

Calorimetric analysis of IgG fragments to define thermal unfolding transitions and probe for domain interactions

Steve Raso & Cliff Entrican

Current Trends in Microcalorimetry

Boston, MA

18 - 21 July 2007

Wyeth
BioPharma

Introduction

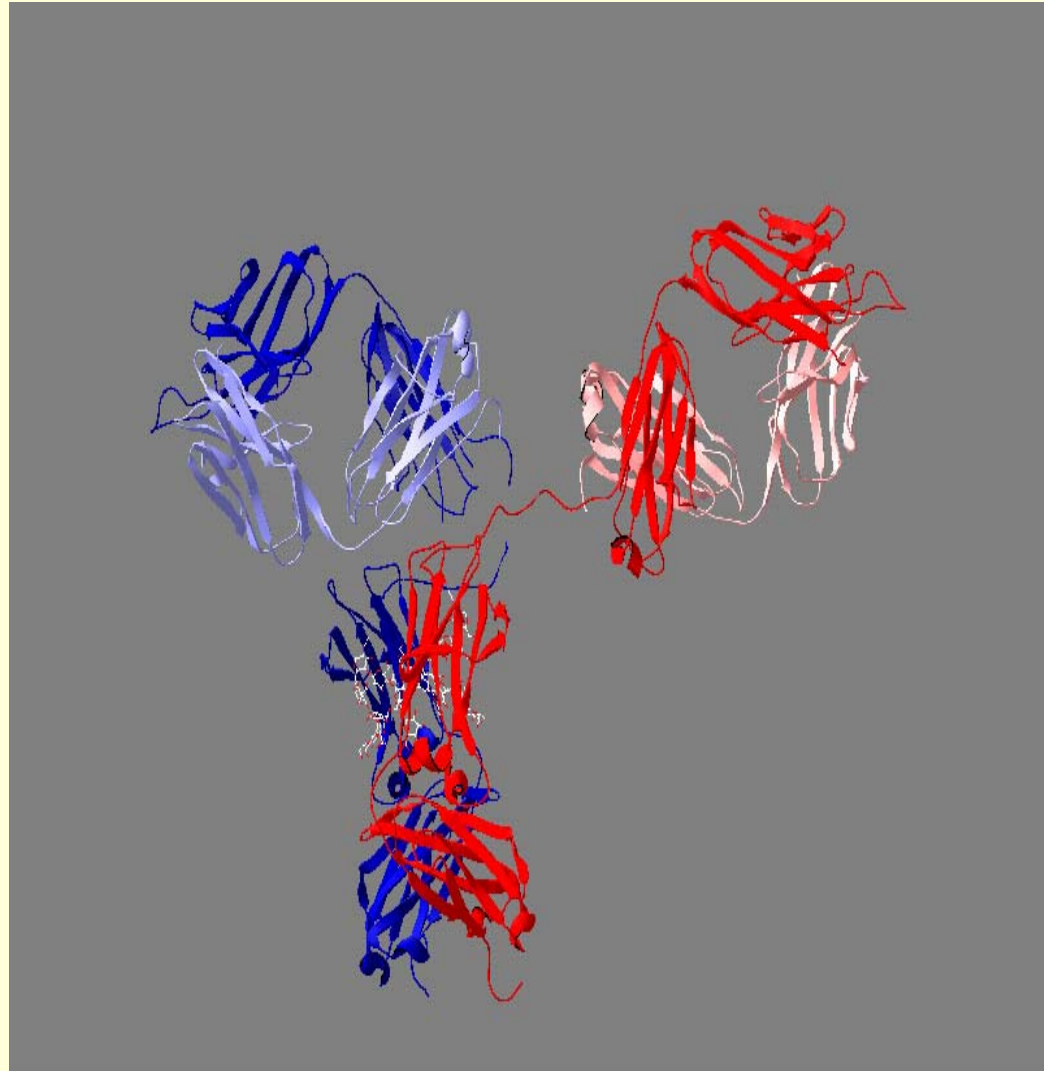
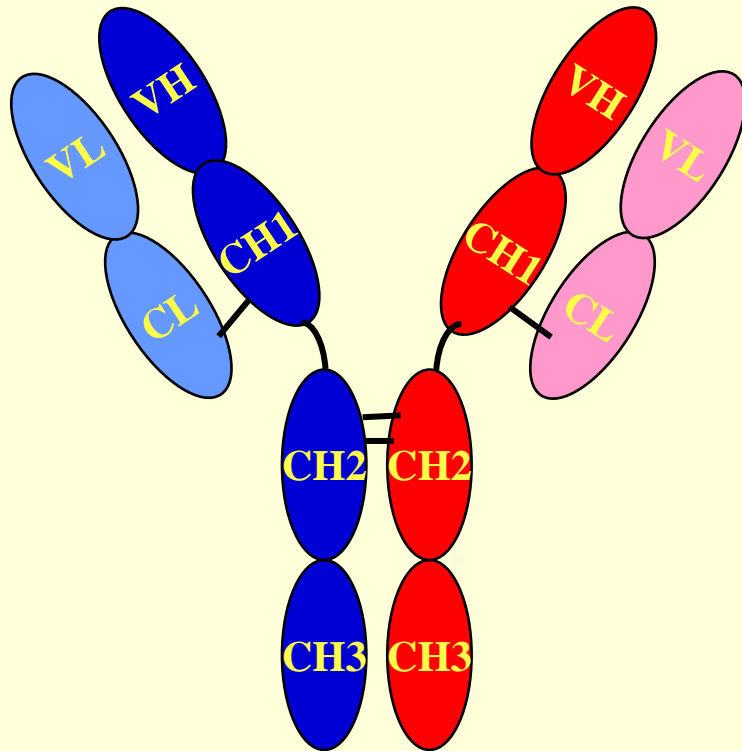
Recombinant antibodies exhibit striking differences in solution behavior, such as aggregation propensity and stability –even within the same subclass. *This disparity is unpredictable and poorly understood.*

DSC analysis of individual antibody domains was performed in order to determine if:

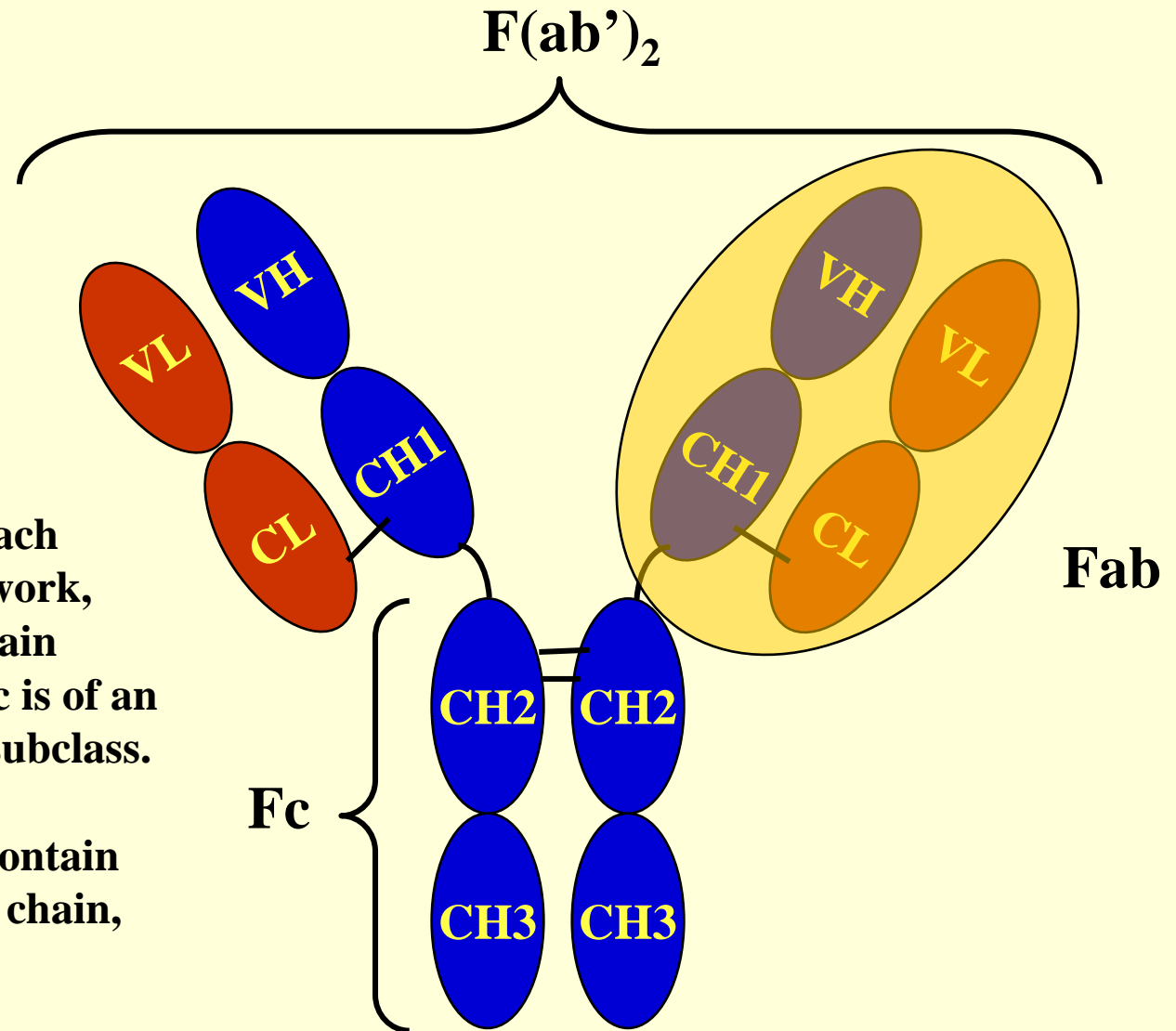
- **Molecule-to-molecule differences can be attributed to variable regions only**
- **Domain-domain interactions within the intact molecule have a *significant* effect on solution behavior**
- **General predictions can be made about antibody, or Fc-fusion protein, solution behavior**

Antibody Domain Structure (IgG1)

Intact IgG comprises 2 Heavy Chains & 2 Light Chains. There are a total of six different domains



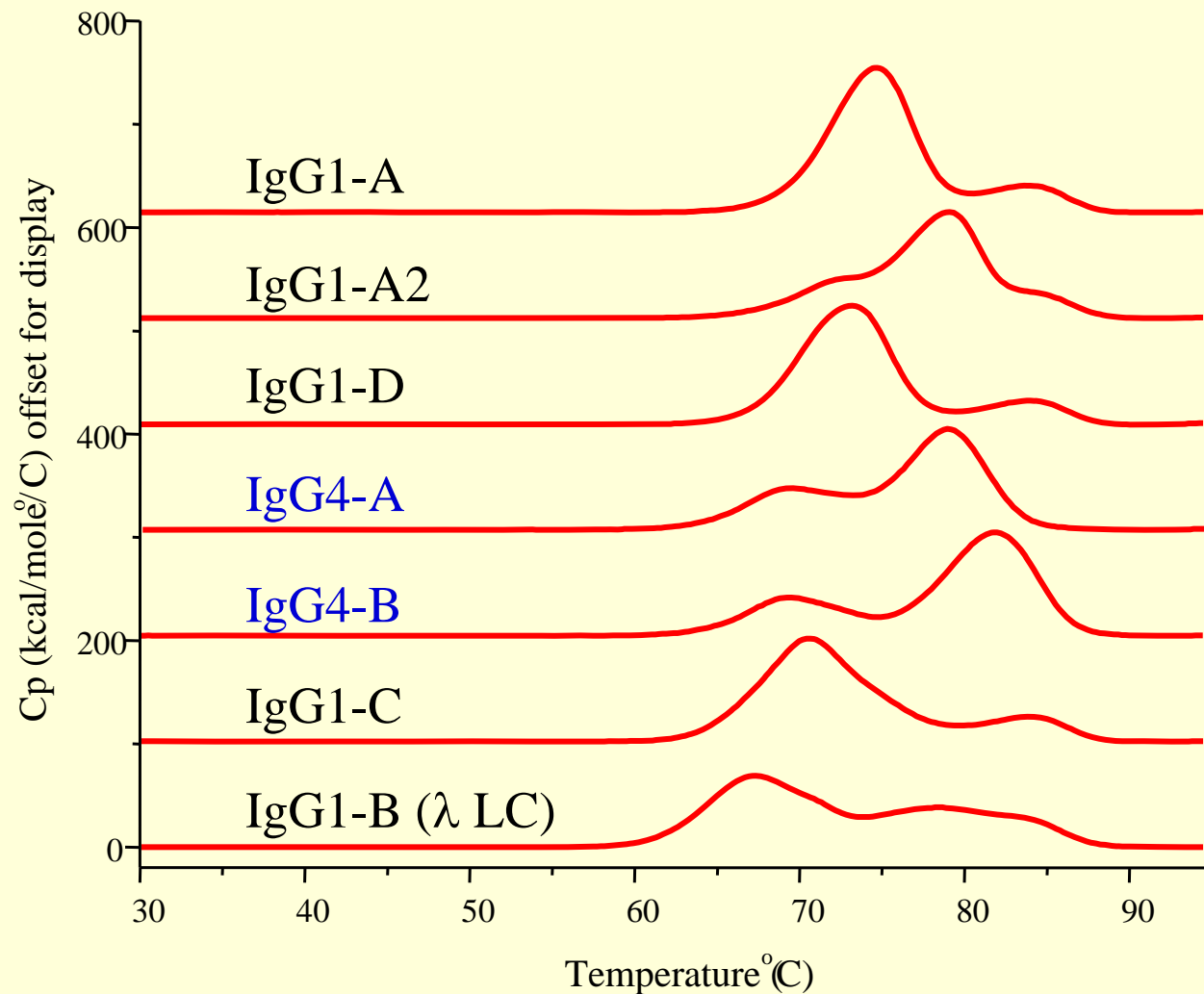
Antibody Domain Structure (IgG1)



