteins containing misincorporated cysteine residues (see Basic Protocol 1) are first unfolded with guanidine·HCl to make the sulfhydryls accessible and then incubated with NTCB. Cyanylated proteins are then precipitated with trichloroacetic acid (Arnold and Ulbrich-Hofmann, 1999). The protein backbone is cleaved upon incubation at increased pH in the presence of ammonia.

Cleavage products are resolved in a Tris·Tricine gel and can subsequently be quantified on a phosphorimager. Cysteine misincorporation occurs at a very low frequency (<1 cysteine per polypeptide, on average). Therefore, this analysis yields a distinct and characteristic cleavage pattern where each band on the gel corresponds to a specific amino acid position in the protein sequence. It is useful to include a background control sample containing a protein that was expressed in the absence of misincorporator tRNA and run it parallel to the sample. Additional control reactions could be performed by

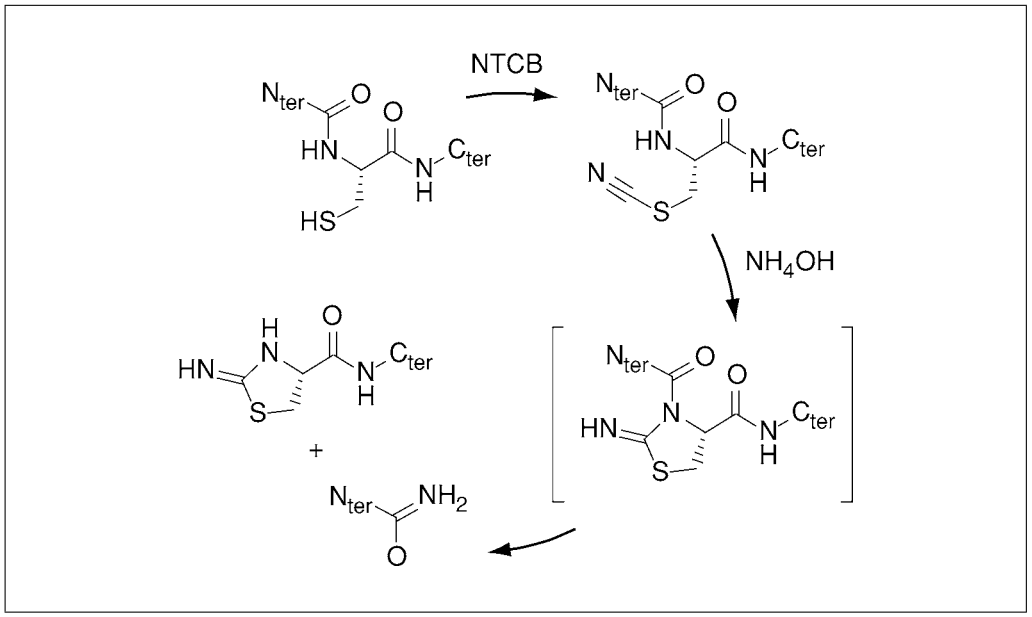

Figure 26.1.2 Cyanyaltion of a cysteine sulfhydryl and subsequent base-mediated site-specific cleavage of the protein backbone.

deleting a reagent, e.g., in absence of CNTB or ammonia, to verify a specific cleavage pattern.

Materials

Cysteine-misincorporated, purified protein (see Basic Protocol 1)
10× PKA buffer (see recipe)
100 mM dithiothreitol (DTT)
1% Triton X-100
10 mg/ml bovine serum albumin
5 U/μl protein kinase A suspended in 1× protein kinase A buffer (see recipe)
10 μCi/μl adenosine triphosphate-γ-^{33}P (3000 Ci/mmol)
10 μM adenosine triphosphate (cold)
2 M Bicine, pH 8.6
8 M guanidine·HCl
1 M 2-nitro-5-thiocyanobenzoic acid (NTCB) in dry dioxane
0.05% (w/v) deoxycholic acid sodium salt
50% (v/v) trichloroacetic acid
Acetone
8 M urea
0.1 M ammonium hydroxide (NH_4OH)
3× Tricine loading dye (see recipe)
Tris·Tricine gel (see Support Protocol 1)

1.5-ml microcentrifuge tubes
Vortex
Speed-vacuum centrifuge

1. Combine 10 ng to 5 μg of cysteine-misincorporated, purified protein in a 1.5-ml microcentrifuge tube with the following reagents:

   5 μl 10× PKA Buffer
   3 μl 100 mM DTT
   2 μl 1% Triton X-100
   2 μl 10 mg/ml BSA
   0.2 μl 5 U/μl protein kinase A suspended in 1× protein kinase A buffer
   H_2O to 44.5 μl

   Then, add:
   0.5 μl 10 μCi/μl ATP-γ-^{33}P (3000 Ci/mmol)
   5 μl 10 μM cold ATP

   Incubate 15 min at 37°C. The addition of 0.04% Triton X-100 is optional and does not interfere with subsequent analysis. Also, the use of ATP-γ-^{33}P is critical because it yields a sharp protein band signal. The total reaction volume is 50 μl.

2. Add an equal volume of 200 mM Bicine in 8 M guanidine·HCl, pH 8.6, and mix. Add 50 mM NTCB (5 μl) from a fresh 1 M stock in dry dioxane and mix. Incubate 5 min at room temperature.

   An "equal volume" in this case would be 50 μl. If the labeling reaction volume is altered, then the "equal volume" added in step 2 should be changed accordingly. Prepare the guanidine·HCl/Bicine mix just prior to addition and check the pH.

3. Add 1 ml of 0.05% sodium deoxycholic acid and mix well. Add 100 μl of 50% trichloroacetic acid, mix by inversion, and centrifuge 1 min at 14,000 × *g*, room temperature.

4. Wash pellets two times with 600 µl acetone and vigorous vortexing at room temperature. Dry in a speed-vacuum centrifuge.

   Heating of the centrifuge is not necessary as the acetone will evaporate within minutes.

   It is important to remove all NTCB in this step. Residual NTCB will cause nonspecific cleavage of the protein backbone when the pH is increased with ammonium hydroxide.

5. Resuspend pellets in 10 µl of 8 M urea, 0.1 M NH_4OH. Incubate 1 hr at room temperature to cleave polypeptides.

   Residual TCA can reduce the pH in this step. Alternatively, the pellets can be resuspended in 10 µl of 8 M urea, 0.8 M NH_4OH. Incubate 1 hr at room temperature and open tubes to allow the NH_4OH to evaporate. Wait ~20 min until the pH < 10. The incubation time can be used to prepare a Tris·Tricine gel (see Support Protocol 1).

6. Add 5 µl of 3× Tricine loading dye. Analyze 5 µl of resultant solution on a Tris·Tricine gel (see Support Protocol 1).

