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Searching for silver bullets: An alternative strategy for crystallizing macromolecules

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Abstract

Based on a hypothesis that various small molecules might establish stabilizing, intermolecular, non covalent crosslinks in protein crystals and thereby promote lattice formation, we carried out three separate experiments. We assessed the impact of 200 chemicals on the propensity of 81 different proteins and viruses to crystallize. The experiments were comprised of 18 240 vapor diffusion trials. A salient feature of the experiments was that, aside from the inclusion of the reagent mixes, only two fundamental crystallization conditions were used, 30% PEG 3350, and 50% Tacsimate™ at pH 7. Overall, 65 proteins (85%) were crystallized. Most significant was that 35 of the 65 (54%) crystallized only in the presence of one or more reagent mixes, but not in control samples lacking any additives. Among the most promising types of reagent mixes were those composed of polyvalent, charged groups, such as di and tri carboxylic acids, diamino compounds, molecules bearing one or more sulfonyl or phosphate groups, and a broad range of common biochemicals, coenzymes, biological effectors, and ligands. We propose that an alternate approach to crystallizing proteins might be developed, which employs a limited set of fundamental crystallization conditions combined with a broad screen of potentially useful small molecule additives.

Keywords: X-ray analysis; Protein structure; Additives; Structural genomics; Crystals; Crosslinks

1. Introduction

Certain chemical compounds or small molecules may have dramatic effects on the success with which individual proteins crystallize. While additives, as they are often called (McPherson, 1976; McPherson, 1982; McPherson, 1999), can be decisive in macromolecular crystallization, their greater use has suffered from lack of any compelling, rational basis for their inclusion in mother liquors.

The most commonly useful class of additives, and the only class of which we have any real understanding, are those which may, for physiological reasons, be bound by the protein with consequent favorable changes in its physical–chemical properties or conformation. These include such things as coenzymes and prosthetic groups, inhibitors, enzymatic products, ions, and other effector molecules. Often the liganded form is structurally defined and stable, while the unliganded form is not, and often the former will crystallize when the latter will not.

Numerous cases have, however, been reported where small, and sometimes large molecules were observed to make crucial interactions between macromolecules in the crystal that either helped guide or secure formation of the lattice. Such small molecules sometimes had a physiological basis for their unexpected presence, but frequently not. They simply provided essential or at least helpful crosslinks within the crystal. A casual poll of crystallographic colleagues using the CCP4 website http://www.ccp4.ac.uk/ccp4bb.php (Collaborative Computational Project, 1994) provided the collection presented in Table 1 of molecules identified (or at least believed to be so), which had been unintentionally incorporated into various protein crystals. There are undoubtedly many more.

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Table 1
Mysterious Small Molecules Observed in Crystals by the Community

Buffers	Acetate	Cacodylate
	MOPS	Hepes
	MES	Tris
	CHES	
Reducing agents	DTT	Mono thiol glycerol
	BME	
Precipitants	Sulfate PEG 400	
	Dioxane	Heptaethylene glycol
	MPD	Tetraethylene glycol
	Phosphate	hexanediol
	Jeffamine	
Cryogens	MPD	Ethylene glycol
	Glycerol	Mannitol
Ions	Sulfate	Phosphate
	Arsenate	Magnesium
	Cadmium	Calcium
	Sodium	Iodide
	Chloride	Bromide
Lipids and detergents: several reports, molecules largely not identified		
Other molecules	Benzoate	Acetyl mannose
	Benzamidine	Formate
	guanosine	Lipopolysaccharide
	Dipeptide	Phenyl–hexane amino acid
	Peptide	Sarcosine
	NADP	Colored dyes
	Nucleic acid	Bound heavy atom
	Non covalently	compounds
	phenol	Oligonucleotides tartrate
	Disaccharide	

Aside from additives that have a rational basis in biochemistry, compounds that might affect protein crystallization probably fall into the following categories.

(1) Molecules, which alter the physical relationship between the surface of the macromolecule and water, the solvent. These include the precipitant, but also chaotropes, osmolytes, cosolvents, and other molecules that perturb or stabilize the hydration of proteins or nucleic acids (Timasheff and Arakawa, 1988; Schein, 1990; Bolen, 2004; Collins, 2004).
(2) Molecules that alter the solubility of the macromolecule, but do not directly affect its physical–chemical properties, such as detergents (Zulauf, 1990), or the sulfobetains (Goldberg et al., 1996).
(3) Molecules, which stabilize the structure or conformation of the macromolecule, in particular certain ions (Trakhanov and Quiocho, 1995; Bolen, 2004; Collins, 2004).
(4) Molecules which play an active role in creating and maintaining the crystal lattice through the formation of intermolecular interactions of a reversible nature, i.e. they serve to crosslink, electrostatically or through hydrogen bonds and hydrophobic interactions, surface groups on neighboring molecules (McPherson, 1999). Examples of these are:
   (a) Cd^{2+} in horse spleen ferritin (Laufberger, 1937; Lawson et al., 1991)
   (b) DNA oligomers in RNase A (McPherson et al., 1986)
   (c) Oligosaccharides in alpha amylase (Larson et al., 1994)
   (d) Tartrate in thaumatin (Ko et al., 1994)
   (e) SO_4^- and PO_4^- (Table 1)
   MPD and PEG
   Glutathione, sarcosine
   Short peptides in proteases

Earlier work with the salts of organic acids (McPherson, 2001) along with the illuminating role of tartrate in the crystallization of the protein thaumatin (Ko et al., 1994), gave rise to the formulation of a new precipitant with the name Tacsimate[1] (Hampton Research, Aliso Viejo, CA). This reagent, which is composed of seven different pH neutralized organic acid salts, has seen increasing success not only because it produces high ionic strengths, but almost certainly because it provides a diverse set of molecules that can form hydrogen bonding or electrostatic, reversible crosslinks between proteins in the crystal lattice. It was this realization that inspired us to examine further the question of additives and the possibility that a more rational basis for their inclusion might be developed. Indeed, observations of difficult to crystallize, intractable proteins that suddenly crystallize under a carelessly wide range of conditions, once some small molecule or compound is included, suggests that this should be a profitable area for investigation. Such "silver bullet" additives have, furthermore, been seen in the presence of virtually all classes of precipitating agents, including salts, PEG, MPD, and other alcohols.

The major problem is that the essential small molecule, or "silver bullet", is generally, to at least some degree, specific to each protein, and one cannot readily guess in advance what it might be. This is sensible, since the surface characteristics of each protein are unique, and the lattice interactions between proteins unique to each crystal as well. We have endeavored here to take some first steps in addressing the problem of selecting potential additives by applying the following tactics:

(1) We attempted to invoke some chemical logic to limit the range of choices of potential compounds. That is, we tried to use the available observations to guide us to groups of compounds that might have general utility.
(2) We focused on a vastly reduced number of basic crystallization conditions in order to limit the number of experimental trials.

[1] Mixture of pH neutralized sodium malonate, sodium acetate, triammonium citrate, succinic acid, DL-malic acid sodium formate and diammonium tartrate.

(3) We used a relatively large set of proteins of various physical, chemical, and physiological properties so that the results might be generalized and be less applicable to only a specific protein.
(4) We tried to find some simplifying principles that reduce the practical problem of setting up large numbers of trials. For example, we take advantage of the fact that crystallization is also a selection process. In growing, a crystal incorporates what it needs to build the most stable lattice; it rejects things that are irrelevant. Thus we screened mixtures of potential additives rather than individual compounds.
(5) We looked for "lead compounds", or groups of compounds, which appeared to show promise, and tried to develop them further.
(6) As the experiments progressed, we refined the set of proteins to contain those which appeared most useful in revealing potentially useful additives.

We describe, here, three experiments intended as initial steps in identifying classes of molecules, and individual compounds that might be generally useful in promoting the crystallization of macromolecules, or that might have utility in increasing the probability that a specific protein crystallizes. In two experiments (I and II, below), we investigated the effects of various reagent mixes, generally having two to eight components, on the crystallization success of 81 different proteins. The compounds were not principally bioactive, but were chemical compounds that might affect the solubilities of the proteins, their stability, surface properties, or their interactions within a crystal lattice. In Experiment III, bioactive and physiologically relevant compounds were tested on 66 proteins with the objective of finding combinations that might cause the proteins to crystallize more readily, or crystallize in a different form than the unliganded protein. In all, 200 compounds were explored as positive additives. Only two fundamental crystallization conditions were used. One of these was based on 30% PEG 3350, the other on 50% Tacsimate, both at pH 7.

2. Materials and methods

The proteins used in the three experiments are shown in Table 2. The proteins were either purchased from the company whose name is shown to their right, or prepared at UC Irvine by the procedure contained in the indicated reference. In all cases the proteins were dialyzed, where necessary, against several changes of deionized water over 24 h to remove salt. By direct dissolution of dry material, or by dialysis, all proteins were made up as 10–40 mg/ml stock solutions in 0.1 M Hepes buffer at pH 7. The viruses were generally 5–8 mg/ml, as were the monoclonal antibodies. In some cases, solubility was limiting and saturated solutions of the protein under the same conditions were used. This was true, for example, for gramicidin (GRM), prostatic acid phosphatase (APHS), and concanavalin B (CONB).

Table 2
Experiment I, II and III

Symbol	Protein	Source
BJ-MLE	Bence Jones Protein—M.L.E., human	[a]
CK	Creatine Kinase, rabbit muscle	Sigma
MLCT	Catalase, micrococcus lutens	Genencor
PMV	Panicum Mosaic Virus, millet	Day et al. (1994)
CAH	Carbonic Anhydrase, bovine	Sigma
M151	Monoclonal Ab IDEC151	Biogen—IDEC
THM	Thaumatin, serendipity berry	Sigma
M159A	Monoclonal Ab, IDEC159A	Biogen—IDEC
PPS	Pepsin, bovine	Sigma
M159B	Monoclonal Ab, IDEC 159B	Biogen—IDEC
BJ-KWR	Bence Jones Protein—K.W.R., human	[a]
SHB	Hemoglobin, ovine	Sigma
HHB	Hemoglobin, human	Sigma
TRX	Xylanase, fungus	Genencor
BL	Lipase, bacterial	Novo Pharm.
PT	Trypsin-benzamidine, porcine	Sigma
ALCT	Alpha Lactalbumin, bovine milk	Sigma
STMV	Satellite Tobacco Mosaic Virus	Koszelak et al. (1989)
BYAD	Alcohol Dehydrogenase, yeast	Worthington
GHB	Hemoglobin, goat	Sigma
GI	Glucose Isomerase, bacterial	Hampton Research
BMV1	Brome Mosaic Virus Protein, barley	Lucas et al. (2001)
CONA	Concanavalin A, Jack bean	Sigma
PA	Alpha Amylase, pig	McPherson and Rich (1972)
STL	Subtilisin, bacterial	Sigma
BC	Catalase, bovine liver	McPherson and Rich (1973)
HRHB	Hemoglobin, horse	Sigma
BLB	Beta Lactoglobulin, bovine milk	Sigma
MYG	Myoglobin, horse	Sigma
PHB	Hemoglobin, pig	Sigma
DNAS	Deoxyribonuclease, bovine	Worthington
SJL	Lectin, Saphora japonica	McPherson et al. (1987)
BTRP	Trypsin-benzamidine, bovine	Sigma
EA	Albumin, hen egg	Sumner and Somers (1944)
ACHY	Alpha Chymotrypsin, bovine	Sigma
XYL	Xylanase, bacterial	Hampton Research
SPMV	Satellite Panicum Mosaic Virus, millet	Day et al. (1994)
BAA	Alpha Amylase, bacterial	Sigma
RSA	Serum Albumin, rabbit	Worthington
TYMV	Turnip Yellow Mosaic Virus, cabbage	Canady et al. (1995)
RNAA	Ribonuclease A, bovine	Sigma
CONB	Concanavalin B, Jack bean	Morrison et al. (1984)
LYZ	Lysozyme, hen egg	Sigma
RTX	Monoclonal Ab—Rituximab	Biogen—IDEC
M231	Monoclonal Ab Mab231	QED Pharm.
Experiment I only		
PEL	Elastase, pig	Sigma
PGHB	Hemoglobin, pigeon	Sigma
IN	Insulin, bovine	Sigma
BMHB	Methemoglobin, bovine	Sigma
HSFR	Ferritin, horse spleen	Sigma
HALB	Serum Albumin, human	Worthington
PTK	Proteinase K, fungal	Sigma

