

The fungal enzyme is stable in the presence of 0.2 M guanidine hydrochloride (Fig. 4), and has a longer half-life, 26 days, at room temperature and a wider stable pH range than bovine liver PDI.

Specificity of the enzyme action: Both the native and recombinant fungal PDIs are similar to bovine PDI in terms of the refolding of scrambled RNase and scrambled lysozyme, the oxidation of reduced BPTI, and the reduction of insulin (Fig. 5). The three enzymes showed no significant differences in substrate specificity. This suggests that the recombinant fungal PDI is also able to catalyze the reduction, oxidation, and isomerization of protein thiol or disulfide with a broad substrate specificity.

On the basis of these properties, the fungal PDI will be useful for the *in vitro* refolding and renaturing of scrambled proteins and for other industrial uses.

[5] Disulfide Bond Catalysts in *Escherichia coli*

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Introduction

The proper oxidation status of cysteine residues is critical for protein stability and activity.¹⁻³ Although many proteins need to be reduced to be active, the presence of disulfide bonds is crucial for the folding and stability of other, mainly secreted, proteins. *Escherichia coli* mutants that are deficient in thioredoxin reductase and in the disulfide bond catalysts DsbA, B, C, and D show altered abilities in disulfide bond formation *in vivo*, demonstrating the important role of these folding catalysts in the cell.^{1,4-8} One advantage of studying these catalysts is that disulfide exchange, both *in vitro* and in the cell, can be frozen at any instant in time using thiol-trapping agents and the reaction intermediates present within the substrate proteins and catalysts separated and quantified. This provides a unique

¹ J. C. A. Bardwell, *Mol. Microbiol.* **14**, 199 (1994).

² H. F. Gilbert, *Adv. Enzymol.* **63**, 69 (1990).

³ N. Darby and T. E. Creighton, *Methods Mol. Biol.* **40**, 219 (1995).

⁴ J. C. A. Bardwell, K. McGovern, and J. Beckwith, *Cell* **67**, 581 (1991).

⁵ J. C. A. Bardwell, J.-O. Lee, G. Jander, N. Martin, D. Belin, and J. Beckwith, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 1038 (1993).

⁶ D. Missiakas, C. Georgopoulos, and S. Raina, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 7084 (1993).

⁷ A. I. Derman, W. A. Prinz, D. Belin, and J. Beckwith, *Science* **262**, 1744 (1993).

⁸ D. Missiakas, F. Schwager, and S. Raina, *EMBO J.* **14**, 3415 (1995).

opportunity to study the kinetics of folding catalysis *in vitro* and *in vivo*. Because proteins rely on disulfide bond formation for their stability the formation of disulfides is energetically coupled with protein folding and provides a powerful tool for studying the folding process itself.³ However, accurately determining the rate of disulfide bond formation within cells presents an experimental challenge because the process normally occurs within seconds,^{4,9} and artifactual oxidation or reduction of proteins can easily occur during sample processing.¹⁰⁻¹²

We describe general methods for rapidly blocking thiol–disulfide exchange and for analyzing cell extracts in ways allowing for the distinction between the oxidized and reduced forms of proteins. A sample labeling protocol specifically designed to detect defects in disulfide bond formation in *E. coli* is detailed as one specific example; however, modifications of this protocol should work with many species. In addition, the trapping and separation techniques described have many applications for the study of disulfide catalysis using purified proteins *in vitro*, and for the study of the folding process itself, in the absence of catalysts. These applications, additional methods, and the theory used to study disulfide exchange have been described in detail.³ We also describe a generally applicable method to determine the equilibrium redox properties of disulfide catalysts.

Trapping Reagents and Conditions

An ideal sulfhydryl trapping agent specifically modifies all sulfhydryls in a protein rapidly, in a stable way, and in a way that allows one to separate the modified forms of the protein from the disulfide-bonded form.¹² The following trapping conditions are rather general and can be used to block thiol–disulfide exchange within or between purified proteins,^{3,11-15} *in vitro* translation reactions,¹⁶ or within cells that have been pulse labeled *in vivo*.^{4,7}

Iodoacetate and Iodoacetamide

Iodoacetate (IAA) reacts fairly specifically with exposed sulfhydryl groups, leaving behind the negatively charged carboxymethyl group

⁹ T. Peters and L. K. Davidson, *J. Biol. Chem.* **257**, 8847 (1982).

¹⁰ A. L. Derman and J. Beckwith, *J. Bacteriol.* **177**, 3764 (1995).

¹¹ J. S. Weissman and P. S. Kim, *Science* **253**, 1386 (1991).

¹² A. P. Pugsley, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 12058 (1992).

¹³ D. M. Rothwarf and H. A. Scheraga, *Biochemistry* **32**, 2671 (1993).

¹⁴ T.-Y. Lin and P. S. Kim, *Biochemistry* **28**, 5282 (1989).

¹⁵ J. W. Nelson and T. E. Creighton, *Biochemistry* **33**, 5974 (1994).

¹⁶ A. Zapun, J. C. A. Bardwell, and T. E. Creighton, *Biochemistry* **32**, 5083 (1993).

