Calorimetric Approaches to Characterizing Effects of Additives on Protein Crystallization

Pt I: Peptergents
Pt II: Microcalorimetry, Crystallization, & Additives

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Membrane Proteins Considerations

For a 3-component system - water, lipid, amphiphilic block copolymer – there are nine isothermal phases [Alexandris (98)]: four cubic, two hexagonal, one lamellar, two micellar.

Nollert, 2005.
Membrane Proteins: unique problem of solubilization
Amphiphilic Microenvironments:

(A)  

(B)  

(C)  

(D)  

(E)  

(F)  

(G)  


Peptergents: Peptide-based Detergents

New Materials for Solubilization & Stabilization of Membrane Proteins

Yeh, Du, Tortajada, Paulo, and Zhang (2005), Biochemistry.
Peptide Materials

Peptergents: self-assembly
Examples of peptide detergents

- A6D
- L6D2
- K2L6
- V6D
- V6D2
- K2V6

(a)  

(b)  

1 nm
Nanostructure formation scheme
Peptergents: solubilization

Table 1: Specific Activity of GlpD under Various Conditions

<table>
<thead>
<tr>
<th>Surfactant</th>
<th>Specific Activity (units/mg) (+/- 10%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Salt Concentration</td>
</tr>
<tr>
<td></td>
<td>0 mM</td>
</tr>
<tr>
<td>CHAPS</td>
<td>7.3</td>
</tr>
<tr>
<td>DM</td>
<td>9.9</td>
</tr>
<tr>
<td>SDS</td>
<td>0.2</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>9.4</td>
</tr>
<tr>
<td>OG</td>
<td>17.4</td>
</tr>
<tr>
<td>V6D</td>
<td>18.7</td>
</tr>
<tr>
<td>A6DA6K</td>
<td>13.3</td>
</tr>
<tr>
<td>pre-solubilized membrane</td>
<td>26.7</td>
</tr>
</tbody>
</table>

1 unit is defined as the reduction of 1 micromole of MTT per minute.
Activity w/ Peptergents: stabilization of membrane and soluble enzymes

Yeh, Du, Tortajada, Paulo, and Zhang (2005), Biochemistry.
Another Membrane Protein: Photosystem I (PS-I)
S. Zhang (MIT)
Peptergents: thermal stabilization PS-I
S. Zhang, MIT

SS-emission @-196 C
characteristic peak @735 nm
dried @683 nm

Emission Spectra dried PS-I
→ A6K particularly stabilizing

Enhanced lifetime of stabilization
Pt. II Correlating Solution Characteristics to Crystallization Behavior
Microcalorimetry in Crystallography

• Most physical or chemical processes have an associated heat effect and this can be used as the basis for a number of analytical techniques and determination of absolute thermodynamic quantities.

• Differential scanning microcalorimetry (DSC) are used mainly for the study of thermal transitions in dilute solutions.

• Isothermal titration calorimetry (ITC) used to study binding and stoichiometry.

• Application of microcalorimetry to characterize crystallization conditions.
Characterizing Crystallization: 
* Differential Scanning Calorimetry (DSC) *

- **Tm:** transition temperatures can give an indication of how the native protein behaves under various conditions.
- **Shifts in Tm** under different conditions and in the presence of additives can characterize effects of various compounds.
ITC directly measures directly the energy associated with a chemical reaction triggered by mixing of two components.

Binding energetics of protein-ligand and protein-protein interactions can be measured.

Useful way to measure coupling of protonation/deprotonation to solution stabilization or effector binding.

quantitation pH effects

Curr Opin Struc Biol 11: 560-566.
Enthalpic versus Entropic Effects

Binding affinity dictated by Gibb’s energy

\[ K_a = e^{\frac{\Delta G}{RT}} \]

- but \( \Delta G = \Delta H - T \Delta S \rightarrow \) both enthalpy and entropy components

- Binding enthalpy primarily reflects strength of ligand-protein

- Entropy change reflects two main contributions: change in solvation and conformation.

\[ \rightarrow ITC \text{ is the only technique that can resolve enthalpic and entropic components of binding affinity.} \]

\[ \rightarrow \text{How does solution thermodynamics relate to crystallizing macromolecules?} \]
Hypothesis:
Conditions that give rise to data quality crystals have a positive effect on the protein’s transition temperature.

Stabilization of Protein Under Crystallization Conditions → shift to higher Tm

Baseline-corrected for buffer -data M. Walsh
Stabilization of Protein Under Crystallization Conditions

-inducing reversibility of transition

Baseline-corrected for buffer
-data M. Walsh
Stabilization of Protein Under Crystallization Conditions

→ shift of states

Baseline-corrected for buffer -data M. Walsh
Summary I:
Positive correlation between solution composition, protein stability, and crystallization behavior.

