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A new protein folding screen: Application to the ligand binding domains of a glutamate and kainate receptor and to lysozyme and carbonic anhydrase

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Abstract

Production of folded and biologically active protein from *Escherichia coli* derived inclusion bodies can only be accomplished if a scheme exists for in vitro naturation. Motivated by the need for a rapid and statistically meaningful method of determining and evaluating protein folding conditions, we have designed a new fractional factorial protein folding screen. The screen includes 12 factors shown by previous experiments to enhance protein folding and it incorporates the 12 factors into 16 different folding conditions. By examining a $1/256^{th}$ fraction of the full factorial, multiple folding conditions were determined for the ligand binding domains from glutamate and kainate receptors, and for lysozyme and carbonic anhydrase B. The impact of each factor on the formation of biologically active material was estimated by calculating factor main effects. Factors and corresponding levels such as pH (8.5) and L-arginine (0.5 M) consistently had a positive effect on protein folding, whereas detergent (0.3 mM lauryl maltoside) and nonpolar additive (0.4 M sucrose) were detrimental to the folding of these four proteins. One of the 16 conditions yielded the most folded material for three out of the four proteins. Our results suggest that this protein folding screen will be generally useful in determining whether other proteins will fold in vitro and, if so, what factors are important. Furthermore, fractional factorial folding screens are well suited to the evaluation of previously untested factors on protein folding.

Keywords: bacterial expression; fractional factorial screen; inclusion bodies; protein folding

Harvesting the fruits of whole, partial, and selective genome sequencing projects frequently requires the heterologous expression of genes and subsequent studies of the gene products. Expression in a bacterial host, such as *Escherichia coli*, is an economical and time efficient method of protein production. However, in many cases expression of foreign proteins in *E. coli* leads to the production of insoluble inclusion bodies. Since a large number of proteins can be folded from inclusion body material and because protein production in the form of inclusion bodies has a number of merits, a method that would answer the question of whether proteins derived from inclusion body material can be folded into a biologically relevant conformation would be useful (Chen & Gouaux, 1997; De Bernardez Clark, 1998). Ideally, the method would answer the folding question with a reasonable degree of confidence using a small number of experiments. If expression in *E. coli* is subsequently deemed untenable, then production of the protein in more expensive and time consuming systems can be justified. In this paper, we present an approach to search for folding conditions and apply it to the folding of four proteins.

The folding buffer should favor the formation of the native state while minimizing the aggregation of folding intermediates. A wealth of experimentation has shown that polar additives (such as arginine), osmolytes, detergents, and chaotropes can minimize aggregation and increase the yield of biologically active material (see Rudolph & Lilie, 1996 for a recent review). Other factors affecting the formation and stability of the folded state are pH, redox environment, ionic strength, protein concentration, presence of ligand, and the mode by which the denaturant concentration is reduced, i.e., by dilution or dialysis, as examples. It is also known that proteinaceous chaperones can promote folding in vitro (Cole, 1996). However, we have devoted our attention to small molecules and polymers in the experiments described here.

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Abbreviations: GluR2, ionotropic glutamate receptor, AMPA specific, subtype 2 or B; GluR2-S1S2, ligand binding domain of GluR2; GFB-S1S2, ligand binding domain of the goldfish kainate binding protein, subtype β; CAB, carbonic anhydrase B; GuHCl, guanidine hydrochloride; GSSG, oxidized glutathione; GSH, reduced glutathione; FF16, 16 condition fractional factorial folding screen; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; KA, kainate; HPLC, high pressure liquid chromatography; PCR, polymerase chain reaction; pNAc, p-nitrophenylacetate; PEG, polyethylene glycol; SEC, size-exclusion chromatography; RT, room temperature.

Different proteins often require distinct conditions for folding. For example, *Mycoplasma* arginine deiminase folds upon dilution into only 10 mM potassium phosphate at pH 7.0 (Misawa et al., 1994), whereas a good condition for urokinase folding requires 50 mM Tris, pH 9.0, 1 M GuHCl, 0.2 M L-arginine, 5 mM EDTA, 0.005% Tween 80, 1.25 mM GSSG, and 0.25 mM GSH (Winkler & Blaber, 1986). In general, determination of folding conditions has involved a trial and error, one factor at a time approach and there is scant information available in the literature on general methods to search for folding conditions. Nevertheless, one approach involved applying a crystallization screen to the search for protein folding conditions (Hofmann et al., 1995). However, one would predict that effective precipitation and crystallization conditions will almost certainly not be useful for protein folding; not surprisingly, this approach was largely unsuccessful. By contrast, optimizations of previously determined folding conditions are more widespread (see Ahn et al., 1997). In an effort to provide a logic to the search for protein folding conditions, we have developed fractional factorial folding screens (Chen & Gouaux, 1997). Here we report an improved version of the original screen and describe its application to a number of structurally diverse proteins.

Since the in vitro folding of a protein may be influenced by a number of factors each bounded by two chemically reasonable levels (i.e., the factor "protein concentration" might have the levels 0.1 and 1.0 mg/mL), an efficient and statistically meaningful method of searching for folding conditions is desired. However, if one chooses 12 factors and each factor is assigned two levels, evaluation of the full factorial would require 4,096 experiments. In many cases, when one is searching for factors that impact the outcome of a particular process, evaluation of a fraction of the full factorial is sufficient to determine which factors have the greatest effects. Indeed, the fractional factorial experiment allows one to estimate main effects and multifactor interactions, depending on the resolution of the particular design (Box et al., 1978). Once the most significant factors have been identified, the folding conditions can be optimized using a subset of the original factors. Although some of the assumptions inherent in fractional factorial experiments, such as the assumptions that the response (i.e., the yield of folded protein) is linearly dependent on the level of the factor and that the factors do not interact, may not be strictly valid, a screen based on a fractional factorial design nevertheless provides a powerful tool by which folding conditions can be screened and factors can be evaluated.