(CH_2COO^-) irreversibly linked to the sulfur in cysteine. This alteration of the net charge of a protein assists in the separation of proteins on urea polyacrylamide gels or native gels, based on the number of free cysteine residues present in the protein at the time of trapping. Because IAA reacts with the thiolate ion, trapping is pH dependent. At a concentration of 0.1 M at pH 8.7 the half-time of reaction between IAA and protein thiols should be about 1–3 sec,³ generally fast enough for quantification of the relative amount of oxidized versus reduced protein.^{14,18–20} At pH 8.7 IAA also reacts slowly with histidines, methionines, and amino groups. To avoid this, quench reactions should be kept short, on the order of 2 min. A quench carried out at pH 7.5 or with lower concentrations of IAA will be somewhat slower, but allows the protein to remain in the presence of the trapping agent for longer periods without significant side reactions. The two major disadvantages of IAA is that buried thiols will not react to an appreciable extent³ and that IAA, even at pH 8.7, may not be fast enough to stop all intramolecular disulfide exchange reactions.^{11,14,18–19,21}

Trapping can be accomplished by addition of 0.2 vol of 1 M IAA stock solution directly to growing cells or to thiol disulfide exchange reactions. Following a 2-min incubation at 25° for solutions at pH 8.7 or >15 min for solutions at pH 7.3, the excess IAA can be removed by desalting on a disposable 10DG column (Bio-Rad, Hercules, CA), and should be, if the samples are to be stored before analysis. If the trapping is carried out in whole cells or within cell extracts the trapping temperature should be lowered to 4° to help slow proteolysis. A closely related blocking agent, iodoacetamide (IAM), can be used interchangeably with IAA, except that it leaves behind an uncharged CH_2CONH_2 group. The lack of charge on this trapping agent increases its ability to pass through cellular membranes.

Stock Solutions

Prepare stock solutions from white crystals just before use.

Iodoacetic acid (1.0 M): Dissolve 184 mg of iodoacetic acid in 0.5 ml of 1 M KOH, then add 0.5 ml of 1 M Tris-HCl, pH 7.3 or 8.7

Iodoacetamide (0.5 M): Dissolve 92 mg of iodoacetamide in 1 ml of 0.5 M Tris-HCl, pH 7.3 or 8.7

Double Trapping with Both Iodoacetate and Iodoacetamide. Using both the acidic iodoacetate, and the neutral iodoacetamide added sequentially,

¹⁷ T. E. Creighton, C. J. Bagley, L. Cooper, N. J. Darby, R. B. Freedman, J. Kemmink, and A. Sheikh, *J. Mol. Biol.* **232**, 1176 (1993).

¹⁸ T. E. Creighton, *J. Mol. Biol.* **87**, 579 (1974).

¹⁹ T. E. Creighton, *J. Mol. Biol.* **87**, 603 (1974).

²⁰ T. E. Creighton, *J. Mol. Biol.* **96**, 767 (1975).

²¹ G. H. Snyder, *Biochemistry* **26**, 688 (1987).

it is possible to count the integral number of cysteine residues and disulfide bonds present in protein and classify their accessibility.^{3,22,23} One protocol involves first adding a negative charge to free sulfhydryl groups with iodoacetic acid in the presence or absence of urea; the second step consists of reducing all disulfides with dithiothreitol (DTT) and then blocking all remaining sulfhydryl groups using the uncharged iodoacetamide. Free sulfhydryls are first trapped by incubation with 30 mM IAA in 50 mM Tris (pH 8.2), 1 mM EDTA in either the presence or absence of 8 M urea for 15 min at 37°. The reaction is stopped by the addition of 0.01 vol of a carrier protein (alkylated lysozyme, 5 mg/ml) followed by precipitation with 10 vol of a cold acetone–1 N HCl mix (98:2, v/v). The precipitate is recovered by centrifugation (3000 g, 5 min, 4°). The precipitates are washed three times by resuspension in a cold acetone–1 N HCl–H₂O mix (98:2:10, v/v/v) and recentrifugation. The next step consists of dissolving the pellet in 8 M urea, 3.5 mM DTT, 50 mM Tris (pH 8.2), 1 mM EDTA and incubating for 30 min at 37° to reduce the remaining disulfides. The newly generated free sulfhydryls are then alkylated by addition of 10 mM IAM and incubated for 10 min at 37°.

When IAA reacts with a cysteine residue it leaves behind the charged carboxymethyl group; IAM leaves behind an uncharged group. This procedure allows the classification of all cysteines into three categories: reactive sulfhydryls, nonreactive sulfhydryls, and disulfide bonded. The total number of IAA molecules incorporated can be determined because migration in urea gels is dependent on charge. This method has the advantage that the effect of protein conformation is minimized. To calibrate the relationship between the mobility and the number of IAA molecules introduced a marker lane in which the protein of interest is modified with various mixtures of IAM and IAA is used.^{22–25} The number of distinct bands present in this marker lane should correspond to one more than the number of half-cystines present in the molecule. This process allows the quantitative analysis of the disulfide status of proteins that contain up to 18 intramolecular disulfides within the complex mixture of proteins present in *in vivo* labeling reactions.²²

N-Ethylmaleimide

N-Ethylmaleimide (NEM) is a highly specific sulfhydryl reagent; blocking of sulfhydryls occurs 1000 times more rapidly than the side reaction

²² N. Takahashi and M. Hirose, *Anal. Biochem.* **188**, 359 (1990).

