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Use of Protein Folding Reagents

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Abstract

The reagents and methods for purification of the most commonly used denaturants guanidine hydrochloride (guanidine-HCl) and urea are described. Other protein denaturants and reagents used to fold proteins are briefly mentioned. Sulfhydryl reagents (reducing agents) and “oxido-shuffling” (or oxidative regeneration) systems are also described.

DENATURANTS

Chemical agents that denature proteins are used to extract proteins from inclusion bodies as well as to study the conformation of purified proteins (Pace, 1986). The most common denaturants are guanidine hydrochloride (guanidine-HCl) and urea. These reagents, methods for their purification and mode of action are described. Also, other protein denaturants are mentioned.

Guanidine Hydrochloride

High-purity guanidine-HCl (also referred to as guanidinium chloride) can be purchased from several vendors as a crystalline solid or concentrated solution (usually 8 M) in water. Because dissolution of the salt is an endothermic process, when preparing concentrated solutions from the solid salt it is convenient to heat the mixture (in a glass container) in a domestic microwave oven. When the best grades of commercial guanidine-HCl (>99% pure) are used, a 6 M solution will be clear and colorless. Concentrated solutions made from practical-grade guanidine-HCl (usually 80% to 90% cheaper) may appear slightly hazy and be colored light brown. For large-scale preparative work, where the purchase of sufficient high-purity reagent would be prohibitive, solutions of practical-grade guanidine-HCl can be cleaned up adequately by filtration with 0.45- to 0.5- μ m filters followed by decolorization with activated charcoal. Other applications, such as folding experiments (especially those involving spectroscopic monitoring), require higher-quality reagent; in such cases it is necessary to recrystallize the salt or purchase the ultrapure grade. Methods for purifying guanidine-HCl by recrystallization have been described by Nozaki (1972).

Because guanidine-HCl is hygroscopic, for critical studies, such as conformational analysis, accurate measurements of molarity should be made using a refractometer (e.g., the Abbe-3L refractometer check on-line for various suppliers including Anton Paar). Using Table A.3.A. 1 as a guideline for quantities, guanidine-HCl and water (or buffer) can be weighed in a

container to give the approximate molarities indicated. A table of the refractive indexes of guanidine-HCl solutions from 0.057 M to 8.51 M (in increments of ~0.06 M) at 25°C is provided by Nozaki (1972).

There have been no reports, at least to the author's knowledge, of specific chemical modification of proteins resulting from exposure to guanidine-HCl or the contaminants found in the commercially purchased forms. However, the common contaminant ammeline, which is characterized by strong UV absorbance, may inhibit certain enzymes (Nozaki and Tanford, 1967; Nozaki, 1972, and references therein).

Guanidine-HCl solutions are stable at room temperature for several days (for longer periods, store at 4°C). Solutions are stable over normal working pH ranges—i.e., pH 2.0 to 10.5. At alkaline pH (>11), biguanidine may form (Nozaki and Tanford, 1967).

Urea

High-purity (>99%) urea can be purchased from many different vendors and is only approximately twice as expensive as practical-grade material. Urea solutions slowly decompose to form ammonium cyanate, which reacts readily with various protein functional groups. An 8 M solution of urea incubated at room temperature >pH 7.0 will contain ~4 mM cyanate after 7 days and ~20 mM at equilibrium. Formation of cyanate occurs faster at higher temperatures (e.g., equilibrium is reached in 30 min at 100°C), and it may therefore be helpful to keep the solution cold. Cyanate reacts readily with the amino, sulfhydryl, imidazole, tyrosyl, and carboxyl groups of proteins. Only the reaction with amino groups results in stable products at alkaline pH (Means and Feeney, 1971). The carbamylation of α -amino (N-terminal) and ϵ -amino groups (lysine side chains) results in the formation of a neutral product, homocitrulline; hence there is a loss of one positive charge for each residue modified.

The standard method for removing cyanate is deionization using a mixed-bed ion-exchange resin (e.g., AG 501-X8, Bio-Rad; see *SUPPLIERS APPENDIX*). The batch method (adding resin to the solution, incubating, then filtering) is recommended for volumes <1 liter, whereas the column method (using resin packed in a chromatography column) is best for larger volumes (see Bio-Rad Bulletin 1825 and *UNIT 8.2*). Cyanate removal can be monitored by determining the solution conductivity; there are also simple specific assays available (Means and Feeney, 1972). For a detailed purification method, involving both recrystallization and deionization, see Prakash et al. (1981). Cyanate can also be removed by acidification to pH 2 with HCl, followed by a 1-hr incubation during which the cyanate breaks down to carbon dioxide and ammonium ions, and neutralization with Tris base. The solution can then be used as is.