BASIC PROTOCOL 3

NATIVE CYSTEINE ALKYLATION

Cysteines introduced by misincorporator tRNAs (see Basic Protocol 1) can be used as structural probes to determine the degree of burial at specific positions in the native protein (Silverman and Harbury, 2002a; Burguete et al., 2004). The chemical reactivity of a cysteine sulfhydryl group with the alkylating agent iodoacetamide provides a measure of residue burial in the native protein. Solvent-exposed sulfhydryls that are alkylated resist modification by NTCB and are not subject to chemical cleavage; buried thiols are protected from alkylation by protein structure and can be revealed by subsequent denaturation and NTCB-driven cleavage (see Basic Protocol 2).

This protocol describes how to monitor the kinetics of iodoacetamide alkylation at all cysteine misincorporation positions in conjunction with NTCB cleavage. A reaction in the absence of iodoacetamide provides a "zero time point" and alkylation in the presence of guanidine·HCl yields maximal alkylation or the "infinite time point." Thus, both extremes of the kinetic reaction are represented.

Materials

Labeled protein (see Basic Protocol 2, steps 1 and 2)
2 M Bicine, pH 8.6
Quench solution (see recipe)
100 mM iodoacetamide
8 M guanidine·HCl
Tris·Tricine gel (see Support Protocol 1)

Prepare labeled protein

1. Label 1 to 10 µg of protein according to Basic Protocol 2, steps 1 and 2, in a total volume of 150 µl. Prepare zero-time-point sample.
2. Combine 5 µl of 2 M Bicine, pH 8.6, and 75 µl water and add 20 µl radiolabeled sample.
3. Add 100 µl quench solution and store on ice.

   For the zero time point, native protein is incubated in the absence of iodoacetamide. Upon denaturation, all cysteines become cyanylated by NTCB and yield cleavage bands that correspond to every misincorporation position in the protein.

   Quenched samples may be stored up to 1 week at −20°C (not longer due to decay of radiolabel) if not analyzed by gel electrophoresis directly after completion of the time course experiment.

Prepare infinite-time-point sample

4. Combine 10 µl of 2 M Bicine, pH 8.6, 10 µl of 100 mM iodoacetamide, 30 µl of 8 M guanidine·HCl, and 30 µl water. Add 20 µl radiolabeled sample and incubate for at least 50 min at room temperature.

   It is important that the pH of the sample is 8.6. Bicine is included because the addition of guanidine·HCl might lower the pH in the sample and cause inefficient alkylation.

   In the so-called, infinite-time-point sample, the protein is unfolded with guanidine·HCl so that all the cysteine sulfhydryls are in contact with solvent and become alkylated. Thus, no sites are available for NTCB modification; this results in the lack of chemical cleavage and the absence of cleaved peptides on the gel.

Determine time course of protein alkylation

5. Combine 25 µl of 2 M Bicine, pH 8.6, 50 µl of 100 mM iodoacetamide, and 325 µl water. Add 100 µl radiolabeled sample. Mix and incubate at room temperature.

6. Remove 100-µl aliquots at intervals (e.g., after 1, 5, 20, and 50 min) and add 100 µl quench solution. Store quenched samples on ice.

7. At the final time point, also add 100 µl quench solution to the infinite-time-point sample.

8. Denature, cleave, and analyze all samples according to Basic Protocol 2, steps 2 to 6.

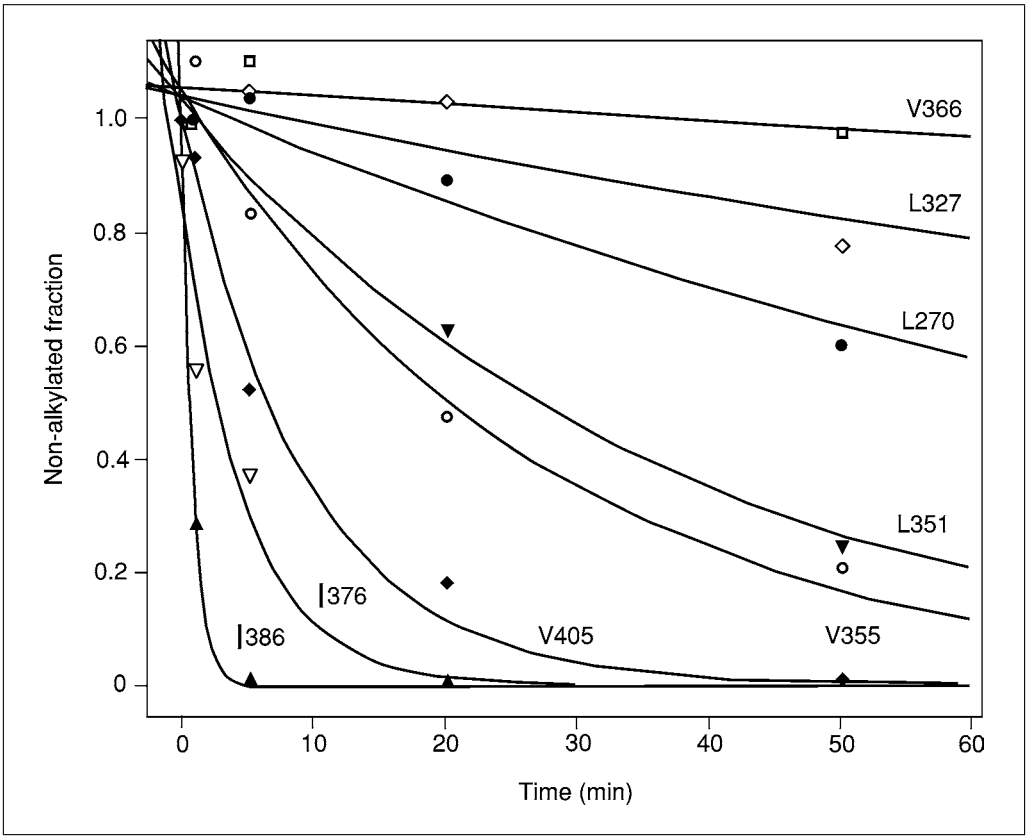

Figure 26.1.3 Representative graph and data fit for a time-course alkylation of eight cysteine substitutions of valine (V), leucine (L), or isoleucine (I) residues in TIP47. (Reprinted with permission from Burguete et al., 2004.)

9. Determine the rate of alkylation at all misincorporated amino acid positions by fitting the data to an exponential decay function. Divide the sec^{-1} rate by 10^{-2} (10 mM iodoacetamide) to determine a rate constant in $M^{-1}sec^{-1}$ units.

   Alkylation will lead to the disappearance of a given band. Thus, bands obtained in the time course experiment will appear weaker with increasing time of alkylation; the rate of their disappearance reflects their relative reactivity to iodoacetamide in the native protein (see Fig. 26.1.3).

BASIC PROTOCOL 4

INTRINSIC ALKYLATION RATE

The protection factor for a given cysteine residue in the folded protein is a measure of its relative burial and thus, accessibility to chemical modification. It can be determined by comparing the rates at which that residue is alkylated in both the native and the unfolded states (Silverman and Harbury, 2002a; Burguete et al., 2004). This terminology is adapted from the nuclear magnetic resonance hydrogen/deuterium exchange literature.