Here we describe a fractional factorial protein folding screen that includes 12 factors and a total of 16 experiments (FF16; Table 1). The effectiveness of the FF16 screen was tested on the ligand binding domains from the rat GluR2 receptor (GluR2-S1S2; Chen & Gouaux, 1997; Arvola & Keinänen, 1996), and from the goldfish kainate binding protein β (GFB-S1S2; Wo & Oswald, 1994), on hen egg white lysozyme, and on bovine carbonic anhydrase B (CAB). GluR2-S1S2 and GFB-S1S2 have low level amino acid sequence identity, related biological activities, molecular weights of ~32 kDa, one disulfide bond, two compact domains, mixed α/β secondary structures and basic isoelectric points; prior to the work from this laboratory, folding conditions for neither GluR2-S1S2 nor GFB-S1S2 had been reported. In contrast, lysozyme (Dobson et al., 1994; Hevehan & De Bernardez Clark, 1997) and CAB (Cleland et al., 1992; Wetlaufer & Xie, 1995; Xie & Wetlaufer, 1996) are both well-studied proteins and have served as model systems for testing factors and methods for protein folding. On the one hand, lysozyme (MW of 14.5 kDa) has four disulfide bonds, a pI of 11.35 and is primarily α-helical. On the other hand, CAB (30 kDa) has no disulfide bonds, a catalytic metal ion center, a central β-sheet that forms the core of the protein and an isoelectric point of 5.9. All four proteins were successfully folded under multiple conditions from the screen.

Results

Statistical analysis of the data

The main effects of each factor were calculated by summing the response (i.e., [^{3}H]-AMPA counts in the case of GluR2-S1S2) obtained when using the "+" level and then when using the "−" level of the particular factor under consideration (Fig. 2). The sum of the "−" experiments were then subtracted from the sum of the "+" experiments and the resulting difference was divided by 8, i.e., Main Effect = $\sum$ "+ level" − $\sum$ "− level"/8 (Box et al., 1978). As shown in Figure 2, polar additive (arginine) at the "+" level, i.e., at 0.5 M, pH at the "+" level (pH 8.5) and protein concentration at the "+" level have some of the strongest favorable effects on the yield of active protein.

To help determine if a given factor had a positive or negative effect for each of the four proteins, the main effects for each protein were scaled in the following manner. For each of the proteins, the values of all factors were scaled such that the main effect of the factor with the greatest effect was set to 1.0. In the case of GluR2-S1S2 the factor is pH, for example. Then, the mean main effects for each factor were calculated. This value is indicated by the unfilled bars in Figure 3. The mean main effects were then ranked in order of decreasing magnitude. As shown in Figure 3, pH, polar additive, chaotrope, and protein concentration have the largest positive mean main effects. Detergent has the largest negative mean main effect. Reduction/oxidation potential has an overall average effect of zero, although the spread in the main effects is large because lysozyme folds well in the presence of GSH/GSSG, i.e., the "+" level of Red./Ox. has a large positive effect (Fig. 2) while GSH/GSSG has a smaller negative effect on the folding of the other proteins.

GluR2-S1S2

As shown in Figure 1, most of the FF16 screen conditions gave significant levels of folded GluR2-S1S2 except #2, #5, #9, and #14. Condition #7 resulted in the highest [^{3}H]-AMPA counts followed by #16 and #6. The main effects plot for GluR2-S1S2 shown in Figure 2 illustrates that the following factors had a positive effect on the folding: pH of 8.5, 0.5 M GuHCl, 0.5 mg/mL protein concentration, and 0.5 M L-arginine. The inclusion of sucrose, divalent cations, and PEG had negligible effects while dialysis, high ionic strength, GSH/GSSG, and detergent had weakly negative effects. The presence of glutamate in the folding buffer had a modestly positive consequence. Based on large scale folding experiments of GluR2-S1S2, which employed folding conditions similar to #7, the yield was ~10% using OD_{280} measurements.

GFB-S1S2

Conditions #7, #3, #4, and #11 gave the largest amount of properly folded GFB-S1S2 as judged by the [^{3}H]-kainate binding results listed in Table 2. The yield of soluble protein was 10–15% as

Table 1. *16 Condition fractional factorial folding screen (FF16)*

Buffer	Pattern[a]	Mode[b]	[Protein] (mg/mL)[c]	Polar add.[d]	Detergent[e]	pH[f]	Red./Ox.[g]	Chao.[h]	Ionic str.[i]	Dival. cat.[j]	PEG (%)[k]	Ligand[l]	NP add.[m]
1	−−−−+−−+−++−	Dil.	0.1	0	0	8.5	1 mM DTT	0	250 mM	EDTA	0.05	10 mM	0
2	−−−+−++−+−−−	Dil.	0.1	0	0.3 mM	6.0	GSH/GSSG	0.5 M	10 mM	Mg, Ca	0	0	0
3	−−+−−++−−+++	Dil.	0.1	0.5 M	0	6.0	GSH/GSSG	0.5 M	10 mM	EDTA	0.05	10 mM	0.4 M
4	−−++−−+−+−−+	Dil.	0.1	0.5 M	0.3 mM	8.5	1 mM DTT	0	250 mM	Mg, Ca	0	0	0.4 M
5	−+−−−+−++−++	Dil.	0.5	0	0	6.0	GSH/GSSG	0	250 mM	Mg, Ca	0	10 mM	0.4 M
6	−+−++−+−−+−+	Dil.	0.5	0	0.3 mM	8.5	1 mM DTT	0.5 M	10 mM	EDTA	0.05	0	0.4 M
7	−++−+−+−+−+−	Dil.	0.5	0.5 M	0	8.5	1 mM DTT	0.5 M	10 mM	Mg, Ca	0	10 mM	0
8	−+++−+−+−+−−	Dil.	0.5	0.5 M	0.3 mM	6.0	GSH/GSSG	0	250 mM	EDTA	0.05	0	0
9	+−−−−−++++−+	Dial.	0.1	0	0	6.0	1 mM DTT	0.5 M	250 mM	Mg, Ca	0.05	0	0.4 M
10	+−−+++−−−−++	Dial.	0.1	0	0.3 mM	8.5	GSH/GSSG	0	10 mM	EDTA	0	10 mM	0.4 M
11	+−+−++−−+−+−	Dial.	0.1	0.5 M	0	8.5	GSH/GSSG	0	10 mM	Mg, Ca	0.05	0	0
12	+−++−−++−−+−	Dial.	0.1	0.5 M	0.3 mM	6.0	1 mM DTT	0.5 M	250 mM	EDTA	0	10 mM	0
13	++−−++++−−−−	Dial.	0.5	0	0	8.5	GSH/GSSG	0.5 M	250 mM	EDTA	0	0	0
14	++−+−−−−++−−	Dial.	0.5	0	0.3 mM	6.0	1 mM DTT	0	10 mM	Mg, Ca	0.05	10 mM	0
15	+++−−−−−−−−+	Dial.	0.5	0.5 M	0	6.0	1 mM DTT	0	10 mM	EDTA	0	0	0.4 M
16	++++++++++++	Dial.	0.5	0.5 M	0.3 mM	8.5	GSH/GSSG	0.5 M	250 mM	Mg, Ca	0.05	10 mM	0.4 M