(*continued on next page*)

Table 2 (*continued*)

Symbol	Protein	Source
BLIP	Lipase, bacterial	Hampton Research
M111	Monoclonal Ab IDEC 111	Biogen—IDEC
BALB	Serum Albumin, bovine	Sigma
M1B8	Monoclonal Ab IDEC M1B8	Biogen—IDEC
M91	Monoclonal Ab, IDEC 9.1E	Biogen—IDEC
M110	Monoclonal Ab IDEC 110	Biogen—IDEC
HSAL	Serum Albumin, horse	McMeekin (1939)
BJAX	Bence Jones Protein Axton, human	[a]
Experiments II and III only		
INV	Invertase, bacterial	Sigma
HXK	Hexokinase, yeast	Sigma
LDH	Lactate Dehydrogenase, rabbit muscle	Sigma
UBQ	Ubiquitin, bovine erythrocytes	Sigma
CNV	Canavalin, Jack bean	McPherson and Campbell Smith (1980)
GRM	Gramacidin, synthetic	Sigma
APHS	Prostatic Acid Phosphatase, bovine	Sigma
GPDH	Glyceraldehyde Phosphate Dehydrogenase	Sigma
LFN	Lactoferrin, bovine milk	Sigma
ALD	Aldolase, rabbit muscle	Sigma
PHS	Phaseolin, kidney bean	McMeekin (1939)
ATRF	Apotransferrin, bovine milk	Sigma
APS	Alkaline Phosphatase, bacterial	Sigma
PRX	Peroxidase, horseradish	Sigma
BLCM	Beta Lactamase, bacterial	Sigma
RNN	Rennin, cow stomach	Sigma
BAMY	Beta Amylase, sweet potato	Sigma
HMCY	Hemocyanin, keyhole limpet	Sigma
PAP	Papain, papaya	Sigma
RB	Ribonuclease B, bovine	Sigma
BLCT	Catalase, bacterial	Genencor

[a] Bence Jones proteins isolated circa. 1962 from patients in Melborne, Australia, kindly provided by Prof. A. Henschen, UCI.

The protein samples were maintained at −80 °C when not in immediate use. In general, at least 30–100 mg of each protein was made as a stock solution so that a defined library of protein solutions would be available for these, and future experiments. Each protein was assigned a short alphabetic code, which is also shown for each protein in Table 2.

The chemical compounds used to make the individual reagents were purchased from a variety of companies, and all were of reagent grade. For Experiments I and II, the compounds were dissolved in 0.1 M Hepes buffer to a concentration of 2 M, if solubility allowed, or made as saturated solutions otherwise. The latter was more often the case, as many of the compounds had limited solubility in aqueous solution. Following dissolution in buffer, the solution of each individual compound was adjusted to pH 7 with either HCl or NaOH as necessary. To make the reagent mixes, equal volumes of several individual reagents, each adjusted to pH 7, were combined in a graduated test tube, and a volume of 30% PEG 3350 in 0.1 M Hepes, at pH 7, that was equal to the total volume of the reagents was added with agitation to insure thorough mixing. For the trials involving Tacsimate as the precipitant, 50% Tacsimate in water at pH 7 was substituted for the 30% PEG in making up the reagent mixes. Thus all reagent mixes were 15% PEG 3350, or 25% Tacsimate. By making the reagent mixes in this way, each individual compound that appeared in a reagent mix was diluted from its initial stock concentration depending on the number of components in the particular reagent mix. The reagent mixes were then assigned sequential numbers and are shown in Table 3.

Crystallization trials were by sitting drops (McPherson, 1999) in Cryschem plates (Hampton Research, Aliso Viejo, CA) sealed with clear plastic tape and maintained at 23 °C, though subject to normal laboratory fluctuations in temperatures (±3 °C). The reservoirs were 15, 20, and 30% PEG 3350, or 25, 45, and 65% Tacsimate in Experiment I, 15 and 30% PEG 3350, or 25 and 50% Tacsimate in Experiment II, and only 30% PEG 3350 in Experiment III. The composition of Tacsimate is 1.36 M malonate, 0.25 M citrate, 0.12 M succinate, 0.3 M DL-malate, 0.4 M acetate, 0.5 M formate, and 0.16 M DL-tartrate. The calculated ionic strength of the 35% Tacsimate solution used in reservoirs is 2.7 M. Conductances of 30% PEG 3350 in 0.1 M Hepes, and 35% Tacsimate were measured experimentally to be 512 μS (micro Siemens) and 80 mS, respectively. Deployment of drops and reservoirs, and all other operations required to formulate the reagents and prepare the plates, as well as inspection and recording of results were performed manually.

The sample drops were 2 μl of a protein stock solution and 2 μl of a reagent mix. The reservoirs were 0.6 ml of the PEG or Tacsimate dehydrants. The Cryschem plates were inspected for crystals under a 20× magnification microscope after 3 weeks, again at 6 weeks, and finally after 10 weeks. When questions arose, specific trials were reexamined using a 200× magnification microscope with polarized light. Every drop was scored as to its contents (clear, light precipitate, heavy precipitate, phase separation, micro crystals, larger crystals, etc.) though in the scoring matrices presented in Section 3, we only show whether crystals were present in a sample or not. In those cases (Experiments I and II) where multiple concentrations of PEG 3350 or Tacsimate were used as reservoirs, and in the duplicate trays in Experiment III, a success in one, two, or three plates was treated as a single success for that reagent and protein in the scoring matrices.

We made a conscious effort in evaluating the results to eliminate false positives. This was straightforward if the protein was colored. If the crystals were sufficiently large, questionable specimens were subjected to X-ray analysis. Small crystals and microcrystals were examined by polarized light, and appropriate controls were composed for all experimental arrays. Even so, some non protein crystals may have escaped our inquiry, and our results, therefore, may contain some incorrect false positives, but we believe those to be very few.

Table 3
The reagent mixes used in the three experiments

Experiment I	Experiment II	Experiment III
1. Spermidine, spermine, cadaverine, putrascine, 1,8-diamino octane	1. α-Schardinger's dextrin	1. *s*-Adenosyl-methionine, β-glycerophosphate, stachyose
2. 6-Aminohexanoic acid, 3-aminopropionic acid, 4-aminobutyric acid	2. NDSB-201 (sulfobetain)	2. Biotin, phosphoenol, pyruvate, melizitose
3. Oxamic acid, sulfanilic acid, 4-aminobenzoic acid	3. NDSB-195, NDSB-201, NDSB-211, NDSB-221, NDSB-256 (sulfobetains)	3. FAD, phosphoglyceric acid, spermine
4. Mix #2 and Mix #3	4. tannic acid	4. Thymine, pyrophosphate, glyceric acid, α-Schardinger's dextrin
5. Glutaric acid, hexadecanoic acid, dodecanedioic acid, fumaric acid	5. Streptomycin	5. NAD, fructose-1,6-diphosphate, spermidine
6. Oxalic acid, maleic acid, oxalacetic acid, terephthalic acid	6. Trimesic acid, phloroglucinol	6. Pyridoxyl-5′-phosphate, pyruvic acid, cyclodextrin
7. Mix #5 and Mix #6	7. Tetraphenylporphorin, cobalt hexamine	7. ADP, AMP, UMP
8. Pentaglycine, triglycine	8. Mellitic acid, myo-inositol, phytic acid	8. dAMP, GDP, naladixic acid
9. Poly-L-lysine, poly-L-ornithine, poly-DL-alanine	9. Mix #6 and Mix #8	9. GTP, dTMP, chlorpromazine
10. Poly-L-glutamic acid, poly-DL-alanine	10. 1, 4-Piperizine ethane sulfonic acid, anthraquinone-1, 5-disulfonic acid	10. ATP, dCMP, estradiol, galactose
11. Mix #9 and Mix #10	11. Resorcinol, tetrahydroxylbenzoquinone	11. Tacsimate
12. Protamine (sigma)	12. Pyromellitic acid, 2,2-thiodiglycolic acid, barbituric acid, terepthalic acid	12. Lipoic acid, creatine, glutathione, pantothenic acid
13. Heparin, dextran sulfate	13. Fumaric acid, maleic acid, glutaric acid, pimelic acid, suberic acid	13. Nicotinamide, batyl alcohol, glutaric acid
14. d(pA)4, d(pA)6	14. Suberic acid, sebacic acid, hexadecanedioic acid, dodecanedioic acid	14. Flavin mononucleotide, triolein, acetyl-choline
15. dp(T)4, d(pt)12	15. Fumaric acid, pimelic acid, sebacic acid, hexadecanedioic acid	15. Pepstatin A, GMP, fumaric acid, glucose
16. Pyrophosphate, tetrapolyphosphate	16. Maleic acid, glutaric acid, suberic acid, sebacic acid, dodecanedioic acid, oxamic acid	16. NADP, tetrahydrofolic acid, ascorbic acid, glucose
17. Ornithine, arginine, asparagine, glutamine	17. Trehalose, sucrose, melibiose, maltose, Cellobiose	17. Riboflavin, phosphatidyl choline, raffinose
18. Tacsimate, PEG 3350	18. Raffinose, maltotriose, melizitose	18. dGMP, cholesterol, thymine, oxamic acid
19. Glycerol, sucrose, sorbitol	19. Melibiose, stachyose	19. Phosphocreatine, *N*-benzoyl-L-arginine ethyl ester, phenobarbital, tetracycline
20. TMAO, proline	20. Cyclodextrin, 2-hydroxypropyl-β-cyclodextrin, 2,6-di-*O*-methyl-β-cyclodextrin	20. UTP, kanamycin, maltotriose, leupeptin
21. Sarcosine, glutamate, glycine, betaine	21. di, tri, tetra, pentaglycine	21. Phosphorylribose-1-pyrophosphate, maleic acid, *n*-acetyl-D-glucosamine
22. Transaconitic acid, glyceric acid, indolbutyric acid, D-hydroxyphenylacetic acid	22. 1,3-Propanediol, 1,2-butanediol, MPD, 1,6-hexanediol	22. 3′-UMP, ribose, phytic acid, palmitic acid
23. Palmitic acid, octanoic acid, sterylamine	23. PEG 3350 (control)	23. The 20 amino acids
24. Barbituric acid, resorcinol, glycerol-2-phosphate	24. Fumaramide, 1,4-diaminobutane, spermine, 1,8-diaminooctane, cadaverine	24. PEG 3350 (control)
25. Praseodymium acetate, gadolinium chloride, cobalt hexamine	25. Pyrophosphate, phosphorous acid triethyl ester, phytic acid	
26. Hexadecyltrimethylammonium bromide	26. Mix #4 and Mix #10	
27. Tacsimate, Mix #8	27. NDSB-201 and Mix #13	
28. Tacsimate, poly-L-ornithine, lysine, glutamic acid, alanine	28. Mix #15 and Mix #16	
29. Tacsimate, Mix #2	29. Phenyl urea, sodium-1-pentanesulfonate, sulfanilic acid, salicin, pentanetricarboxylic acid	
30. Tacsimate, Mix #3	30. Oxamic acid, fumarate, fumaramide, putracine, pentanetricarboxylic acid	
31. Tacsimate, Mix #5	31. Trans-aconitic acid, PABA, sulfanilic acid, 3-indolebutyric acid, pentanetricarboxylic acid	
32. Tacsimate, Mix #5	32. β-Thiooctylglucoside, Anapoe-305, MEGA-9, nonyl-β-D-glucoside	
33. Tacsimate, Mix #1	33. Trimesic acid, mellitic acid, pyromellitic acid, terepthalic acid	
34. Tacsimate, Mix #19	34. Phloroglucinol, phytic acid, anthroquinone-1,5-disulfonic acid, barbituric acid, tetraphenyporphorin	