This protocol describes how to determine the alkylation rate for fully exposed, misincorporated cysteines (see Basic Protocol 1) in proteins that are denatured in 2.4 M guanidine·HCl. The iodoacetamide concentration is kept low (0.8 mM) to achieve a logistically feasible time frame for the experiment. For the infinite time point, the iodoacetamide concentration is increased to 10 mM. Keep in mind that a ten-fold increase or decrease in iodoacetamide concentration results in a ten-fold faster or slower alkylation rate, respectively. The alkylation rate is also dependent on the pH and will be ten times slower for every pH unit below 9. It is therefore extremely important to perform the reactions at the recommended pH 8.6.

Note that the observed rate reflects pseudo-first order kinetics with units of sec^{-1} and needs to be converted to a rate constant with the units $M^{-1}sec^{-1}$ for comparison with alkylation rate constants of native protein.

It is convenient to determine the intrinsic alkylation rate in a sample containing only a few misincorporated cysteines, at both buried and exposed positions. Similar rates of alkylation at buried and exposed sites confirm that the protein is indeed fully denatured.

As a control, the rates of alkylation at the same positions should be determined in the native protein.

Materials

Labeled protein
2 M Bicine, pH 8.6
8 M guanidine·HCl
Quench solution (see recipe)
100 and 8 mM iodoacetamide
Tris·Tricine gel (see Support Protocol 1)

Label protein

1. Label 1 to 10 μg of protein according to Basic Protocol 2, steps 1 and 2, in a total volume of 150 μl.

Prepare zero-time-point sample

2. Combine 10 μl of 2 M Bicine, pH 8.6, 30 μl of 8 M guanidine·HCl, and 40 μl water. Add 20 μl radiolabeled sample.
3. Add 100 ml quench solution and store on ice.

Prepare infinite-time-point sample

4. Combine 10 µl of 2 M Bicine, pH 8.6, 10 µl of 100 mM iodoacetamide, 30 µl of 8 M guanidine·HCl, and 30 µl water. Add 20 µl radiolabeled sample and incubate at room temperature.

Alkylate over time

5. Combine 40 µl of 2 M Bicine, pH 8.6, 40 µl of 8 mM iodoacetamide, 120 µl of 8 M guanidine·HCl, and 120 µl water. Add 80 µl radiolabeled sample. Mix and incubate at room temperature.

   It may be necessary to adjust the iodoacetamide concentration to fit the measurable range for the particular system studied. If cysteines remain unalkylated at the last time point, try increasing the iodoacetamide concentration slightly. If all cysteines become alkylated within the first time point, try lowering the iodoacetamide concentration. Keep in mind that a ten-fold increase or decrease in iodoacetamide concentration results in a ten-fold faster or slower alkylation rate, respectively. The alkylation rate is also dependent on the pH and will be ten times slower for every pH unit below 9. Make sure to perform the reactions at the recommended pH.

6. Remove 100-µl aliquots after 10, 30, and 60 sec and add 100 µl quench solution. Store quenched samples on ice.

   Prepare tubes containing 100 µl quench solution each before the experiment and keep on ice. This facilitates the addition of aliquots at the desired time point.

7. At the final time point, add 100 µl quench solution to the infinite-time-point sample.

8. Denature, cleave, and analyze all samples according to Basic Protocol 2, steps 2 to 6.

9. Determine the intrinsic rate of alkylation by averaging the decay rates of all detected cysteine probes.

   For example:

   This example illustrates the determination of the intrinsic alkylation rate constant as reported in Burguete et al. (2004). The pseudo-first order rate constants (the rate constants at a fixed iodoacetamide concentration) for cysteines at five isoleucine positions in a denatured sample were determined at 0.8 mM iodoacetamide.

Residue	Pseudo-first order rate constant k (sec^{-1})
I333	0.018
I348	0.020
I376	0.030
I386	0.032
I425	0.038

   The first order rate constant can then be calculated (I333 in this example) as:

   $0.018\ sec^{-1}/(0.8 \times 10^{-3}\ M) = 22.5\ M^{-1}sec^{-1}$

   The rate constants for the other residue positions in the sample and their average are then determined as follows:

Residue	First order rate constant ($M^{-1}sec^{-1}$)
I333	22.5
I348	25.0
I376	37.5
I386	40.0
I425	47.5

And therefore, the intrinsic rate constant (average) is as follows:

$(22.5 + 25.0 + 37.5 + 40.0 + 47.5)/5 = 34.5\ M^{-1}sec^{-1}$

10. Determine a protection factor according to the following formula:

Protection factor $= k_{intrinsic}/k_{observed}$

Example:

Calculate the protection factor for I333:

$34.5\ M^{-1}sec^{-1}/1.19\ M^{-1}sec^{-1} = 29$

Residue	First order rate constant in native protein ($M^{-1}sec^{-1}$)	Protection factor
I333	1.19	29
I348	0.18	192
I376	0.25	138
I386	1.85	19
I425	16.67	2

Protection factors range from 1 (for a cysteine sulfhydryl that is fully exposed to solvent) to 10^4 or greater (for a cysteine sulfhydryl that is fully buried within the protein structure).

BASIC PROTOCOL 5

PREDICTION OF BINDING INTERFACES

When analyzing MPAX data, it is useful to plot the protection factor against residue position in the amino acid sequence (see Fig. 26.1.4). This type of graph provides useful information about protein topology and allows ready detection of buried and or solvent-exposed regions in the polypeptide (Burguete et al., 2004). Protection factors obtained by nuclear magnetic resonance (*UNIT 17.5*) hydrogen exchange measurements have been used in an analogous way in a number of studies to characterize protein topology (Raschke and Marqusee, 1998).

Aromatic and hydrophobic residues are enriched at binding interfaces (Lo Conte et al., 1999). MPAX provides a rapid means to identify amino acid residues that have the potential to contribute to hydrophobic protein-protein interactions. The cores of proteins of known structures have a defined amino acid composition (Janin, 1979). Thus, different amino acids have different probabilities of being buried in a folded protein. Measured protection factors for different residue positions can be directly compared to the burial propensity of the amino acid that was replaced. This type of plot readily identifies candidate residues that are more solvent-exposed than would be expected from their occurrence in protein cores of known structures. Such residues have a high burial propensity but a low protection factor (Burguete et al., 2004).

BASIC PROTOCOL 6

SELECTION OF MUTANTS WITH ALTERED LIGAND OR PARTNER BINDING

In this protocol, an ensemble preparation of proteins containing different, randomly misincorporated cysteine residues (see Basic Protocol 1) are screened as a pool for alterations in their ability to bind ligand (Burguete et al., 2004). The mutant proteins are allowed to bind to a ligand (or partner protein), which is then captured on a solid support and unbound material is washed from the column. Bound proteins are eluted and subjected to NTCB-mediated chemical cleavage (see Basic Protocol 2) and separation by gel electrophoresis (see Support Protocol 1). The eluted protein containing a mixture of individual mutants is then directly compared to the total starting protein preparation.