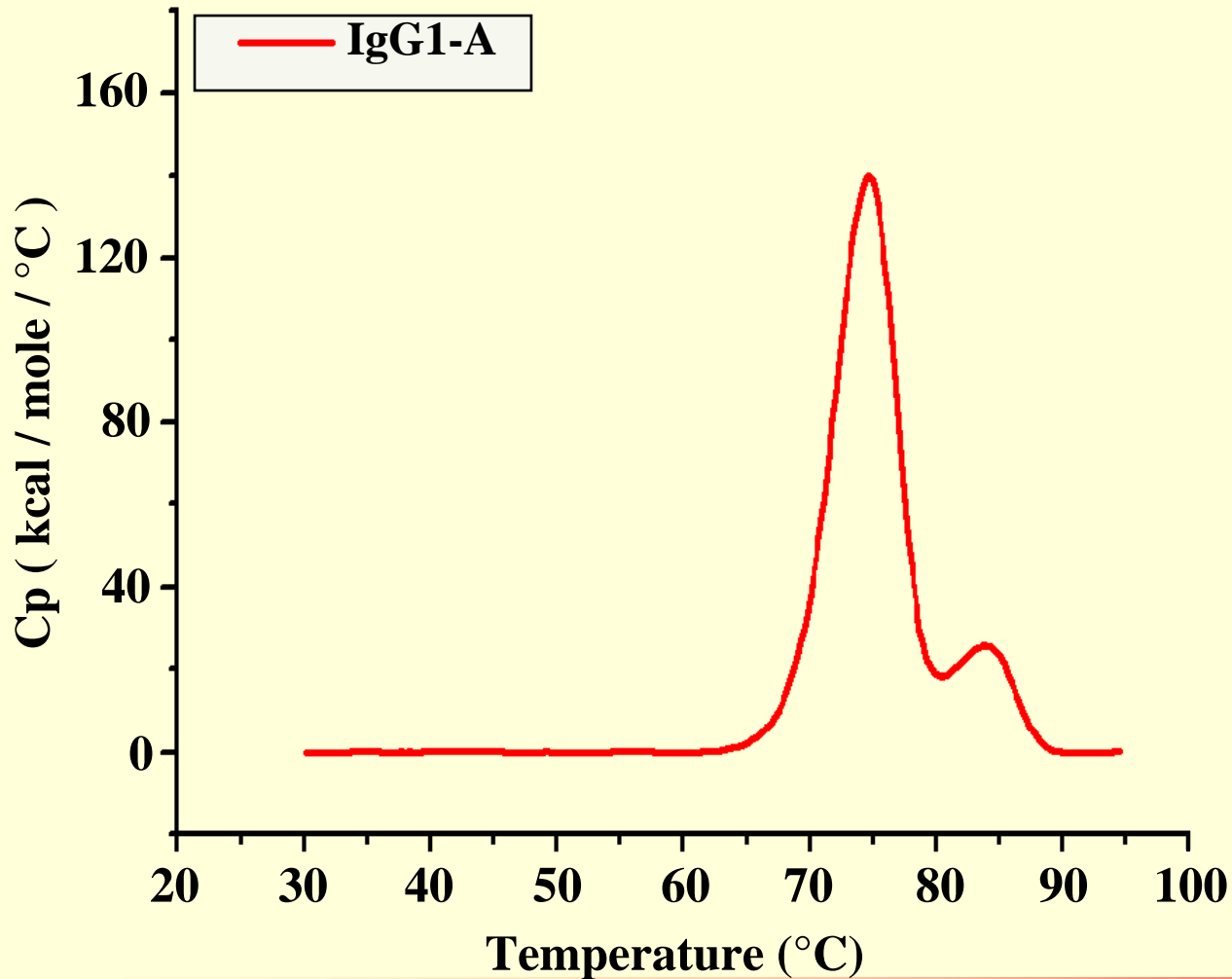
Four subclasses of IgG, each having a different framework, but the same overall domain structure. This schematic is of an IgG1, the most common subclass.

IgG molecules may also contain two distinct types of light chain, designated κ or λ .

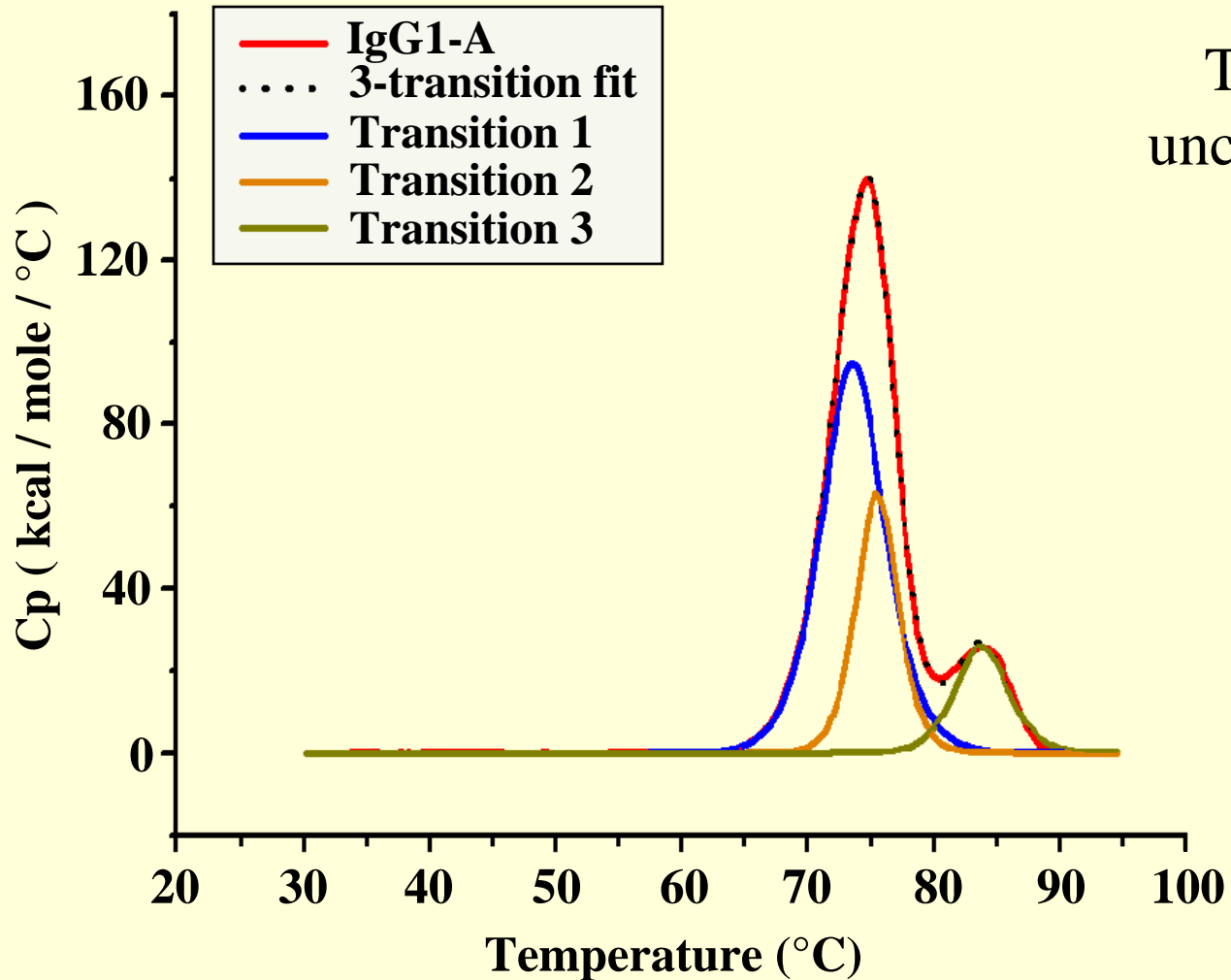
Representative Thermograms for Intact Antibodies in PBS



Thermogram of IgG1-A in PBS

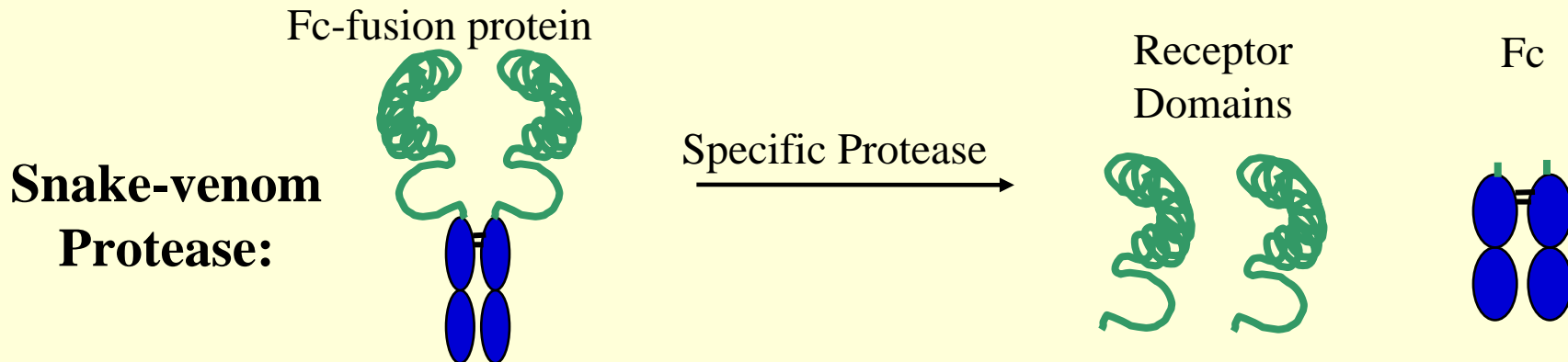
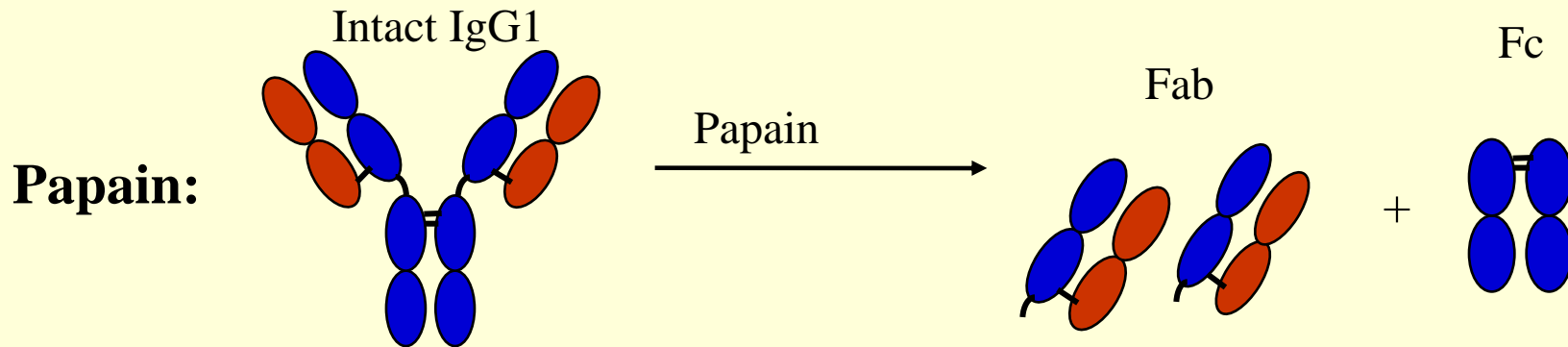
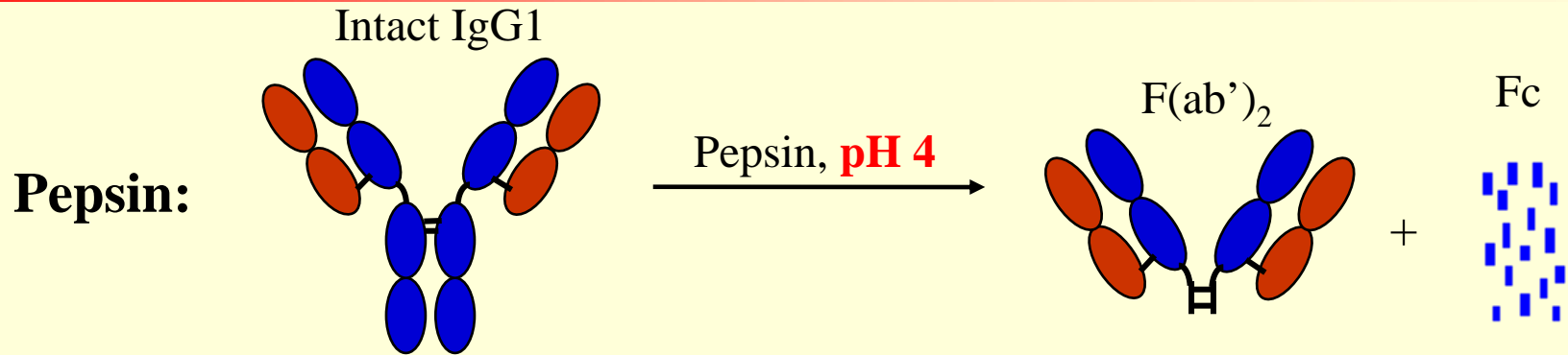


