Differential Scanning Fluorimetry (DSF)

Brief description DSF is a rapid and inexpensive screening method to identify low-molecular-weight ligands that bind and stabilize purified proteins. The temperature at which a protein unfolds is measured by an increase in the fluorescence of a dye with affinity for hydrophobic parts of the protein, which are exposed as the protein unfolds. Its generic applicability represents DSF as one of the most attractive methods used for ligand and condition screening:

- What is the thermal stability of the protein?
- Is the flexibility of the protein, expressed as the number of differently stable conformational states, high or low?
- If a protein comprises two domains, do they unfold cooperatively?
- Can the thermal stability be improved by addition of generic additives such as salts, nucleotides, etc.?
- How high is the thermal stability at different pH or different concentrations of additives such as salts?
- By how much does a known ligand stabilize the protein?
- Does a known ligand influence the mode-of-unfolding for the protein (e.g. of multi-domain proteins)?

- DSF can rapidly determine the stability of proteins in high throughput, and allows to compare directly different proteins or the same protein under different conditions to be studied.
- The range of temperature over which unfolding occurs reports on the flexibility of the protein: steep transitions are indicative for highly cooperative unfolding, shallow transitions indicate high flexibility.
- A monophasic unfolding transition observed with multi-domain proteins indicate cooperative unfolding (so-called: two-state unfolding). More complex transitions show that unfolding of the domains does not occur in concerted manner.

<u>What is needed?</u>

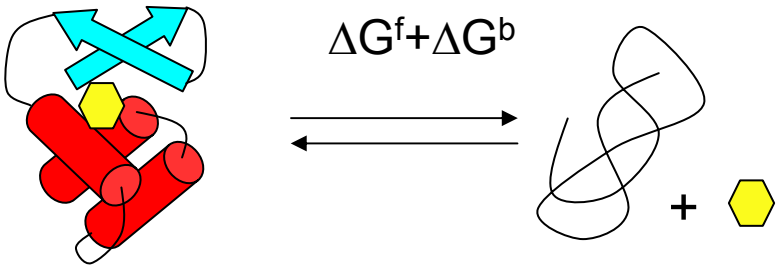
- Buffer 10-100 mM to maintain pH (for compound screening, lower strengths are preferred)
- Electrolytes dependent on the protein, e.g. 0.1 – 0.5 M NaCl
- ≥ 0.5 mM concentrations of each compound
- 4.2 ml of sample at about 0.1 mg/ml (96 conditions)