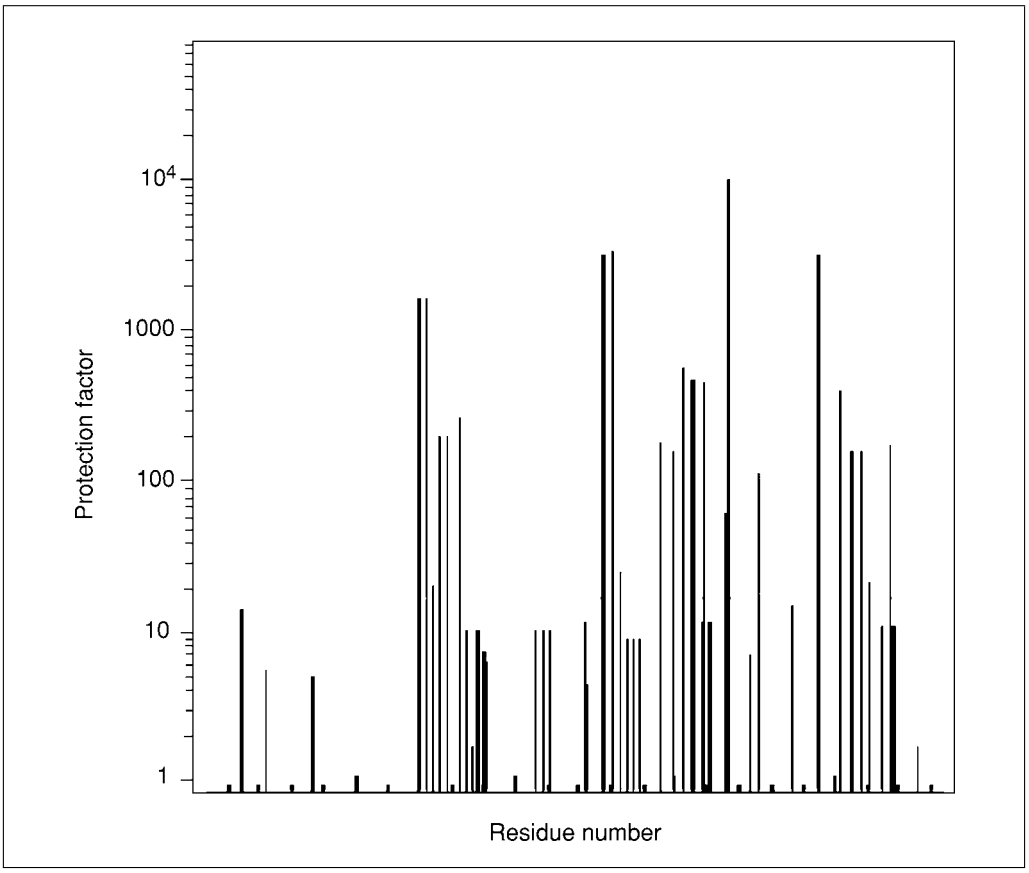

Figure 26.1.4 Protection factor map of 48 cysteine substitutions in TIP47 replacing valine, leucine, isoleucine, phenylalanine, and glutamine residues. (Reprinted with permission from Burguete et al., 2004.)

Individual cysteine mutants with increased or decreased ligand binding, as monitored by their enrichment or loss from the ligand-bound fraction, are readily detected in this comparison. This protocol requires that the ligand be coupled to a solid support using a different means than that used to purify mutant proteins. For example, the misincorporated protein could bear a 6-histidine tag for purification purposes while the binding partner could be immobilized using an antibody epitope tag.

It is important to verify the biochemical properties of selected mutant proteins. For this purpose, individual mutants can be prepared by conventional site-directed mutagenesis of the wild type gene. The resulting protein preparation, in which every molecule carries the same mutant residue, can then be analyzed for binding to known ligands or partners by established protocols to confirm the MPAX findings. In addition, the folded nature and overall secondary structure can be confirmed by circular dichroism or other methods.

Materials

Radiolabeled protein (see Basic Protocol 2)
Ligand or partner protein
Solid support
Binding buffer
4 M guanidine·HCl
100 mM sodium Bicine, pH 8.6
1 M 2-nitro-5-thiocyanobenzoic acid (NTCB) in dry dioxane
Tris·Tricine gel (see Support Protocol 1)
Scintillation counter

1. Radiolabel protein according to Basic Protocol 2, steps 1 and 2.
2. Bind to ligand or partner protein according to established protocol.
3. Bind ligand/protein complex to a solid support according to individual protocol.

   Glutathione S-transferase (GST) is a commonly used tag for immobilization and/or purification of proteins. The tagged protein is supported on glutathione agarose beads according to the manufacturer's instructions. A GST tag has been used successfully in this approach (Burguete et al., 2004). In some cases, it may be preferable to invert the order of steps 2 and 3, depending upon the specific application.

4. Wash in binding buffer to remove unbound protein.
5. Elute in 4 M guanidine·HCl and 100 mM sodium Bicine, pH 8.6.

   The protein can alternatively be eluted according to other protocols and chemical cleavage performed according to Basic Protocol 2, steps 2 to 6.

6. Add 50 mM NTCB from a fresh 1 M stock in dry dioxane. Incubate 5 min at room temperature.
7. Perform Basic Protocol 2, steps 3 to 6.
8. Determine radioactivity in eluted protein and total protein in a β-scintillation counter. Load equal counts of eluted and total protein on a Tris·Tricine gel (see Support Protocol 1).
9. Quantify band intensities corresponding to different amino acid residue positions and determine signal ratios internally within a single sample.

   It is useful to identify a reference band that does not change in intensity between samples (assuming equal loading of different samples in different lanes). The relative intensity of other bands in comparison with the reference band in a given lane is then determined as a percentage of the reference band intensity.

10. Compare relative band intensities from eluted and total protein samples.

    A mutant that binds better to ligand will be overrepresented in the bound fraction and the ratio of eluted/total mutant protein will be >1. Alternatively, if a cysteine mutant displays decreased ligand affinity, the corresponding band will be underrepresented in the bound fraction and the ratio of eluted/total mutant protein will be <1. Specific cysteine mutants can then be prepared individually by conventional site-directed mutagenesis of the wild type gene for further characterization.

SUPPORT PROTOCOL 1

TRIS·TRICINE GEL TO RESOLVE SMALL PEPTIDES FROM MPAX EXPERIMENTS

This protocol makes use of Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a discontinuous buffer system to give high-resolution separation of small peptides (Schagger and von Jagow, 1987; also see Alternate Protocol 1 of *UNIT 10.1*). It is highly recommended to use this protocol to analyze samples from Basic Protocols 2, 3, 4, and 6. This gel consists of four different layers, which include resolving, intercalating, stacking, and comb gels that are essential for the resolution of peptides that only differ in a few amino acids in size.