Curve Fit of IgG1-A Thermogram

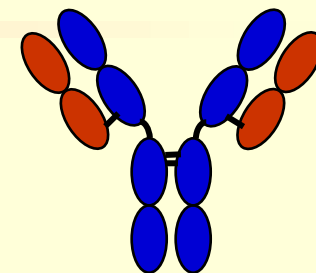
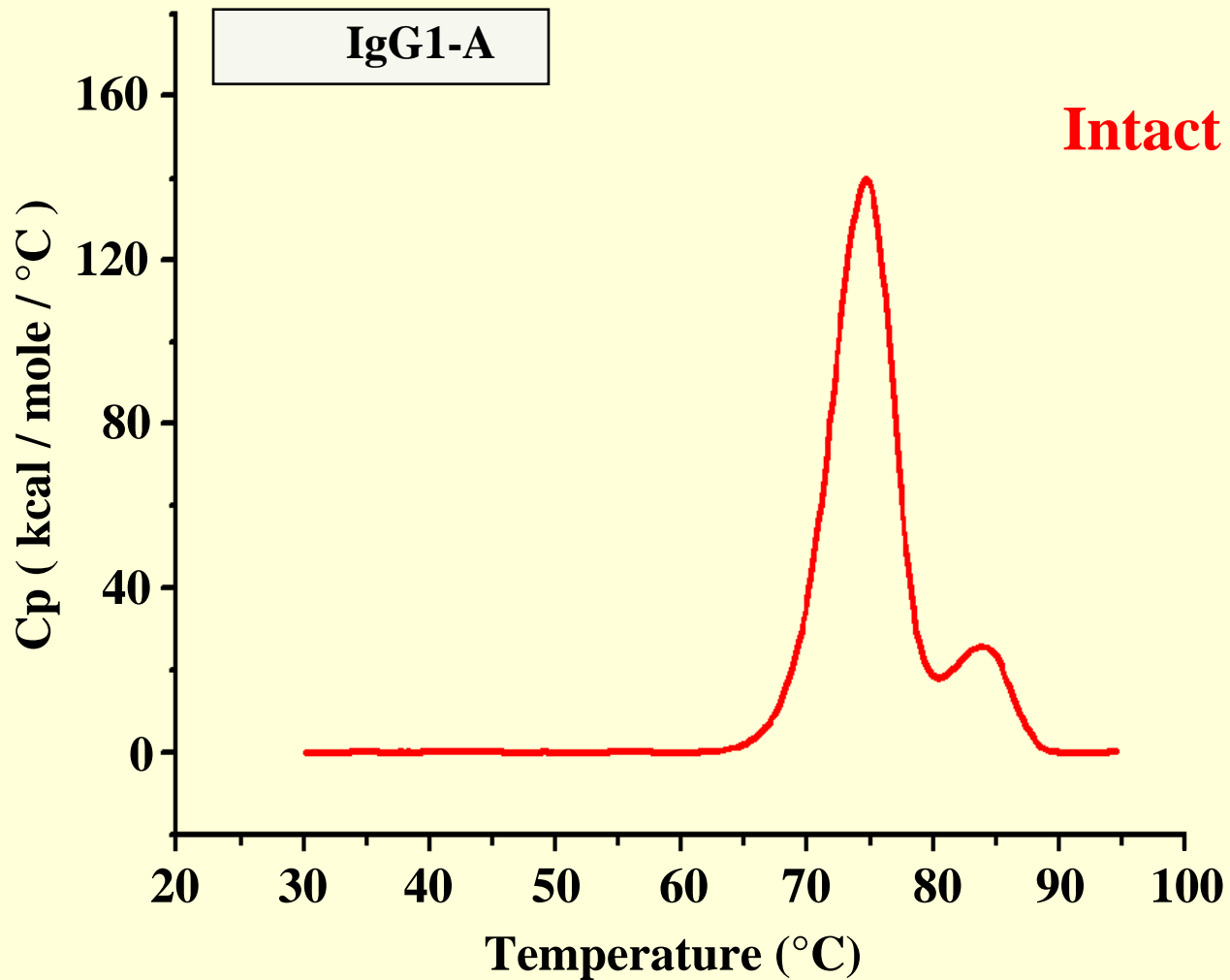


There is an inherent uncertainty in the curve-fitted transitions.

Enzymatic Fragmentation of mAbs and Fc-fusion Proteins

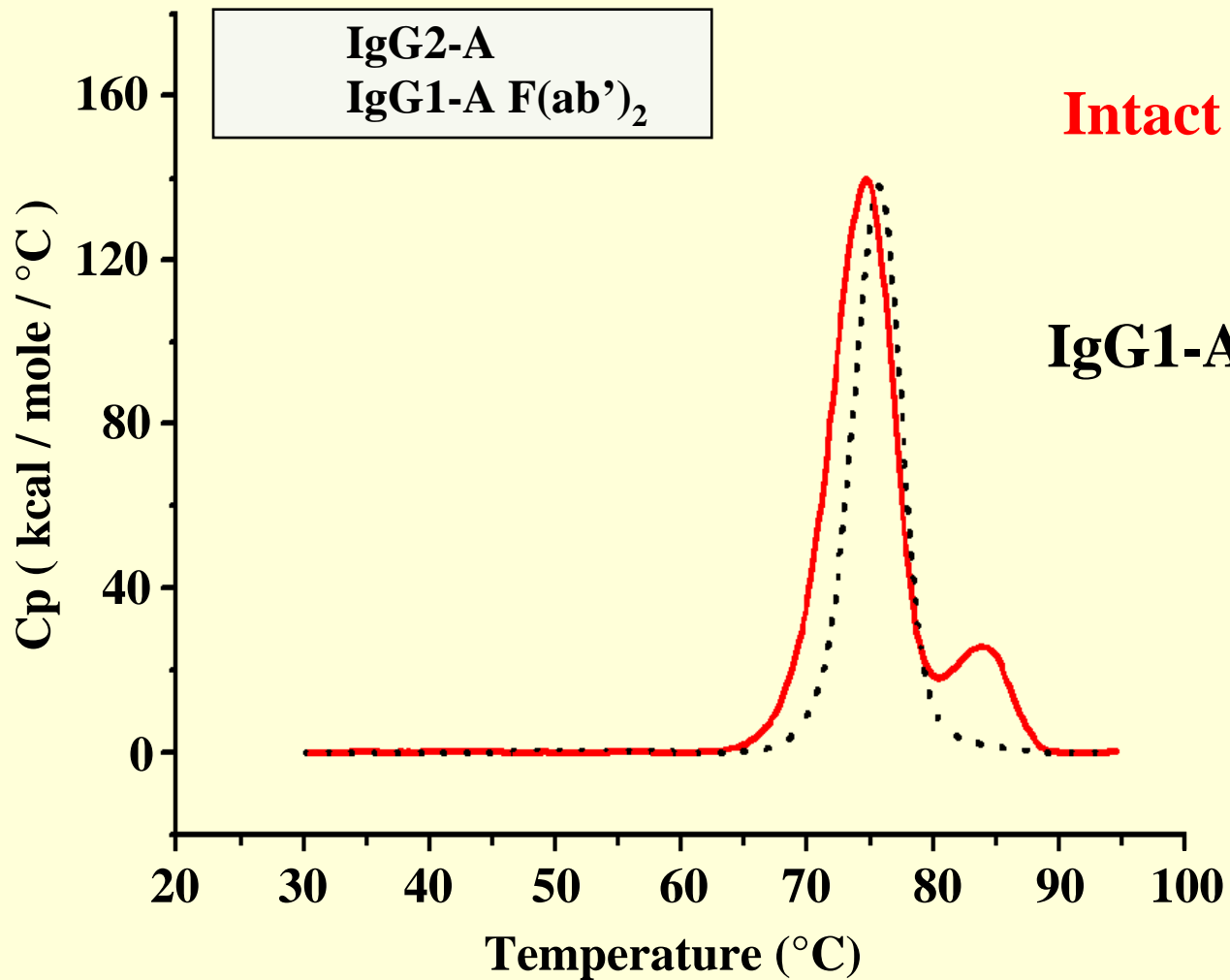


Intact IgG1-A

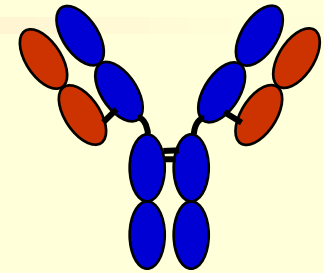


Intact IgG1-A

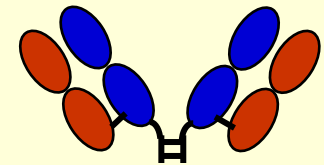
Intact IgG1-A and F(ab')₂ from Pepsin Digest