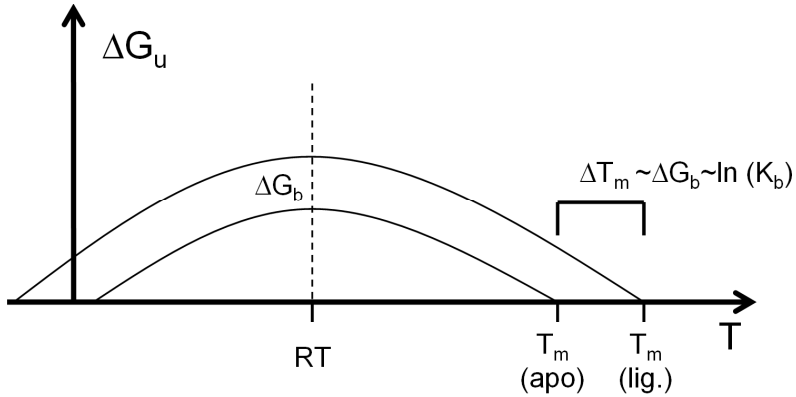
<u>Disadvantages</u>

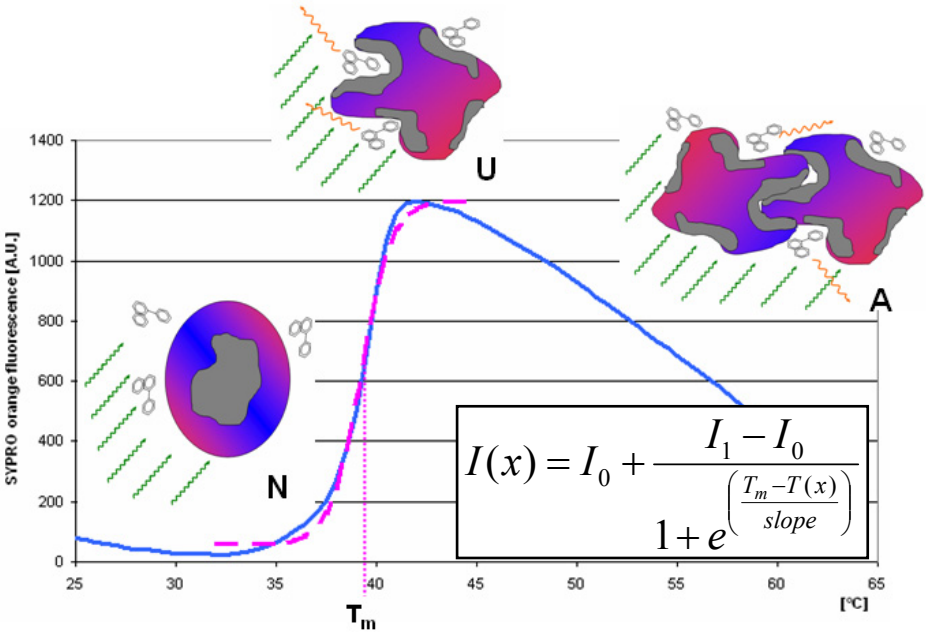
- Interactions between compounds and unfolding-monitoring dye may mask stabilization or give rise to artifacts.
- Coloured compounds may interfere with optical detection of fluorescence.
- Ambiguous results for stabilization of multi-domain proteins that show non-two state unfolding by compounds.
- Not applicable to conditions comprising hydrophobic additives such as detergents.

Instrumentation & Method Principle

The stability of a protein is related to its Gibbs free energy of unfolding, ΔG_u which is temperature-dependent. The stability of most proteins decreases with temperature; as the temperature increases the ΔG_u decreases and becomes zero at equilibrium where the concentration of folded and unfolded protein are equal. At this point the temperature is considered as melting temperature (T_m). If the protein unfolds in a reversible two-state manner the equilibrium thermodynamics models will apply. If a compound binds to a protein, the free energy contribution of ligand binding in most cases results in an increase in ΔG_u, which may cause an increase in the T_m.