(*continued on next page*)

Table 3 (*continued*)

Experiment I	Experiment II	Experiment III
35. Tacsimate, Mix #17	35. Tacsimate (control)	
36. Tacsimate, Mix #13	36. Tacsimate and Mix #2	
37. PEG 3350 (control)	37. Tacsimate and Mix #6	
38. Tacsimate, d(pA)4, d(pA)6	38. Tacsimate and Mix #8	
39. Tacsimate (control)	39. Tacsimate and Mix #10	
40. Tacsimate, d(pT)12, d(pT)4	40. Tacsimate and Mix #12	
41. Mix #8, Mix #9	41. Tacsimate and Mix #13	
42. Mix #8, Mix #9	42. Tacsimate and Mix #14	
43. Mix #1, Mix #5	43. Tacsimate and Mix #15	
44. Mix #1, Mix #3	44. Tacsimate and Mix #19	
45. The 20 amino acids	45. Tacsimate and Mix #20	
46. Tacsimate, glutamic acid, aspartic acid, arginine, lysine, the 20 amino acids	46. Tacsimate and Mix #26	
47. Tacsimate, the 20 amino acids	47. Tacsimate and Mix #24	
48. Tacsimate, protamine (sigma)	48. Tacsimate and Mix #21	

Experiment III differed from Experiments I and II, though it was operationally the same. In Experiment III, the biologically active molecules making up the reagent mixes were anticipated to bind at sites on individual proteins having substantially higher affinities than would sites binding arbitrary small molecules linking proteins in a crystal lattice. As a consequence, the individual compounds used to compose the reagent mixes were made in 0.1 M Hepes, pH 7, at 0.2 M concentration, or saturation when 0.2 M was unattainable because of solubility limitations. For some hydrophobic compounds, such as phosphatidyl choline and batyl alcohol, the final reagent solutions were only a few millimolar. From the individual biological reagents the reagent mixes, shown in Table 3, were formulated with 30% PEG 3350 as described above. No Tacsimate based reagent mixes were explored in Experiment III, though Tacsimate itself was included as one reagent mix.

In Experiment III, the droplets were again 2 μl of stock protein solution plus 2 μl of a specific reagent mix, with 0.6 ml reservoirs of 30% PEG 3350. The protein set was the same as used for Experiment II (with the inadvertent omission of TYMV). In Experiment III, unlike the earlier two, duplicate plates were made for each protein investigated.

Identical sets of proteins were not used in Experiments I and II because we attempted to correct deficiencies we thought present in the initial protein set, and because we believed we could compose a more challenging set that would better identify the characteristics we sought. In the revised set of proteins used in Experiments II and III, half of the monoclonal antibodies were eliminated along with four proteins which did not crystallize at all in Experiment I (and whose purity and integrity we felt were suspect), and several proteins [for example, insulin (IN) and proteinase K (PTK)] which crystallized regardless of what was present in the trials, i.e. yielded 30 to 40 successes in Experiment I. These latter, because of their ease of crystallization gave virtually no useful information regarding the value of a reagent mix. The most useful information is given by proteins which can be crystallized, but not easily. With this in mind, we added 21 new proteins that we felt provided more substantial challenges.

X-ray diffraction data were collected on some of the crystals grown in the experiments to measure unit cell parameters, identify space groups, and define resolution limits. The data sets were collected using a laboratory apparatus consisting of a Rigaku RU-200 generator fitted with Osmic mirrors, and an *R*-axis detector. Data were collected at room temperature on crystals conventionally mounted in quartz capillaries so that the evaluations would not be affected by the consequences of freezing. Diffraction intensity measurements were reduced to structure amplitudes using the program d*Trek (Pflugrath, 1999).

3. Results

Results of three separate experiments, comprising a total of 18 240 crystallization trials, involving 81 proteins, and 200 chemical compounds were obtained in this investigation. In the scoring matrices presented below, the proteins are designated with alphabetic codes defined in Table 2, and the reagent mixes by their numeric codes, which are defined in Table 3.

We need first reiterate that in Experiments I, II, and III, only two fundamental, or base crystallization conditions were used. All crystallization trials were derived from only these two conditions by combination with the reagent mixes. The two base conditions were 30% PEG 3350 buffered with 0.1 M Hepes at pH 7, and 50% Tacsimate also at pH 7. The droplets were all initially at one quarter the reservoir concentration. While reservoirs of 15, 20, and 30% PEG 3350, and 20, 35, and 50% Tacsimate were used in Experiment I, the results indicated those to be unnecessarily redundant, and they were reduced to two concentrations each of PEG 3350 and Tacsimate in Experiment II, and finally to only a single concentration of PEG 3350 in Experiment III. Thus we feel it fair to contend that, effectively, only two base crystallization conditions were used throughout the experiments.

The scoring matrix for Experiment I, which consisted of 8640 individual crystallization trials is shown in Fig. 1, and a condensation of statistics for all of the three Experiments I through III is found in Table 4. Of the 60 proteins whose crystallization was attempted in Experiment I, 48/60, or 80% were crystallized using the two base conditions sequentially combined with 48 different reagent mixes. Of the 12 proteins that did not crystallize at all, seven were intact monoclonal antibodies, which are unusually difficult to crystallize. If these are removed from consideration, then only five proteins, or 8% of the total, failed to crystallize at all. Twenty-one, or 35% of the

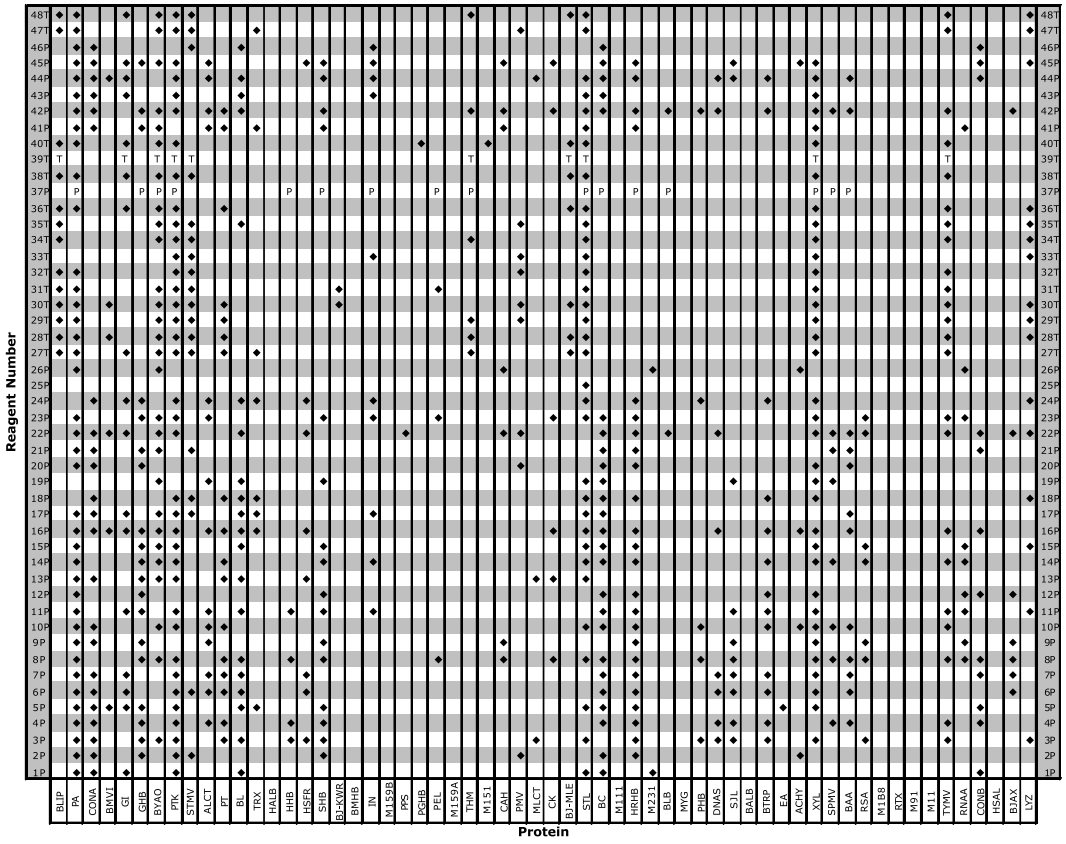

Fig. 1. Scoring matrix for Experiment I. The 60 proteins investigated are along the horizontal axis according to their alphabetic designation in Table 2. The reagent mixes comprising the 48 trials, defined according to their numeric designation in Table 3 are along the vertical axis. A diamond denotes that crystals were observed in the trial, open space indicates that no crystals were seen by light microscopy. P and T indicate crystals for the PEG and Tacsimate controls, respectively.

Table 4
Statistics for Experiments I, II and III

	All experiments	Experiment		
		I	II	III
1. How many proteins were investigated	81	60	67	66
2. How many proteins crystallized	65	48	50	50
3. How many proteins crystallized				
in PEG controls	20	11	14	13
Tacsimate controls	6	5	4	—
Both controls	5	5	3	—
Total controls	31	21	21	13
4. How many proteins crystallized only in the presence of some reagent mix	35	28	29	37
5. How many reagent mixes exceeded				
PEG controls		11	7	4
Tacsimate controls		7	1	—
6. How many proteins that did not crystallize in controls, crystallized in only				
1 reagent mix		4	7	13
2 reagent mix		2	4	9
3 reagent mix		1	4	6

proteins investigated crystallized in the controls, the base conditions with no added reagent mix. No protein, however, crystallized only in control samples without also crystallizing in at least some samples containing reagent mixes. Significantly, 28 proteins, 47% of those investigated, crystallized from samples that contained various reagent mixes, but did not crystallize at all from either PEG 3350 or Tacsimate controls lacking a reagent mix. Of the 48 proteins yielding crystals in Experiment I, 21/48, or 44% crystallized in the controls and 56% only in the presence of a reagent mix. Of those which did crystallize in controls, 11/21, or 52% crystallized only in PEG controls and 5/21, or 24% crystallized only from Tacsimate controls. 5/21 or 24% crystallized in both PEG and Tacsimate controls. Some of the crystals obtained from Experiment I are shown in Fig. 2.

The number of proteins crystallized for each reagent mix is presented in Fig. 3. No reagent mix failed to yield crystals of any protein. The average number of proteins crystallized for the reagent mixes was 13. There were 11 different reagent mixes based on PEG 3350 that yielded more crystals than the PEG controls, some significantly more. Seven Tacsimate based reagent mixes produced more crystals than the Tacsimate controls.

The compounds that seem to produce the greatest benefit are small molecules that contain multiple charged and polar groups, and, indeed, this is what we might have anticipated if our original hypothesis was correct. Reagent mixes containing molecules bearing carboxyl groups, such as 3, 5, 6, 7, 22, and 42, several of which exceeded control success rates by significant amounts, are examples. Most of the compounds comprising these mixes possess two or more carboxyl groups, or in some cases a single carboxyl or sulfonyl group combined with uncharged polar groups, such as hydroxyls or amides, or positively charged amino groups. Thus, they all have the potential of acting as multivalent, electrostatic grappling hooks to link proteins together.