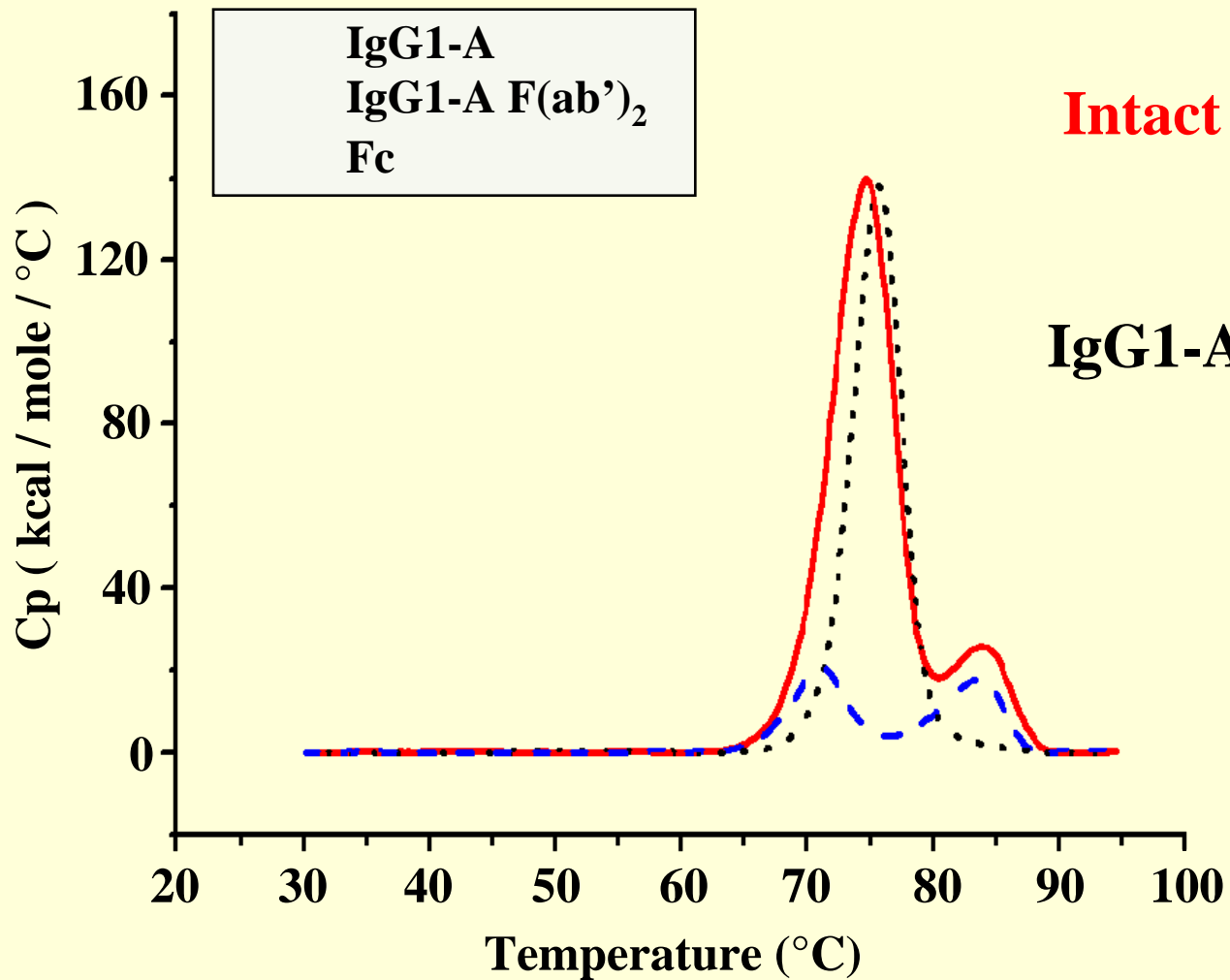
Intact IgG1-A



IgG1-A F(ab')₂



Intact IgG1-A, F(ab')₂, and Fc

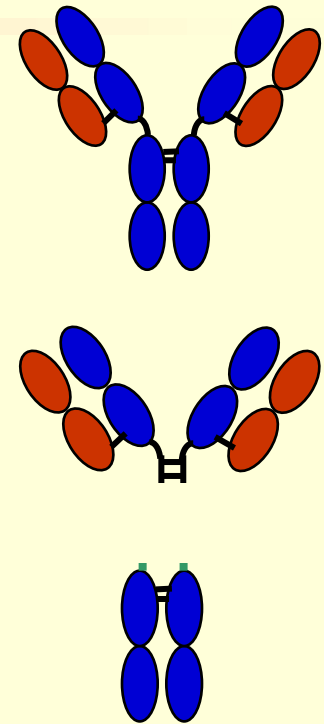


Intact IgG1-A

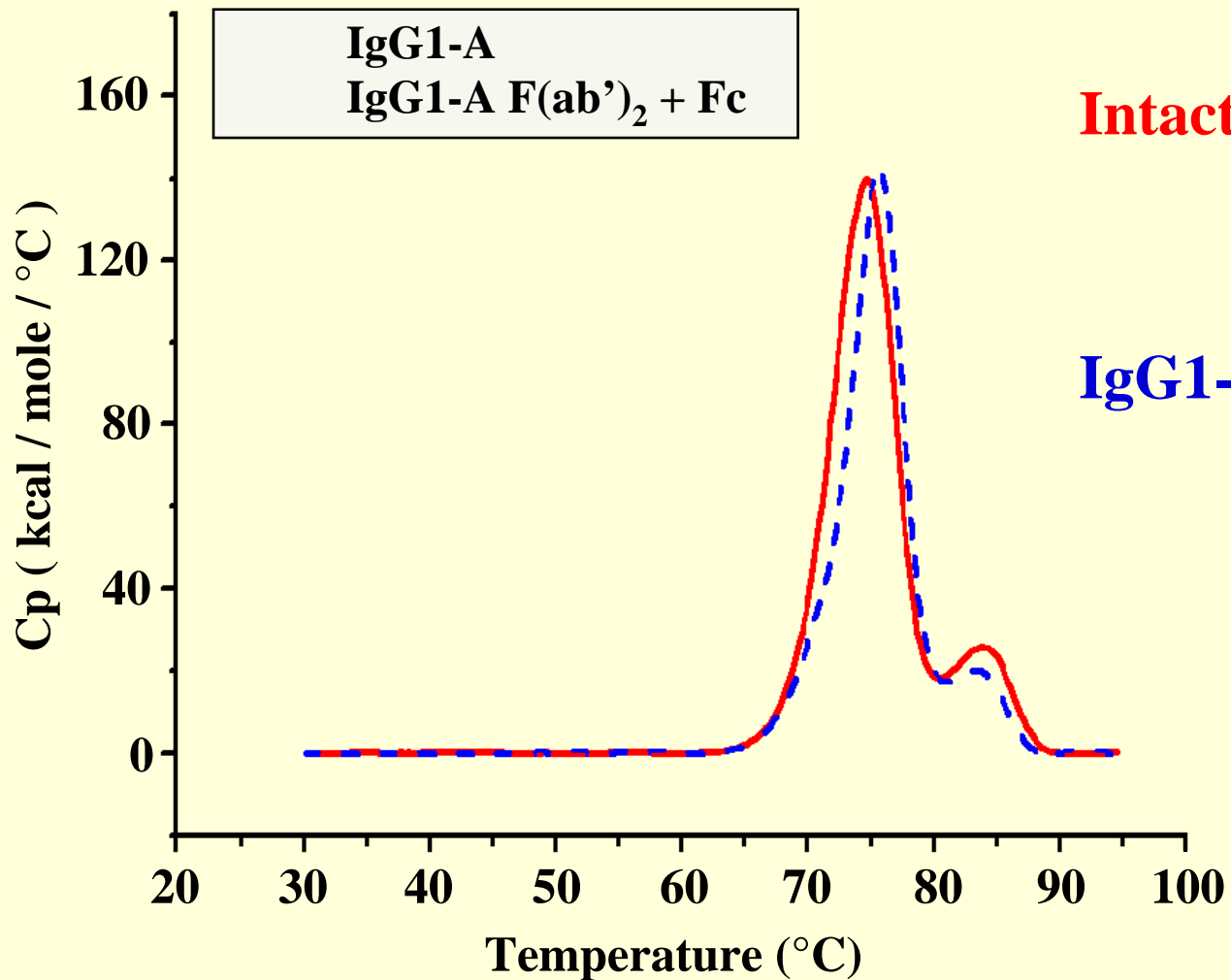
IgG1-A F(ab')₂

Fc

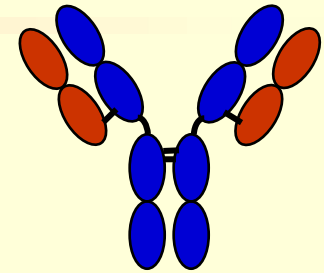
Additive?



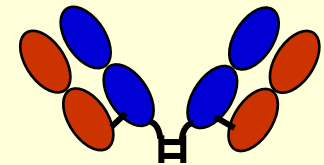
Intact IgG1-A Compared with Added Thermograms of F(ab')₂ and Fc