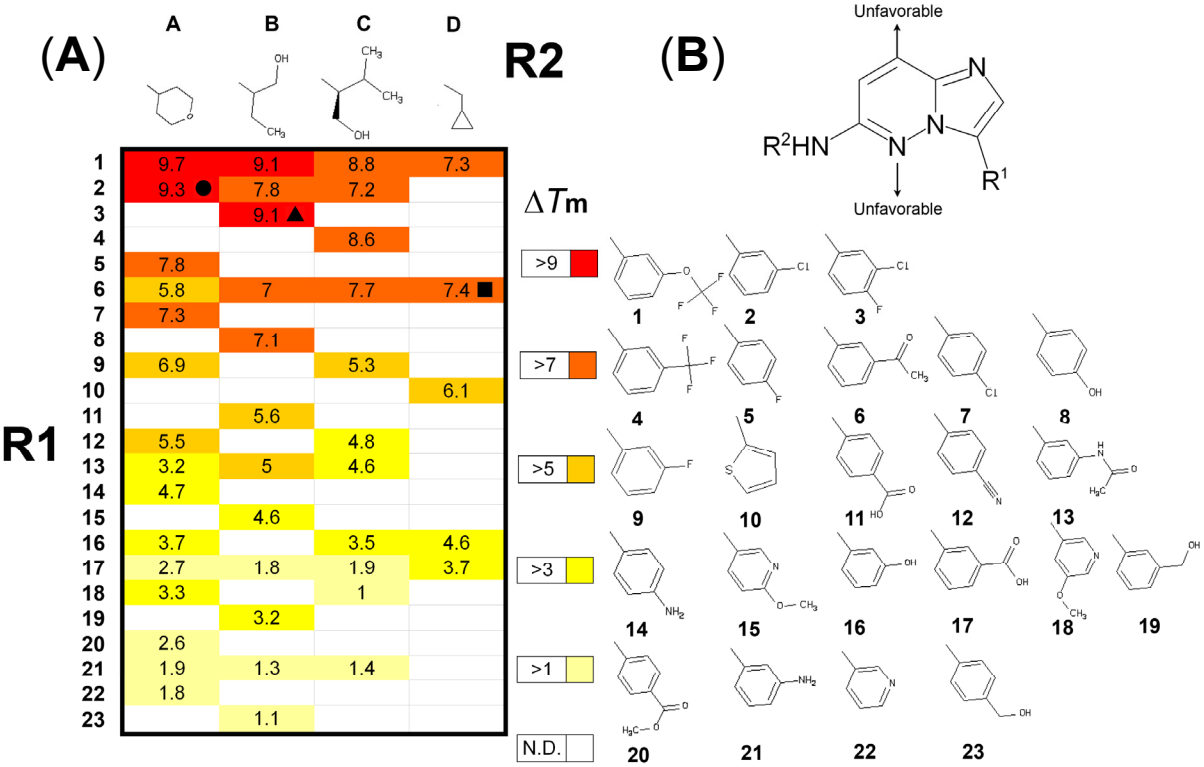


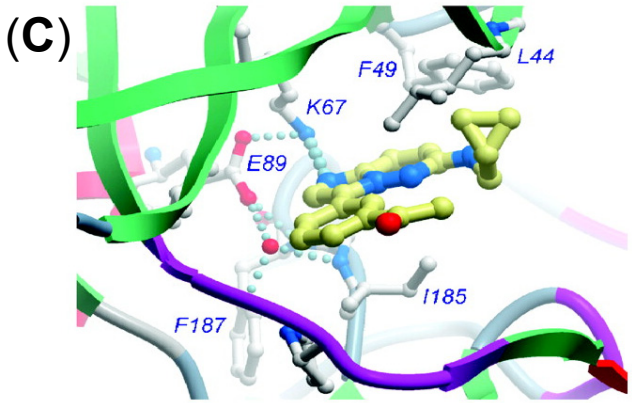
DSF monitors thermal unfolding of proteins in the presence of a fluorescent dye and is typically performed by using a real-time PCR instrument. The fluorescent dyes that can be used for DSF are highly fluorescent in non-polar environment, such as the hydrophobic sites on unfolded proteins, compared to aqueous solution where the fluorescence is quenched.
The fluorescence intensity is plotted as a function of the temperature; this generates a sigmoidal curve that can be described by a two state transition. The inflection point of the transition curve (T_m) is calculated using simple equations such as that of Boltzmann.

F. Niesen, H. Berglund & M. Vedadi, *Nature Protocols* 2(9),2212 (2007)

CASE STUDY – Identification and classification of inhibitors

Using protein stability shift assays, we identified a family of imidazo[1,2-b]pyridazines to specifically interact with and inhibit PIM kinases with low nanomolar potency. The high-resolution crystal structure of a PIM1 inhibitor complex revealed that imidazo[1,2-b]pyridazines surprisingly interact with the NH(2)-terminal lobe helix alphaC rather than with the kinase hinge region. Thus, the identified inhibitors are ATP competitive but not ATP mimetic compounds.