The data suggest that multiple carboxyl, or acidic groups appear more effective than either multiple positively charged molecules, such as the polyamines in reagent mixes 1, or mixtures of negative and positive charges. Reagent mix 44, however, which is a complex of polyamines (reagent mix 1) and carboxyl containing molecules (reagent

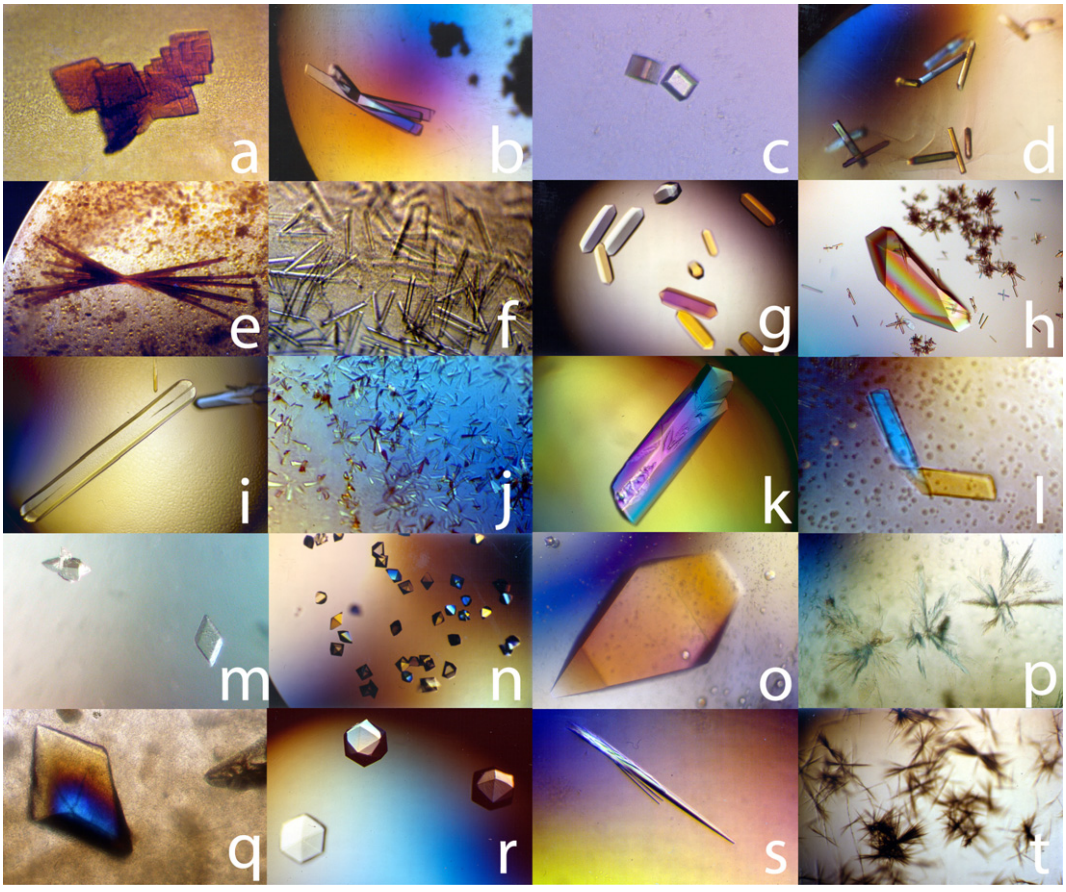

Fig. 2. Light microscope photographs, using polarized light, of a sampling of crystals grown in Experiment I. They are (a) sheep hemoglobin (b) bovine carbonic anhydrase (c) jack bean concanavalin B (d) jack bean concanavalin A (e) goat hemoglobin (f) Bence Jones Protein KWR (g) fungal lipase (h) hen egg lysozyme (i) bovine milk α lactalbumin (j) bacterial xylanase (k) RNase A (l) Bence Jones Protein MLE (m) satellite panicum mosaic virus (n) Proteinase K (o) porcine trypsin (p) bovine milk β lactoglobulin (q) bacterial alpha amylase (r) turnip yellow mosaic virus (s) porcine elastase (t) yeast alcohol dehydrogenase.

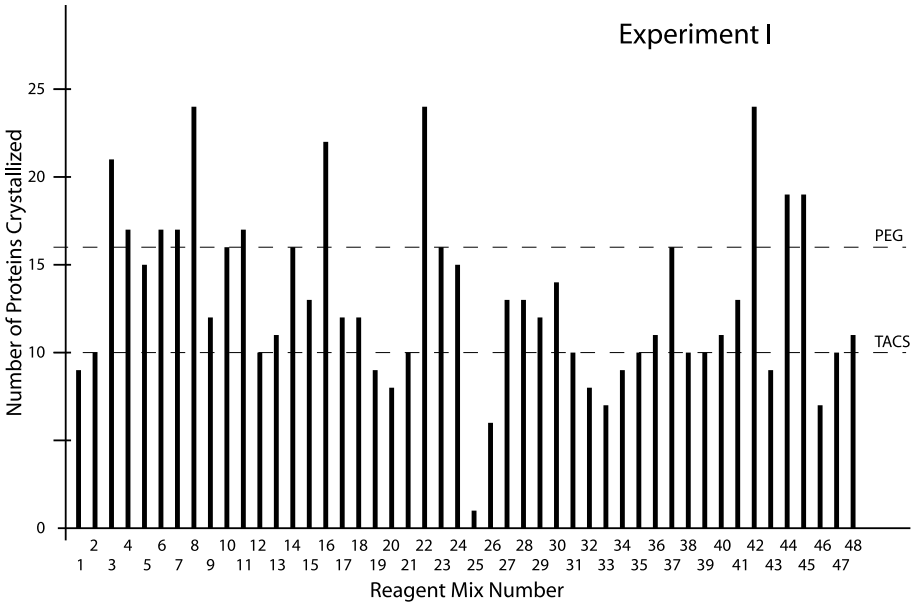

Fig. 3. A display of the number of proteins for which crystals were observed for each of the 48 reagent mixes in Experiment I. The reagent mixes are denoted by their numerical code and their composition defined in Table 3. The values for the PEG and Tacsimate controls (reagent mixes 37 and 39, respectively) are indicated by the horizontal lines.

mix 3) shows well in the experiment. Multiple carboxyl, and other acidic groups, perhaps combined in the same molecule with positive amino and other uncharged polar groups, thus appear good candidates for further investigation.

Other reagent mixes that emerged as beneficial were those containing oligomers of amino acids, such as reagent mix 8 which was tri- and pentaglycine, and longer polyamino acids as exemplified by reagent mixes 10, 11, and 42 (the most generally successful of all of the reagent mixes tested). For short oligomers, oppositely charged groups at the two termini may be crucial in bringing protein molecules together, but the intervening, periodic array of hydrogen bond donors and acceptors would likely be of importance as well. This is supported by the successes of longer amino acid polymers whose terminal charge groups are less important.

A third, general class of molecules whose mixes proved effective include multi-phosphates, such as pyrophosphate and tetraphosphate in reagent mix 16. The polyphosphates possibly serve as negative charge centers that organize proteins about them in an ordered manner. This class also contains the oligonucleotides, as in reagent mixes 14 and 15. Such oligomers provide even more intricate, periodic alternations of charged, uncharged polar, and hydrophobic centers.

A final group of promising reagent mixes from Experiment I are those containing mixtures of amino acids, such as mixes 17, 35, 45, and 47. Reagent mixes 45 and 47 are particularly intriguing since they are both simply mixtures of the 20 amino acids found in proteins, but combined with PEG and Tacsimate, respectively. Possibly, the panoply of amino acids is effective because it provides an expansive array of small molecules, each containing a positive and negative charge separated by a fixed distance.

Somewhat disappointing, in a general sense, were the osmolytes and chaotropes in reagent mixes 19, 20, 21, 32, and 34. It may very well be, however, that in mixtures of compounds like these, negative interactions and counter effects among the constituents, and with the proteins, obscure the usefulness of the individual components. The sampling of odd surfactants in reagent mixes 23 and 26, though not encouraging, is probably too small to be meaningful. Also appearing of little value, at least at the concentrations used, were the triply positively charged lanthanide ions in reagent mix 25, which precipitated most proteins immediately. Biological polymers containing charged groups, such as heparin and dextran sulfate in reagent mixes 13 and 36 did not appear useful.

As Fig. 3 shows, reagent mixes, in general, promoted crystallization more effectively for PEG than for Tacsimate based reagents. This should probably have been anticipated, because the reagent mix components carrying charged and polar groups would, on purely physical–chemical grounds, be expected to be less effective in inducing proteins to associate in the strong electrolyte solutions. In addition, Tacsimate already contains many effective cross-bridging molecules in high concentration.

3.1. Experiment II

Experiment II was an attempt to explore additives that might have general utility, but in a somewhat more focused manner. Again, compounds rich in hydrogen bonding groups, and those groups potentially involved in electrostatic interactions were given particular attention,

especially those having multiple carboxyl, phosphate, and sulfonyl groups. Because symmetry is an essential feature of crystals, compounds having rotational symmetry that could occupy special positions within a crystal were targeted for inclusion.

Based on the outcomes of Experiment I, final concentrations of PEG 3350 in the reservoirs were 15 and 30%, or 25 and 50% Tacsimate, without any intermediate concentration. The protein set for Experiment II was, we believe, more challenging, deliberately so, by the inclusion of proteins whose successful crystallization was uncertain or which, from experience, we found to be difficult or infrequent. On the other hand, we eliminated four of the intact monoclonal antibodies that failed to crystallize in Experiment I with the intention of improving balance, and four proteins (HSAL, BALB, HALB, and BMHB) which had uniformly failed to crystallize.

The scoring matrix for Experiment II, which was comprised of 6432 individual crystallization trials, is presented in Fig. 4. Using this second array of reagent mixes, 50/67, or 75% of the total number of proteins were crystallized. 21/67, or 31% of the proteins in Experiment II crystallized in controls containing no reagent mix. Significantly, 29/67, or 43% of the proteins failed to crystallize in controls, but did crystallize in the presence of one or more reagent mixes. In Experiment II, 14/67 (21%) and 4/67 (6%) crystallized only in the PEG and Tacsimate controls, respectively, and 3/67 (4%) crystallized in both controls. Fig. 5 presents the successes, in terms of proteins crystallized, for each of the 46 reagent mixes, plus the PEG and Tacsimate controls. As in Experiment I, no reagent mix failed to produce any crystals, and seven PEG based reagent mixes gave more successes overall than the PEG control. Only one Tacsimate based reagent mix produced more crystals than the corresponding control, and this was only marginally so. Fig. 6 presents some of the crystals grown in Experiment II. If Experiments I and II are taken in total, then 65/81 or 80% of the 81 unique proteins involved were crystallized, with 31/81 (38%) crystallized in controls, and 34/81 (42%) only in the presence of reagent mixes. The inclusion of reagent mixes increased the overall success rate by greater than twofold over the two base conditions lacking any additives.