[a] +/− factor levels are: Mode, dil = −, dial = +; [Protein], 0.1 mg/mL = −, 0.5 mg/mL = +; Polar Additive, 0 = −, 0.5 M = +; detergent, 0 = −, 0.3 mM = +; pH, 6.5 = −, 8.5 = +; Red./Ox., 1 mM DTT = −, GSH/GSSG = +; chaotrope, 0 = −, 0.5 M = +; ionic strength, 10 mM = −, 250 mM = +; divalent cations, 1 mM EDTA = −, Mg, Ca = +; PEG, 0 = −, 0.05% = +; ligand, 0 = −, 10 mM = +; nonpolar additive, 0 = −, 0.4 M = +.

[b] This factor was only included for GluR2-S1S2 and GFB-S1S2 folding experiments.

[c] Lysozyme experiments were performed using protein concentrations of 0.1 and 1.0 mg/mL.

[d] L-arginine.

[e] Detergent: lauryl maltoside

[f] pH 6.0, 50 mM MES; pH 8.5, 50 mM Tris-HCl; pH was measured at 4 °C.

[g] 1 mM reduced (GSH) and 0.1 mM oxidized (GSSG) glutathione.

[h] Guanidine hydrochloride.

[i] Molar ratio of NaCl to KCl was 25:1.

[j] 1 mM EDTA or 2 mM $MgCl_2$, 2 mM $CaCl_2$; except for CAB buffers that contained 1 mM $ZnCl_2$ instead of $MgCl_2$ and $CaCl_2$.

[k] PEG $MW_{average}$ = 3,550 Da; the concentration was weight/volume.

[l] The ligand was L-glutamate for GFB-S1S2 and GluR2-S1S2 buffers; this factor was excluded from the folding buffers for lysozyme and CAB.

[m] Sucrose.

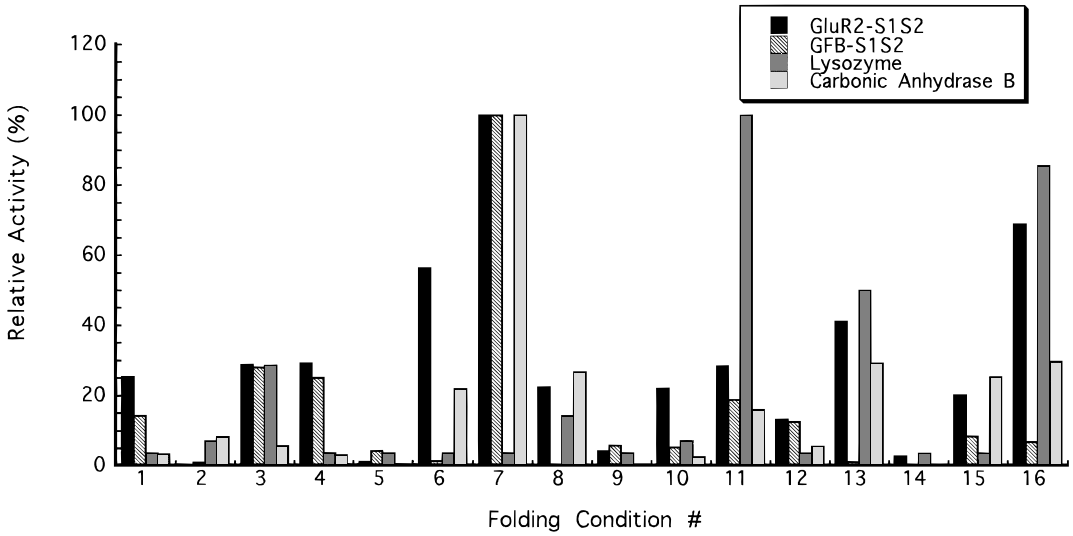

Fig. 1. Relative activity chart. The relative activity was calculated with respect to the condition that resulted in the greatest amount of active material for each protein; all other conditions are given as a percentage of the best condition. Condition #7 resulted in the largest amount of properly folded GluR2-S1S2, GFB-S1S2, and CAB, whereas condition #11 yielded the greatest amount of active lysozyme.