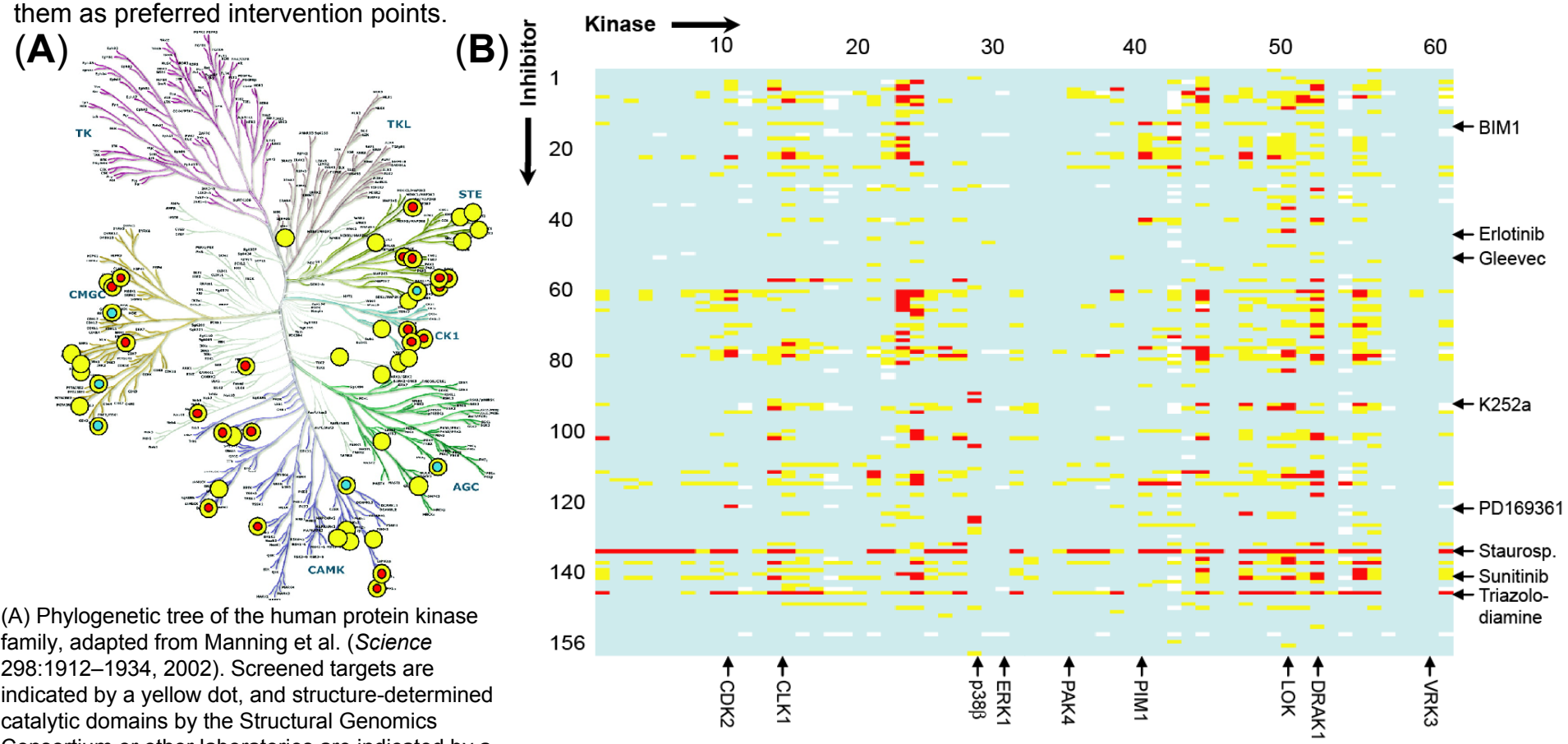


(**A**) Structure-activity relationship of the imidazopyridazine inhibitor derivatives screened by temperature shift assays. Structure-activity relationship identified on the imidazopyridazine scaffold. Shown are *T*m shift data as a function of the chemical moieties in R1 and R2. The *T*m shift data are color coded. The different chemical moieties studied are shown on the right side (R1) and on top (R2). Moieties present in the three inhibitors studied in cells are indicated (*triangle,* K00486; *circle,* K00152; *square,* K00135). (**B**) General scaffold of the studied imidazopyridazine inhibitor showing the location of the two varied R groups. (**C**) A view from the hinge region (highlighted in magenta). *Dotted lines,* hydrogen bonds. Only side chains that make significant contact with the inhibitor are shown.