The crystals obtained in both Experiments I and II were, in many cases, micro crystals, but this was not, by any means, always the case, as is evidenced by Figs. 2 and 6. For an appreciable number of proteins, crystals were obtained that were immediately suitable for X-ray diffraction analysis. For examples, bacterial amylase (BAM) yielded a complete data set (in space group $P2_12_12_1$ with $a = 77$ Å, $b = 92$ Å, $c = 150$ Å) and α lactalbumin (ALCT, space group C2 with $a = 98$ Å, $b = 120$ Å, $c = 77$ Å, and $\beta = 107°$). Indeed, we have collected data sets for more than two dozen of these. We observed, with considerable

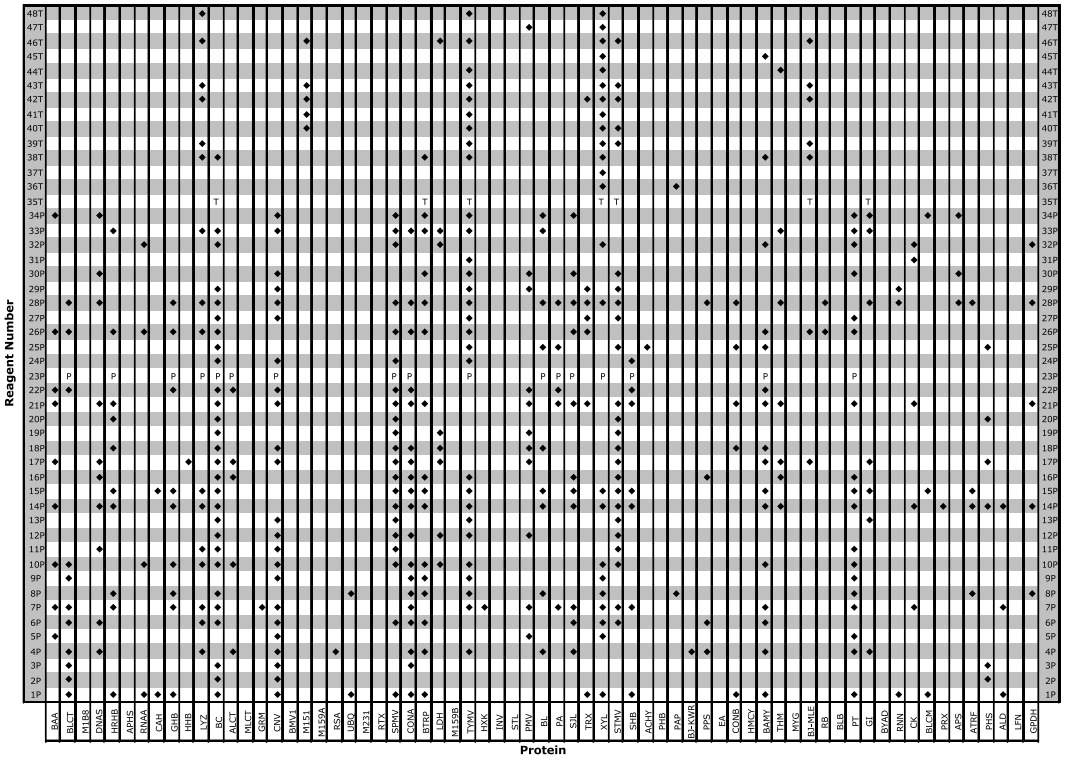

Fig. 4. Scoring matrix for Experiment II. The 67 proteins investigated are along the horizontal axis according to their alphabetic designation in Table 2. The reagent mixes comprising the 48 trials, defined according to their numeric designation in Table 3, are along the vertical axis. A diamond denotes that crystals were observed in the trial, open space indicates that no crystals were seen by light microscopy. P and T indicate crystals for the PEG and Tacsimate controls, respectively.

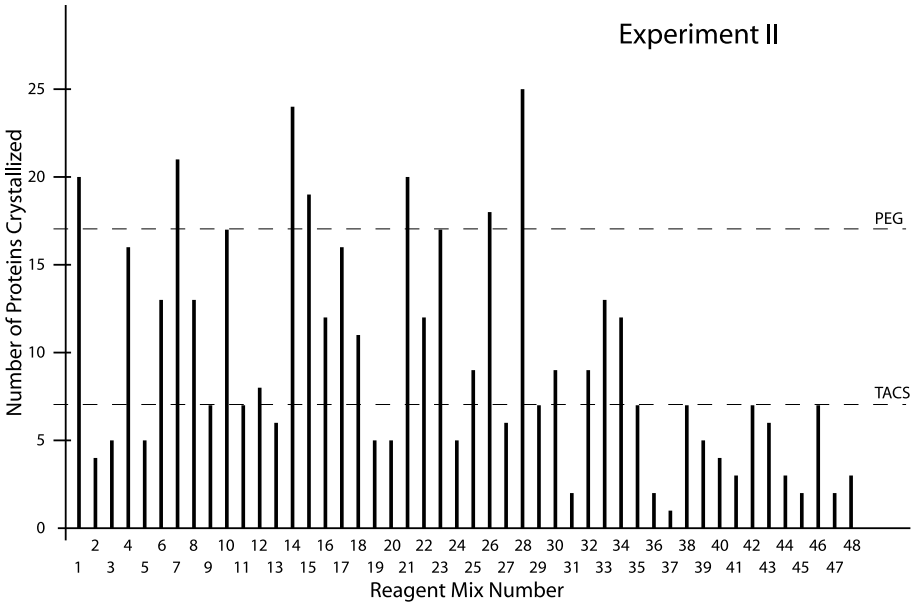

Fig. 5. A display of the number of proteins for which crystals were observed for each of the 48 reagent mixes in Experiment II. The reagent mixes are denoted by their numerical code, and their composition defined in Table 3. The values for the PEG and Tacsimate controls (reagent mixes 23 and 35, respectively) are indicated by the horizontal lines.

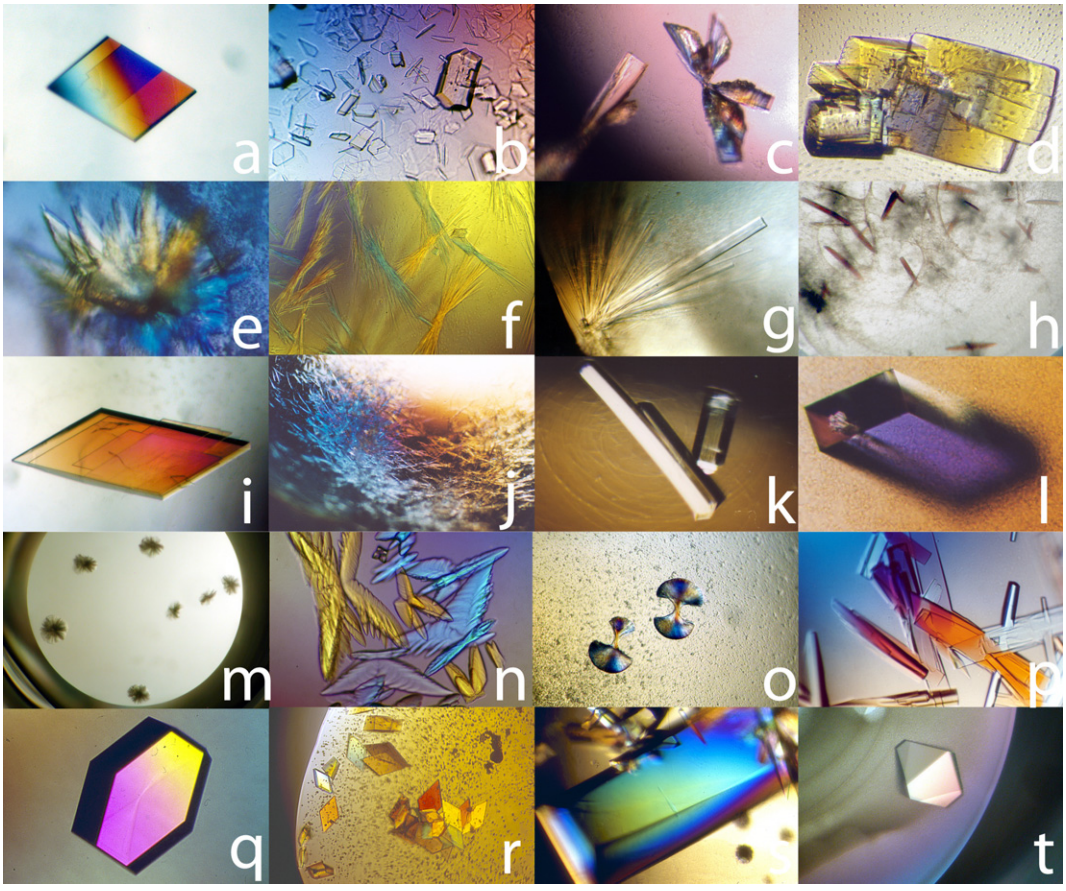

Fig. 6. Light microscope photographs, using polarized light, of a sampling of the crystals grown in Experiment II. They are (a) bacterial alpha amylase (b) turnip yellow mosaic virus (c) fungal xylanase (d) Rnase A (e) rabbit serum albumin (f) rabbit lactate dehydrogenase (g) intact IgG IDEC 151 (h) sheep hemoglobin (i) porcine trypsin (j) bovine carbonic anhydrase (k) bovine milk alpha lactalbumin (l) rabbit glyceraldehyde-3-phosphate dehydrogenase (m) papain (n) sweet potato beta amylase (o) Bence Jones Protein MLE (p) bovine apotransferrin (q) hen egg lysozyme (r) jack bean concanavalin A (s) bovine trypsin (t) bacterial glucose isomerase.

frequency, that different crystal forms appeared depending on the reagents present in the sample. A few examples of this polymorphism are shown in Fig. 7.

The different crystal forms are not simply alterations of habit or morphology, but, in many cases, significant changes in the crystallographic unit cell. For example, the crystals of rabbit muscle creatine kinase (CK) in Fig. 7e are of space group $P2_1$ ($a = 54$ Å, $b = 114$ Å, $c = 145$ Å, $\beta = 91°$), while those in Fig. 7f are of space group I222 ($a = b = 200$ Å, $c = 71$ Å). The crystals of thaumatin (THM) seen in Fig. 7l are of space group $P4_12_12$ ($a = b = 58.6$ Å, $c = 151.8$ Å), while those in Fig. 7k are of orthorhombic space group $P2_12_12_1$ ($a = 44.3$ Å, $b = 63.7$ Å, $c = 72.7$ Å). Porcine trypsin (PT) in Figs. 7p, s and o had unit cells C2 ($a = 127$ Å, $b = 52$ Å, $c = 72$ Å, and $\beta = 99°$), $P2_12_12_1$ ($a = 47$, $b = 55$ Å, $c = 78$ Å), and $P4_12_12$ ($a = b = 59$ Å, $c = 139$ Å). Bovine catalase (BC) crystals were grown of space groups $P2_12_12_1$ ($a = 89$ Å, $b = 140$ Å, $c = 231$ Å) and $P3_121$ ($a = b = 173$ Å, $c = 237$ Å). Bovine trypsin (BTRP) crystallized in three unit cells, $P3_121$ ($a = b = 55$ Å, $c = 109$ Å), P3 ($a = b = 138$ Å, $c = 151$ Å), and $P2_12_12_1$ ($a = 55$ Å, $b = 59$ Å, $c = 68$ Å). Ribonuclease A (RNAA) was obtained in at least two different unit cells, C2 ($a = 33$ Å, $b = 101$ Å, $c = 74$ Å, and $\beta = 90.2°$) and P3 ($a = b = 55$ Å, $c = 39$ Å), as was canavalin (CNV), in space group R3 ($a = b = 137$ Å, $c = 76$ Å, hexagonal setting) and $C222_1$ ($a = 137$ Å, $b = 150$ Å, $c = 133$ Å).

We also noted, that the diffraction quality of crystals of the same unit cell also varied depending on the particular reagent mixes from which they were grown. For example, tetragonal lysozyme (LYZ) crystals grown in the presence of reagent mix 11 in Experiment II (space group $P4_32_12$, $a = b = 79$ Å, $c = 38$ Å) diffracted only to about 1.8 Å resolution, while those from reagent mix 28 diffracted to beyond 1.4 Å on the laboratory X-ray system. Concanavalin A (CONA) crystals grown in the presence of reagent mix 4 from Experiment II diffracted to less than 4 Å resolution, while those grown from reagent mix 16 in the same

Fig. 7. Light microscope photographs, using polarized light, of some instances where multiple crystal forms were observed for the same protein as a consequence of the particular reagent mix present.

experiment ($P2_12_12_1$, $a = 69$ Å, $b = 118$ Å, $c = 123$ Å) diffracted to about 2.2 Å.