²³ R. A. Reisfield, U. J. Lewis, and D. E. Williams, *Nature (London)* **195**, 281 (1962).

²⁴ A. Zapun, D. Missiakas, S. Raina, and T. E. Creighton, *Biochemistry* **34**, 5075 (1995).

²⁵ T. E. Creighton, *Nature (London)* **284**, 487 (1980).

with amino groups at pH 7.²⁶ *N*-Ethylmaleimide reacts more rapidly than iodoacetate with exposed sulfhydryl groups; however, it suffers from the disadvantage that it reacts even more slowly than iodoacetate with sulfhydryls that are buried or partially exposed, which leads to reactions that are not completed for 100 min or more.²⁷ The pH of the reaction with *N*-ethylmaleimide is critical. The rate of reaction drops off rapidly below pH 6. Above neutrality the rate of undesirable side reactions is increased and the reagent itself breaks down.²⁷ Adducts of *N*-ethylmaleimide and thiol are stable at pH values below 9.5, but unstable above pH 10.²⁸ The adduct it leaves behind is a rather bulky but uncharged C₆NO₂H₈ group. Stock solutions are 1 *M* in ethanol and can be stored at -20°. Crystals that form on storage should be dissolved before use. Blocking reactions are for 20 min using 20 *mM* *N*-ethylmaleimide at pH 7.

2-Aminoethyl Methanethiosulfonate

The reaction rate of the blocking reagent 2-aminoethyl methanethiosulfonate¹³ (AEMTS) is about seven orders of magnitude greater than that of IAA. It is sufficiently rapid to gain access to essentially buried protein thiols owing to local fluctuations or "breathing" of the protein structure during the blocking period. The use of AEMTS should be contemplated if rapid intramolecular disulfide exchange reactions are anticipated. This blocking reagent leaves behind the positively charged cysteamine moiety, which facilitates the separation, in a predictable manner, of proteins that contain different numbers of blocked thiols. Unfortunately, AEMTS is not commercially available; however, it can be synthesized.²⁹ Before use it should be freshly dissolved in 100 *mM* Tris, 2 *mM* EDTA, pH 8.0. AEMTS appears to be specific; no side reaction modifications are observed using up to 100 *mM* AEMTS at pH 5 and above.¹³ At a minimum, it should be used in at least fivefold molar excess relative to free thiols. Following a 2-min incubation at 25° (or 5 min at 4°) at pH 8 this blocking agent can, if desired, be removed by desalting on a disposable 10DG column (Bio-Rad).

Acid

Acid quenching is fast,¹⁴ occurring with a rate constant greater than 10⁹ sec⁻¹ M⁻¹, and is effective even when the thiol groups are relatively

²⁶ G. E. Means and R. E. Feeney, "Chemical Modification of Proteins." Holdan-Day, San Francisco, 1971.

²⁷ J. F. Riordan and B. L. Vallee, *Methods Enzymol.* **XI**, 541 (1967).

²⁸ J. Nishiyama and T. Kuninori, *Anal. Biochem.* **200**, 230 (1992).

²⁹ G. L. Kenyon and T. W. Bruce, *Methods Enzymol.* **47**, 407 (1977).

inaccessible. However, pH quenching does not stop thiol–disulfide exchange; it just slows it down by reducing the concentration of the reactive thiolate ion, necessitating the timely processing of samples.^{14,18} This reversibility is also an advantage, because it allows one to isolate individual folding intermediates and reinitiate thiol–disulfide exchange by raising the pH.¹¹ Acid quenching can be accomplished by addition of various acids, i.e., a 1/4 vol of 1 M HCl, or an equal volume of 10% (w/v) trichloroacetic acid.

AMS (4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid)

AMS (Molecular Probes, OR) reacts specifically and irreversibly with sulfhydryl groups conjugating a large 490 Dalton, negatively charged moiety to the free cysteines. This major change in molecular weight can be used to clearly separate proteins on SDS polyacrylamide gels based on the number of AMS moieties added onto the protein at the time of trapping. In addition to the large increase in molecular weight, AMS also has the added advantage of being more cysteine specific and reacting faster with cysteines as compared with the haloacetates.

Combination Quench Techniques Using Acid and AMS

Internal disulfide rearrangement reactions and disulfide catalysts such as DsbA are capable of rapid disulfide exchange reactions, which are difficult to quench using IAA. Acid quenching is sufficiently fast but necessitates acidic pH conditions for sample processing. This is possible if high-performance liquid chromatography (HPLC) separation techniques are used but not if any of the electrophoresis techniques discussed below are used. A combination of the acid and chemical quench techniques^{30,31} can make use of the best properties of both techniques. It involves first mixing the sample to be quenched with an equal volume of ice-cold 20% (w/v) trichloroacetic acid. This both rapidly quenches disulfide exchange and also denatures proteins present, including any disulfide catalysts, inhibiting their function. The precipitates of denatured proteins are pelleted by centrifugation (17,000 g for 20 min at 4°). The pellet is washed twice with 100% ethanol, dried in a dessicator, and then redissolved in freshly prepared 10 mM AMS in a 50-mM Tris-HCl (pH 8.1), 0.1% SDS, 1 mM EDTA solution. After 30 min of reaction, these samples can be resolved in nonreducing SDS gels. 90% acetone has been used in place of ethanol in the washing step, however, this can lead to poor recovery. Carrier proteins can be used to increase the size of the pellet, and a cysteine-free carrier protein has been successfully

³⁰ J. C. Joly and J. R. Swartz, *Biochemistry* **36**(33), 10067 (1997).