CAUTION: Note that the voltages and currents used during electrophoresis are dangerous and potentially lethal. It is extremely important to read *UNIT 10.1* to understand how to run a gel and to follow the "Safety Considerations" outlined under "Electricity and Electrophoresis."

Materials

1.5% (w/v) agarose solution (see recipe)
40% (v/v) 19:1 acrylamide
3× gel buffer (see recipe)
3× comb buffer (see recipe)
Glycerol
1% (w/v) Coomassie brilliant blue G250
10% (w/v) ammonium persulfate
TEMED
Butanol
1× cathode buffer (see recipe)
10× anode buffer (see recipe)
Glass plates (20 × 20–cm and 18 × 20–cm)
Spacers (0.5-mm)
Clamps
Whatman filter paper
Comb (0.5-mm; 19 wells ~0.5-cm wide)
Gel-running apparatus
Capillary pipet tips
Power supply
Plastic wrap
Heated vacuum gel dryer
Phosphor image plate and casette
Phosphorimager

Prepare Tris·Tricine gel

1. Pre-assemble two scrupulously clean glass plates with 0.5-mm spacers on the side and bottom edges. Hold in place with clamps.
2. Heat 1.5% agarose solution in a microwave without lid for 1 min or until boiling. Cool for 1 min.
3. Use a glass pipet to seal the spacers by applying warm 1.5% agarose along the outside edges.

   The warm agarose will enter the space by capillary force.

4. Combine resolving gel components (see Table 26.1.1) and pour quickly into the preassembled gel sandwich until 3.5 cm from top edge. Carefully add a layer of neat butanol (~ 2 mm) and leave for 10 min or until fully polymerized.

   Add ammonium persulfate and TEMED just prior to pouring. Butanol provides a barrier to oxygen, which will inhibit polymerization. Do not use propanol or ethanol as these tend to mix with the gel solution.

5. Rinse with water to remove the butanol. Hold gel upside down and insert a small piece of filter paper between the glass plates parallel to one of the spacers to absorb remaining water. Be careful not to disturb the gel surface.
6. Repeat steps 4 and 5 for intercalating and stacking layers. Make these layers each ~1 cm high.
7. Combine comb-gel reagents and fill the rest of the chamber.
8. Quickly insert the comb as close to the stacking layer as possible without disturbing it. Avoid trapping air bubbles.

Table 26.1.1 Comb, Stacking, Intercalating, and Resolving Gel Components

Order of layers	4 (top)	3	2	1 (bottom)
Reagent	Comb	Stacking	Intercalating	Resolving
40% 19:1 acrylamide	1.0 ml	0.5 ml	1.3 ml	8.0 ml
3× gel buffer	–	1.2 ml	1.7 ml	6.0 ml
3× comb buffer	1.7 ml	–	–	–
Glycerol	–	–	0.5 ml	1.8 ml
dH_2O	2.3 ml	3.3 ml	1.5 ml	2.2 ml
1% Coomassie brilliant blue G250	15 µl	–	–	–
Add just prior to pouring:				
10% ammonium persulfate	50 µl	50 µl	50 µl	100 µl
TEMED	7 µl	7 µl	7 µl	20 µl

9. Allow the comb gel to polymerize (~10 to 20 min) at room temperature and then remove bottom spacer.

   Keep side clamps attached while removing bottom spacer to inhibit accidental displacement of the plates.

Run and dry gel

10. Place gel in running apparatus and fill the cathode (upper) chamber with 1× cathode buffer. Fill the anode (lower) chamber with 1× anode buffer.

11. Remove comb and carefully rinse wells with a pipet.

12. Load 5 to 10 µl of sample at the bottom of each well with a capillary pipet tip.

    Loading larger volumes will result in band thickening and suboptimal resolution. Loading samples in every other well will increase resolution.

13. Run gel at 145 V, 145 mA, and 100 W for 16 hr or until dye front reaches bottom of gel.

14. Transfer the gel to a piece of Whatman filter paper and cover with plastic wrap. Dry the gel on a heated vacuum gel dryer for 2.5 hr at 75°C. Cool to room temperature before breaking the vacuum.

    Position the gel sandwich on a flat surface with the smaller glass plate facing up. Insert a metal spatula 0.5 cm into the comb gel and gently split the two glass plates apart. Carefully remove the small plate (the gel usually sticks to the bottom, large plate). Cover gel with a 30 × 30–cm piece of Whatman filter paper. Do not apply pressure on the filter paper as the gel will attach to the glass plate. Turn the sandwich upside down. Carefully remove the large glass plate and apply a 30 × 30–cm piece of plastic wrap onto the gel. Avoid trapping air bubbles. Cut off excess paper at edges and dry the gel in a preheated dryer to avoid cracking the gel.

15. Expose the dried gel on an image plate overnight to 2 days and quantify bands on a phosphorimager.

SUPPORT PROTOCOL 2

GENE ASSEMBLY

This protocol describes a procedure to assemble a synthetic gene (Stemmer et al., 1995) containing codons complementary to those of specific misincorporator tRNAs (see Basic Protocol 1 and Table 26.1.2). Misincorporation seems to follow normal wobble base pairing rules. As an example, the Val(GAC) tRNA misincorporates cysteine at the same frequency at GUC or GUU codons, but not at GUG or GUA codons (Silverman and Harbury, 2002a). Hence, if a wild-type gene is co-expressed with the Val(GAC) tRNA, cysteine will be misincorporated at fewer than all possible valine positions.

Table 26.1.2 Suggested Codon Usage for Misincorporation Proton-Alkyl Exchange

Amino acid	Misincorporator tRNA anticodon	RNA codon	Codon usage for DNA gene assembly
Tyr	GUA	UAC	TAC
Ile	GAU	AUC	ATC
Val	GAC	GUC	GTC
Leu	GAG	CUC	CTC
His	GUG	CAC	CAC
Phe	GAA	UUC	TTC
Trp	CCA	UGG	TGG
Ser	GCU	AGC	AGC
Asp	GUC	GAC	GAC
Ala	AGC	GCU	GCT
Arg	ACG	CGU	CGT
Thr	AGU	ACU	ACT
Asn	GUU	AAC	AAC
Gln	UUG	CAA	CAA
Glu	UUC	GAA	GAA
Gly	ACC	GGU	GGT
Lys	UUU	AAA	AAA
Pro	AGG	CCU	CCT

Maximal misincorporation can be achieved by expressing a gene carrying exclusively codons that match those of the co-expressed misincorporator tRNAs. This protocol describes the design and assembly of such a gene from oligonucleotides using the polymerase chain reaction (PCR). Note that synthesis and purification of oligos (steps 2 through 16) can be omitted by ordering full-length gel-purified oligos from a synthesis facility.

The assembled gene is cloned into either of two vectors, *pET28a pH6_PKA* or *pET28a PKA_pH6* (Harbury laboratory, Stanford University), using the restriction sites *Eco*RI and *Hind*III or *Bam*HI and *Hind*III, respectively. The restriction enzymes used in this protocol are intended for cloning into the *pET28a pH6_PKA* vector.