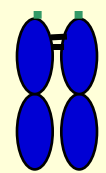
Intact IgG1-A



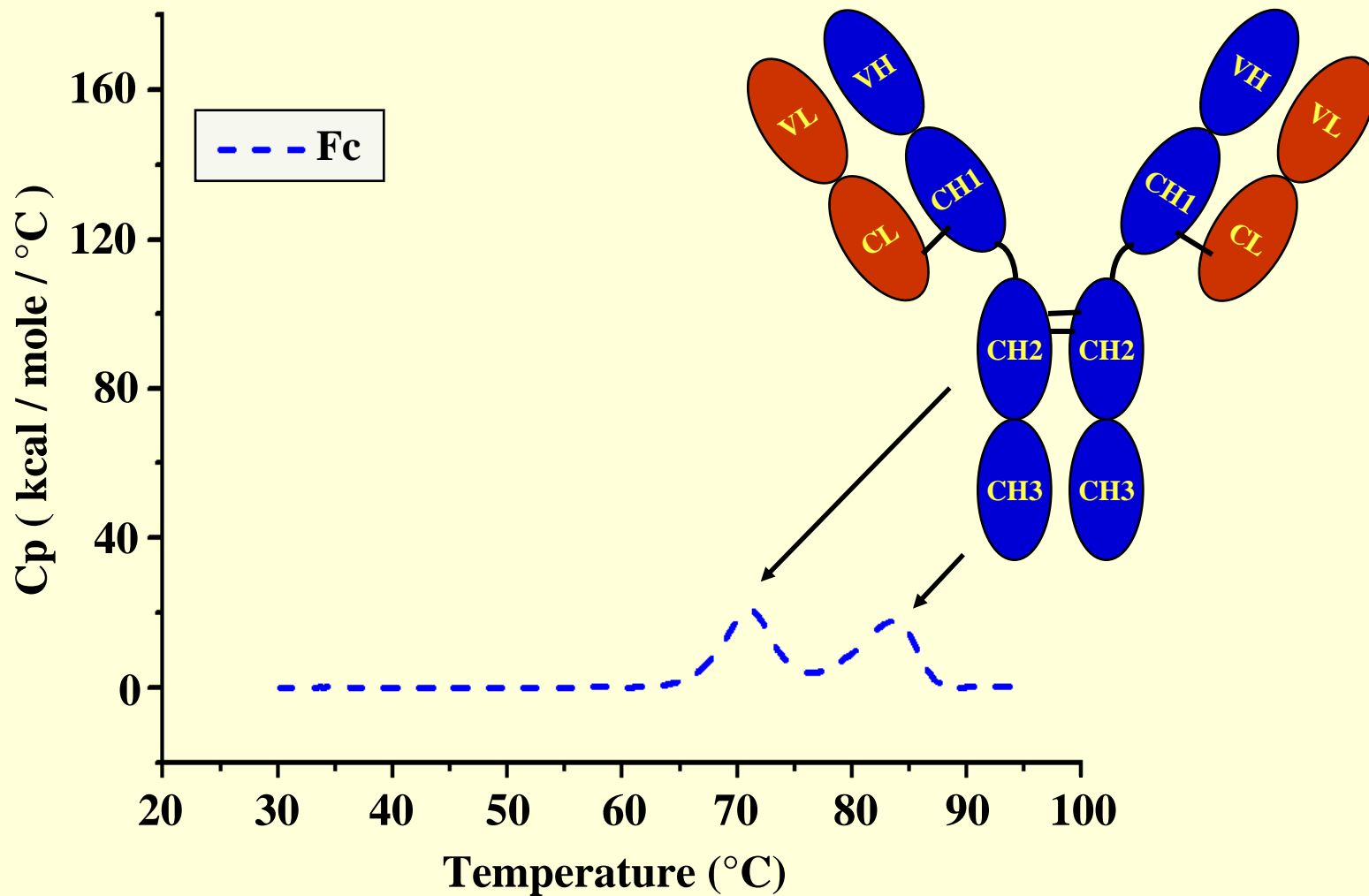
IgG1-A F(ab')₂



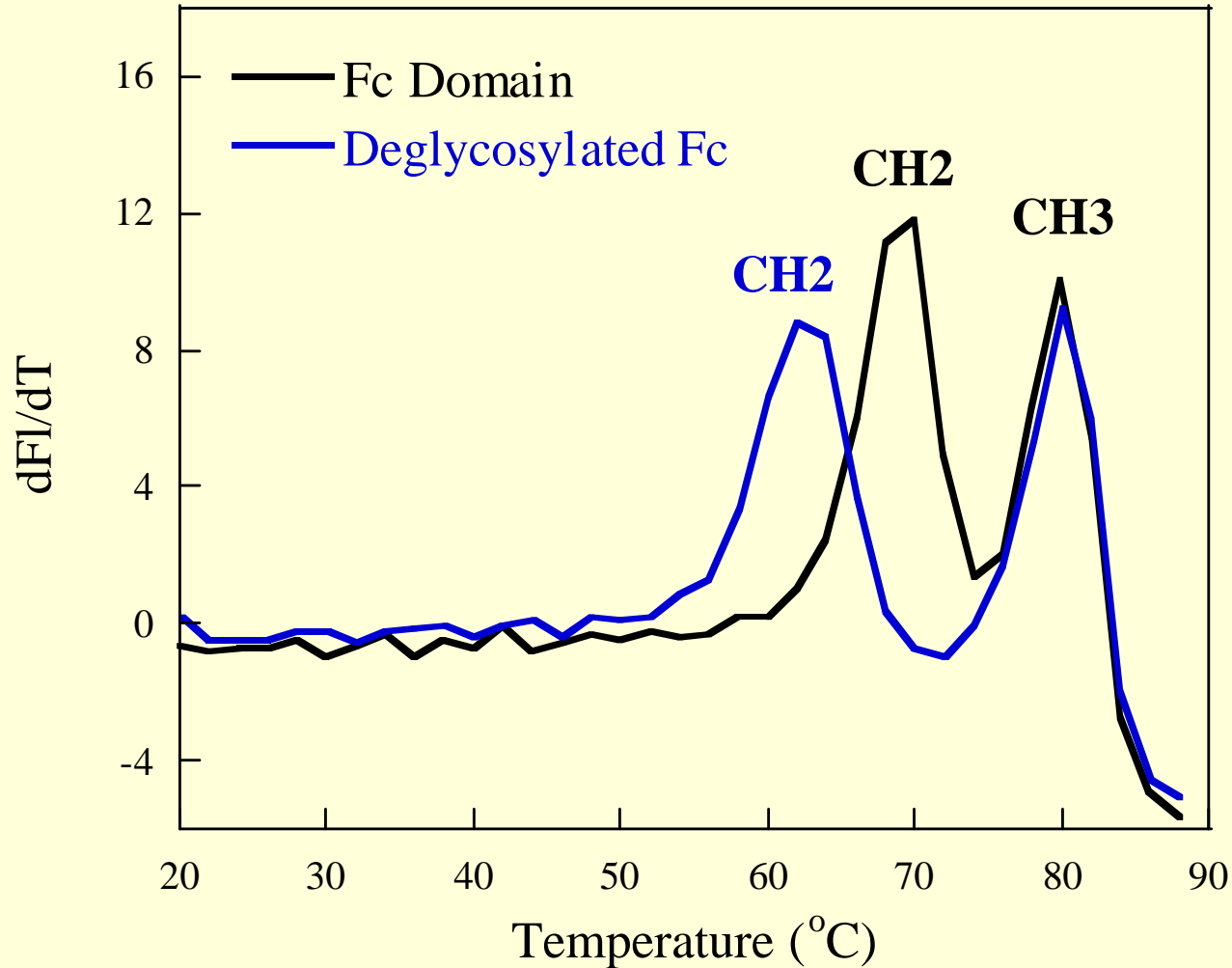
**+
Fc**



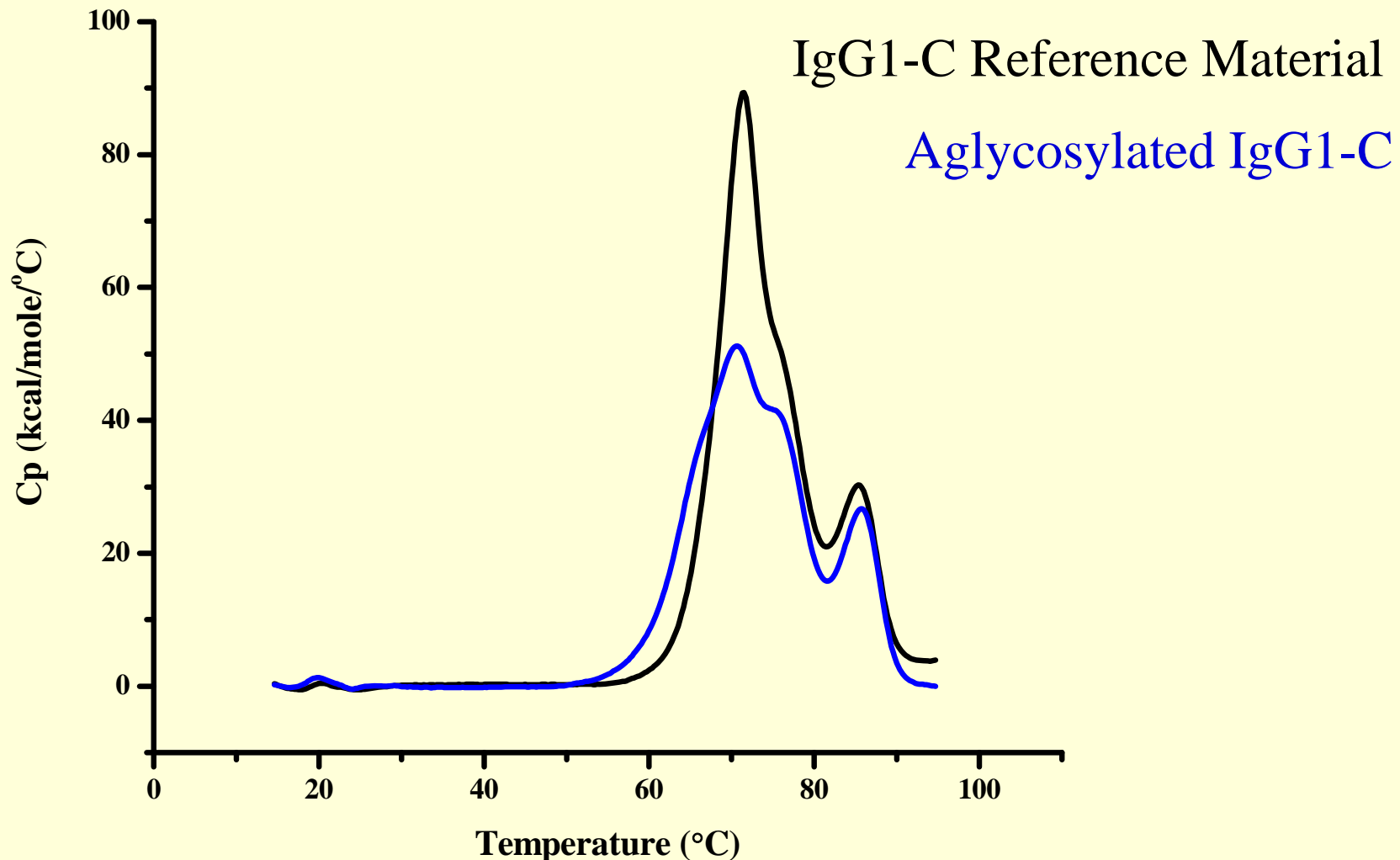
Fc Thermogram assignments



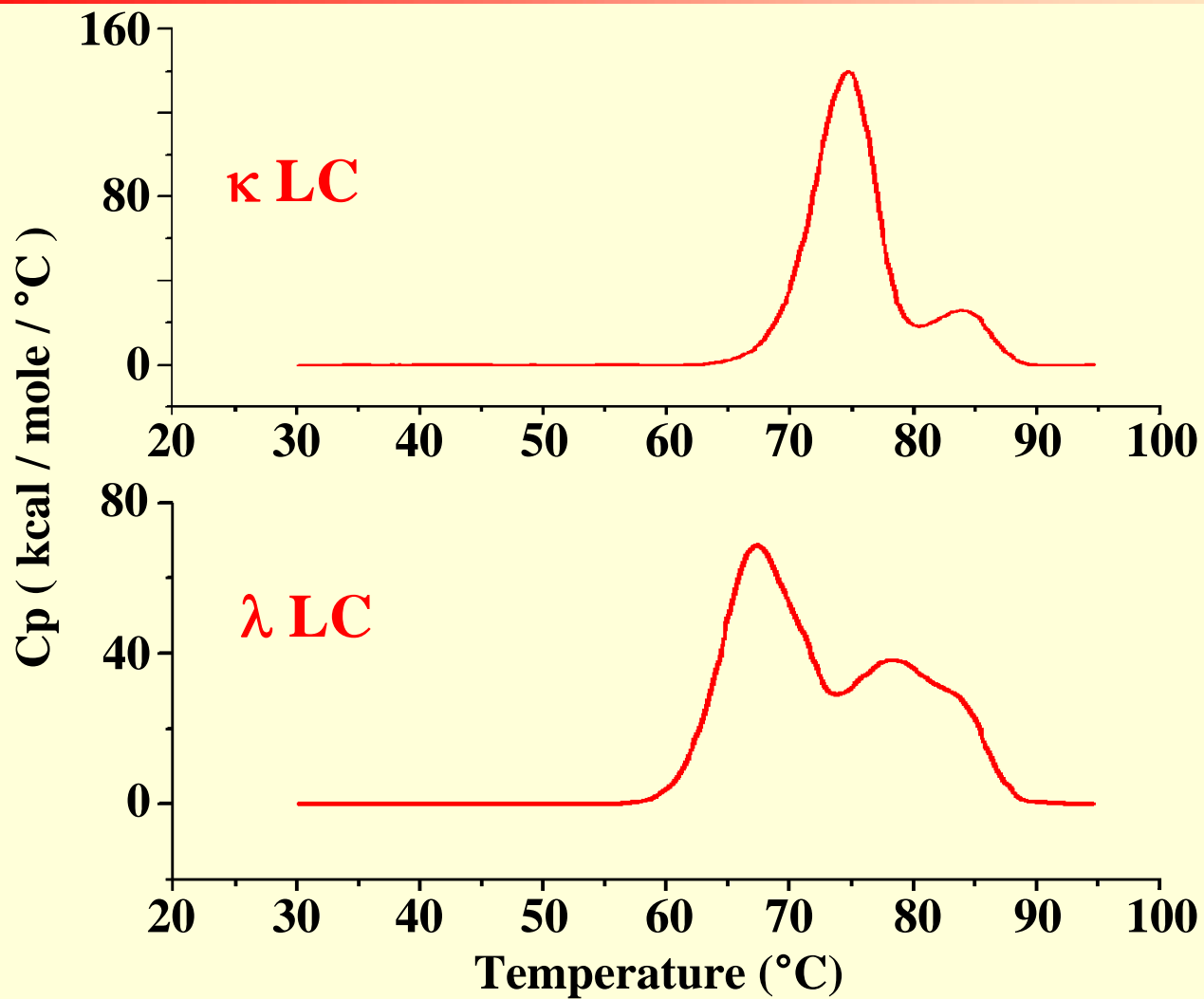
Thermal Denaturation of Deglycosylated Fc Domain Monitored by Tryptophan Fluorescence



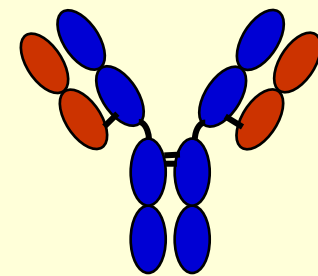
Thermogram of IgG1-C in Formulation Buffer Compared to the Aglycosylated Molecule