³¹ T. Kobayashi. *et al.*, *PNAS*, in press.

used in this procedure. Because AMS is a light-sensitive reagent, all steps involving AMS are carried out in the dark.

Safety

All of the thiol-trapping agents described above are rather toxic and should be handled accordingly.

In Vivo Labeling and Trapping

See Bardwell *et al.*⁴ for details.

1. Dilute 1:100 an overnight culture of *E. coli* cells that had been grown in M63 minimal medium³² supplemented with glucose (0.4%, w/v) and amino acids (20 $\mu\text{g/ml}$ each except for methionine and cysteine), into the same medium.

2. Let the culture grow until the cells are in the logarithmic phase (OD_{600} 0.3).

3. Label the cells for 40 sec by addition of [³⁵S]methionine (50 $\mu\text{Ci/ml}$; 29 TBq/mmol).

4. Chase with unlabeled methionine should be initiated by addition of cold methionine to a final concentration of 20 mM.

5. Immediately after addition of the cold methionine, transfer 1 ml of cells into a prechilled Eppendorf tube that contains 200 μl of ice-cold 100 mM IAM, vortex, and transfer to an ice-water bath.

6. After 1, 5, and 15 min, transfer additional 1-ml aliquots of the pulse-chased culture into Eppendorf tubes containing the trapping reagent.

7. After the samples have been incubated for 15 min on ice, recover the cells by centrifugation for 5 min at 10,000 rpm in a cooled Eppendorf centrifuge. Remove the supernatant.

8. The samples can now either be further processed by cellular fractionation, immunoprecipitation, and gel electrophoresis or frozen at -70° in a dry ice-ethanol bath before further processing.

Comments

This simple protocol is adequate for trapping disulfide bond formation in many periplasmic proteins; if more rapid trapping is desired use one of the alternate trapping agents and conditions previously described.

Immunoprecipitation

Specific visualization of the substrates for disulfide catalysts requires separation of the substrates from the total pattern of proteins synthesized.

³² T. J. Silhavy, M. J. Berman, and L. W. Enquist, "Experiments with Gene Fusions." Cold Spring Harbor Press, Cold Spring Harbor, New York, 1984.

This can be easily accomplished by immunoprecipitation. Perhaps the most suitable protein for measuring the rates of disulfide bond formation in *E. coli* is the outer membrane protein OmpA.⁴ OmpA has the fortunate quality that it is stable *in vivo* in the absence of its disulfide bond. The oxidized and reduced forms of OmpA are easily distinguished because the reduced form can be cleaved by trypsin to a discrete fragment.

1. Resuspend the frozen pellets from the pulse chase (sample protocol above) in 0.5 ml of ice-cold spheroplast buffer (100 mM Tris, pH 8.0; 100 mM KCl; 0.5 mM EDTA; 0.5 M sucrose).

2. Add EDTA and lysozyme to final concentrations of 7 mM and 60 $\mu\text{g/ml}$, respectively, and incubate for 15 min on ice.

3. Divide each sample into two equal aliquots, and lyse the cells by the addition of Triton X-100 to 1% (v/v). To one aliquot, add trypsin to a final concentration of 10 $\mu\text{g/ml}$. Proteolysis is carried out on ice for 20 min and stopped by addition of phenylmethylsulfonyl fluoride (1 mM final concentration) and trypsin inhibitor (2.5 mg/ml, final concentration).

4. Add an equal volume of KISDS buffer [100 mM Tris-HCl (pH 8.0), 300 mM NaCl, 4% (v/v) Triton X-100, 2 mM EDTA, 0.06% (w/v) SDS] and freeze-thaw three times to disrupt the spheroplasts completely. Pellet the debris for 5 min at 10,000 rpm in an Eppendorf centrifuge and pipette the soluble cell extract into a fresh tube.

5. OmpA is immunoprecipitated by incubation with rabbit polyclonal anti-OmpA antibody (obtained from C. Kumamoto, Tufts Medical School, Boston, MA) overnight at 4°.

6. Fifty microliters of immunoabsorbent *Staphylococcus aureus* (10%, w/v; Sigma, St. Louis, MO) is added, incubated for 20 min on ice with occasional inversion.

7. Pellet *Staphylococcus aureus* by centrifuging at 8000 g for 30 sec at 4°, resuspend in 750 μl of HS buffer [1% (v/v) Triton X-100; 1 M NaCl; 50 mM Tris-HCl, pH 8.0; 1 mM EDTA], by vortexing for 1 min.

8. Repeat the preceding wash step. Complete resuspension is important; do not spin longer or faster than required to pellet the *Staphylococcus aureus* suspension.