Reading *APPENDIX 4E* of this manual and *UNITS 2.1A, 2.11, & 2.12* in Current Protocols in Molecular Biology (CPMB) for directions on purification of DNA and synthesis of oligonucleotides is recommended. In addition, *APPENDIX 4I* and *APPENDIX 4J* of this manual and *UNITS 3.1, 3.16, & 3.17* in CPMB can be consulted for instructions on how to digest DNA with restriction endonucleases, and to subclone and amplify DNA.

CAUTION: Voltages and currents used during electrophoresis are dangerous and potentially lethal. Before undertaking this protocol, read *UNIT 10.1* to understand how to run a gel and follow the "Safety Considerations" outlined under "Electricity and Electrophoresis."

Materials

Sense and antisense oligonucleotides (5-nmol scale)
Ammonia
Formamide loading dye (see recipe)

Urea-acrylamide gel (see Support Protocol 3)
1× TBE (see recipe)
Ethidium bromide solution in TBE (see recipe)
Sep-Pak C18 cartridge (Vac 1 cc, 100 mg; Waters)
50% (v/v) acetonitrile
2 M and 100 mM triethylammonium acetate
*Eco*RI and *Hind*III restriction enzymes
pET28a pH6_PKA plasmid modified to provide an N-terminal histidine tag and a C-terminal protein kinase A recognition site
2-ml screw-cap tubes
50°C incubator
Speed vacuum centrifuge
Power supply
Metal spatula
Low-intensity UV source
Scalpel
Metal spatula
Rocker
Glass fiber paper (Whatman)
Additional reagents and equipment for chloroform extraction and ethanol precipitation (*APPENDIX 4E*)

Design and deprotect oligonucleotides

1. Design a gene using a single codon for each amino acid according to Table 26.1.2 and replace any cysteine codons with GCT (alanine). Avoid any *Eco*RI and *Hind*III restriction sites as these will interfere with later cloning procedures.

   The amino acid codons in Table 26.1.2 match the anticodons in the mutant cysteine misincorporator tRNAs used for co-expression. It is recommended that cysteine codons be removed, as they may complicate the biochemical readout of cysteine misincorporation (see Strategic Planning and Critical Parameters).

2. Synthesize sense and antisense oligonucleotides on a 5-nmol scale), 40 bp long, and with a 20-bp overlap to cover the entire gene.

3. Resuspend each oligonucleotide in 40 µl water and transfer 2 µl to a 2-ml screw-cap tube.

4. To deprotect the oligonucleotides, add 0.5 ml ammonia, cap tightly, and incubate overnight at 50°C.

5. Cool to 22°C and evaporate the ammonia in a speed vacuum centrifuge.

Isolate full-length oligonucleotides

6. Resuspend oligonucleotides in 7 µl formamide loading dye.

7. Pre-run a urea-acrylamide gel for 45 min at 600 V in 1× TBE.

8. Load oligonucleotides in separate lanes and run gel at 600 V for ~1 hr or until the loading dye runs out from the bottom of the gel.

9. Turn off the power supply and carefully lift the front glass plate with a metal spatula.

10. Carefully transfer the gel to a tray containing ethidium bromide in 1× TBE and incubate 15 min on a rocker to stain DNA.

11. Visualize DNA with a low-intensity UV source and excise and pool the top, full-length oligonucleotides with a clean scalpel.

Avoid exposing unprotected skin and eyes to intense UV sources. If the UV light is aimed upwards, wear a UV protective face shield when standing near the source.

12. Homogenize gel pieces in 2 ml water with a metal spatula, place in a screw-cap tube and rotate overnight at 37°C.

13. Filter through Whatman glass fiber paper and collect liquid.

Desalt oligonucleotides

14. Wash a Sep-Pak C18 cartridge with 2 ml of 100% acetonitrile and then with 2 ml of 2 M triethylammonium acetate.

15. Load sample slowly and reapply flow-through onto the column several times.

16. Wash with 2 ml of 100 mM triethylammonium acetate, 1 ml water, and elute with 1 ml of 50% acetonitrile.

Assemble gene

17. Use the desalted primer mix to assemble the gene in a PCR reaction (*APPENDIX 4J*). Set up the thermal cycler using the following parameters:

36 cycles:	30 sec	94°C
	40 sec	42°C
	60 sec	72°C.

When analyzed by agarose gel electrophoresis (APPENDIX 4F) the product should appear as a smear ranging from small to large sized DNA.

18. Reamplify the PCR product in a second reaction generated by 5′ and 3′ end primers containing restriction sites for *Eco*RI and *Hind*III, respectively, using the following parameters:

36 cycles:	30 sec	94°C
	40 sec	52°C
	60 sec	72°C.

Agarose gel electrophoresis analysis should reveal a blurry band around the predicted size of the gene.

19. Chloroform extract and ethanol precipitate the second PCR product (*APPENDIX 4E*).

20. Cleave concatamers with restriction endonucleases *Eco*RI and *Hind*III (*APPENDIX 4I*) to generate a single gene assembly product.

Agarose gel electrophoresis analysis should reveal a single sharp band of the predicted size.

21. Clone the assembled gene into a *pET28a pH6_PKA* plasmid, modified to provide an N-terminal histidine tag and a C-terminal protein kinase A recognition site (Silverman and Harbury, 2002a), generating *pH6_GENE_PKA*.

UREA-ACRYLAMIDE GEL

SUPPORT PROTOCOL 3

This protocol provides a method to purify oligonucleotides by use of a urea-acrylamide gel.

Materials

Urea-acrylamide gel (see recipe)
TEMED
Glass plates, spacers, and combs (see Support Protocol 1)

1. Assemble glass plates according to Support Protocol 1, steps 1 to 3.
2. Mix and pour urea-acrylamide gel quickly into the preassembled gel-sandwich.
3. Quickly insert a 0.5-mm comb with ~ 0.5-cm wide wells.
4. Allow the gel to polymerize (~10 to 20 min) at room temperature and then remove bottom spacer.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent for the preparation of all buffers. For common stock solutions, see ***APPENDIX 2E****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Agarose solution, 15% (w/v)

1.5 g agarose
Add H_2O to 100 ml
Heat to dissolve
Store ≤1 week at room temperature

Anode buffer, 10×

242 g Tris base (2 M final)
Add H_2O to <1 liter
Adjust pH to 8.8 with HCl
Correct volume to 1 liter with H_2O
Store ≤3 months at room temperature

Cathode buffer, 1×

12 g Tris base (100 mM final)
18 g Tricine (100 mM final)
1 g SDS (1 g/liter final)
Add H_2O to 1 liter
Store ≤3 months at room temperature

Comb buffer, 3×

36 g Tris base (0.3 M final)
54 g Tricine (0.3 M final)
3 g SDS (3 g/liter final)
Add H_2O to 1 liter
Store ≤3 months at room temperature

Ethidium bromide solution in TBE

4 μl ethidium bromide (10 mg/ml in water)
100 ml 1× TBE (see recipe)
Prepare fresh for each experiment

CAUTION: Ethidium bromide is a powerful mutagen and moderately toxic. Gloves should be worn when working with solutions that contain this dye.