Of the seven reagent mixes that exceeded the success rate of the PEG controls (only one exceeded the Tacsimate control, and even that by only a barely significant amount) four of these, 14, 15, 26, and 28 were mixtures (4–9 components) of small molecules principally containing two or more carboxyl groups. It should be noted, however, that reagent mix 26 contained molecules carrying sulfonyl groups as well. Reagent mix 22 from Experiment I, among the most successful mixes tested in that experiment, also contained a component bearing a sulfonyl group. The results here for reagent mixes 14, 15, 26, and 28, along with similar mixes 6, 8, 10, 16, and 33, which also contain multiple carboxyl groups and some sulfonyl groups, are consistent with what was seen in Experiment I.

Another reagent mix that exceeded controls, as a corresponding mix did in Experiment I, was the oligoglycine containing mix, 21. It was different from the mix in Experiment I by the addition of diglycine and tetraglycine. When combined with Tacsimate in reagent mix 48, however, it did not prove effective. Because of availability problems, we could not include the polyphosphates and the oligonucleotide class properly in Experiment II. Their representation is so meager in Experiment II that we do not feel any meaningful conclusions can be drawn.

Among the most productive of the reagent mixes was 7, cobalt hexamine plus tetraphenylporphorin. The latter component was in such low concentration, however, that we do not feel it could have contributed significantly to the success of the mixture. Thus cobalt hexamine, which has previously been used as an additive, emerges here as a useful adjunct. Its value was likely obscured in Experiment I, reagent mix 25, where it was combined with the strongly precipitating lanthanide salts.

A conscientious effort was made to evaluate the potential of the sulfobetains. These are non detergent, solubilizing agents for proteins (Goldberg et al., 1996) that have recently received attention with regard to membrane proteins. As the results for reagent mixes 2, 3, 27, and 36 show however, they were not impressive in Experiment II. There were some curious reagent mixes for which we have rather little understanding, but that nevertheless caught our attention. Prominent among these was reagent mix 1, Schardinger's dextran, which exceeded controls significantly.

We were struck that tannic acid, a heterogeneous mixture of polyphenol based oligomers from plants that is used to tan leather by crosslinking the constituent protein molecules, inspired the crystallization of 16 proteins, almost all of them brown or green in color. Similarly, crystals grown in reagent mixes 6, 11, and 12 also took on brown colors considerably darker than the surrounding mother liquor. Displays of color by crystals as a consequence of the reagent mix were also observed in numerous instances in Experiment I as well. Some examples from both experiments are shown in Fig. 8. These observations are consistent with binding of the reagent molecules to proteins in the crystal lattices.

There are too many intriguing results particular to individual proteins within the 18 240 crystallization trials to review all of them here, but a few examples may be in order. These would include yeast hexokinase (HXK) with cobalt hexamine, concanavalin A (CONA) with tannic acid, lysozyme (LYZ) with trimesic acid and phloroglucinol, lactate dehydrogenase (LDH) with oligosaccharides, and glyceraldehyde-3-phosphate dehydrogenase (GPDH), which crystallized in space group $P2_12_12_1$ ($a = 82$ Å, $b = 99$ Å, and $c = 186$ Å), and apotransferrin (ATRF), both of which crystallized in reagent mix 28, a combination of aliphatic dicarboxylic acids. Crystals of several proteins that we feel are difficult to crystallize, at least using conventional screening approaches, were also produced. These included gramicidin (GRM) from aqueous solution, bovine ubiquitin (UBQ), rabbit serum albumin (RSA), and the intact antibody designated IDEC151 (Kuznetsov et al., 2000). A number of proteins were crystallized which currently have no entry in the PDB, and these include rabbit serum albumin (RSA), bovine ubiquitin (UBQ), and the enzymes from rabbit muscle, lactate dehydrogenase (LDH) and aldolase (ALD).

3.2. Experiment III

Experiment III had a somewhat different rationale than the two prior experiments. In Experiment III, a screen of 24 novel reagent mixes (one being a control) was composed of 100 small molecules of biochemical relevance. These included common coenzymes, nucleotides, prosthetic groups, metabolic intermediates, inhibitors, drugs, and effectors of various sorts. The underlying hypothesis was that particular proteins might selectively bind a single component of one of the reagent mixes, and that the complex so formed might then crystallize more readily, or in a different crystal form, than the unliganded protein. The reagent set used in Experiment III is shown in Table 3 and the protein set was identical to that used for Experiment II, with the exception of TYMV, which was inadvertently omitted. Only one starting and reservoir concentration of PEG was used in Experiment III, and no Tacsimate samples were included. Duplicate plates were set up for each protein, however.

The scoring matrix for Experiment III is shown in Fig. 9. Of the 66 proteins included in the experiment, 50 were crystallized; representing 76% of all the proteins. This was using only a single base condition, 30% PEG 3350 in 0.1 M Hepes at pH 7, but supplemented with the various biochemical reagent mixes. Of the 50 proteins which crystallized, 13 crystallized in controls lacking any reagent mix, as well as in others that did, or 20% of the total. There were 37 proteins, or 56% of the total that crystallized only in samples containing some reagent mix, but not in controls. In terms of only those proteins that crystallized, 26% appeared in controls, while 74% were seen only in

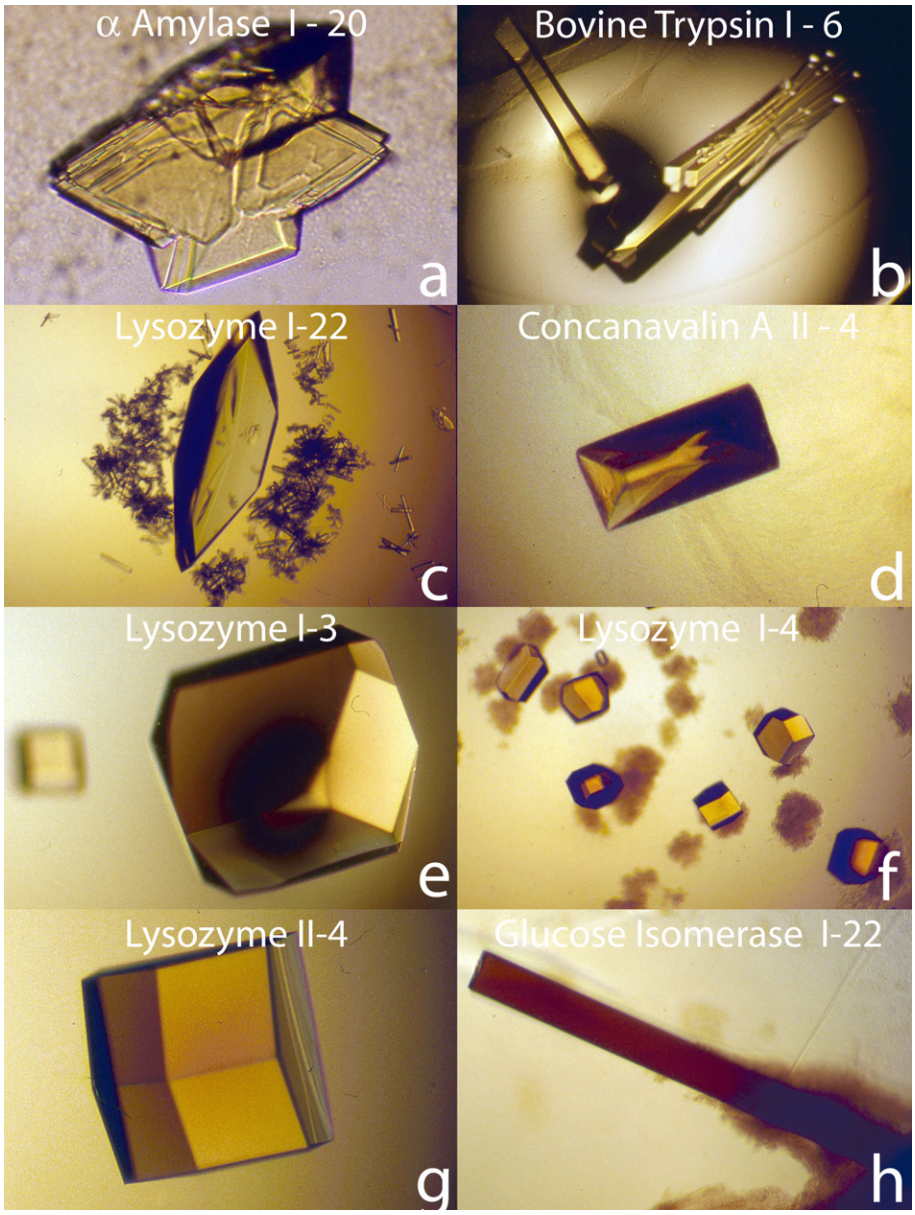

Fig. 8. Protein crystals became colored in excess of the mother liquor in the presence of various reagent mixes, the color becoming particularly pronounced with time. Some examples are shown here. The protein name is given, along with the Experiment I or II, and the number of the reagent mix from which the crystal was grown.

the presence of some reagent mix. The degree of reproducibility between the two identical trays for each protein was very high, better than 90% overlap of successes. Given the stochastic nature of protein crystallization, we found this quite encouraging. It was all the more remarkable that in those cases where only a single reagent mix was successful in yielding crystals (RA, OVL, RSA, HHU, BMV, for examples), there was exact duplication in the two trays of samples. Duplication essentially eliminates the possibility that the results were simply a consequence of random chance. A comparison of success totals for each reagent mix is shown in Fig. 10.

Only four reagent mixes surpassed the number of proteins crystallized in the controls. This might suggest that the general utility of this set of reagent mixes was less than those used in Experiments I and II, but this is deceptive. This set, having only 24 members (versus 48 in Experiments I and II), nonetheless crystallized 50 of the 66 proteins investigated, and used only a single base condition combined with reagent mixes. This is substantially more proteins crystallized per reagent mix than for either of the two reagent mix screens of Experiments I and II.

The broad statistics that might be derived from Fig. 10 belie the remarkable successes that were observed for individual proteins in the experiment. With almost complete fidelity between duplicate trays, 14 proteins crystallized from only a single reagent mix, and failed to do so in the other 23, including controls. Some of these are shown in

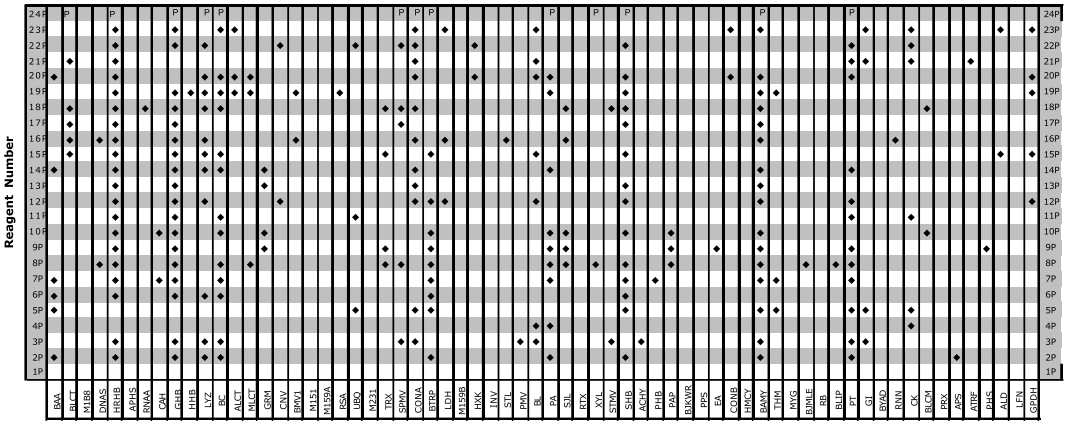

Fig. 9. Scoring matrix for Experiment III. The 66 proteins investigated are along the horizontal axis according to their alphabetic designation in Table 2. The reagent mixes comprising the 24 trials, defined according to their numeric designation in Table 3, are along the vertical axis. A diamond denotes that crystals were observed in the trial, open space indicates that no crystals were seen by light microscopy. P indicates crystals for the PEG control.