9. Remove the salt by washing with 750 μl of 50 mM Tris-HCl, pH 7.5.

10. Resuspend the pellet in 50 μl of SDS-PAGE sample buffer [2.5 mM DTT, 2% (w/v) SDS, 12.5 mM Tris-HCl (pH 8.0), 15% (v/v) glycerol]. Boil for 5 min to release the antigen from antibody and *Staphylococcus aureus*, spin *Staphylococcus aureus* down, and transfer the supernatant into a new Eppendorf tube. Store the supernatant at -20° before electrophoresis.

11. Of the various electrophoresis conditions described below for separating the oxidized and reduced forms of OmpA, a nonreducing 9% (w/v)

acrylamide–Tricine–SDS gel³³ has been found to be the one that gives the highest separation between oxidized and reduced forms. Split samples into two aliquots; to one aliquot add 2 mM DTT to illustrate the migration position of reduced OmpA, boil the samples for 5 min, and apply reduced sample to one-half of the gel and nonreduced sample to the other half. The reduced nonproteolyzed form of OmpA will run slightly slower than the oxidized form, and the trypsin fragment of the reduced form will run much faster than the oxidized form.⁴

Gel Separation of Oxidized and Reduced Protein

Methods used for the separation of trapped species with purified proteins, such as reversed-phase HPLC and ion-exchange chromatography,³ are not generally suitable for studies of disulfide bond formation *in vivo*, in which the limited amount of the proteins of interest is contaminated with much higher amounts of nonspecific cellular components.

Gel electrophoresis is one of the most useful techniques for analysis of disulfide bond formation *in vivo* because it readily allows the rapid, simultaneous separation of multiple samples and the detection of the small amounts of proteins produced by *in vivo* labeling reactions. Visualization can be achieved by immunoprecipitation before gel electrophoresis or by Western blotting.⁵ Native gels, urea gels, and nonreducing SDS acrylamide gels have all been used to resolve the oxidized and reduced forms of proteins. The best choice for any particular protein should be determined experimentally.

Nonreducing Sodium Dodecyl Sulfate Gel Electrophoresis

Nonreducing SDS gel electrophoresis is a simple modification of the conventional Laemmli gel system,³⁴ in which samples on adjacent gel lanes alternatively contain or do not contain a reducing agent. This gel system separates reduced from oxidized proteins primarily on the basis of the conformational state of the SDS-solubilized proteins. The oxidized forms of proteins are often slightly more compact and thus migrate slightly faster than the reduced form.⁴ Small proteins with multiple disulfide bonds that create large loop sizes will generally show large differences in mobility between oxidized and reduced forms. Subtle mobility differences found with larger proteins with small numbers of disulfides can be emphasized by running very long protein gels in apparatus normally used to electrophorese

³³ H. Schagger and G. von Jagow, *Anal. Biochem.* **166**, 368 (1987).

³⁴ D. M. Bollab and S. J. Edelman, "Protein Methods." Wiley-Liss, New York, 1991.

DNA sequence samples, modified only by the substitution of thicker (0.8 mm) spacers. Large sheets of Mylar obtained relatively inexpensively from local plastic suppliers can be cut cleanly with a sharp paper cutter to construct inexpensive spacers of any length (1/32-inch-thick stock is equivalent to 0.8 mm). Small differences in mobility between adjacent lanes can be better visualized by minimizing the width of the teeth on the comb used to cast the stacking gel.

Reducing agents tend to diffuse into adjacent lanes during electrophoresis and cause reduction of proteins in these lanes. Diffusion of 2-mercaptoethanol is relatively rapid and can pass through several lanes during prolonged electrophoresis. This necessitates the grouping of samples that have been treated with 2-mercaptoethanol on one portion of the gel. Substitution of 2 mM DTT, which diffuses slower than the 700 mM 2-mercaptoethanol normally present in SDS sample buffer, will greatly reduce this problem. The transition between oxidized and reduced protein will show up as a band containing a step or spur connecting the oxidized and reduced versions of the same protein. This feature can be helpful in spotting small differences in mobility because it usually occurs within a band instead of between lanes. This phenomenon is also useful in identifying the oxidized and reduced forms of a protein that exhibits a major shift in mobility, such as a protein linked by intermolecular disulfides.³⁵ SDS-PAGE gels run in the presence of Tricine instead of glycine³³ run more slowly but may give better resolution of oxidized and reduced forms.

Native Gel Systems

One simple native gel system is identical to conventional SDS-PAGE except that SDS is eliminated from all solutions.³⁴ Performing the electrophoresis at a slightly elevated pH (pH 9.5) has the advantage that more thiol groups will be ionized, and thus cause a mobility shift. Here separation is proportional to net charge and hydrodynamic volume.³ Differences in charge between oxidized and reduced proteins can be introduced by the use of trapping agents such as IAA and AEMTS that leave a charged group covalently linked to the free sulfhydryl. Native gels are primarily useful for analyzing small purified proteins or extracts that consist entirely of soluble proteins, such as periplasmic extracts. The presence of membranes and other cellular components in an extract tends to make the bands smear.