For most applications it is recommended that solutions be made with ultrapure urea and used within 24 hr. A scavenger should be included to react with slowly forming cyanate; buffers containing primary amines, such as Tris and glycine, are suitable for this purpose. It should be noted that urea may increase the measured pH of aqueous solutions (see Bull et al., 1964).

As with guanidine-HCl, refractometry can be used to accurately determine the concentration of solutions, although solutions prepared by weight (Table A.3.A.2) are normally accurate enough for most applications (Pace, 1986).

Effectiveness

According to Pace (1986) and references therein, guanidine-HCl is generally 1.5 to 2.5 times more effective per mole as a protein denaturant than urea.

Mechanism of protein destabilization with urea and guanidine.HCl

In solution guanidinium ions can transiently stack and in this flattened conformation can coat protein hydrophobic surfaces especially those with planar amino acid side chains (Arg, Trp Gln) as well as aliphatic side chains. The effect of coating or covering hydrophobic surfaces of proteins reduces the unfavorable exposure to water (solvent) as would be encountered in the denatured or unfolded protein. Urea does not appear to undergo the stacking process but concentrates around exposed peptide groups due to direct H-bonding to peptide NH groups and probably to CO groups. Both guanidine and urea, therefore, weaken the hydrophobic effect which is a major contribution to the stability of native protein conformations (Lim et al., 2009; England and Haran, 2011).

Denaturation curves using urea and guanidine.HCl

Protein denaturation using guanidine.HCl and urea (and often temperature) are monitored using various physical methods such as circular dichroism (units 7.6; 28.3) and are used to determine the conformational stability of the protein (unit 28.4). For many proteins, the curves are sigmoidal in shape and consist of a region (pre-transition) where the protein is its native conformation, a transition region where equilibrium between folded and unfolded proteins exists and the post transition region where only unfolded (denatured) protein exists (Bajaj et al., 2004). For example, with a hypothetical protein and using urea as the denaturant, native protein may exist 0 – 3M urea, the unfolding transition region 3 – 6M and denatured protein > 6M urea. The thermodynamic analysis of these types of curves is described in detail in unit 28.4. In practical terms, these curves indicate of the concentrations of denaturant required for the solubilization of aggregated protein, for example, in inclusion bodies (> 6M urea). They also indicate the concentration range of denaturant compatible with folded protein. Although urea is a denaturant it can be used in limited concentrations as a co-solvent to assist in protein folding by maintaining solubility; with our hypothetical protein, this would be between 0 – 3M urea (the folding of BGH described in unit 6.5 (basic protocol 1) is an example of using this approach).

Other Protein Denaturants

Guanidine thiocyanate is another denaturant of interest that is often used to elute proteins from immunoaffinity columns. Although guanidine thiocyanate is rarely used in preparative folding of recombinant proteins or in conformational analysis of proteins in general, it is 2.5 to 3.5 times more effective than guanidine-HCl, and is available (albeit expensive) in high purity. Sodium dodecyl sulphate (SDS) is a well known and powerful protein denaturant and is being increasingly used in preparative protein folding (Unit 28.6). A related denaturant is n-

lauroyl sarcosinate (sarkosy) and has been successfully used to extract and fold *E.coli* inclusion body aggregates (Burgess, 2009). This reagent has a relatively high critical micellar concentration (14.4 mM) and binds less tightly to proteins than SDS making its controlled removal by dialysis etc., more practical. Both SDS and sarcosine are fairly inexpensive and can be purchased in high purity.

The chemical reagents mentioned above are the most commonly used to extract recombinant proteins but it should be mentioned that in experimental protein folding to determined mechanisms and pathways, often temperature is used to unfold proteins. Also, pH is another important parameter in protein stability and solubility. Denaturation curves established as a function of temperature and pH can greatly assist in protein extraction and purification. For example, thermostability and acid stability can be exploited to selectively extract proteins from complex extracts.