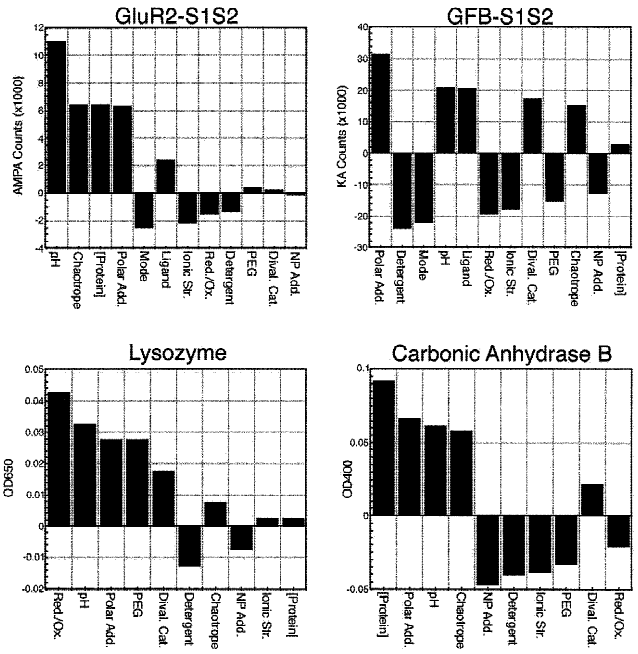

Fig. 2. Plot of the factor main effects. The main effects for each factor were calculated using the equation: ($\sum$ "+ level" − $\sum$ "− level")/8 (Box et al., 1978). The factors are plotted in order of decreasing main effects, starting from the left. A positive value on the chart indicates that the "+" level (i.e., pH 8.5 or 0.5 M L-arginine) is estimated to enhance protein folding, whereas a negative value reflects a decrease in folding due to the "+" level of the factor. The calculations of main effects do not take into account multifactor interactions, which, if present, may alter the estimation of factor main effects; to estimate multifactor interactions, higher resolution screens must be performed. The 1σ uncertainty for estimation of the main effects are 502 for GluR2-S1S2, 1980 for GFB-S1S2, 0.008 for lysozyme, and 0.005 for carbonic anhydrase B.

estimated by SDS-PAGE. The fewest counts were recorded for conditions #2, #6, #8, #13, and #14. The strongest positive factors were 0.5 M L-arginine followed by dialysis, pH 8.5, and 1 mM L-glutamate (Fig. 2). The presence of detergent had a strong negative effect while glutathione, high ionic strength, PEG, and sucrose had moderately negative consequences. Interestingly, protein concentration appeared to have no effect on GFB-S1S2 folding showing that a higher protein concentration did not yield more folded material. This in turn suggests that nonproductive folding and aggregation occurs at protein concentrations above 0.1 mg/mL. Although most conditions in the FF16 screen were sufficient to fold GFB-S1S2, only one of the best four folding conditions were shared with its close relative GluR2-S1S2. Likewise, the chaotrope and protein concentration factors, which were important for GluR2-S1S2 folding, were relatively neutral factors for GFB-S1S2 folding. While these two proteins have many common physical characteristics and functional properties, these similarities do not confer equivalence in terms of factor main effects.

Lysozyme

Lysozyme did not yield active material under as many conditions of the FF16 screen as compared to GluR2-S1S2 and GFB-S1S2. This result is probably due to the requirement of lysozyme for conditions that promote disulfide bond formation and rearrangement. In fact, none of the eight conditions that contained 1 mM DTT resulted in active lysozyme, indicating that lysozyme folding is greatly disfavored under reducing conditions. The two best conditions for lysozyme folding were #11 and #16, respectively. Like GFB-S1S2, protein concentration was a neutral factor in lysozyme folding. Since the data presented here measured the total amount of active protein and conditions with different protein concentrations, such as #11 (0.1 mg/mL) and #16 (1.0 mg/mL), resulted in similar

Table 2. *Results of folding GluR2-S1S2, GFB-S1S2, lysozyme, and CAB using FF16*

Buffer	GluR2-S1S2 ^{3}H-AMPA binding[a]	GFB-S1S2 ^{3}H-KA binding[a]	Lysozyme activity[b]	CAB activity[c]
1	8,055 ± 704	21,603 ± 393	<0.01	0.013 ± 0.001
2	<200	<2,400	0.01 ± 0.007	0.032 ± 0.004
3	9,152 ± 1,061	42,557 ± 3,116	0.04 ± 0.009	0.022 ± 0.006
4	9,295 ± 2,054	38,177 ± 1,628	<0.01	0.012 ± 0.003
5	355 ± 10	6,320 ± 500	<0.01	<0.01
6	17,920 ± 1,035	<2,400	<0.01	0.084 ± 0.016
7	31,785 ± 310	152,130 ± 1,060	<0.01	0.384 ± 0.010
8	7,130 ± 925	<2,400	0.02 ± 0.009	0.103 ± 0.010
9	1,340 ± 87	8,829 ± 572	<0.01	<0.01
10	7,001 ± 428	8,069 ± 1,048	0.01 ± 0.005	0.010 ± 0.001
11	9,039 ± 390	28,714 ± 5,108	0.14 ± 0.021	0.061 ± 0.017
12	4,201 ± 530	19,222 ± 3,900	<0.01	0.021 ± 0.002
13	13,075 ± 2,435	<2,400	0.07 ± 0.023	0.112 ± 0.001
14	890 ± 55	<2,400	<0.01	<0.01
15	6,430 ± 75	12,730 ± 1,961[d]	<0.01	0.097 ± 0.013
16	21,920 ± 4,955	10,318 ± 2,280	0.12 ± 0.033	0.114 ± 0.050

[a]The units are cpm. For conditions that include a protein concentration of 0.5 mg/mL, the original measurements were multiplied by five to account for the differences in protein concentration (see Materials and methods). For all measurements, the error is presented as one standard deviation.

[b]The units are OD. Lysozyme activity is given as the decrease in OD_{650} over a 5 min period.

[c]The units are OD/min. Carbonic anhydrase activity is given as the increase in OD_{400} over a 5 min period divided by the time for reaction.

[d]This measurement was not duplicated; the standard deviation given for this measurement is the mean of all the GFB-S1S2 standard deviations.

amounts of folded product, condition #11 gave a higher yield of active lysozyme, based on the amount of starting material. By comparing the FF16 results to an assay using native lysozyme, condition #11 had a yield of ~37% and condition #16 yielded ~4% of the activity based on the amount of original material.