Urea Polyacrylamide Gels

All of the preceding separation techniques are easiest to interpret if the protein of interest has only one possible disulfide resulting in only two

³⁵ R. J. Allore and B. H. Barber, *Anal. Biochem.* **137**, 523 (1984).

bands, oxidized and reduced. Proteins that have more than one disulfide will have multiple intermediates in the protein-folding pathway and may migrate as multiple, poorly resolved bands on native and nonreducing gels. These different forms can be separated by urea PAGE. Oxidized and reduced forms of the same protein are separated on urea gels almost entirely on the basis of charge introduced by the trapping agent.

Urea Polyacrylamide Gel Protocol. In the urea polyacrylamide gel protocol,²² proteins should be electrophoresed on a discontinuous acrylamide slab gel. Both stacking and resolving gels contain 8 M urea. The stacking gel contains 0.12 M Tris-HCl buffer, pH 6.8 and the resolving gel contains 0.037 M Tris-HCl buffer, pH 8.8. The reservoir contains 0.025 M Tris, 0.192 M glycine (pH 8.3). The concentration of acrylamide in the separating gel should be 9% (w/v); the stacking gel uses 2.5% (w/v). Electrophoresis should be carried out at 4°. For additional gel protocols see Ref. 3.

Enzymatic Assays to Detect Defects in Disulfide Bond Formation in *Escherichia coli*

Protein folding in the cell is a competition between two processes: correct folding leads to active protein and incorrect folding leads, at a minimum, to inactive protein but more often to degraded or aggregated protein *in vivo*. As a result, one can get an idea of the effectiveness of protein-folding catalysts simply by comparing the enzymatic activity of their substrate proteins between wild-type strains and strains that are deficient in these folding catalysts.

Of the large number of substrates whose activity is affected by mutations in the disulfide catalysts¹ only two are described here in detail: alkaline phosphatase and the MalF- β -galactosidase #102 fusion protein. Other genetic tests that can be used to distinguish between wild-type strains and those that form disulfides slowly are acid phosphatase levels,³⁷ sensitivity to 7 mM DTT,⁶ sensitivity to benzylpenicillin (15 μ g/ml),⁶ and motility in LB plates containing 0.3% (w/v) agar.³⁸

Alkaline Phosphatase

Disulfide bond formation in both the periplasm and cytoplasm can be assayed by measuring specific alkaline phosphatase activity. Two intrachain disulfide bonds are necessary for the activity of alkaline phosphatase as phosphomonoesterase. Mutations that decrease the rate of disulfide bond

³⁶ B. J. Davis, *Ann. N.Y. Acad. Sci.* **121**, 404 (1964).

³⁷ P. Belin, E. Quéméneur, and P. L. Boquet, *Mol. Gen. Genet.* **242**, 23 (1994).

³⁸ F. E. Dailey and H. C. Berg, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 1043 (1993).

formation in the periplasm will result in a loss of activity of this periplasmic enzyme.^{4,6,37} Mutations that increase the rate of disulfide bond formation in the cytoplasm will result in activation of artificially cytoplasmically located alkaline phosphatase.⁷

Alkaline Phosphatase Activity Assay. Grow cultures overnight in NZ medium. Dilute cultures 1:100 (v/v) into M63 salts³² supplemented with 0.4% (w/v) glucose and a 50- $\mu\text{g}/\text{ml}$ concentration of all amino acids except cysteine and methionine. When the OD_{600} is between 0.3 to 0.6, wash the cells twice by centrifuging at 10,000 g for 5 min at 25° and resuspending in an equal volume of TS buffer [10 mM Tris (pH 8), 150 mM NaCl] at room temperature. Do not leave the cells on ice at any time, as this can result in artificially high levels of alkaline phosphatase, although this problem can be prevented by addition of 10 mM IAM to the TS buffer.¹⁰ Use 800 μl to determine the OD_{600} of the cell suspension following the wash. To determine alkaline phosphatase activity, mix 100 μl of cells with 900 μl of 1 M Tris-HCl, pH 8.0, containing 0.1 mM ZnCl_2 . Add 25 μl of 0.1% (w/v) SDS and 25 μl of chloroform; vortex. Incubate for 5 min at 28° and add 100 μl of 0.4% (w/v) *p*-nitrophenyl phosphate (PNPP in 1 M Tris-HCl, pH 8.0). Record the starting time. Incubate at 28° until a light yellow color develops. Stop the reaction by adding 120 μl of 2.5 M K_2HPO_4 ; record the time. Spin for 5 min at 10,000 g in an Eppendorf microcentrifuge to remove cells. Read the OD_{420} and calculate the alkaline phosphatase (AP) activity by the following equation:

$$\text{AP activity units} = \frac{\text{OD}_{420} \times 1000}{\text{Time} \times \text{OD}_{600} \times \text{ml of cell culture used}}$$

The steady level of active alkaline phosphatase found in *dsb*⁻ strains is a complex function of growth conditions, and especially of availability of oxygen and cystine. To obtain reproducible values it is important to grow the cultures in exactly the same way each time, in the absence of added cystine and with the same rate of aeration. The accuracy of this procedure is improved by using *phoR*⁻ strains, in which the synthesis rate of alkaline phosphatase is high and constitutive.⁴

Disulfides are not normally formed in cytoplasmic proteins; as a result, if alkaline phosphatase is forced to stay in the cytoplasm by elimination of its secretion signal, it fails to form its disulfides and is inactive.^{7,30} However, a mutation in the gene for thioredoxin reductase leads to an increased rate of disulfide bond formation in the cytoplasm and up to 25% of the total alkaline phosphatase becoming active.⁷ The formation of disulfides in the cytoplasm can therefore be monitored and quantified either by measuring the specific activity of the alkaline phosphatase or by immunoprecipitation and separation of reduced and oxidized forms of alkaline phosphatase

by nonreducing SDS-PAGE. Antibody to alkaline phosphatase can be obtained from 5 Prime → 3 Prime (Boulder, CO).