Purification of Unfolded Proteins

Proteins in denaturing concentrations of urea and guanidine can be purified by gel filtration; if the proteins are His-tagged, they can also be purified by metal chelate chromatography (see unit 6.3). Proteins in urea can be purified by ion-exchange chromatography. HPLC with a TFA/acetonitrile solvent system is also compatible with urea and guanidine denatured proteins. SDS denatured proteins can be purified by gel filtration, hydroxyapatite chromatography, and, if hist-tagged, maybe purified by metal chelate chromatography depending on the manufacturer of the resin and the concentration of SDS in the sample (consult vendors on-line descriptions).

Protein Folding Co-Solvents

Protein folding from denatured protein may require no more than dilution of the denaturant or its removal by dialysis against a suitable buffer. In many cases folding requires various co-solvents which assist in folding by either suppressing the aggregation of folding intermediates or by stabilizing the folding conformation. Small intracellular organic molecules which can protect the cell against water stress conditions are known as protective osmolytes, for example proline, glycinebetaine, sorbitol, sucrose, N-methylglycine (sarcosine) and trimethylamine *N*-oxide (TMAO). Osmolytes influence protein folding by pushing the folding equilibrium toward the native fold. The molecular basis for this effect is under investigation (Street et al. 2006). Practically, these small molecules, at up to molar concentrations, are used as co-solvents to assist in in-vitro protein folding (Bondos and Bicknell, 2003) and there are many examples of this application in the literature.

Another widely used co-solvent is arginine which acts to suppress aggregation of folded intermediates and folded proteins. Arginine (0.1- 1M) appears to increase the solubility of both folded and unfolded proteins (Tisher et al, 2010). Arginine at very high concentrations (up to 2M) can also be used to extract *E.coli* inclusion bodies (Tsumoto et al, 2008).

SULFHYDRYL REAGENTS AND OXIDO-SHUFFLING SYSTEMS

Sulfhydryl reagents (reducing agents) are used to prevent the oxidation of protein thiol groups. When proteins are extracted from inclusion bodies it is standard practice to include a

reducing agent to prevent random oxidation of cysteine residues. Denatured proteins are subsequently folded under conditions that favor native disulfide formation. This process, referred to as oxidative folding, is carried out using “oxido-shuffling” (or oxidative regeneration) systems consisting of low-molecular-weight thiol/disulfide pairs (*UNITS 6.4 & 6.5*). The thiols and disulfides commonly used to reduce and oxidize recombinant proteins are shown in Table A.3.A.3 and discussed in this section. Further details on these and other reducing agents are reviewed by Jocelyn (1987).

Dithiothreitol

Dithiothreitol (DTT), also known as Cleland’s reagent, is the preferred reductant for protein reduction. DTT and its isomer dithioerythritol (DTE)—which can also be used as a protein reductant—have similar properties (and prices), except that at lower pH the former is more effective. This is because the pK_a values for DTE are higher (9.0 and 9.9) than those of DTT (8.3 and 9.5); hence, a higher pH is required to ionize DTE to its functional form. The reducing capacity of both reagents increases greatly over the pH range 7 to 9.5. On the other hand, the reagents are quenched by acidification to $<pH$ 3.0.

DTT is very soluble in water, and stock solutions of 0.1 to 0.2 M can be prepared and stored at 4°C for a day or so or frozen for longer periods. For critical work, solid DTT should be added to solvents and buffers immediately before use to achieve a final working concentration in the range of 1 to 20 mM. Because DTT complexes metal ions, EDTA is usually included in the reduction buffer. Air oxidation of DTT solution can be monitored by measuring the increase in absorbance at 283 nm (see Table A.3.A.3).

2-Mercaptoethanol

2-Mercaptoethanol (2-ME) is also commonly used as a protein reducing agent. 2-ME is a volatile liquid that should be stored at 4°C and dispensed under a fume hood. A 100-mM stock solution of 2-ME can be made by diluting 6.99 ml of the reagent with solvent to 1 liter. 2-ME is not as potent a reductant as DTT, as it has a higher pK_a (Table A.3.A.3), and is used in the concentration range of 5 to 100 mM. Air oxidation of 2-ME is best monitored by measuring the increase in absorbance at 260 nm (Table A.3.A.3). Freshly opened bottles contain 0.1% to 6.7% of the oxidation product 2-hydroxyethyl disulfide (HED; Wetlaufer et al., 1987).