Carbonic anhydrase B

CAB folded to yield catalytically active protein under many of the conditions in FF16. However, as was determined from the folding of GluR2-S1S2 and GFB-S1S2, condition #7 clearly gave the highest yield per volume of protein folding solution. Both condition #7 and condition #11 resulted in a folding yield of ~60% based on the amount of starting material. It was striking that the six conditions for which the highest CAB activity was measured all had protein concentrations of 0.5 mg/mL, which made high protein concentration the strongest positive factor. The presence of 0.5 M L-arginine, pH 8.5, or 0.5 M GuHCl also had a positive effect on CAB folding. PEG has previously been shown to increase the yields of CAB folding (Cleland et al., 1992). However, in this screen the presence of PEG had a moderately negative effect. This disparity could be due to multifactor interactions, since the folding buffers used by Cleland et al. included only 1 M GuHCl, 0.5 mg/mL CAB, and PEG (Cleland et al., 1992).

Discussion

Utilization of protein produced as inclusion bodies from *E. coli* is a valuable strategy if the protein folds in vitro. Searching for folding conditions by trial and error, particularly in light of the substantial number of factors that may facilitate folding, is a daunting and potentially fruitless task. To answer the questions of (1) whether the protein of interest will fold and (2) what factors are most influential, we have designed and tested a fractional factorial protein folding screen. The screen answers the question of whether the protein will fold using a small number of highly varied conditions and it facilitates the determination of factors, and their respective levels, which enhance or diminish productive folding.

FF16 was tested on four denatured and reduced proteins, GluR2-S1S2, GFB-S1S2, lysozyme, and carbonic anhydrase B. These proteins differ in isoelectric point, secondary structure, the number of disulfide bonds, and molecular mass. All four proteins were successfully folded under multiple conditions. Although the degree to which each factor affected the folding of these proteins differed, there were discernable trends estimated by comparing the main effects of each factor, as shown in Figure 3 (Box et al., 1978). The main effects of the pH and polar additive factors were substantial in all of the folding experiments and productive folding was favored by high pH (8.5) and the presence of arginine. These results are consistent with the folding behavior of many other proteins (Rudolph & Lilie, 1996). For proteins that contain disulfide bonds, high pH will certainly enhance disulfide bond exchange. However, for proteins that do not contain disulfide bonds, it is unclear why high pH facilitates folding. Perhaps proteins that contain cysteines but do not have essential disulfide bonds nevertheless fold more effectively under conditions permissive to cleavage of "incorrect" disulfide bridges. Alternatively, high pH may reduce the aggregation of folding intermediates.

Higher protein concentration was a positive factor in the folding of GluR2-S1S2 and for carbonic anhydrase but in the instances of

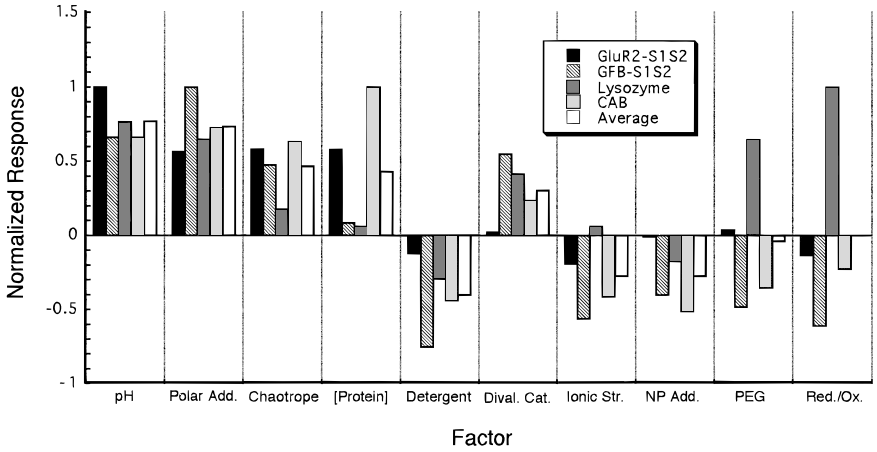

Fig. 3. Comparison of normalized main effects. All of the factors for each of the four proteins were normalized with respect to the most influential factor from the main effects plot. The factors are listed in order of decreasing average main effect (positive or negative) from left to right; pH has the largest average effect on protein folding and Red./Ox. environment has the least overall influence on renaturation. Although the average main effect of a particular factor may be minimal, such as in the case of the Red./Ox. factor, there may be a significant variation in the responses to the factor that fortuitously cancel. In other words, even though the Red./Ox. factor is generally not very important, it plays a critical role in the folding of lysozyme, which has four disulfide bonds.

lysozyme and GFB-S1S2, using a higher final protein concentration in the folding reaction did not result in a larger amount of active material. These results suggest that in the folding of lysozyme and GFB-S1S2, protein aggregation is a serious problem and that species along the folding pathway may (1) have a low solubility and/or (2) be prone to aggregation.

The presence of chaotrope exerts a favorable influence on folding with all of the proteins although the relative importance varies from significant (GluR-S1S2 and CAB), to possibly significant (GFB-S1S2), and to insignificant (lysozyme). It is striking that chaotrope would have the least positive effect in the instances (lysozyme and GFB-S1S2) where aggregation and/or insolubility may be most severe. It appears that in the cases of lysozyme and GFB-S1S2 arginine plays a more substantial role that guanidine hydrochloride in promoting correct folding.

The factors of PEG (Cleland et al., 1992) and detergent (Tandon & Horowitz, 1987; Zardeneta & Horowitz, 1994a) were included in the screen because previous experiments had demonstrated that they had a positive effect on protein folding. These studies suggested that PEG and detergent may help to prevent the aggregation of folding intermediates. From the experiments on lysozyme, it is clear that PEG has an important positive effect although for the other proteins the effects of PEG are less substantial. In contrast to experiments on rhodanese at protein concentrations up to 0.2 mg/mL (Zardeneta & Horowitz, 1994b), lauryl maltoside does not play a strong positive role in the folding of the four proteins examined here and in the case of GFB-S1S2, lauryl maltoside appears to have a significant negative effect. The basis for this behavior is not clear and it may be that particular proteins will benefit from the presence of detergent while others will not. Use of detergents in protein folding may include cyclodextrins in the folding process (Rozema & Gellman, 1995), as well as involve optimization of detergent type and concentration (Stockel et al., 1997).