MalF-β-Galactosidase #102 Fusion Protein

MalF-β-galactosidase #102 fusion protein, an artificial substrate for detection of disulfide bond formation *in vivo*, is particularly useful because it shows a large difference in activity between strains with defects in disulfide bond formation and wild-type strains; a big enough difference to allow the selection of mutants that show decreased levels of disulfide bond formation.^{4,5} In this disulfide indicator protein, β-galactosidase, a normally cytoplasmic protein, is fused to the large periplasmic domain of the *E. coli* integral membrane protein MalF. In strains that are wild type for the *dsb* genes the fusion has virtually no β-galactosidase activity. The Dsb proteins are apparently able to oxidize and thus inactivate the portion of this fusion protein that is exposed to the periplasm. However, this fusion protein has substantial β-galactosidase activity if the strain in which this fusion protein is expressed contains a null mutation in the genes for the disulfide catalyst DsbA or DsbB. This increase in β-galactosidase activity can be selected for using lactose minimal plates³² or can be screened for using LB plates that incorporate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 60 μg/ml). The MalF-β-galactosidase #102 fusion protein is expressed from λ integrated by homology at the maltose locus. Because the fusion is under the maltose promoter, one needs to induce the maltose promoter to see substantial levels of β-galactosidase. This can be done either by introducing the *malT^c* mutation, which constitutively expresses the maltose genes, or by growing cells in the presence of 0.4% (w/v) maltose. It is also helpful to stabilize the λ MalF-β-galactosidase #102 fusion lysogen in the chromosome by introduction of the *recA* mutation into the strain. A semiquantitative test for Dsb function *in vivo* has been developed by exploiting the observation that partially active *dsb* mutants isolated in a strain that contains the MalF-β-galactosidase #102 fusion protein vary in their ability to overcome millimolar concentrations of DTT added to the growth medium.³⁹

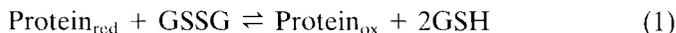
Determination of Redox Equilibrium with Glutathione

The relative oxidizing power of disulfide catalysts or indeed of any disulfide bond can, in principle, be measured against glutathione (GSH) as a standard.^{2,40-42} This redox scale compares the ability of oxidized gluta-

³⁹ U. Grauschopf, J. Winther, P. Korber, T. Zander, P. Dallinger, and J. C. A. Bardwell, *Cell* **83**, 947 (1995).

⁴⁰ M. Wunderlich and R. Glockshuber, *Protein Sci.* **2**, 717 (1993).

thione (GSSG) to oxidize protein thiols as measured by the equilibrium constant K_{ox} for the following reaction:



$$K_{\text{ox}} = \frac{[\text{Protein}_{\text{ox}}][\text{GSH}]^2}{[\text{Protein}_{\text{red}}][\text{GSSG}]} \quad (2)$$

K_{ox} can be measured by any technique that detects changes in the concentration of the reduced protein. One convenient method exploits the quenching effect of disulfides on the fluorescence of adjacent tryptophan residues.^{40,41} A number of disulfide exchange enzymes (thioredoxin, DsbA, calf liver protein disulfide isomerase, and the periplasmic domain of TlpA) exhibit a much higher fluorescent yield of reduced as compared with oxidized proteins.^{40,41} The fluorescence emission properties of oxidized proteins, and proteins reduced by the presence of 10 mM DTT, can be measured by exciting a 1 μM solution of the purified protein at 295 nm in a scanning fluorometer and recording emission spectra between 300 and 400 nm. A significant difference in fluorescent yield between reduced and oxidized protein allows their relative quantification. The wavelength that shows the biggest difference in fluorescence between fully oxidized and fully reduced proteins (wavelength Q) should be used to monitor the oxidation status of the protein. This quantification is greatly simplified if the protein of interest contains only one disulfide bond. As the number of sulfhydryl groups that participate in thiol–disulfide exchange increases, analysis of the multiple redox states present at equilibrium becomes increasingly difficult.^{2,41} Our analysis applies only to the simplest case, in which the protein contains one disulfide bond. It is also important to show that the protein does not show a significantly altered secondary structure on reduction as judged by near- and far-UV circular dichroism measurements,^{40,41} because unfolding transition equilibria will complicate the measurement of redox equilibria.