Pt II. Microcalorimetry and Additives & Optimization:

Additives can salt-in or salt-out macromolecules that are insoluble or especially soluble in the presence of the major precipitant used.

Enhancements: (1) larger size, (2) increased resolution of diffraction, and/or (3) lowered mosaicity.
Possible Mechanism of Additives:

• stabilization of crystal contacts (specific intra- and inter-molecular interactions).
• enhancement of conformity or homogeneity of the macromolecule.
• modification of the rate of nucleation and/or growth rate to enhance or depreciate the equilibration rate. Modulation of solvent structure around the protein molecule.
• stabilization of protein conformation is achieved not by specific binding but by preferential exclusion from the protein surface and the consequent preferential hydration of the protein.
General Classes of Additives:

(1) Ions
(2) Organics
(3) Linkers, multivalent tethers
(4) Detergents
(5) Cofactors, chelators, reducing agents
(6) Chaotropes
(7) Kosmotropes

→ Effects of additives mediated through numerous mechanism including direct interactions with proteins (electrostatic, covalent, H-bonding) or may influence indirectly by modulating solvent interactions.
Classes of Additives – effectors:

  → intermolecular bridges, cofactors (effectors)

Classes of Additives - linkers:

- Electrostatic cross-linking agents bridge and stabilize intermolecular contacts in crystals. This group includes small and/or charged polymers to increase size, reduce nucleation sites, and improve crystal quality. Multivalent linkers may influence association kinetics and stabilize interactions.
  → facilitates non-covalent, electrostatic interactions
Classes of Additives – organics, sugars:

- *Organic additives* include phenol, e.g. stabilize helix structure in zinc insulin crystals (Derewenda et al., 1989); 1,10-phenanthroline stabilized intermolecular contacts, e.g. in membrane-bound receptor (Yeh et al., 1996). Polyols can stabilize proteins via amphiphilic interactions and may contribute to stabilizing crystal packing via decreasing conformational mobility (Saraswathi et al, 2002). Sugars may be effectors and osmolytes (affecting hydration; Bolen et al, 2004).

Classes of Additives – amino acids and peptides:

L-cysteine used as reducing agent, L-arginine to prevent aggregation (Machius et al 2001), and glycine based osmolytes have all been reported (Santoro et al, 1992). Peptides can act as amphiphiles and recent short peptide detergents molecules can also solubilize (Yeh et al, 2005).
Classes of Additives - detergents:

- *Detergents*: used in crystallization of not only membrane proteins but also of soluble proteins, tRNAs, and protein-nucleic acid complexes. Detergents, particularly βOG, can in general have positive and at times, strikingly favorable effects, on parameters affecting crystal growth (McPherson et al., 1986). Peptergents can solubilize membrane proteins and stabilize soluble proteins (Yeh et al, 2005).

- Numerous others including reducing agents, surfactants, chelators, etc. To clearly distinguish their effects, categorize according to basic mechanism and chemistries.
When are additives used to improve crystallization and diffraction characteristics?

(1) When microcrystals are observed but does not grow into larger crystals
(2) When crystals have poor morphology
(3) When crystals possess good morphology but are very small
(4) When crystals look nice but diffracts poorly
(5) When reproducibility of a particular condition is erratic
(6) To alter conformation of a particular protein for crystallization
Important Parameter: concentration

Typical final concentrations are roughly:
(1) Ions: 1 mM to 100 mM
(2) Organics: 0.1% to 5%
(3) Detergents: 1 mM to 200 mM
(4) Linkers: 2 mM to 150 mM
(5) Chaotropes: 1 mM to 100 mM
(6) Kosmotropes: 1 mM to 200 mM
(7) Cofactors, reducing agents: 1-15 mM
(8) Glycerol: 5-20%
Applying DSC to Characterize Additive Effects: 
*shifts in Tm with additives*

Yeh, Beale, Walsh (2006)
Effects of Additives 1

Starting from microcrystals

Control - H232A

H232A + 1,12-dodecanedioic acid

H232A + hexaglycine

H232A + poly-L-asp acid
Starting from single crystals with decent morphology but small
Effects of Additives 3

Starting from ‘ugly’ crystals

Control - KinR - cond. 1
KinR + 1,2,3-heptanetriol

Starting from tiny crystals

Control - KinR - cond. 2
KinR + 1,8-diaminoocctane
Tabulation of *Tm shifts under various conditions*