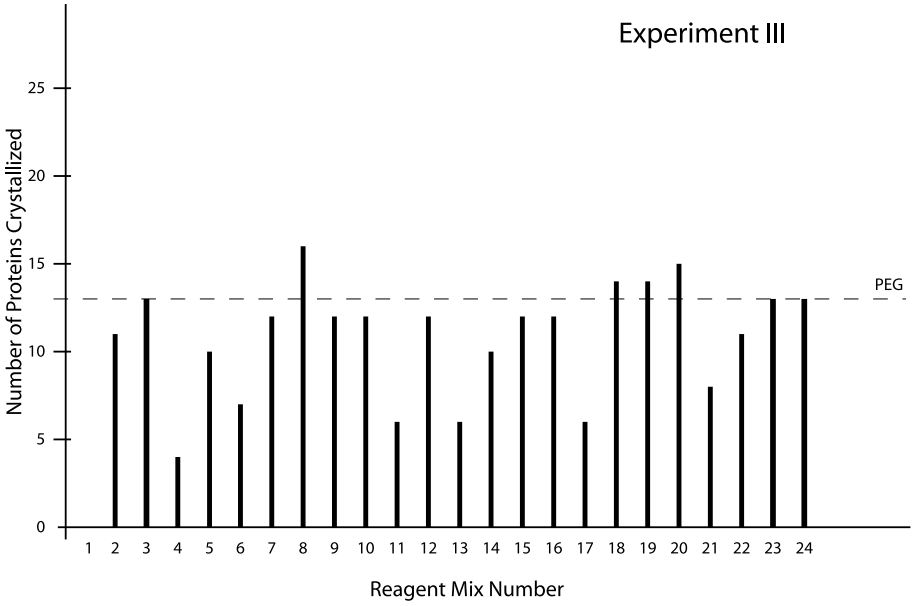

Fig. 10. A display of the number of proteins for which crystals were observed for each of the 24 reagent mixes in Experiment III. The reagent mixes are denoted by their numerical code and their composition defined in Table 3. The control (reagent mix 24) is indicated by a horizontal line.

Fig. 11. Another 15 proteins crystallized in only two or three reagent mixes, and in no others. In cases where only one to three reagent mixes promoted crystallization, some common elements of their composition could be discerned. For example PAP crystallized only in reagents 8, 9, and 10 with 100% agreement in duplicate trays. Those reagent mixes all contain mono, di and tri nucleotides (which are, so far as we know, completely unrelated to the function and biochemistry of papain, a sulfhydril protease).

In some cases, the unique reagent mix(s) contained some individual component that could, by at least some stretch of the imagination, explain why it was successful with a given protein. Ribonuclease A (RA) crystallized only with reagent mix 18. Reagent mix 18 contained dGMP, which could very well bind to the active site of the enzyme. Rabbit serum albumin (RSA) crystallized only from reagent mix 19, and this contained two drug molecules, phenobarbital and tetracycline. Serum albumin is known to form complexes with such drugs as part of its carrier function. Hexokinase (HXK) crystallized from reagent mix 20. Reagent mix 20 contains a nucleotide (UTP) and maltotriose, both potential ligands of hexokinase. Ovalbumin (OVL) crystallized in duplicate trays only in the presence of reagent mix 9. That reagent mix contained chlorpromazine, a drug molecule that previously served to aid the crystallization of another glycoprotein, α1-acid glycoprotein (McPherson et al., 1984).

In other cases, however, it is difficult to discern any correspondence between a successful reagent composition and the biological, physiological, or known biochemical properties of the protein whose crystallization it promoted. PAP, HHU, and CNV are cases in point, but there are

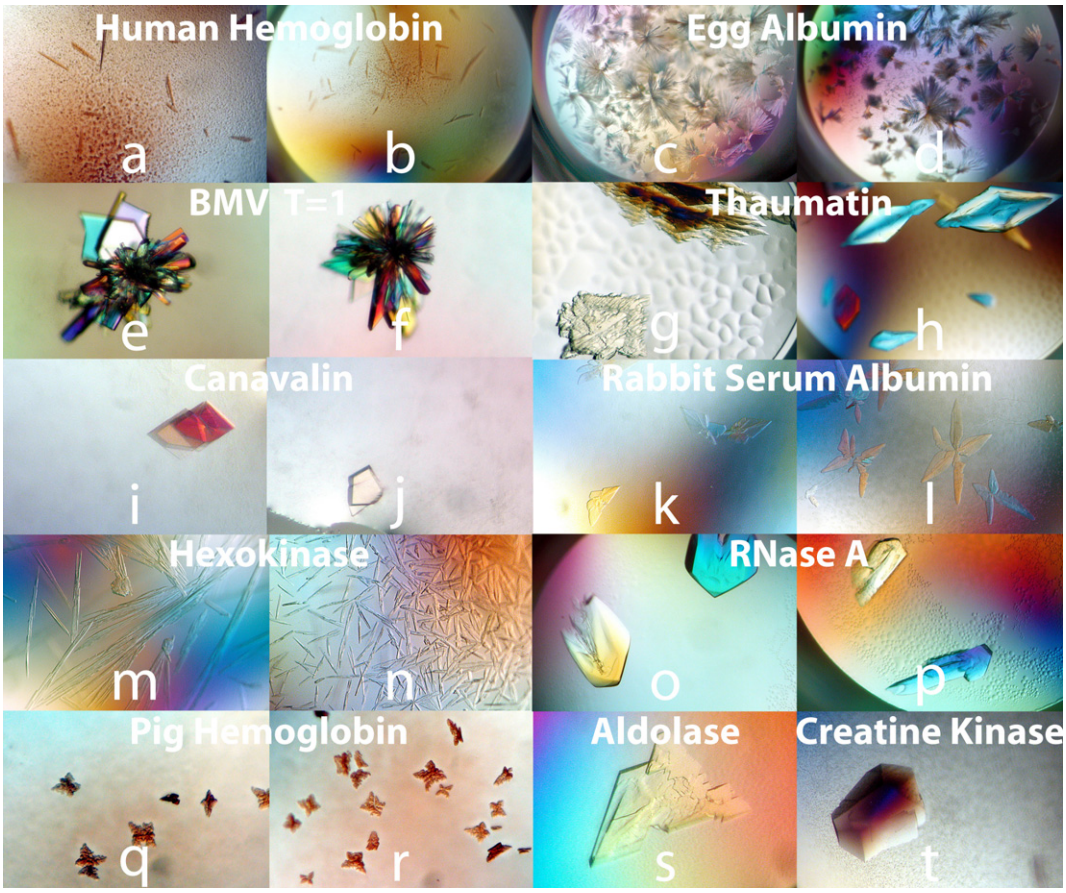

Fig. 11. Light microscope photographs of crystals observed in corresponding samples in duplicate sets of trials in Experiment III. For human and pig hemoglobin, BMV T=1 protein, hexokinase, canavalin, egg albumin, rabbit serum albumin, and RNase A, these were the only samples from which the protein crystallized at all in the experiment.

others. These examples make us suspect that some other effects, of a physical, chemical, or biochemical nature might be in play that we did not anticipate.

4. Discussion

Additives that are used in protein crystallization or that might be appropriate for use in crystallization fall into eight categories.

1. Physiologically or biochemically relevant small molecules such as coenzymes, substrate analogues, inhibitors, metal ions, prosthetic groups, etc. These bind at the active sites of enzymes, or at specific sites elsewhere on protein molecules, and may promote more stable, homogeneous conformations, or they may induce conformational changes into alternate states. In any case, the ultimate protein–ligand complex may exhibit a more monodisperse, less dynamic character. The pertinent molecules are, here, specific to the individual protein under study, and their selection for inclusion in mother liquors is amenable to rational analysis informed by the enzymology and biochemistry of the protein under study. That is, one considers all of the possible ligands of the protein and includes them in the screen of potential crystallization conditions.
2. Chemical protectants. These include reductants such as BME, DTT, heavy metal ion scavengers such as EDTA and EGTA, and compounds intended to prevent microbial infection such as sodium azide, phenol, or chlorobutanol. These too are generally included for well-understood reasons, their effects are predictable, and their impact on the crystallization process usually (but not always, see Table 1) of marginal significance.
3. Solubilizing agents and detergents. These include quaternary ammonium salts (Mirzabekov et al., 1972), sulfobetains (Goldberg et al., 1996), chaotropes like urea (Bolen, 2004), and a range of surfactant and detergent molecules (Neugebauer, 1990; Zulauf, 1990; Wiener, 2004). Because of the interest in membrane proteins, this class of additives has received extensive study, and has been broadly applied to many proteins. Remarkably, there is still no consensus on which are most useful, which should be included in screening conditions, or even how they function in the solubilization of macromolecules.

4. Poisons, as they have traditionally been called (McPherson, 1982; McPherson, 1999), were originally employed to reduce twinning. These are generally low concentrations, 1–5% w/v, of common organic solvents. They include compounds such as ethanol, DMSO, acetone, dioxane, butanol, or MPD. Their role in the crystallization process, even after 50 years of use, remains obscure. They likely enhance the solubility of the proteins and slightly reduce the degree of supersaturation in the mother liquor, as well as lower the dielectric constant of the medium, but they may have other effects as well.
5. Osmolytes, co-solvents, and cosmotropes are compounds that exert their effects at relatively high concentrations, one molar or more, and include a wide range of molecules that include sucrose and other sugars, proline, TMAO, glycine, betaine, taurine, sarcosine, and a host of others (Collins and Washabaugh, 1985; Bolen, 2004; Collins, 2004). The effect of their inclusion in the mother liquor is to stabilize (or destabilize) the native conformation of the protein by altering the interaction of the protein's surface with water, or by altering the hydration layer and possibly the structured waters.
6. It has been proposed that the conformations of proteins might be stabilized, and their dynamic character reduced, by providing the proteins with small molecules that could reversibly crosslink charged groups (carboxyl and amino groups) on the protein's surface, or form intramolecular hydrogen bonding networks using surface polar groups (Maclean et al., 2002). The molecules that have been explored are usually multivalent molecules such as diamino or dicarboxylic acid containing molecules, or aliphatic moieties of various lengths carrying some combination of charged groups. It is not known whether the stabilization of proteins by this means is significant enough to affect their crystallization or not. This potential mechanism of altering crystallization behavior must, however, be kept in mind in evaluating the experimental results presented here, as it may indeed be pertinent.
7. The class of compounds useful for stabilizing proteins through non covalent intramolecular bonds, as described above, may also help create and stabilize protein crystals by interposing themselves between protein molecules and forming intermolecular crosslinks (McPherson, 1999). These cross bridges may involve purely electrostatic interactions, or they may rely on hydrogen bonding arrangements as well. The compounds most favorable for forming such "lattice interactions" are, again, likely to be multivalent, charged compounds, but we might expect that their length, or "reach" would need be greater, since they would have to extend from one protein molecule to another.
8. The final class of additives would be those materials or compounds that somehow serve to enhance nucleation, including unique surfaces. What are in mind here are low concentrations of PEG (Ray and Puvathingal, 1986), or other polymeric substances such as jeffamine emulsified in solutions of high salt concentration (Kuznetsov et al., 2000; Kuznetsov et al., 2001). The micro droplets of the polymeric phase serve to concentrate the protein locally and provide an interface for nucleation to occur. This category should probably also include things like the gel used in cubic lipidic phase crystallization (Caffrey, 2003; Nollert, 2004), and surfaces which promote epitaxy and heterogeneous nucleation (McPherson and Schlicta, 1988; Chayen et al., 2006).