The specific fluorescence of a protein is used to measure the equilibrium concentrations of the oxidized and the reduced form of the protein in the presence of different ratios of oxidized and reduced glutathione. The protein (1 μM) is incubated at 30° under a nitrogen atmosphere in the presence of degassed glutathione redox buffers that contain constant concentrations of 0.01 mM GSSG, 100 mM sodium phosphate (pH 7.0) and 1 mM EDTA and various concentrations of GSH ranging from 0.005 to 125 mM until equilibrium is reached. R , the relative amount of reduced protein present at equilibrium, can be calculated from the measured fluorescence intensity using the following formula:

⁴¹ H. Loferer, M. Wunderlich, H. Hennecke, and R. Glockshuber, *J. Biol. Chem.* **270**, 26178 (1995).

⁴² R. P. Szajewski and G. M. Whitesides, *J. Am. Chem. Soc.* **102**, 2011 (1980).

$$R = (F - F_{\text{ox}})/(F_{\text{red}} - F_{\text{ox}})$$

where F is the measured fluorescence intensity at the wavelength Q , and F_{red} and F_{ox} are the fluorescence intensities of completely reduced or oxidized protein, respectively. The reactions are judged to be in equilibrium when further incubation does not result in a change in intensity and when the intensity is the same independent of whether one approaches equilibrium starting with reduced or oxidized protein.

Determination of GSH and GSSG Ratios

Degassing buffers, flushing with nitrogen, and then incubating under nitrogen atmosphere will reduce air oxidation of GSH. However, to account for air oxidation and correct for the small amount of GSSG contaminating commercial GSH, GSH and GSSG concentrations actually present in the various glutathione mixtures at equilibrium should be measured in conjunction with fluorescence measurements.⁴¹ The equilibrium concentrations of GSH can be determined by the method of Ellman⁴³; an ϵ_{412} of 14,140 $M^{-1} \text{ cm}^{-1}$ can be used for the thionitrobenzoate anion. The equilibrium concentrations of GSSG can be quantified enzymatically using yeast glutathione reductase (EC 1.6.4.2; Boehringer GmbH, Mannheim, Germany). NADPH is added to a final concentration of 200 μM to a degassed reaction at 25° that contains the GSSG to be assayed, 100 mM sodium phosphate (pH 7.0), and 1 mM EDTA. Reactions are started by addition of 1 U glutathione reductase. After a 10-min incubation the rate of the decrease in absorption at 340 nm is recorded ($\epsilon_{\text{NADPH}}^{340 \text{ nm}} 6220 M^{-1} \text{ cm}^{-1}$). A linear calibration curve should be obtained with GSSG concentrations ranging from 1 to 200 μM .

Calculation of K_{ox}

Once the proportion of the protein present in the reduced form at equilibrium (R) and the GSH and GSSG concentrations actually present in the various redox buffers have been determined the redox equilibrium constant, K_{ox} , of the protein can be calculated. This is done by plotting R against the $[\text{GSH}]^2/[\text{GSSG}]$ ratio and performing a nonlinear regression fit using the following formula:

$$R = \frac{[\text{GSH}]^2/[\text{GSSG}]}{K_{\text{ox}} + [\text{GSH}]^2/[\text{GSSG}]}$$

⁴³ P. W. Riddles, R. L. Blakeley, and B. Zerner, *Methods Enzymol.* **91**, 49 (1983).

This analysis contains the simplifying assumption that the mixed disulfide between glutathione and the protein is present at equilibrium in negligible quantities. This has been shown to be true for DsbA.¹⁶ To check if this is true for the protein to be tested it may be necessary to quench the protein–glutathione equilibrium reactions by addition of HCl (to about pH 2). Separation, identification, and quantification of all the thiol and disulfide species to determine their concentration at equilibrium may be attempted by reversed-phase HPLC analysis.^{3,15} This type of analysis provides an alternative method for determining K_{ox} values. If the K_{ox} value of the tested protein is high, equilibrium constants may be measured by using DTT redox buffer instead of glutathione redox buffers.^{2,40} A number of other techniques used in the investigation of disulfide catalysts are not discussed here but are explained in detail in Refs. 15–17, 24, and 42–48.

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⁴⁴ M. Wunderlich and R. Glockshuber, *J. Biol. Chem.* **268**, 24547 (1993).

⁴⁵ M. Wunderlich, A. Otto, R. Seckler, and R. Glockshuber, *Biochemistry* **32**, 12251 (1993).

⁴⁶ M. Wunderlich, R. Jaenicke, and R. Glockshuber, *J. Mol. Biol.* **233**, 559 (1993).

⁴⁷ M. Wunderlich, A. Otto, K. Maskos, M. Mücke, R. Seckler, and R. Glockshuber, *J. Mol. Biol.* **247**, 28 (1995).

⁴⁸ A. Zapun and T. E. Creighton, *Biochemistry* **33**, 5202 (1994).

[6] Yeast Immunophilins: Purification and Assay of Yeast FKBP12

By GREGORY WIEDERRECHT and JOHN J. SIEKIERKA

Introduction

The immunophilins constitute a class of ubiquitously expressed proteins named for their ability to bind the immunosuppressive drugs cyclosporin A (CsA), FK506 (Prograf), and rapamycin (RAPA).^{1,2} The genes for five

¹ S. L. Schreiber and G. R. Crabtree, *Immunol. Today* **13**, 136 (1992).

² J. J. Siekierka, *Immunol. Res.* **13**, 110 (1994).