Formamide loading dye

0.2 ml 0.5 M EDTA (10 mM final)
0.1% (w/v) bromphenol blue
0.1% (w/v) xylene cyanol FF
10 ml deionized formamide
Store ≤4 months at room temperature

Gel buffer, 3×

363 g Tris base (3 M final)
3 g SDS (3 g/liter final)
Add H_2O to <1 liter
Adjust pH to 8.6 with HCl
Correct volume to 1 liter with H_2O
Store ≤ 3 months at room temperature

M63 medium containing 30 μg/ml kanamycin and 100 μg/ml carbenicillin

14 g $(NH_4)_2SO_4$ (100 mM final)
2 g glucose (11 mM final)
100 μl 0.5% (w/v) thiamine HCl in H_2O (15 mM final)
1 ml 1 M $MgSO_4$ (1 mM final)
1 mg $FeSO_4$ (0.1% w/v final)
50 ml 1 M KH_2PO_4 pH 7.5 (50 mM final)
1 ml 30 mg/ml kanamycin (30 μg/ml final)
1 ml 100 mg/ml carbenicillin (100 μg/ml final)
Add H_2O to 1 liter; filter sterilize
Store ≤2 days at room temperature

Phosphate buffered saline (PBS), 10×

160 g NaCl (1.37 M final)
4 g KCl (26.8 mM final)
4 g KH_2PO_4 (14.7 mM final)
43.4 g $Na_2HPO_4 \cdot 7H_2O$ (80.9 mM final)
Add H_2O to 2 liters
Adjust pH to 7.5
Store ≤3 months at room temperature if sterile

Protein kinase A buffer, 10×

500 μl 1 M Tris·Cl, pH 7.5 (0.5 M final)
100 μl 1 M $MgCl_2$ (0.1 M final)
400 μl H_2O
Store ≤12 months at −20°C

Quench solution

25 mM β-mercaptoethanol
0.1 mg/ml BSA
Prepare fresh for each experiment

TBE, 10×

215.7 g Tris base (0.89 M final)
110 g Boric acid (0.89 M final)
14.9 g $Na_2EDTA \cdot 2H_2O$ (0.02 M final)
Add H_2O to just <2 liters
Adjust pH to 8.2 to 8.5
Correct volume to 2 liters with H_2O
Store ≤3 months at room temperature

Tricine loading dye, 3×

250 µl 4 M Tris·Cl, pH 6.5 (1 M final)
250 µl glycerol (25% v/v final)
480 µl 10% (w/v) SDS (4.8% w/v final)
20 µl 1% (w/v) Coomassie brilliant blue G250 (0.02% w/v final)
Store ≤12 months at −20°C

Urea-acrylamide gel

12.6 g urea (7 M final)
3 ml 10× TBE (see recipe)
11.25 ml acrylamide, 19:1 (40% w/v)
Add H_2O to 30 ml
Then add:
150 µl 10% (w/v) ammonium persulfate in H_2O (0.05% w/v final)
15 µl TEMED (0.05% v/v final)
Prepare fresh for each experiment

COMMENTARY

Background Information

Methods for the identification of protein interaction domains often rely on truncated or mutated recombinant proteins. A disadvantage of this approach is that binding interfaces represent three-dimensional surfaces and cannot be fully identified using protein truncations or point mutations. Knowledge of the three-dimensional structure of the proteins under study is helpful, but in the absence of a co-crystal of two binding partners, a significant amount of work is still required to define binding interfaces.

Previous methods for mapping protein structure through the measurement of amino acid side chain solvent accessibility were based on acylation of lysine residues, oxidation of methionine residues, and alkylation of cysteine residues (Doonan and Fahmy, 1975; de Arruda et al., 1992; Hanai and Wang, 1994; Doering and Matsudaira, 1996; Tu and Wang, 1999). However, data collection was limited to a few naturally occurring probes. Recently, a technique named misincorporation proton-alkyl exchange (MPAX) was developed, which makes use of translational misincorporation to introduce multiple structural probes into a protein structure (Silverman and Harbury, 2002a). This method was named analogous to nuclear magnetic resonance hydrogen/deuterium exchange and makes use of the reactivity of cysteine sulfhydryls to an alkylating reagent as a reporter of protein structure.

The approach described here makes use of MPAX to rapidly identify surface hydrophobic residues that contribute to protein-protein interaction interfaces. Small quantities (micrograms) of protein are required to perform all of the protocols described in this unit. This, in combination with the short time required for each assay, makes the method a highly attractive complement to high-resolution structural techniques such as X-ray crystallography and nuclear magnetic resonance. The technique is not limited by protein size or solubility. It has also proven very useful for proteins that are difficult to crystallize (Burguete et al., 2004). A central advantage lies in the rapid generation of a complete set of single amino acid mutant proteins that can be analyzed simultaneously for alterations in ligand binding or partner protein interaction.

Mutation to a cysteine residue does not generally disrupt normal protein topology because cysteine is small and amphiphilic and can easily be accommodated on the surface or within the core of folded proteins. The relative burial of cysteine residues in folded proteins correlates well with the expected burial of the particular amino acids that were replaced (Silverman and Harbury, 2002a,b; Burguete et al., 2004).

A further advantage of this technique, compared with crystallography, is that it detects the dynamics of a molecule in solution rather than a single, immobile, structural form. Unstructured regions of the protein and/or flexible loops are fully "visible" with this technology (Burguete et al., 2004). Moreover, residues that seem buried in a crystal structure may unexpectedly exchange with solvent due to protein dynamics in solution.

An alternative use of MPAX for detection of protein interfaces has been described (Silverman and Harbury, 2002a). Amino acids in binding interfaces were detected through direct protection from solvent by ligand binding.

Such residues displayed a slowed alkylation rate in the presence of ligand as compared to reactions carried out in its absence. It should be possible to apply this method to the detection of conformational changes, in the presence or absence of cofactors and/or ligands. In addition, Basic Protocols 2 and 3 can be performed in the presence of any number of additional proteins that do not contain a PKA recognition site. The methods described here are thus also applicable to large protein complexes, partially folded proteins, and membrane proteins.

With regard to the specific chemistry utilized herein, solvent-accessible cysteine thiols are reactive to alkylation by iodoacetamide. The alkylating reagent is quenched with a 2.5-fold molar excess of β-mercaptoethanol together with BSA. Non-alkylated cysteines that are buried in the protein can be detected by unfolding in guanidine·HCl and subsequent reaction with 2-nitro-5-thiocyanobenzoic acid (NTCB). A four-fold molar excess of NTCB over β-mercaptoethanol is used to cyanylate cysteine sulfhydryls that have not been selectively alkylated. Subsequent exposure to pH 8 to 9 results in cleavage at the amino acid that has been modified by NTCB (see Fig. 26.1.2; Jacobson et al., 1973).