While many factors may enhance protein folding by minimizing nonspecific aggregation, stabilization of the states near or at the final folded form is probably the mechanism by which ligand increases the folding yield for GluR2-S1S2 and GFB-S1S2. Unliganded GluR2-S1S2 is much more sensitive to proteolysis and thermal denaturation compared to the liganded species (Chen et al., 1998). Interestingly, another factor that acts by stabilizing the native state, the nonpolar additive sucrose (Timasheff, 1993), does not have a positive effect for any of the proteins. For the proteins studied, deleterious effects of sucrose on folding intermediates may be more costly than potential stabilizing effects on the folded states. Ionic strength appears to exert a negative effect on protein folding although the basis for this result is not apparent and multifactor interactions may obscure the true effect of differences in ionic strength. Finally, the mode of carrying out the folding is a factor that we have not explored as thoroughly as the other factors primarily because screening by dilution is (1) rapid, (2) trivial to execute, and (3) generally successful. For preparative folding, a dialysis approach using the conditions determined analytically may prove useful (Chen et al., 1998).

Because each of the proteins studied here probably has a unique folding scheme composed of a number of pathways, the reaction conditions that promote folding vary. Nevertheless, condition #7 produced the largest amount of active material for three out of the four proteins tested. Lysozyme did not fold under condition #7, probably due to the presence of 1 mM DTT. However, it would be interesting to measure lysozyme folding in a version of buffer #7, which contained a mixture of GSH and GSSG instead of DTT. The similarities between buffers #7 and #11, which differ in protein concentration, redox conditions, chaotrope, and PEG, support the conclusion that lysozyme would fold in a modified version of buffer #7. Other conditions in which three out of the four proteins folded well were #11, #13, and #16. Conditions that did not yield active proteins were #2, #5, #9, and #14, all of which contain divalent cations, no L-arginine, and have a pH value of 6.0.

Despite the importance of determining protein folding conditions, there has been little work in the area of protein folding screens. A previous study from this lab reported the predecessor of the screen presented here (Chen & Gouaux, 1997) and experiments described by Hofmann et al. involved the application of a sparse matrix crystallization screen to search for protein folding condi-

tions (Hofmann et al., 1995). Since the sparse matrix screen was not designed in a statistically meaningful fashion, one cannot determine any main effects or multifactor interactions. By contrast, main effects can be estimated from the FF16 screen reported here.

In comparison to the paucity of reports on protein folding screens, there are numerous reports on the optimization of folding conditions. For example, folding conditions for human tissue inhibitor of metalloproteinases-2 (Williamson et al., 1996), tissue-plasminogen activator (Grunfeld et al., 1992), and citrate synthase (Zhi et al., 1992) were optimized by varying one parameter while keeping the remaining factors constant. A weakness of this approach is that two factor interactions cannot be estimated unless the proper fraction of the full factorial is performed. Ahn et al. attempted to circumvent this problem by applying response surface methodology to the folding of *Pseudomonas fluorescens* lipase in which they optimized only the factors, pH, protein concentration, and GuHCl concentration from 36 experiments (Ahn et al., 1997). This type of optimization strategy might be most useful after two preliminary fractional factorial experiments: the first screen would involve using the 16 experiment screen described in this paper, while the second screen would be a 5 factor, 16 experiment screen of resolution V. The former screen would allow one to determine the five most important factors and from the second screen one could determine main effects and all two factor interactions. With this information in hand, one could then optimize the most important factors using, as an example, a response surface approach.

One of the strengths of a fractional factorial screen is that it allows for the logical testing of additional folding agents and the systematic modification of conditions to account for particular proteins. For example, if one knew that the current protein of interest contained disulfide bonds in the folded state, DTT could be replaced by a different ratio of GSH/GSSG. In this way, one would not only be carrying out the folding using 16 conditions that contained GSH/GSSG, the results from such an experiment would also allow one to determine the better ratio of GSH/GSSG. Furthermore, one could also modify the protein prior to folding, by sulfitolysis for example, and use either only modified protein, or modified and unmodified protein in the folding screen. Under the latter conditions, the effect of using the sulfite-derivatized or unmodified protein could then be determined. Alternatively, if one were working with a protein where temperature might be included as a factor, such as in the case of a thermophilic protein, one could replace the "mode" factor with a "temperature" factor. Finally, a statistically grounded folding screen, such as that reported here, provides the framework for testing the effects of other factors, such as novel small molecules, ions, detergents, lipids, and polymers, as well as protein chaperones, on protein folding.

Materials and methods

Materials

The vector containing the GFB gene (pGFKARβ) was generously provided by Dr. Robert Oswald (Cornell University, Ithaca, New York). Restriction enzymes were purchased from New England Biolabs (Beverly, Massachusetts). Cloned *Pfu* polymerase was from Stratagene (La Jolla, California). The Rapid DNA Ligation Kit from Boehringer Mannheim (Mannheim, Germany) was employed for all ligations. The folding screens were designed and analyzed using JMP statistics software (SAS Institute, Cary, North Carolina). The GluR2-S1S2 plasmid, pHS1S2, was constructed in the Gouaux lab (Chen & Gouaux, 1997). The GFB expression vector, pETNA, is a pET30b derivative (Novagen, Madison, Wisconsin), which had the NcoI and EcoRV sites removed and a DNA sequence encoding a His_8 tag and a thrombin cleavage site inserted into the multiple cloning site; thus the GFB protein has the sequence MHHHHHHHHSSGLVPRGS appended to its N-terminus. Arginine (>98% pure), PEG 3350, MES, Tris, L-glutamate, hen egg white lysozyme, bovine erythrocyte carbonic anhydrase B (CAB), *Micrococcus luteus* lyophilized cells, and p-nitrophenyl acetate were purchased from Sigma (St. Louis, Missouri). [^{3}H]-AMPA and [^{3}H]-KA were purchased from New England Nuclear (Wilton, New Hampshire). GSWP 02500 0.22 μM filters were purchased from Millipore (Bedford, Massachusetts) and Whatman (Maidstone, Kent, UK) GF/B filters were purchased from Fisher (Springfield, New Jersey). Guanidine hydrochloride (GuHCl) was purchased from ICN (Cosa Mesa, California) or Fisher and was >99% pure. Lauryl maltoside was obtained from Anatrace (Maumee, Ohio) and contained <2% of the α anomer. Dialysis chambers (200 μL) were provided by Cambridge Repetition Engineers (Cambridge, England).