<table>
<thead>
<tr>
<th>Protein Concentration</th>
<th>Additive Concentration</th>
<th>Tm (buffer)</th>
<th>Tm (crystallization solution)</th>
<th>Tm (+additive)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GK</td>
<td>Glycerol</td>
<td>42</td>
<td>65.7</td>
<td>69.1</td>
</tr>
<tr>
<td></td>
<td>Poly-L-aspartic acid</td>
<td></td>
<td>57.2</td>
<td>59.1</td>
</tr>
<tr>
<td>GlpO</td>
<td>Fructose-6-phosphate</td>
<td>50</td>
<td>55</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Hexaglycine + glycerol</td>
<td></td>
<td>52</td>
<td>51.6</td>
</tr>
<tr>
<td>Npx</td>
<td>1,12-dodecanedioic acid</td>
<td>47</td>
<td>57</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>hexaglycine</td>
<td></td>
<td>58</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>ε-amino-n-caproic acid</td>
<td></td>
<td>53</td>
<td>59</td>
</tr>
<tr>
<td>GlpD</td>
<td>1,2,3-heptanetriol</td>
<td>57</td>
<td>73.6</td>
<td>77.7</td>
</tr>
<tr>
<td></td>
<td>1,8-diaminoctane</td>
<td></td>
<td>75.2</td>
<td>75.5</td>
</tr>
<tr>
<td></td>
<td>dextran sulfate</td>
<td></td>
<td>63</td>
<td>63</td>
</tr>
</tbody>
</table>
Working hypothesis correlating crystallization behavior, additives, and solution thermodynamics

What stabilizes proteins? (thermophiles vs mesophiles)

1. Decrease in solvent exposed surface area
2. Increase in packing density
3. Increase in core hydrophobicities
4. Decrease in length of surface loops
5. Increase in number of hydrogen bonds
6. Increase in number of ion pairs

Solution conditions may influence / increase one or more of these interactions in a protein
Working Hypothesis – Additive ‘Mechanism’ and Annealing (flash / in-situ annealing): decreasing domain structure

Conclusions from Calorimetric Study of Four Proteins (Initially)

- Positive correlation between increased Tm and crystallization condition.
- Positive correlation between increased Tm and additive effects.
- Application of DSC and ITC can be used to differentiate enthalpic and entropic contributions to binding.
- ITC can quantitate and correlate effect of pH, solution components, and small molecule compounds to crystallization behavior.

→ For HT applications: capillary DSC permits screening using 96-well format.
Structural Biology at Univ. of Pittsburgh Medical School

Basic Science Tower 3

a state-of-the-art research building on 5th Ave.

- Structural Biology
- Computational Biology
- Genomics and Proteomics core
- Pittsburgh Institute for Neurodegenerative Diseases
- Drug Discovery Institute
X-ray Crystallography Facility at Univ. of Pittsburgh Medical School

I. Biomaterial Production
   Molecular Biology Core → Protein Production Core

II. Crystallization Screening
   High-Throughput Crystallization Robot → Crystal Plate Imaging

III. Data Collection
   Crystal Diffraction Screening (ACTOR Robot) → X-ray Data Collection

IV. Atomic Structure
   Crystal Structure Determination → Applications

V. Other Resources

   Departmental
   600, 700, 800, 900 MHz NMR
   Cryo Electron Microscopy
   Microcalorimeters (ITC / DSC)
   Biacore (SPR)
   Light Scattering
   Circular Dichroism
   Mass Spectrometry
   Imaging Facilities
   Computational Cluster

   Institutional Resources
   Institute of Nanoscience :
   SEM, TEM, AFM, STM
   Electron beam lithography (Raith e-Line)
   Microfabrication
   Atom probe field ion microscope
   Electron microscope
Acknowledgments

Collaborators

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Same Beale – Brown University