V. Pogacic, A. Bullock, O. Fedorov, P. Filippakopoulos, C. Gasser, A. Biondi, S. Meyer-Monard, S. Knapp & J. Schwaller. *Cancer Research* 67(14), 6916 (2007)

CASE STUDY – Specificity and cross-reactivity of inhibitors

Intensive research is focused on inhibitors of protein kinases of which many play a pivotal role in cell signaling, and their dysregulation has been linked to disease development. Moreover, kinase inhibitors are widely used as specific probes to study cell signaling. Because systematic studies describing selectivity of these reagents across a panel of diverse kinases are, however, largely lacking, we evaluated the specificity of 156 validated kinase inhibitors, including inhibitors used in clinical trials, against 60 human Ser/Thr kinases using a thermal stability shift assay. Our analysis revealed many unexpected cross-reactivities for inhibitors thought to be specific for certain targets. We also found that certain combinations of active-site residues in the ATP-binding site correlated with the detected ligand promiscuity and that some kinases are highly sensitive to inhibition using diverse chemotypes, suggesting them as preferred intervention points.

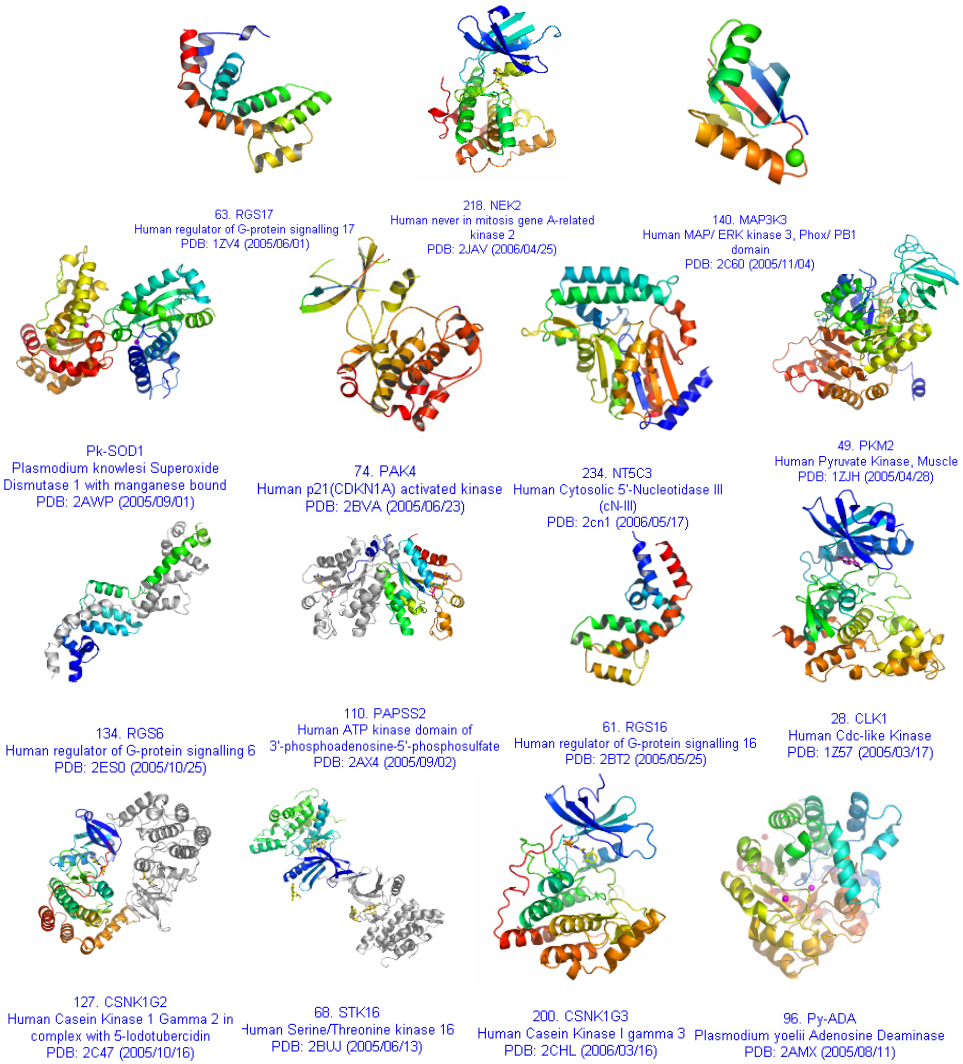


(A) Phylogenetic tree of the human protein kinase family, adapted from Manning et al. (*Science* 298:1912–1934, 2002). Screened targets are indicated by a yellow dot, and structure-determined catalytic domains by the Structural Genomics Consortium or other laboratories are indicated by a red dot and a blue dot with a yellow sphere, respectively. (B) Inhibitor array of the screened kinases. Yellow fields indicate a *T*m shift >4°C and red field of >8°C, respectively. White fields represent data that have not been determined.