Construction of the GFB-S1S2 plasmid

The DNA fragments, S1 and S2, were amplified in separate PCRs. The primers for the S1 reaction were PRM1: 5′-CGGGATCC GCAAGGCAAGAACTGATAGTTACC-3′ and PRM2: 5′AGTGG TACCTGCACCGTTATCGTCGCTAGCAGACGGCTCCCCAGA GAAGGGGGAG; the primers for amplification of S2 were PRM3: 5′-CGATAACGGTGCAGGTACCACTACTCCTGCACCGCTC ATGATT-3′ and PRM4: 5′-CCGCTCGAGTCATCAGCCATG AGCCCTCAGAGAGC-3′; the underlined sequences are complementary to the template. Each 100 μL PCR used 100 ng of pGFKARβ template, 250 ng of each primer, 200 μM of each dNTP, 5 U of polymerase, and 20 cycles of amplification. The major products were of the predicted size and were gel purified. Because the full S1-S2 construct proved difficult to clone, the S1 and S2 portions were cloned and sequenced separately and then combined. The S1 product and the vector, pETNA, were digested with BamHI and KpnI while the S2 product and pETNA were digested with KpnI and XhoI. Subsequently, all digest products were gel purified; S1 and S2 were ligated independently into the pETNA vector to generate pGFS1 and pGFS2. After the sequences were verified, both pGFS1 and pGFS2 were digested with KpnI and XhoI. The vector from the former and the insert from the latter digests were gel purified. The S2 insert was ligated into the plasmid containing the S1 insert to generate pGFS1S2.

GluR2-S1S2 and GFB-S1S2 expression and purification

The GFB-S1S2 protein contained a 12 residue hydrophilic linker (-PSASDDNGAGTT-), which was derived from a loop connecting β-strands in γ-chymotrypsin (Cohen et al., 1981) and which connected the S1 and S2 segments. S1 spanned Ala1–Glu126 and S2 included Thr219–Gly372 (numbered according to the mature sequence). GluR2-S1S2 and GFB-S1S2 proteins were overexpressed in BL21(DE3) cells. Culture growth, harvesting, cell lysis, inclusion body isolation, and solubilization in 8 M GuHCl were carried out as previously described (Chen & Gouaux, 1997). Prior to folding, 7 mL of a 20 mg/mL solubilized inclusion body solution was slowly diluted with 28 mL of 3 M GuHCl, 20 mM NaOAc, pH 4.5, 1 mM EDTA, and 1 mM DTT, with vigorous stirring at

4 °C. The resulting solution was concentrated to ~20 mg/mL before centrifugation to remove precipitate (125,000 × *g*, 1 h). Monomeric protein was isolated by size-exclusion chromatography (SEC) using a 1.6 cm × 50 cm column packed with Superose 12 resin and equilibrated with a solution containing 4 M GuHCl, 20 mM NaOAc, pH 4.5, and 1 mM DTT. Fractions corresponding to the expected size of the monomer were collected and the protein was diluted or concentrated to yield stock solutions of 5 and 25 mg/mL (1 $OD_{280} \cong$ 1 mg/mL).

Preparation of denatured lysozyme and CAB

Lyophilized lysozyme and CAB were dissolved in 8 M GuHCl, 0.1 M Tris-HCl, pH 8.5, and 0.3 M β-mercaptoethanol at concentrations of 40 and 30 mg/mL, respectively. After stirring for 24 h at RT, 2 mL of each protein were dialyzed against 50 mL of 4 M GuHCl, 0.1 M NaOAc (pH 4.5), and 10 mM DTT (total dialysis time: 18 h; buffer changed every 6 h); to ensure removal of the catalytic zinc ion, 5 mM EDTA was included in the CAB dialysis. The dialyzed protein solutions were centrifuged at 125,000 × *g* for 30 min to remove precipitate. The protein concentrations were estimated spectrophotometrically: lysozyme, $A_{280}^{1\%} = 23.7$ (Wetlaufer et al., 1974); CAB, $A_{280}^{1\%} = 18.3$ (Wong & Tanford, 1973).

16-Condition folding screen

Denatured GluR2-S1S2, GFB-S1S2, lysozyme, and CAB were each incubated for 18–20 h under the 16 folding conditions listed in Table 1. The mode factor (i.e., dialysis vs. dilution) was included in the GluR2-S1S2 and GFB-S1S2 folding screens. Under the conditions using dialysis, protein (0.1 or 0.5 mg/mL) was placed in 200 μL dialysis chambers covered with dialysis membrane (10 kDa MW cutoff). For dialysis conditions containing detergent, lauryl maltoside was added directly to the protein solution (final concentration of 0.3 mM) and was included in the dialysis buffer. The dialysis chambers were then placed in 15 mL polypropylene tubes with 10 mL of the appropriate folding buffer. Folding by dilution involved the addition of 20 μL of either a 5 or a 25 mg/mL protein stock solution to 1 mL of folding buffer at a rate of 3 μL/min. Lysozyme and CAB folding reactions were performed by dilution and in the lysozyme experiments the final protein concentrations were 0.1 and 1.0 mg/mL. The divalent cation factor in the CAB folding buffers was 1 mM $ZnCl_2$. The ligand factor was only included in the GluR2-S1S2 and GFB-S1S2 experiments. Folding was carried out at 4 °C for GluR2-S1S2 and GFB-S1S2 and at 20 °C for lysozyme and CAB for 12–18 h.