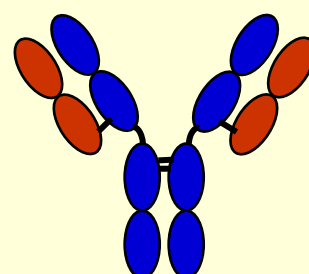
Intact IgG1-A and IgG1-B



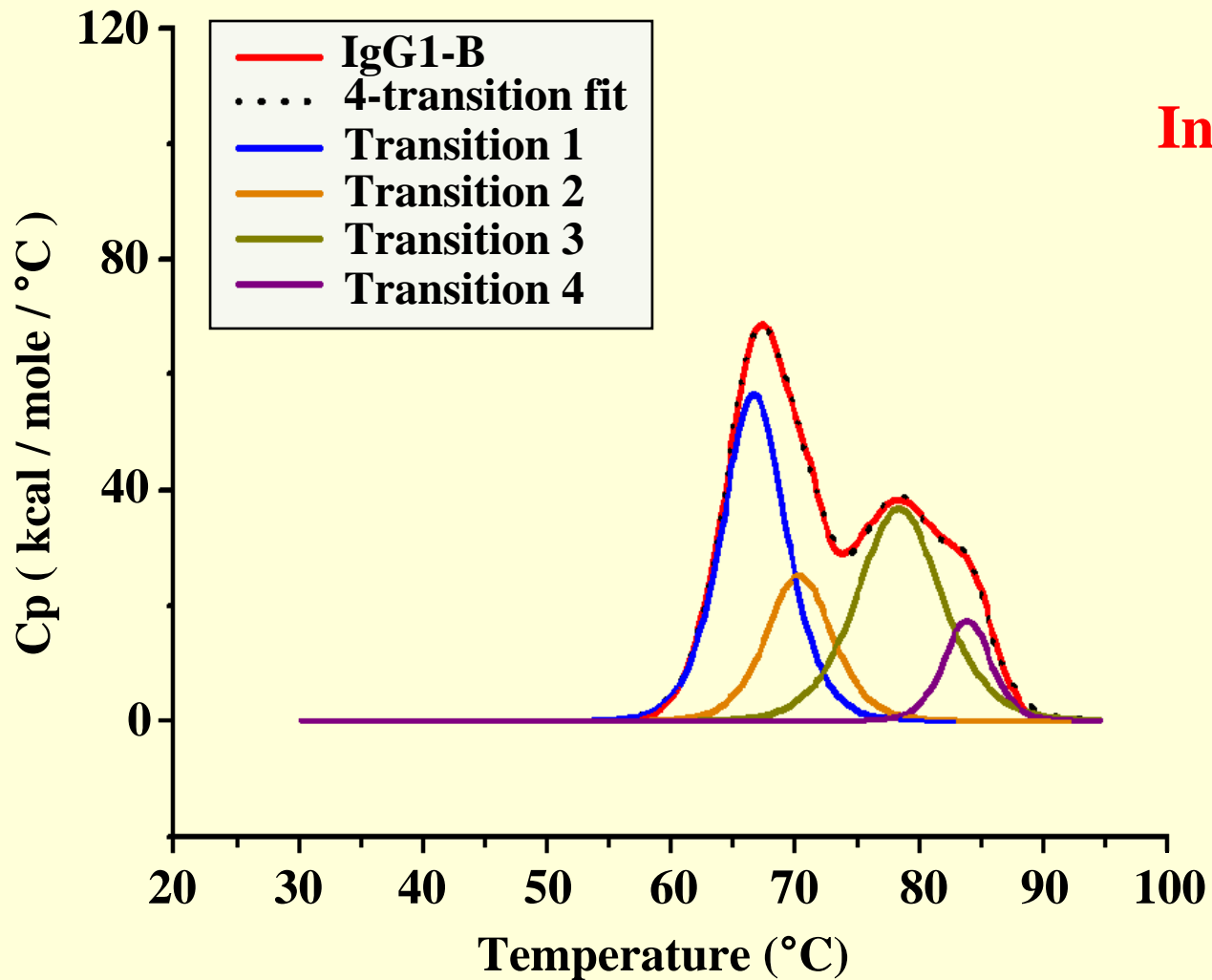
— IgG1-A



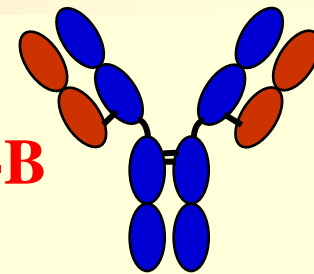
— IgG1-B



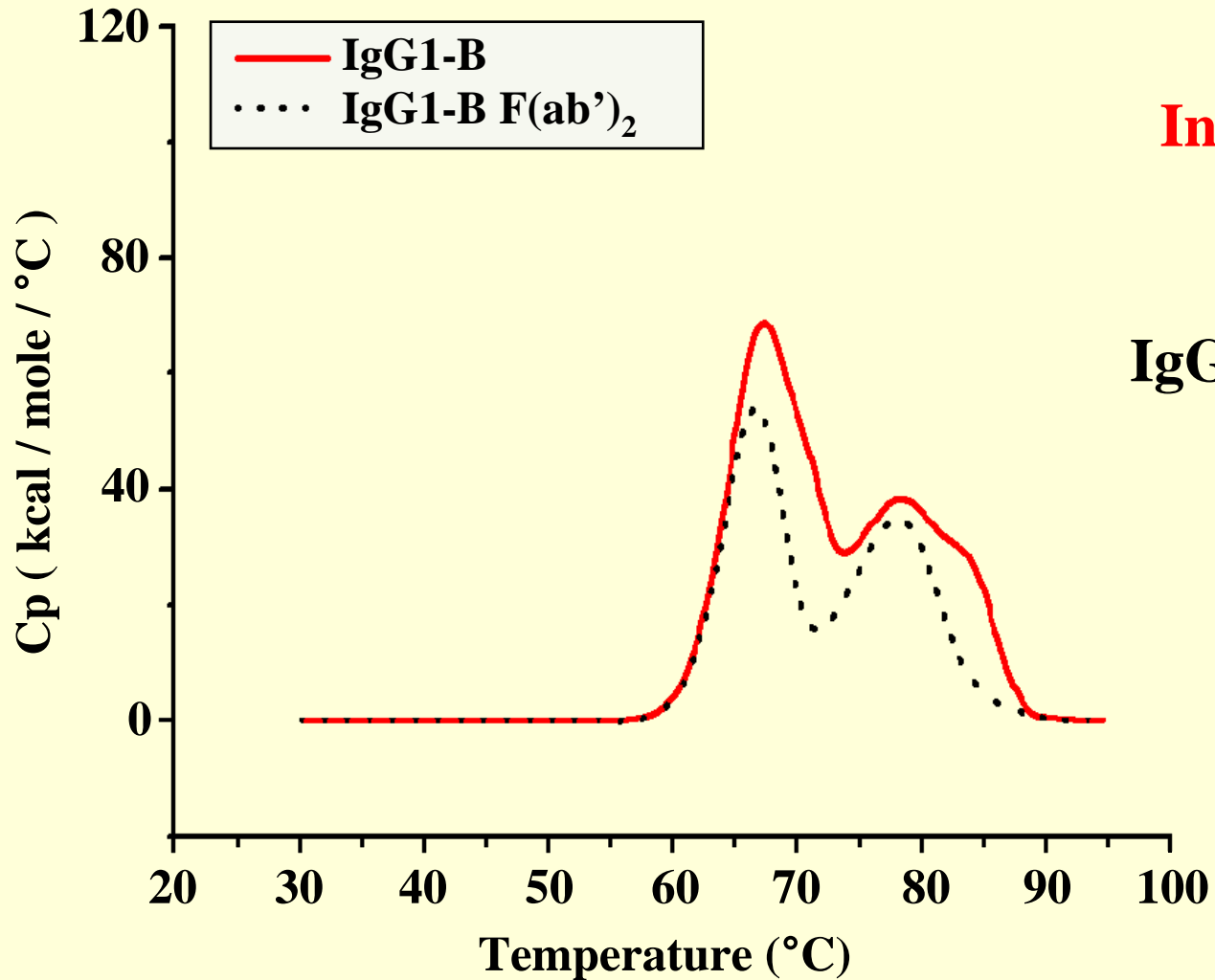
Curve Fit of IgG1-B Thermogram



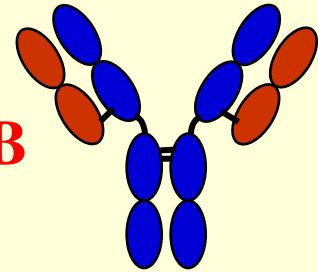
Intact IgG1-B



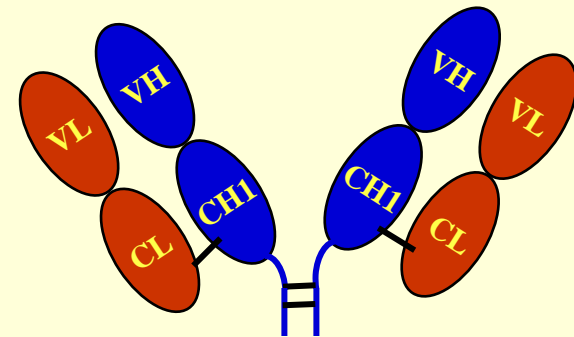
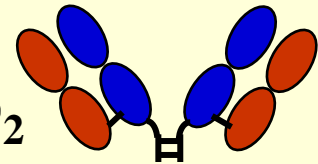
Intact IgG1-B and F(ab')₂



Intact IgG1-B

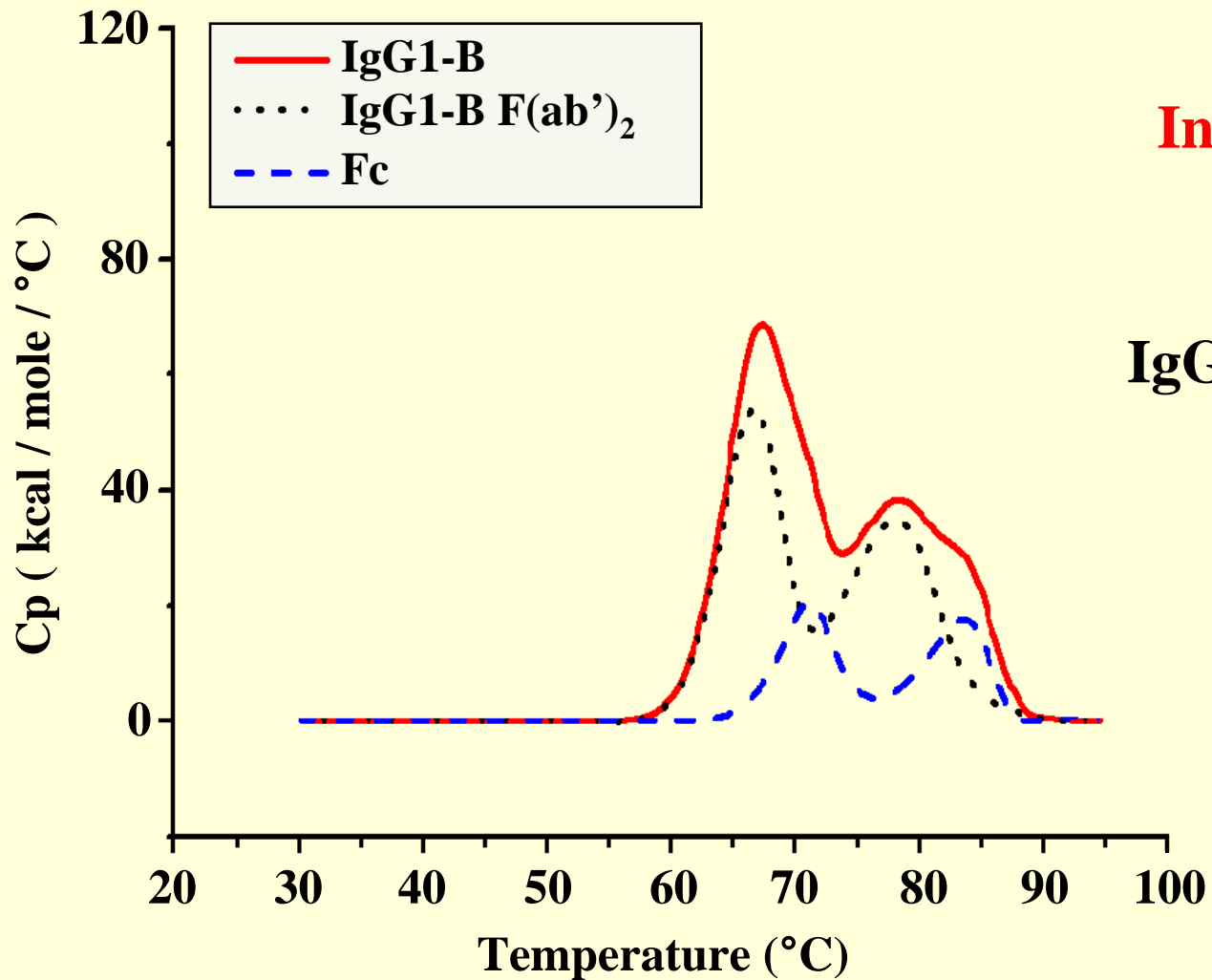


IgG1-B F(ab')₂

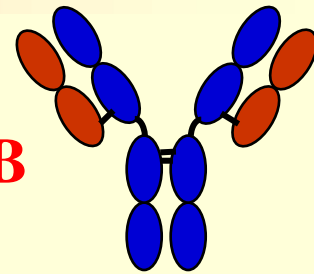


IgG1-B F(ab')₂ exhibits two very distinct domain stabilities.

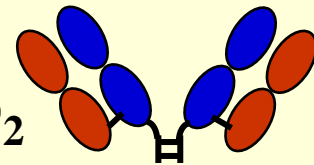
Intact IgG1-B, F(ab')₂, and Fc



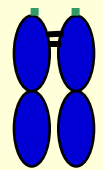
Intact IgG1-B



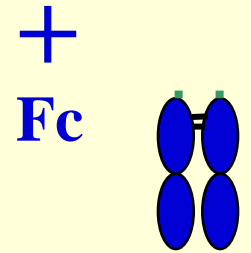
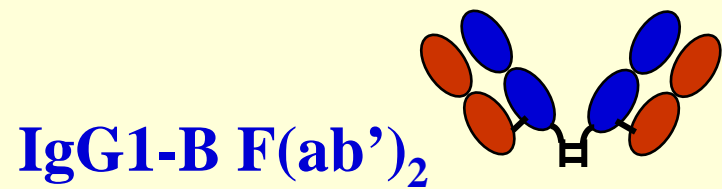
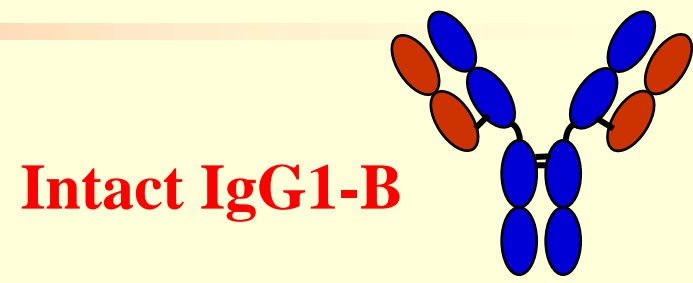
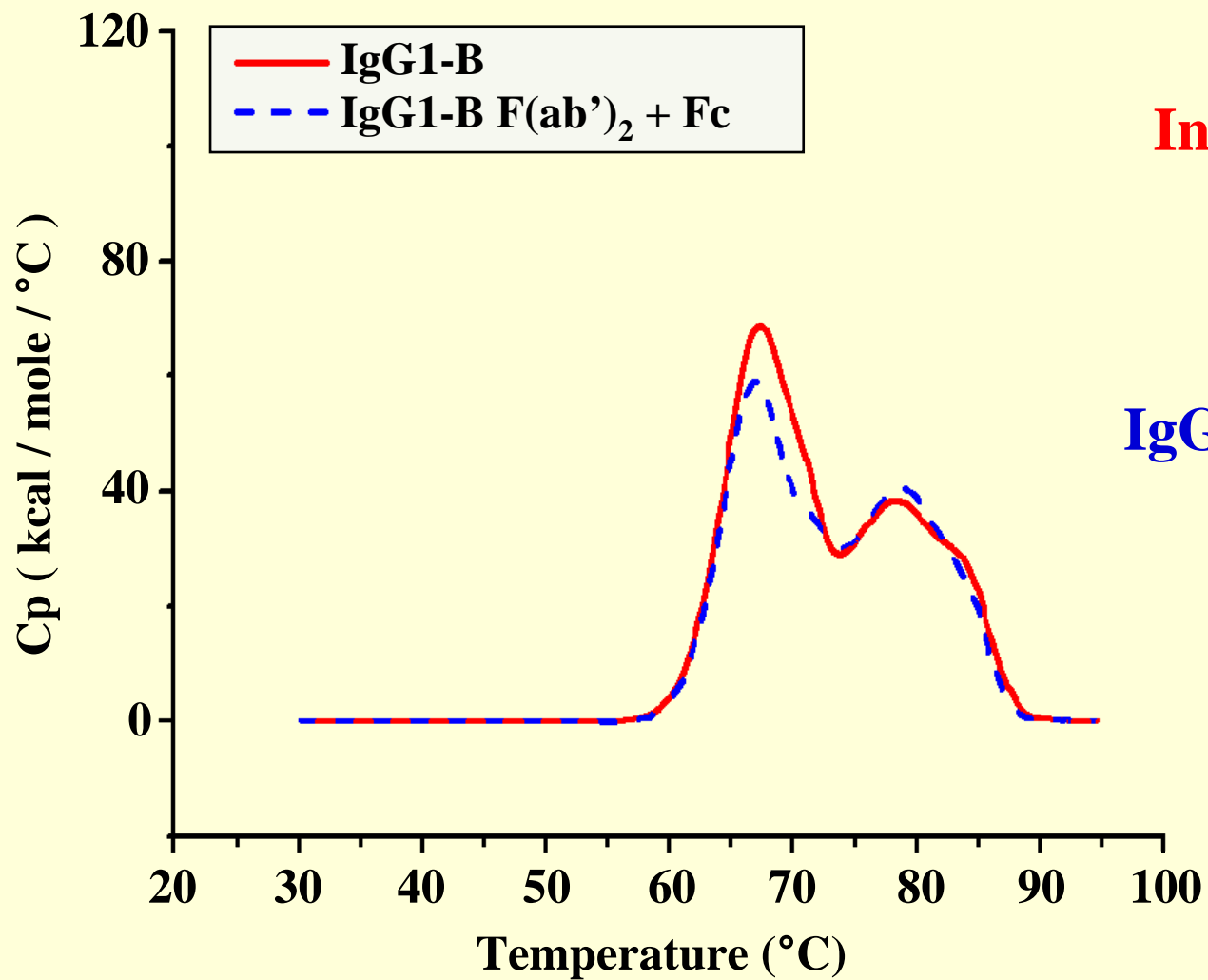
IgG1-B F(ab')₂