Critical Parameters

While the general MPAX strategy of replacing hydrophobic residues with cysteine residues does not appear to significantly disturb protein stability, the effect of replacing conserved cysteine residues with alanine may be large in some cases (Silverman and Harbury, 2002b). It is, therefore, recommended to avoid replacing conserved cysteine codons when preparing the gene of interest (see Strategic Planning). A protein of interest must be expressed in minimal medium to achieve efficient misincorporation. Furthermore, it is critical to use ATP-γ-^{33}P to obtain sharp protein bands upon phosphorimaging.

The accuracy of the iodoacetamide concentration is critical to obtain reproducible results and to compare rate constants determined at different concentrations (native versus intrinsic rates). The alkylation rate is directly proportional to the iodoacetamide concentration (a ten-fold increase in concentration results in a ten-fold faster alkylation rate and vice versa). Because the cysteine alkylation chemistry is pH-sensitive (it requires the deprotonated form of the sulfhydryl, pK_a ~9), it is critical to perform the assay at the indicated pH. In addition, it is important to exclude any reagents containing free sulfhydryls in significant concentrations, as they will quench the alkylating reagent.

Troubleshooting

Nonspecific background backbone cleavage

If excess NTCB has not been completely removed before raising the pH of the protein solution with ammonia, the NTCB will cleave the protein backbone nonspecifically. It is important to remove excess NTCB fully during the TCA precipitation, if necessary, with additional acetone washes. Vortexing for prolonged periods of time can help dissolve the TCA pellet.

Autophosphorylation of PKA and subsequent nonspecific cleavage can cause background bands in the absence of a PKA substrate. Include a control sample with protein expressed in the absence of misincorporator tRNA to verify that no spurious bands are generated. Always include BSA in labeling reactions. Full length PKA and BSA do not enter the resolving gel and will not interfere with the analysis.

Bands appear at the infinite alkylation time point

Addition of guanidine·HCl in the infinite time point can lower the pH of the solution. The Bicine concentration is therefore doubled in samples that are alkylated in the presence of guanidine·HCl (see Basic Protocols 3 and 4). It is important to check the solution pH after guanidine·HCl has been added.

Chemical cleavage is not efficient

TCA that remains in the pellet after the acetone washes in Basic Protocol 2, step 5, might reduce the pH in the cleavage reaction. If this is a problem, the pellets can be resuspended in 10 μl of 8 M urea, 0.8 M ammonia in step 5. After cleavage, the ammonia is evaporated in open tubes before the samples are loaded onto the Tricine gel. High ammonia concentrations in samples will interfere with gel analysis.

Band intensities cannot be fitted to exponential decay functions

This is a typical result of loading unequal protein quantities from samples corresponding to different time points. Analyze equal amounts of radioactivity for different samples on the Tris·Tricine gel. The signals from individual bands can also be normalized for total lane intensity to facilitate comparison.

Bands appear blurry

It is critical to label the protein with ^{33}P (and not ^{32}P) to obtain the sharpest possible band signals from peptides that are resolved in the Tris·Tricine gel. In addition, this problem can be addressed by carefully loading a small volume (~5 μl) of sample into the bottom of the well of the Tris·Tricine comb gel. Avoid getting sample on the side of the wells. This can be done using a capillary pipet tip. Dry the gel immediately after peptide separation. Make sure that gel interfaces are smooth and that air bubbles are not trapped in the gel. Since any movement of the gel on the image plate results in a distortion of the signal, it is recommended to tape the dried gel onto the image plate.

Anticipated Results

Cysteine misincorporation and chemical cleavage at cysteine are highly reproducible techniques. The variation of measured alkylation rates between individual experiments is very small. MPAX in combination with affinity chromatography has previously been used to identify residues in binding interfaces and to detect surface hydrophobic residues that are likely candidates for protein-protein interaction (Burguete et al., 2004). Presuming that the protein of interest can be purified in microgram quantities, these methods are very likely to reveal amino acids that contribute to protein-protein interaction.

Time Considerations

Preparation of LB plates and medium prior to starting Basic Protocol 1 should take no longer than 1 to 2 hr. Bacterial expression of protein together with misincorporator tRNAs in minimal medium requires approximately the same time as expression without misincorporator tRNAs in rich medium. This includes transformation of *E. coli* and plating onto agar on day 1, which can be done in 1.5 hr. Inoculation of LB medium by individual bacterial colonies on day 2 should take no longer than 20 min. On day 3, cells are inoculated into minimal medium (20 min) and grown for 4 to 6 hr. Induction with IPTG takes 10 min and the expression time varies according to individual protocols and can be 1 hr to overnight. The duration of purification on day 4, again, varies according to individual protocols. As an example, a normal histidine-tagged protein can be purified in 4 to 6 hr and dialysis can be set up to proceed overnight. Thus, the total hands-on time for preparation of protein containing misincorporated cysteine should be no longer than 11 hr within a total time frame of 4 days.

Chemical cleavage at cysteine positions is typically performed within 3 hr, with the addition of 1.5 hr for the native cysteine alkylation protocol or the intrinsic alkylation rate protocol (see Basic Protocols 2 to 4). Tris·Tricine gels are prepared in 1 to 2 hr depending on the time required for polymerization (see Support Protocol 1). Gel set-up, counting of samples, and loading requires ~2 hr and gels are run overnight. The total drying time for gels is ~3.5 hr on the subsequent day. Thus, MPAX assays require 1 full day of hands-on time with the addition of a total of 20 min on day 2 for gel drying.

The exposure time for radioactive gels on image plates can range from a few hours to 2 days, depending on signal intensity. Data analysis including gel scanning, band quantification, data fitting using software, and determination of protection factors can take 1 to 3 days, depending on the skill and experience of the investigator.

The protocol describing how to select residues that contribute to binding interfaces (see Basic Protocol 6) can be performed in 1 day, assuming that purified proteins are available and with the addition of gel-processing time as described above.

Budget ~2 weeks for the gene assembly process (see Support Protocols 2 and 3), excluding the time required for oligonucleotide synthesis. Additional time will be required for correction of any point mutations detected in the assembled gene, sequencing, and cloning.

Finally, plan 1 to 3 weeks for designing a gene that will be useful for the planned experiments considering the points raised under Strategic Planning.

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Key References

Burguete et al., 2004. See above.

This is the first "blind" study where protein structure is mapped using the MPAX protocol and subsequently compared to crystallographic data. A method to select for residues that contribute to protein-interaction interfaces is developed and MPAX technology is also used to enable prediction of residues that lie in protein-protein interfaces. Residues that are highly likely to contribute to partner protein binding are selected by screening a population of different cysteine misincorporation mutants in a single affinity chromatography step.

Silverman and Harbury, 2002a. See above.

MPAX technology to footprint protein structure at a single amino acid resolution is developed and described here for the first time. The substrate-binding site for triosephosphate isomerase $(\beta/\alpha)_8$ barrel is accurately mapped and the stability of the barrel is determined. In addition, a mass spectrometry method to measure the alkylation rate at misincorporated cysteines is described.

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