GluR2-S1S2 folding analysis

After incubation at 4 °C, protein that was folded by dilution was transferred into 200 μL dialysis chambers. All folding experiments were then dialyzed against AMPA binding buffer (30 mM Tris-HCl, pH 7.2, 100 mM KSCN, 2.5 mM $CaCl_2$, and 10% glycerol) for 36 h with 4–5 buffer changes. The dialyzed solutions were centrifuged (14,000 × *g*, 1 h, 4 °C) to remove precipitate. To verify the presence of soluble protein, 80 μL (0.1 mg/mL conditions) or 16 μL (0.5 mg/mL conditions) of the folding solution (supernatant) was precipitated with ethanol, resuspended in gel loading buffer, and analyzed by SDS-PAGE (12% gel). For the [^{3}H]-AMPA binding assay, 20 μL (0.1 mg/mL conditions) or 4 μL (0.5 mg/mL conditions) of dialyzed protein was combined with AMPA (2 nM [^{3}H]-AMPA, 53 Ci/mmol; 8 nM cold AMPA) and an appropriate amount of the dialysis buffer to make a final volume of 500 μL. These solutions were incubated on ice for 1 h prior to filtration through GSWP 02500 membranes. The membranes were washed twice with 3 mL of dialysis buffer, placed into scintillation vials with 6 mL of scintillation fluid, and counted on a scintillation counter. Nonspecific ligand binding was estimated by including 1 mM glutamate in the binding and filtration steps and was ~200 cpm. All reported counts were corrected for nonspecific binding. The ligand binding experiments were repeated twice using protein from the same folding experiment. Reported in Table 2 are the means of both sets of measurements.

GFB-S1S2 folding analysis

GFB-S1S2 folded by dilution was transferred into dialysis chambers. All dialysis chambers were then placed in kainate binding buffer (20 mM HEPES, pH 7.4, 100 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 10% glycerol, and 0.1% Tween-20). Dialysis proceeded for 36 h at 4 °C with 4–5 buffer changes. The protein was removed from the dialysis chambers with a sterile syringe, transferred to 1.5 mL microcentifuge tubes and centrifuged (14,000 × *g*, 1 h, 4 °C). To estimate the amount of soluble protein remaining in the supernatant, SDS-PAGE analysis was performed as described for GluR2-S1S2. Conditions that showed strong bands by SDS-PAGE were also analyzed by HPLC to determine the protein aggregation state. For the HPLC runs, 50 μL of protein solution was loaded onto a TSK-GEL G3000SW column equilibrated in a buffer composed of 100 mM sodium phosphate, pH 6.8, 200 mM sodium sulfate, 10 mM glutamate, 1 mM DTT, 2 mM EDTA, and operating at a flow rate of 0.75 mL/min. [^{3}H]-KA binding assays and controls were performed in a fashion similar to the [^{3}H]-AMPA binding experiments described above with the following exceptions: kainate binding buffer was used in the binding and washing steps, the folding mixtures were incubated with 20 nM [^{3}H]-KA (58 Ci/mmol), and GF/B filters soaked in 0.3% polyethyleneimine were used in place of the GSWP 02500 membranes. All of the values in Table 2 have been corrected to account for nonspecific binding. The GFB-S1S2 experiments were duplicated as described for GluR2-S1S2 and the ^{3}H counts are the mean of the two measurements.

Lysozyme activity assay

All folding reactions were transferred to dialysis chambers and dialyzed extensively against a buffer containing 0.1 M Tris-HCl, pH 7.8, and 0.1 M NaCl. To measure lysozyme activity, a turbimetric assay was employed where the decrease in OD_{650} of a suspension of *M. luteus* cells was measured photometrically (Saxena & Wetlaufer, 1971; Holtje, 1996). Dry *M. luteus* cells were suspended in 0.1 M sodium phosphate, pH 6.2, and 0.1 M NaCl to a final concentration of 0.25 mg/mL. Following centrifugation of each of the folding reactions (14,000 × *g*, 10 min, 4 °C), 50 μL of each protein solution was added to 450 μL of *M. luteus* cells in a plastic cuvette. Each reaction was allowed to proceed for 5 min before the OD_{650} was measured on a Shimadzu BioSpec-1601 spectrophotometer, which held a reference cell containing the lysozyme dialysis buffer alone. To correct for a decrease in absorbance that was not due to lysozyme, the background lysis of *M. luteus* cells in dialysis buffer was subtracted from the measurements obtained from the folding reactions. The lysozyme folding

and assay experiments were duplicated. The activity reported in Table 2 is the mean of the two measurements.

CAB activity assay

CAB activity was measured by the hydrolysis of p-nitrophenylacetate (Wetlaufer & Xie, 1995; Rozema & Gellman, 1996). All of the CAB folding samples were transferred to dialysis chambers and dialyzed extensively against 20 mM Tris-sulfate, pH 7.5. The dialyzed solutions were centrifuged (16,000 $\times$ *g*, 10 min) before the activity assay. To quantitate CAB activity the hydrolysis of p-nitrophenylacetate (pNAc) was followed by monitoring the increase in OD_{400}, which is due to the production of p-nitrophenolate. p-Nitrophenylacetate was dissolved in an aqueous solution supplemented with 4% acetone. The spectrophotometer was blanked with 1.6% acetone, 12 mM Tris-sulfate, and 1 mM $ZnCl_2$. To satisfy the requirement of Zn^{2+} for catalytic activity 100 μL of each CAB solution was pre-incubated for 30 min with 1 mM $ZnCl_2$, since one-half of the folding conditions contained EDTA rather than Zn^{2+}. The folding solutions were then combined with 500 μL of 20 mM Tris-sulfate, pH 7.5, and 400 μL 5 mM pNAc, in a quartz cuvette and were thoroughly mixed by rapid inversion. Background p-NAc hydrolysis was measured simultaneously on a reference sample containing 2 mM pNAc, 12 mM Tris-sulfate, pH 7.5, and 1 mM $ZnCl_2$. The OD_{400} was recorded every minute for 5 min and for each time point the background hydrolysis was subtracted. The enzyme activity was calculated as the change in absorbance (OD_{400}) divided by the reaction time (min). The CAB folding and assay experiments were carried out twice, and the activity reported is the mean of the two measurements.

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