O. Fedorov, B. Marsden, V. Pogacic, P. Rellos, S. Müller, A. Bullock, J. Schwaller, M. Sundstrom & S. Knapp. *Proc Natl Acad Sci USA* 104(51), 20523 (2007)

CASE STUDY – HT screening of compounds and solvent conditions

The 3D structures of human therapeutic targets are enabling for drug discovery. However, their purification and crystallization remain rate determining. It is known that the expression and purification of a protein can be improved significantly by the addition of a specific ligand, which serves to stabilize the protein, thereby reducing its propensity to unfold, aggregate, or succumb to proteolysis. Using thermal shift screening we identified small-molecule ligands that promoted protein purification, concentration, and crystallization, thereby contributing significantly to our ability to generate crystal structures of proteins from diverse families.



Protein	Situation before screening	Condition derived and applied	PDB code
Cdc2-like kinase CLK1	solubility limited, insufficient for crystallization	addition of L-Arg and L-Glu; co-crystallization with 10Z-Hymenialdisine	1Z57
Pyruvate kinase	poor crystal diffraction	addition of L-Phenylalanine	1ZJH
Regulator of G protein signaling, RGS17	solubility limited, insufficient for crystallization	higher pH, lower NaCl conc.	1ZV4
Adenosine deaminase	no crystals formed	addition of deoxyguanosine	2AMX
Calpain 1	poor crystal diffraction	lower NaCl conc.	2ARY
Fe-superoxide dismutase	poor crystal diffraction	addition of MnCl2	2AWP
PAPS synthetase	poor crystal diffraction	addition of ATP at high conc.	2AX4
Regulator of G protein signaling, RGS16	solubility limited, insufficient for crystallization	higher pH	2BT2
Serine/Threonine kinase 16	poor crystal diffraction	Staurosporine	2BUJ
Casein kinase I γ2	poor crystal diffraction	5-Iodotubercidin	2C47
p21(CDKN1A) activated kinase 4	poor crystal diffraction	Cdk1 Inhibitor (CGP74514A)	2CDZ
Casein kinase I γ3	poor crystal diffraction	Cdk1/2 Inhibitor III	2CHL
Never in mitosis kinase 2	poor crystal diffraction	SU11652	2CL1
MAP/ERK kinase 3 (kinase domain)	poor crystal diffraction	Staurosporine	2CLQ
Cytosolic 5'-nucleotidase	solubility limited, insufficient for crystallization	lower pH, lower NaCl conc.	2CN1
Regulator of G protein signaling, RGS6	solubility limited, insufficient for crystallization	lower pH	2ES0
p21(CDKN1A) activated kinase 5	poor crystal diffraction	Cdk1 Inhibitor (CGP74514A)	2F57
NAD-dependent deacetylase sirtuin 5	no crystals formed	addition of suramine	2FZQ

All proteins (until May-2006), for which structure determination was dependent on the presence of a ligand and/or a condition during expression, purification, concentration, or crystallization that was identified using thermal shift screening. Structures taken from the SGC website, http://www.thesgc.com/SGC-WebPages/sgc-structures.php

M. Vedadi, F. Niesen, A. Allali-Hassani, O. Fedorov, P. Finerty jr., G. Wasney, R. Yeung, C. Arrowsmith, L. Ball, H. Berglund, R. Hui, B. Marsden, P. Nordlund, M. Sundstrom, J. Weigelt & A. Edwards. *Proc Natl Acad Sci USA* 103(43),15835-15840 (2006)

FURTHER INFORMATION - LITERATURE

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- Poklar, N., Lah, J., Salobir, M., Maĉek, P., and Vesnaver, G. (1997) pH and temperature-induced molten globule-like denatured states of equinatoxin II: a study by UV-melting, DSC, far- and near-UV CD spectroscopy, and ANS fluorescence.

Useful Resources

ftp://ftp.sgc.ox.ac.uk/pub/biophysics/

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