Glutathione

Glutathione (GSH) and its oxidized form GSSG are present in most cells at concentrations of 1 to 10 mM. In *E. coli*, the ratio of GSH/GSSG is 50:1 to 200:1 (Hwang et al., 1992), and the system is responsible for maintaining cytoplasmic proteins in a reduced state. GSH is not normally used as a reductant in analytical protein chemistry—its main utility is as a component of oxidative redox buffers (see discussion of Oxido-Shuffling Systems). However, GSH can be used to maintain unfolded protein in inclusion-body extracts in the reduced state (analogous to its role in vivo).

Stock solutions of 0.1 M GSH can be prepared in water or the reagent can be directly added to buffers. In either case the pH should be adjusted with base to the required pH.

Tris (2-carboxyethyl) phosphine hydrochloride (TCEP)

Tris (2-carboxyethyl) phosphine hydrochloride, CAS 5961-85-3 (TCEP) is a thiol-free reductant for protein and peptide disulfide bonds. Compared to dithiothreitol and β -mercaptoethanol, TCEP is odorless, a more powerful irreversible reducing agent (the end product of TCEP-mediated disulfide cleavage is two free thiols/cysteines), more hydrophilic, and more resistant to oxidation in air. It also does not reduce metals used in immobilized metal affinity chromatography. TCEP reacts rapidly (<5 min) with disulfides at room temperature in dilute (1 mM) solutions over a wide range of pH (Burns et al., 1991). It is noteworthy that TCEP is reactive at acid pH values as low as 1.5 (Hun and Hun, 1994).

TCEP is not particularly stable in phosphate buffers, especially at neutral pH, for example, it completely oxidizes within 72 hours in 0.35M phosphate-buffered saline, pH 7.0. If TCEP is used with phosphate buffers it should be added fresh and used immediately (see Thermo Scientific web site product #20490 for more details). In Unit 7.13 (see Figure 7.13.1), the extinction profile of common reductants in the ultraviolet range is shown: TCEP is the preferred reductant for spectroscopic studies and especially for analytical ultracentrifugation using UV optics due to the low extinction at 280 nm where most proteins are measured.

One disadvantage of TCEP is that it is rather expensive compared to the other commonly used reductants and many investigators purify proteins using, for example, DTT in the buffers and then switch to TCEP for storage and for protein characterization studies.

Dithiobutylamine, (DTBA)

DTBA has thiol pK_a value (~ 8.2) that is ~1 unit lower than that of DTT (~ 9.2) and, therefore, functions at lower pH values. DTBA has been shown to reduce the disulfide in oxidized glutathione 5x faster and reduce (activate) the cysteine protease papain 14x faster than the commonly used DTT. The presence of the primary amine group in DTBA, as opposed to the two hydroxyl groups of DTT, leads to lower pK_a values for the two thiols and contribute to its enhanced biological reducing activity. Another advantage of the primary amine group is that it allows for easy separation of DTBA by cation-exchange. (Lukesh et al., 2012).

DTBA appears to have a high affinity for metal ions such as Zn^{2+} etc., (higher than, for example DTT) this does limit the effectiveness of the reductant using metalloproteins; however, it does make DTBA a useful agent for competition studies with metalloproteins (Adamczyk, 2015). DTBA can be obtained from Aldrich-Sigma and, similar to TCEP, is rather expensive.

Cysteine-HCl

Cysteine hydrochloride (Cys-HCl) CAS 52-89-1; is used to make sulfhydryl assay standards and can be used in protein refolding experiments.

2-Mercaptoethylamine-HCl

2-aminoethanethiol (2-MEA-HCl, also called cysteamine-HCl), CAS 156-57-0; selectively reduces antibody hinge-region disulfide bonds (see Therm Scientific instructions for use 20408).

Oxido-Shuffling Systems

The commonly used in-vitro oxido-shuffling systems used in folding and oxidizing recombinant proteins are GSH/GSSG, 2-ME/HED, and DTT/oxidized DTT (Creighton, 1984, and Wetlauffer, 1984), which are shown in Table A.3.A.3. All six reagents are commercially available (the scarcest, HED, can be obtained from Aldrich; see *SUPPLIERS APPENDIX*) and are normally used as purchased, without further purification. For details of protein folding and oxidation see reviews by Vallejo and Rinas (2004) Mamathambika and Bardwell (2008).