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Yeh Lab
http://www.biophysics.pitt.edu/structuralbiology/Yeh/index.htm
<table>
<thead>
<tr>
<th>Additive</th>
<th>Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cadmium Chloride dihydrate</td>
<td>Ion</td>
</tr>
<tr>
<td>Zinc Chloride</td>
<td>Ion</td>
</tr>
<tr>
<td>D-fructose-6-phosphate</td>
<td>Organic, non-volatile</td>
</tr>
<tr>
<td>Ethylene Glycol</td>
<td>Organic, non-volatile</td>
</tr>
<tr>
<td>Glycerol anhydrous</td>
<td>Organic, non-volatile</td>
</tr>
<tr>
<td>1,6 Hexanediol</td>
<td>Organic, non-volatile</td>
</tr>
<tr>
<td>2-Methyl-2,4-pentanediol</td>
<td>Organic, non-volatile</td>
</tr>
<tr>
<td>1,3-Propanediol</td>
<td>Organic, non-volatile</td>
</tr>
<tr>
<td>Hexaglycine</td>
<td>Linker</td>
</tr>
<tr>
<td>6-Aminocaproic acid</td>
<td>Linker</td>
</tr>
<tr>
<td>Additive</td>
<td>Class</td>
</tr>
<tr>
<td>----------------------------------</td>
<td>----------</td>
</tr>
<tr>
<td>1,8-Diaminooctane</td>
<td>Linker</td>
</tr>
<tr>
<td>1,12-Dodecanedioic acid</td>
<td>Linker</td>
</tr>
<tr>
<td>Spermidine</td>
<td>Linker</td>
</tr>
<tr>
<td>poly-L-aspartic acid</td>
<td>Linker</td>
</tr>
<tr>
<td>1,2,3-Heptanetriol</td>
<td>Linker</td>
</tr>
<tr>
<td>NDSB 195</td>
<td>Chaotrope</td>
</tr>
<tr>
<td>Trimethylamine HCl</td>
<td>Chaotrope</td>
</tr>
<tr>
<td>Ammonium Sulfate</td>
<td>Kosmotrope</td>
</tr>
<tr>
<td>EDTA sodium salt</td>
<td>Chelator</td>
</tr>
<tr>
<td>1,10-phenanthroline</td>
<td>Chelator</td>
</tr>
<tr>
<td>NAD</td>
<td>Cofactor</td>
</tr>
<tr>
<td>ATP disodium salt</td>
<td>Cofactor</td>
</tr>
</tbody>
</table>
Experimental Output:

- End up with a titration plot of heat (μJ or μcal)/sec vs. time.

- You can then process the data to get a binding isotherm (middle panel):
  
  1) Integrate the area under each peak (gives you heat released at each titration point, ΔQ).
  2) Divide by the diluted concentration of ligand added at each titration point (gives ΔQ/Δ[X]; y-axis)
  3) Calculate the running concentration of ligand added (x-axis).

Importantly,

\[ \Delta H^\circ = \frac{\Delta Q}{\Delta[X_{\text{bound}}](\text{volume})} \]  

\[ \Delta \text{ moles of bound complexes} \]

- Alternatively, we can plot the data in an integrated form, where we keep track of the running total of heat released.

FIG. 1. Binding of the trisaccharide epitope Gal[Ab]ManOMe by monoclonal antibody Sc13.4. The thermogram (a) and binding isotherms in the differential heat mode (b) and in the integral heat mode (c) have shown. The lines in (b, c) have been obtained from nonlinear regression according to Eqs. (10) and (11), respectively.
Theoretical Titration Curves:

What you can get out of the plot depends upon the concentration of the titration vs. $K_a$ for the binding interaction.

The parameter that we use to describe this is $c$:

$$c = (M_{tot}) (K_a), \quad \text{where } M_{tot} = \text{concentration of the macromolecule in the reservoir.}$$

Note that $c<1$ implies very weak binding.

$C>1000$ implies very little free ligand (i.e., must use a competition experiment to get a meaningful fit – see below).

FIG. 3. Simulated binding isotherms for various values of the parameter $c$ (equal to the product of the binding constant times the total macromolecule concentration), presented in derivative format. See text for details.
If $1 < c < 1000$ then the isotherms can reasonably be fit to determine $n$, $\Delta H$, and $K_a$.

In the general case of $n$ non-interacting binding sites, the integrated binding isotherm (the total amount of heat released $(Q)$ after some total concentration of ligand $(X_t)$ has been added to a reservoir containing a known concentration of protein $(M_t)$ is given by the equation below. Note that heat evolved increases with increasing $X_{tot}$, $n$, $\Delta H^o$, $M_t$, and $K_a$.

We know $M_t$, $V_o$, Measure $Q$ vs. $X_t$, Fit to get $K_a$, $n$, and $\Delta H$.

The total heat content $Q$ of the solution contained in $V_o$ (determined relative to zero for the unliganded species) at fractional saturation $\Theta$ is

$$Q = n\Theta M_t \Delta H V_o$$

(8)

where $\Delta H$ is the molar heat of ligand binding. Solving the quadratic equation (7) for $Q$ and then substituting this into eq. (8) gives

$$Q = \frac{nM_t \Delta H V_o}{2} \left[ 1 + \frac{x}{nM_t} + \frac{1}{nKM_t} \right] - \sqrt{\left(1 + \frac{x}{nM_t} + \frac{1}{nKM_t}\right)^2 - \frac{4x}{nM_t}}$$

(9)

The value of $Q$ above can be calculated (for any designated values of $n$, $K$, and $\Delta H$) at the end of the $i$th injection and designated $Q(i)$. The parameter of interest for comparison with experiment, however, is the change in heat content from the completion of the $i-1$ injection to completion of the $i$ injection.


Notice that this equation has the same form as the general expression we derived for titration-type experiments (Lecture 2, notes pages 20-21).