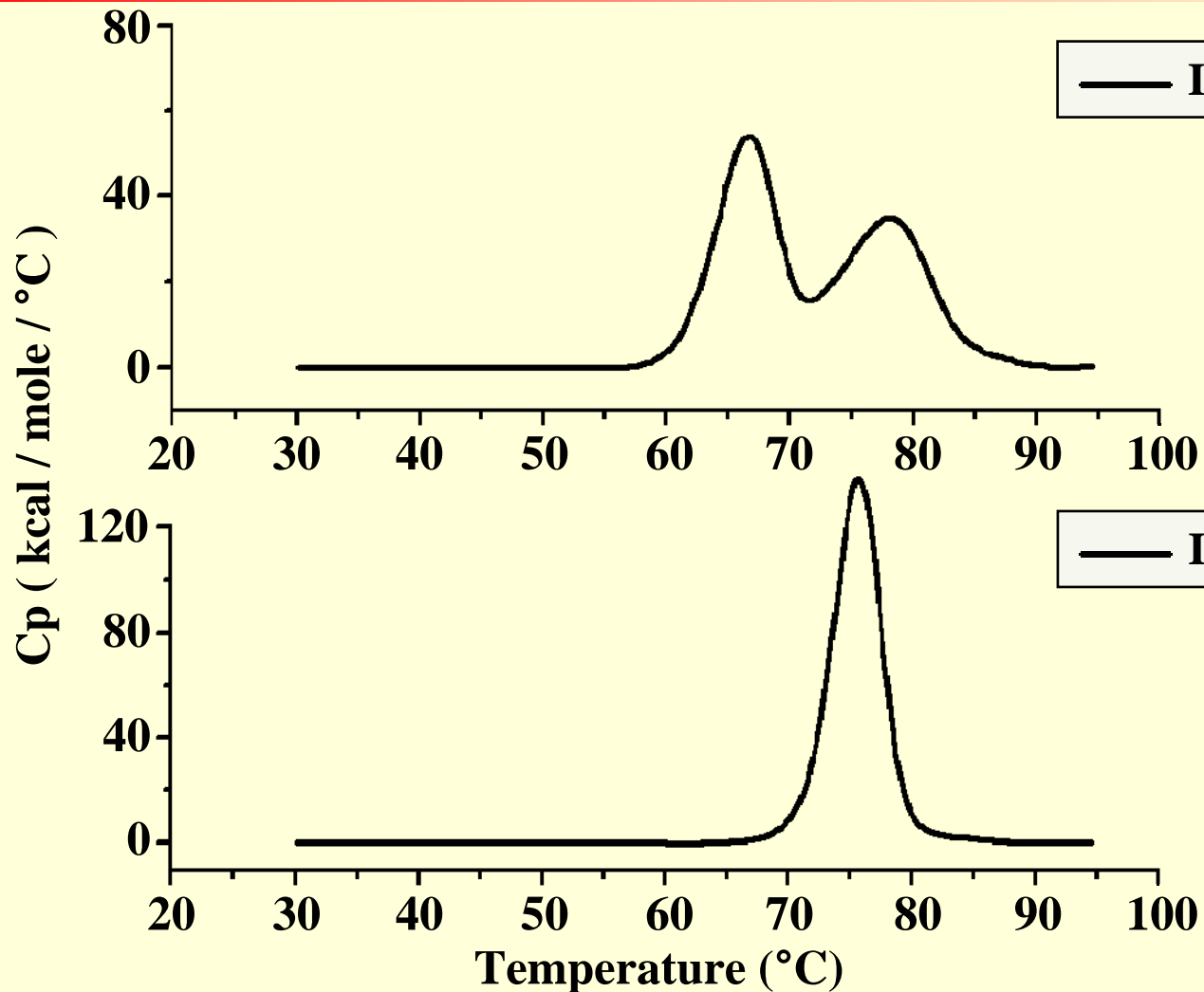
Fc



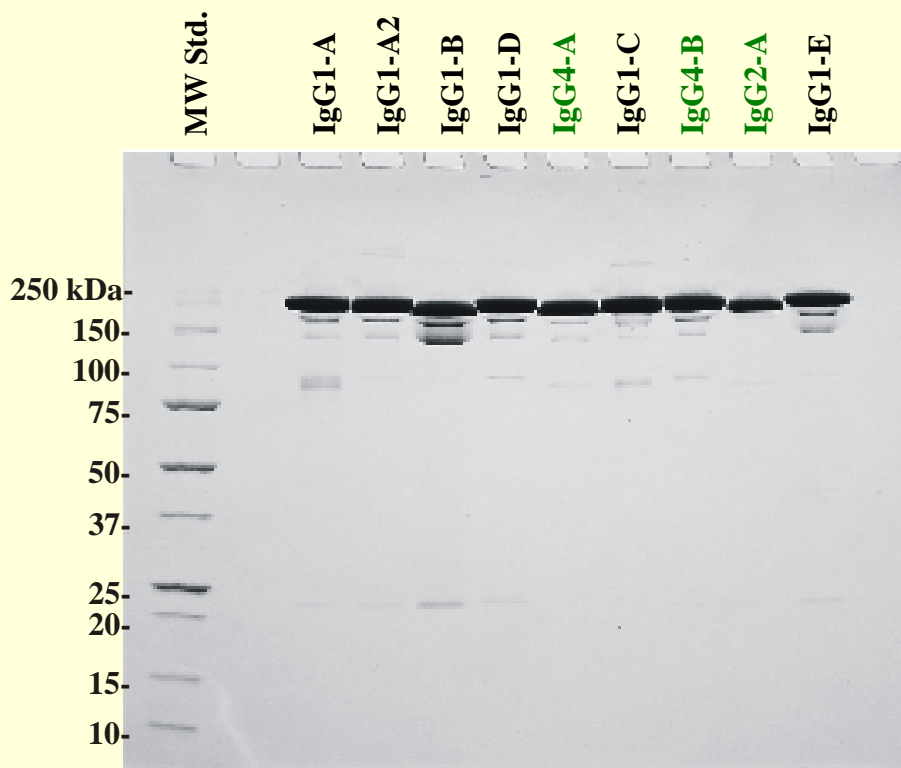
Intact IgG1-B Compared with Added Thermograms of F(ab')₂ and Fc