Molecular chaperones and foldases aid in-vivo folding and oxidation (Kim et al, 2013) The most well known *E. coli* chaperones include GroEL-GroES, DnaK-DnaJ-GrpE (Hsp70) and also ClpA/ClpB (Hsp100). While molecular chaperones can promote correct folding, foldases accelerate the process and include: peptidyl prolyl cis/trans isomerases (PPI's), disulfide oxidoreductase (DsbA) and disulfide isomerase (DsbC) (which promote disulfide bonds, *found in E. coli*) and protein disulfide isomerase (PDI) a eukaryotic protein catalyzing oxidation and isomerization (Tu and Weissman, 2004). PDI has been used for in-vitro folding/oxidation also chaperones and foldases can be co-expressed with proteins of interest (Baneyx and Palumbo, 2003).

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Table A.3.A.1

Properties of Urea and Guanidine-HCl Solutions

Property	Urea	Guanidine-HCl
Molecular weight	60.06	95.53
Solubility (at 25°C) ^a	10.49 M	8.54 M
Melting point (°C)	133–137	186.5–188.5
A ₂₆₀ (6 M in water)	<0.06	<0.03

^aSolubilities at 5°C: urea, ~8 M; guanidine-HCl, ~8 M.

Table A.3.A.2

Preparation of Urea and Guanidine HCl Solutions

Molarity	Urea			Guanidine-HCl		
	Quantity ^a	Vol (ml) ^b	Concentration (g/liter) ^c	Quantity ^a	Vol (ml) ^b	Concentration (g/liter) ^c
1 M	0.63	10.52	60.06	1.03	10.82	95.53
2 M	1.32	11.04	120.12	2.23	11.71	191.06
3 M	2.09	11.61	180.18	3.65	12.76	286.59
4 M	2.93	12.24	240.24	5.36	14.05	382.12
5 M	3.88	12.96	300.30	7.45	15.63	477.65
6 M	4.95	13.76	360.36	10.09	17.63	573.18
7 M	6.16	14.68	420.42	13.51	20.23	668.71
8 M	7.54	15.73	480.48	18.14	23.77	764.24
9 M	9.15	16.95	540.54	—	—	—
10 M	11.02	18.38	600.60	—	—	—

^a Given weight of reagent added to 10 g water to generate a solution of the indicated molarity.

^b The final volume resulting from the addition of indicated amount of reagent to 10 g water was determined using the specific gravity of the solution calculated as described by Kawahara and Tanford (1966). Volume estimates can also be made by assuming 1 g of urea or guanidine-HCl increases the volume by 0.763 ml (i.e., the partial specific volume = 0.763). The density of water at 25°C is 0.9971 (10 g = 10.029 ml).

^c Weight of reagent made up with water or buffer to a final volume of 1 liter.

Table A.3.A.3

Reduced and Oxidized Sulfhydryl Reagents

Reagent	MW ^a	MP (°C)	Absorbance (nm)	(liter/mol/cm) ^e	SH/ pK _a	E'° (mV) ^g
GSH	307.33	192–195	260 (5.8)		8.63 (α)	–240
			280 (2.3)		9.45 (β)	
GSSG	612.63	178	250 (342)		—	
			260 (271)			
			280 (111)			
			300 (30)			
2-ME ^b	78.13	—	>260 (≈ 0, if pure)		9.5	
HED ^c	154.25	25–27	245 λ _{max} (380)		—	
			260 (310)			
			280 (200)			
DTT	154.25	42–44	>270 (≈ 0)		8.3 9.5	–330
Oxidized DTT ^d	152.20	132	283 λ _{max} (273)			—
			310 (110)			

^a Abbreviations: DTT, dithiothreitol; GSH, reduced glutathione (γ-L-glutamyl-L-cysteinylglycine, anionic thiol); GSSG, oxidized glutathione; HED, 2-hydroxyethyl disulfide; MP, melting point; MW, molecular weight.

^b Pure liquid is 14.3 M (density = 1.114 g/ml) with a boiling point of 157° – 158°C.

^c Liquid is 8.2 M (density = 1.261 g/ml).

^d Oxidized DTT (dihydroxy-1,2-dithiane) can be prepared from DTT by oxidation with ferricyanide (Cleland, 1964). Commercial material can be purified according to Creighton (1984).

^e The values in parenthesis are the molar absorbencies at the wavelengths indicated.

^f In amino thiols, the pK_a of the amine and thiol groups overlap; relevant pK_a's are indicated as α and β.

^g The standard redox potential (at pH 7, 25°C); refers to the reduction potential for thiol-disulfide pairs: 2RSH = R-S-S-R + 2H⁺⁺ + 2e[–]. Note that the lower the E'°₀, the stronger the reductant—e.g., DTT is a better reductant than GSH.