The experiments carried out here concentrate principally on classes 5, 6, and 7 in Experiment I and II, and the bioactive compounds of class 1 in Experiment III. Though not unequivocal, the experiments do yield, we feel indications as to which groups of reagents may be most helpful in a general sense. They also demonstrate that it is possible to find compounds that have a pronounced effect on the crystallization of individual, specific proteins. In addition, it is clear from the outcomes that many reagents can profoundly affect the way in which individual proteins crystallize, often inducing the appearance of alternate crystal forms.

4.1. The control samples

It is informative to look simply at what happened to control samples lacking any of the reagent mixes. Only two fundamental crystallization conditions were utilized in Experiments I and II, one based on 30% PEG 3350, and the other on 50% Tacsimate, and the PEG 3350 condition alone was used in Experiment III. If only the control samples for the three experiments are considered, then the percentage of test proteins crystallized in Experiments I, II, and III, respectively, were 35%, 31%, and 20%. Because these sets of control successes were non congruent, at least a third to a half of all the proteins tested could be crystallized from no more than two basic conditions. This, we feel, is a pleasantly surprising outcome in itself, particularly when it is considered that this applies to a heterogeneous array of 81 proteins.

4.2. Useful groups of small molecules

Experiments I and II tend to support, as did the original work with organic acid salts (McPherson, 2001), and the successes with Tacsimate, the idea that molecules rich in charged groups, particularly negatively charged carboxyl groups, might be of general utility. This is consistent with our expectations regarding the reversible crosslinking of proteins in a lattice. The results suggest that sulfonyl groups and phosphate groups might serve as well; perhaps even better. There are some indications that more constrained constellations of these groups may hold some advantages. For example, charged groups linked to benzene rings rather than at the ends of aliphatic chains, but this is still uncertain. Multiple amino groups appear, overall, to be less effective (but still useful in some cases), as were molecules containing both a positive and negative

charge group (with the exception of reagent mixes 45 and 47 in Experiment I, which were the 20 amino acids in PEG and Tacsimate respectively). Polyalcohols were statistically unimpressive in the screens.

An interesting class of compounds in Experiment I that produced a high overall success rate was the polyphosphates, either as some form of polyphosphate itself, or in the form of polynucleotides.

The consistent successes of reagent mixes containing multiple carboxyl groups suggests the possibility that other kinds of negatively charged groups might be as useful, or even more so. Thus, sulfonyl groups (which did appear in several successful reagent mixes) and phosphate groups, which carry two negative charges at pH 7, might be superior to the carboxylate compounds used in many of the reagent mixes in our experiments. Aliphatic moieties with phosphates or sulphonyls at both ends, or a phosphate or sulfonyl at one end and a positively charged amino group at the other all seem interesting possibilities. They are particularly attractive because they could be synthesized in a wide range of lengths.

The class of molecules that includes the polyamino acids, such as the oligo glycines yielded noteworthy results in both Experiments I and II. Again, access to a greater variety of these molecules limited our investigation, but they do appear promising. The 20 amino acids mix remains intriguing, though why it is effective remains something of a mystery.

Osmolytes and surfactants, the sulfobetains, and detergent mixes, though we could explore only a limited set, did not appear to promote crystallization to any discernable extent. Another generality is that PEG seems to far exceed Tacsimate, and probably other salt precipitants, in its effectiveness in crystallizing proteins. It is also clear, however, that some macromolecules will only crystallize (or at least readily) from salts, but not PEG. Thus salt-based precipitants cannot be eliminated from the standard screens. Any advantage of symmetry in the reagent molecules was not evident in the experimental results.

4.3. Overall vs. individual successes

The observations and conclusions above pertain to general trends and statistically significant results taken over all the experiments. Indeed, an important question is, which reagent mixes exceeded controls? To do so implies that they are comprehensively useful. That is, in their presence, more overall successes would be achieved than otherwise. That kind of analysis, however, masks the consequences of given reagent mixes for specific proteins. Statistical measures fail to reveal the "silver bullets" that inspire the crystallization of selected proteins. An equally important measure is how many, or what percentage of the test proteins crystallized, which would not have done so in the absence of a reagent mix, i.e., how many proteins crystallized, but not in controls?

That number is impressive for both Experiments I and II where it was 47% and 43%, respectively (42% of the 81 unique proteins investigated in all experiments). It was even more striking in Experiment III where it reached a high of 56%. This measure suggests the need of some proteins for one or more specific small molecules to satisfy their individual requirements for crystallization. Thus we have two classes of potentially useful reagents, those of broadly useful character, and those specific to individual proteins. While illustrated most clearly by the results of Experiment III, the effects of the reagent mixes on individual proteins are demonstrated as well by the numerous occasions where protein crystals were obtained in completely different forms, depending only on the reagent mix, as was illustrated in Fig. 7.

As an estimate of the potential of a matrix of reagent mixes for producing "silver bullets" (high specificity and low probability, but dramatic enhancement of crystallization behavior) we might consider the number of proteins successfully crystallized from only 1, 2, or 3 reagent mixes in the set. In Experiment I, four proteins crystallized from only a single reagent mix, two proteins from two mixes, and one protein from three different reagent mixes. In Experiment II, the number of silver bullets was greater, seven proteins crystallized from only a single reagent mix, and four proteins each from both two and three mixes. The array of reagent mixes in Experiment III was by far the most impressive. 14 proteins crystallized from only one reagent mix in the set of 24 (see Fig. 10 for some of these), nine proteins crystallized from only two reagent mixes, and 6 proteins crystallized from three. The number of proteins crystallized in three or less reagent mixes for Experiments I, II, and III in total were, respectively, 7, 15, and 29. While Experiment I produced the greatest number of generally useful reagent mixes based on statistical averages, Experiment II second, and Experiment III last, the order is exactly reversed in terms of silver bullet content.

The results of Experiment III are particularly encouraging, as the degree of success in the experiment considerably exceeded our expectations. In fact, they surpassed our most optimistic predictions by such a degree, that we feel the logic we employed in conducting the experiment in the first place is insufficient to explain its success. Seventy-seven percent of the 66 proteins were crystallized using, excluding the reagent mixes, only a single basic crystallization condition. Many of the proteins which crystallized showed distinct specificities for the reagent mixes from which they could be crystallized, and some, acutely so; 14 crystallizing from only a single reagent mix.

One important point should be acknowledged. The experiments described here were based largely, though not entirely, on macromolecules that had previously been demonstrated to crystallize. Were we to have employed a set of completely arbitrary proteins, as might be done, for example, in a structural genomics, high throughput search of conditions, the results likely would have been less

positive. Nonetheless, we are confident that they would still support our general conclusions.

4.4. Formulation of reagent mixes

The question arises as to whether the positive effects of the reagent mixes are a consequence of only a single component of the reagent mix, or if there is synergy. Synergy would imply that the particular reagent formulation was important. Our initial hypothesis was that a protein crystal, in nucleating and growing, would select the critical component from its mother liquor, and that the other compounds present in the mix would have no relevance. We believe that this is probably so. On the other hand, we also suspect that for some reagent mixes there may indeed be complementarity. Thus the indications for a particular component may depend on its companions. For example, in Experiment II, the reagent mixes of variable length aliphatic dicarboxylic acids (6, 8, 10, 14, 15, 16, 26, 28) were statistically noteworthy. While the unique component hypothesis may well be valid here as well, our inclination is toward a more extensive network of intermolecular cross-links involving multiple components. This may be true as well for the oligomers of glycine and the polyphosphates, and perhaps for the mixture of the 20 amino acids.

One negative example shows that while formulation of mixes may not be decisive, it cannot be ignored. In Experiment I, cobalt hexamine was combined in reagent mix 25 with gadolinium and praseodymium salts, a collection of positive charge centers. Virtually every protein was immediately precipitated by the lanthanide salts, and any positive effects the cobalt hexamine might have had were completely disguised. In Experiment II, however, cobalt hexamine was included in a different formulation (reagent mix 7), and here it proved to be one of the most successful of the reagent mixes. Aside from cases illustrated by this example, however, we believe that the effects of individual components within reagent mixes are likely to be independent and additive. By including individual reagents in two or more mixes, so that the reagent mixes are overlapping, the actual value of a specific component is more likely to become evident.

4.5. False positives

With so many small molecules, often having limited or sensitive solubilities, the appearance of false positives is almost inevitable. We were surprised, however, that in Experiment I there were virtually no false positives. There were some false positives in Experiment II, however. In Experiment II, the appearance of the same clusters of large lath like crystals for reagent mixes 8 and 9, and occasionally 33 and 38, regardless of the protein involved, told us at once that the crystals were of mellitic acid. The appearance of the same needle crystals in reagent mix 8 containing samples of Experiment III, independent of the protein, told us that naladixic acid was crystallizing. The false positives can generally be identified by their repeated appearance for multiple proteins, and by setting up the screen of reagent mixes in the absence of any protein, or with some non crystallizable protein such as casein. This was done for the experiments we report. Although some conventional, small molecule or salt crystals may have, nonetheless, been misassigned as protein crystals, these are likely to be few in number. We do not believe that they would significantly affect our results or conclusions.

Investigators should be very cautious in indiscriminant combination of the reagents and reagent mixes that we studied here, with other precipitants, with buffers at different pH, or in mother liquors containing other ions. To do so would invite a large number of false positives. The combination of the reagents with Ca^{2+}, Mg^{2+}, or other divalent ions, we have confirmed by secondary experiments, will cause many of the small molecules to crystallize. Lower or higher pH would likely do the same. Similar sensitivity might also be expected for organic solvent precipitants such as isopropanol. Extension of the approach presented here to other precipitants, and other crystallization screens, must be accompanied by appropriate control samples, which would indicate the occurrence of false positives.

4.6. A novel strategy

While it might appear that identifying specific molecules that promote the crystallization of a particular protein is a hopeless task, there being an impossibly vast number of chemical compounds, this is not really the case, for several reasons. First, we are not obliged to evaluate the compounds individually, but can do so in groups. For example, one of the most successful reagent mixes in Experiment II was number 28 which was composed of nine different compounds. By grouping compounds into various formulations, a 96-sample matrix could be devised to test 200–300 chemicals. The only chemicals that need be considered would be those of relatively good solubility in water that do not denature proteins. Further, it is highly likely that the most suitable compounds will be those bearing groups that can be involved in electrostatic and/or hydrogen bonding interactions with proteins.

The underlying idea that serves as the foundation for the approach presented here is the following. Any one compound or reagent mix may have a very small chance of promoting the crystallization of a specific protein, say one in a hundred. The probabilities of success contributed by each reagent mix in a large set are, however, additive. Even if the probability for a given reagent with a specific protein is only 0.01, if the set of reagents tested is several hundred, then the overall chance of a winner hiding in there somewhere becomes quite significant. The problem currently facing us is to identify those additives, those molecules and compounds that can serve, for at least some proteins, to occasionally enhance, even by a small amount, the probability of a successful outcome.

It may be argued that the approach to crystallizing proteins described here is not scientifically rigorous, given that it relies to a large extent on chance. That may be true, but current methodologies continue to depend, perhaps even more, on chance. Presently, our efforts are directed at simply maximizing the number of potentially useful trials for a given amount of protein, and doing this in the most efficient manner. The strategy suggested here shares those features, but addresses the problem along what we might term an orthogonal direction.

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