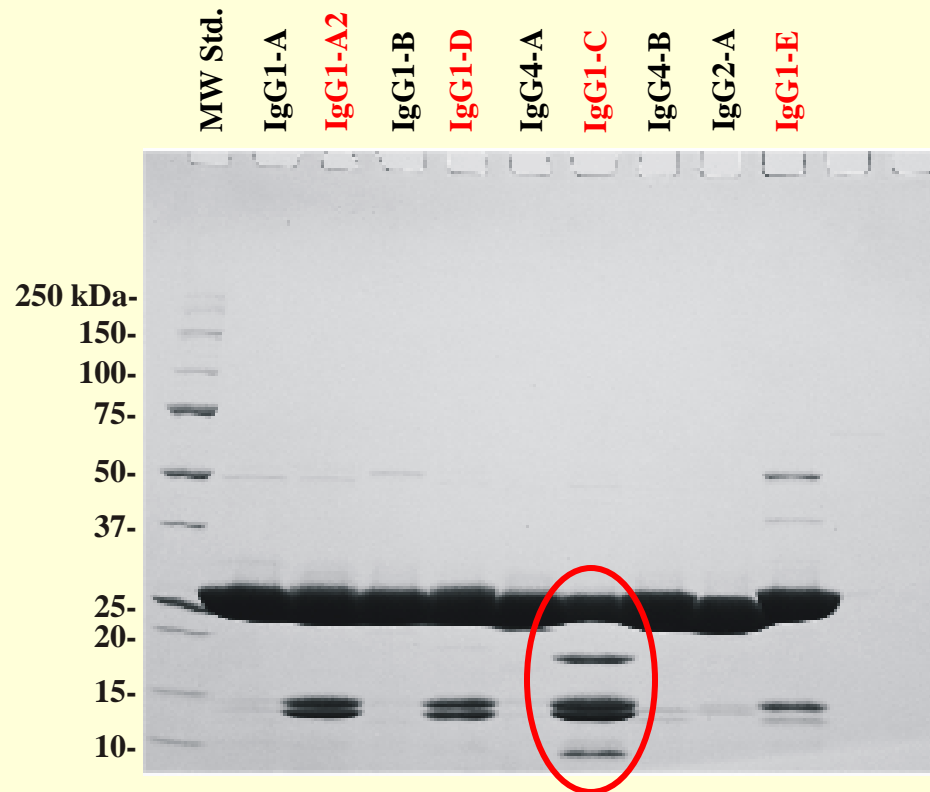
IgG1-A and IgG1-B F(ab')₂ Comparison



Unexpected Pepsin Cleavage within the Fab Domains of Some IgG1 Molecules

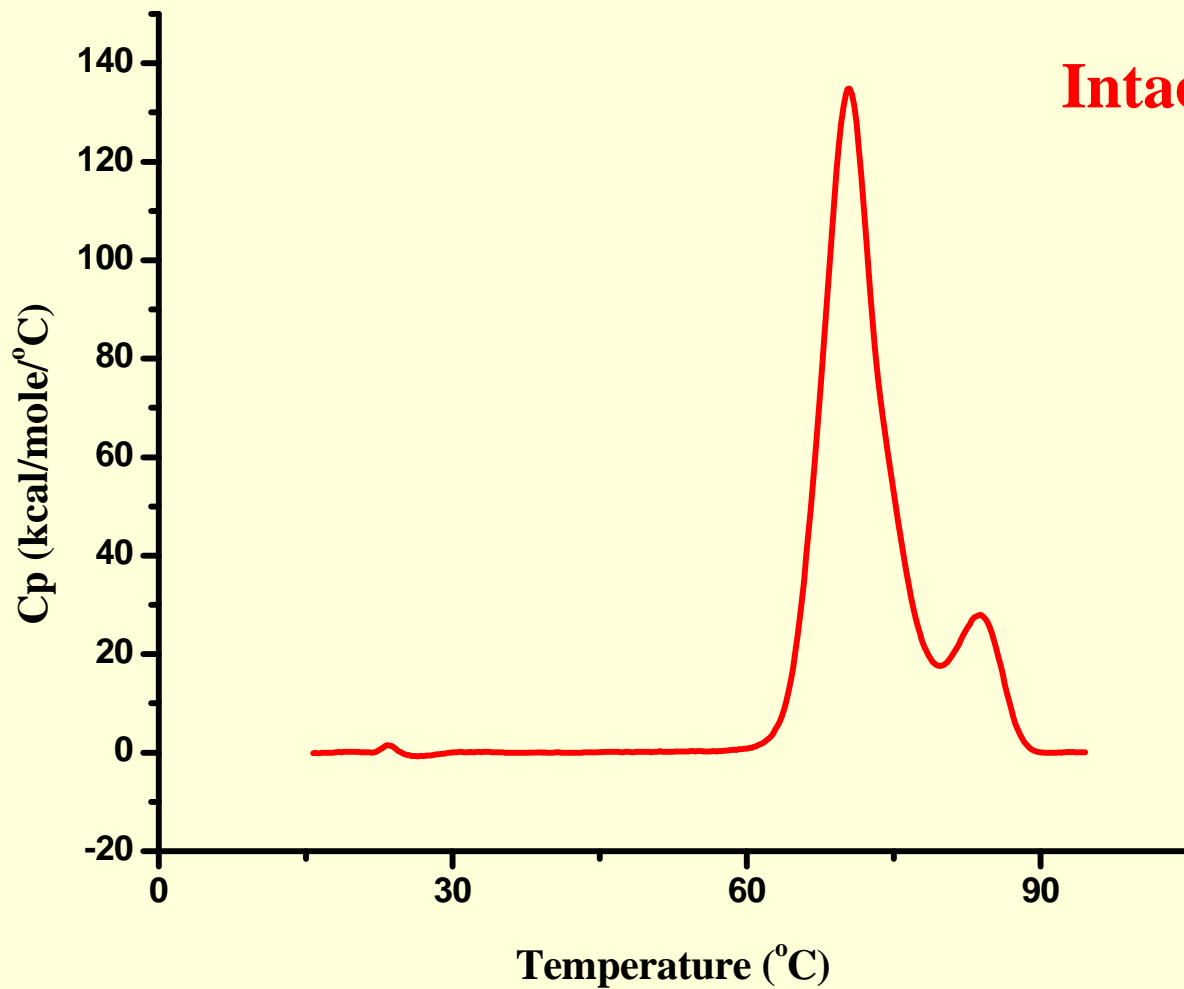


Intact mAbs, nonreduced

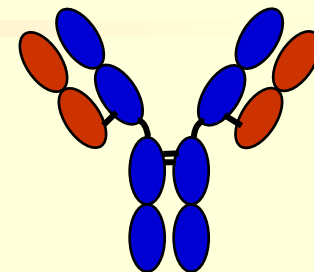


Pepsin digested, reduced

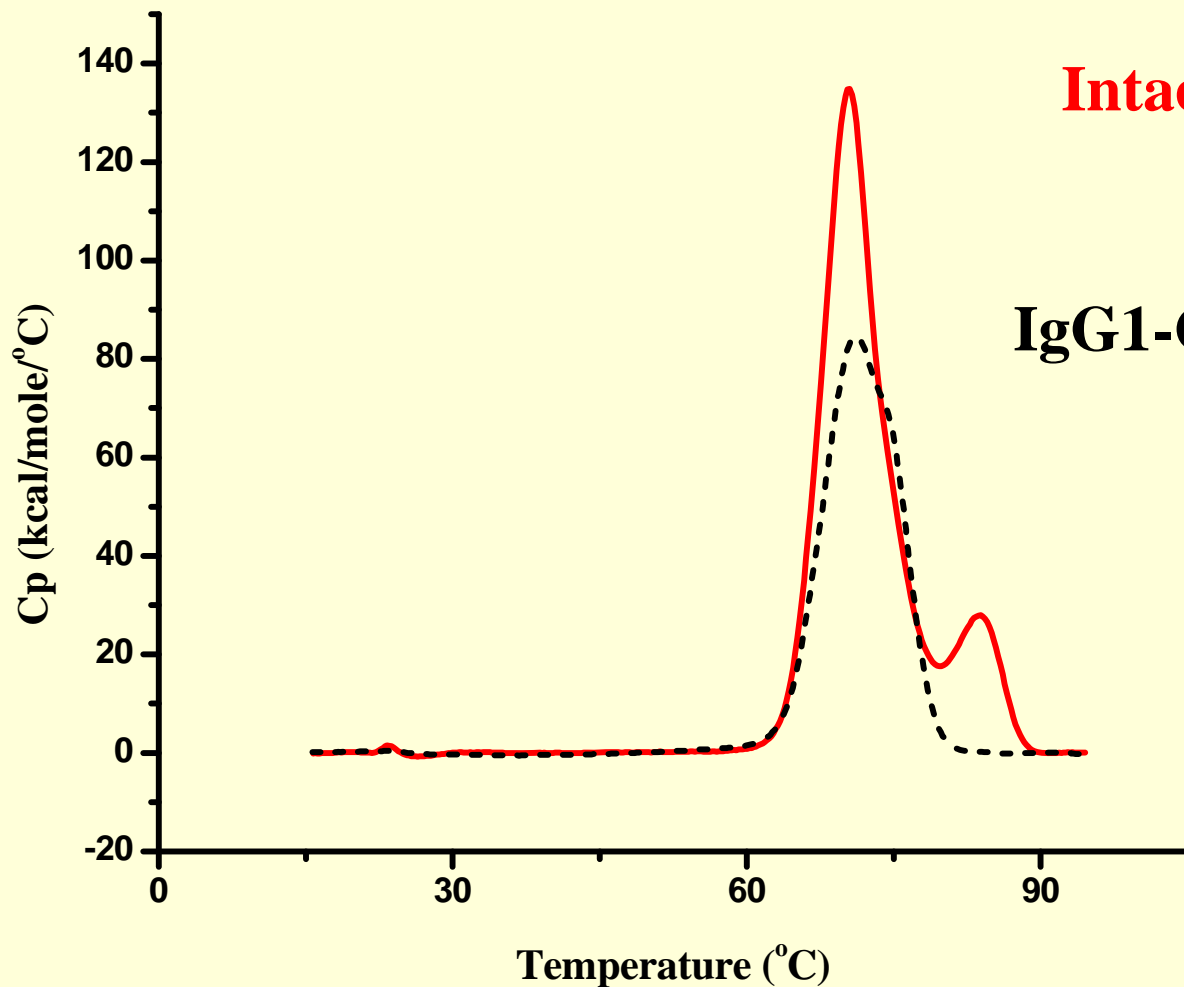
Thermogram of IgG1-C in PBS



Intact IgG1-C

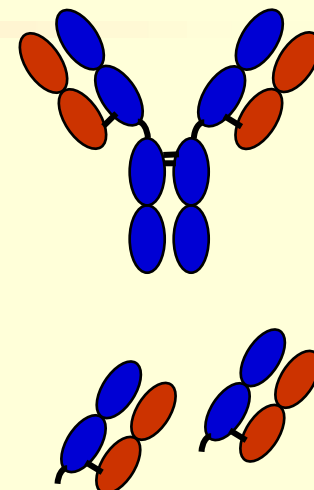


Intact IgG1-C in PBS with Fab from Papain Digestion

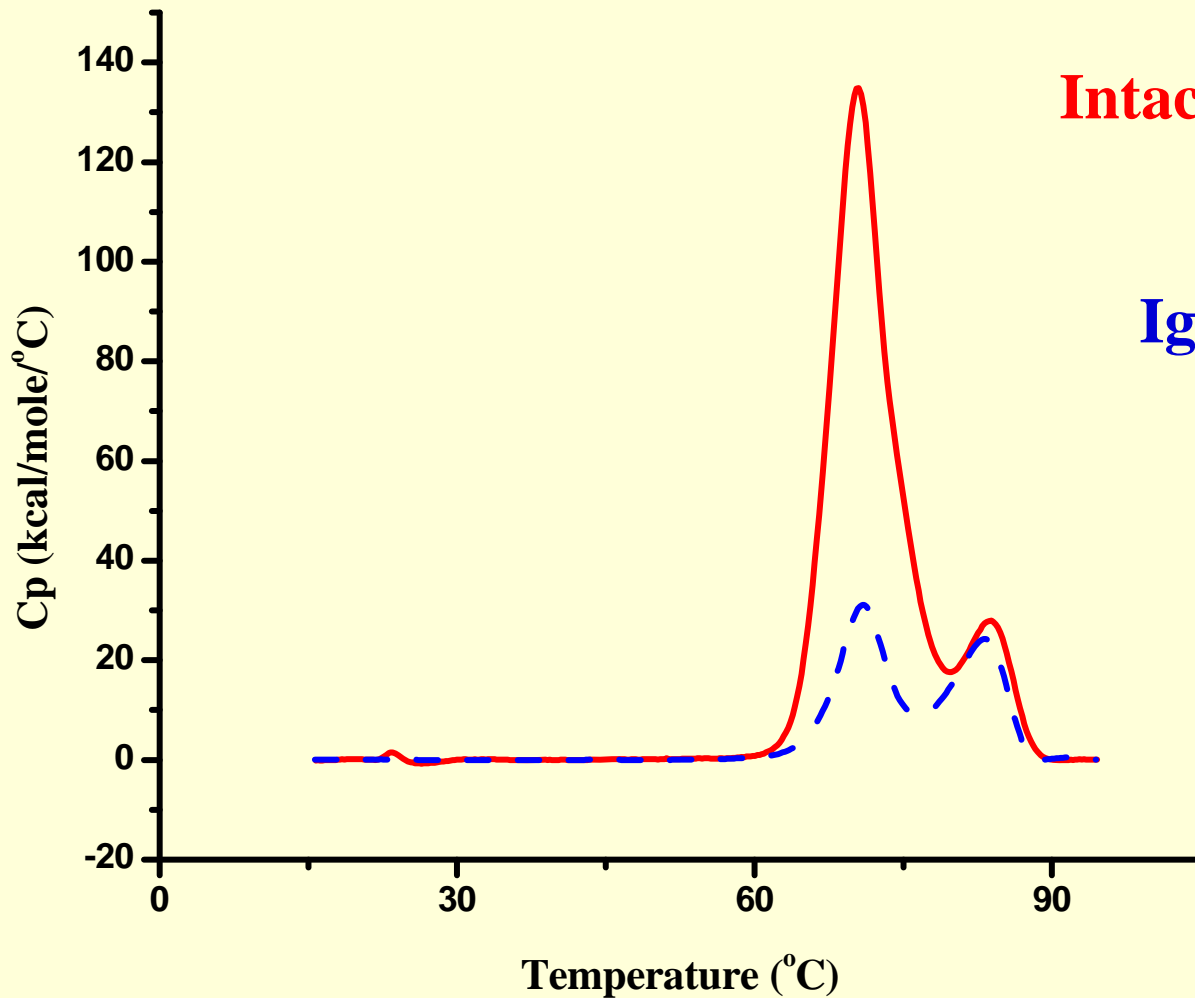


Intact IgG1-C

IgG1-C Fab (2x)

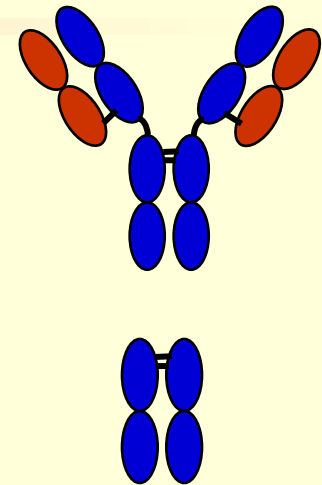


Intact IgG1-C in PBS with Fc using Papain

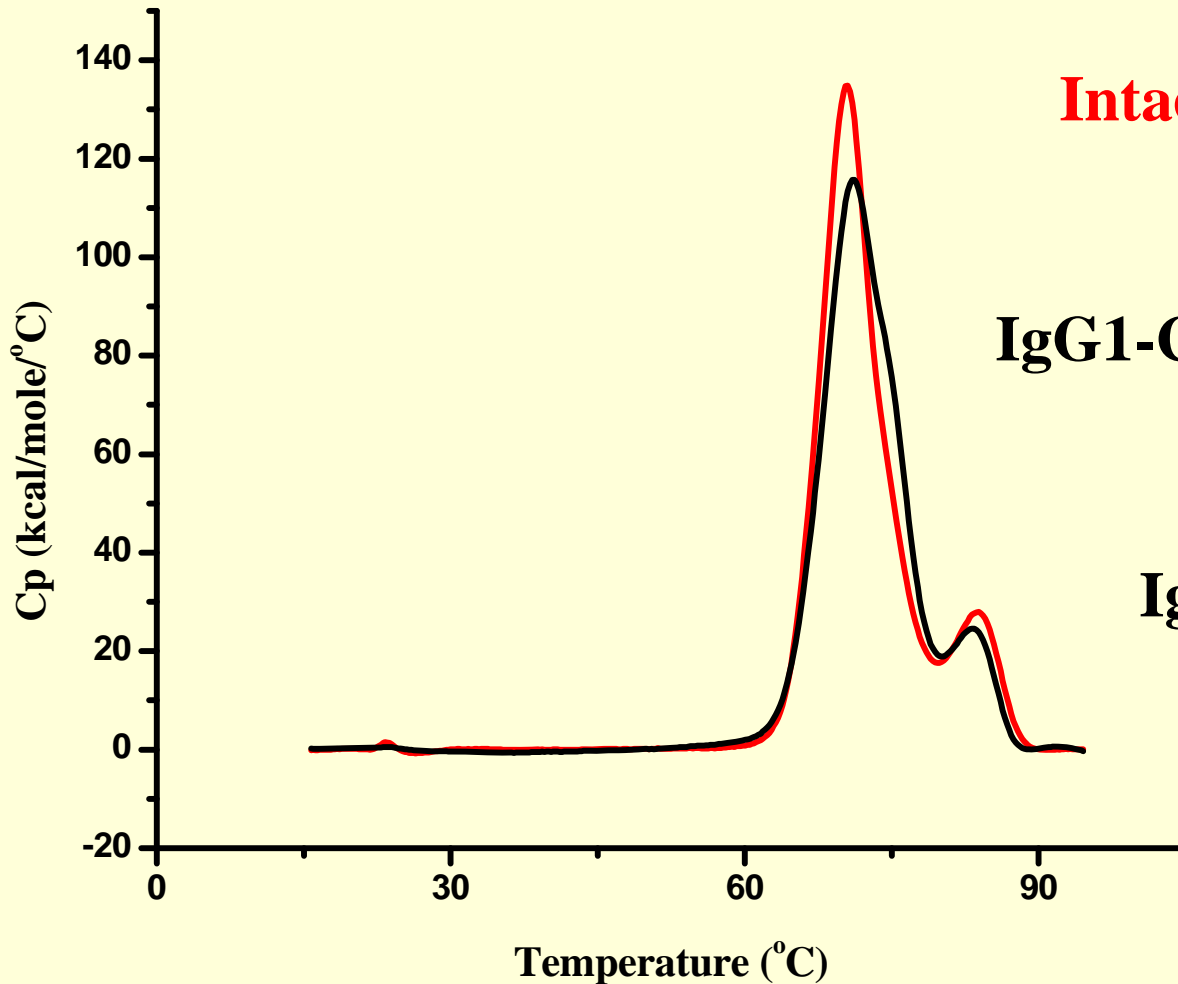


Intact IgG1-C

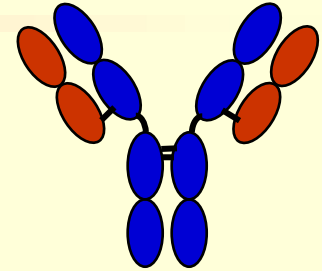
IgG1-C Fc



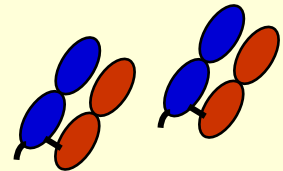
Intact IgG1-C Compared with Added Thermograms of Fab (2x) and Fc from Papain Digest



Intact IgG1-C

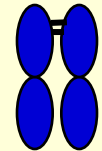


IgG1-C Fab (2x)

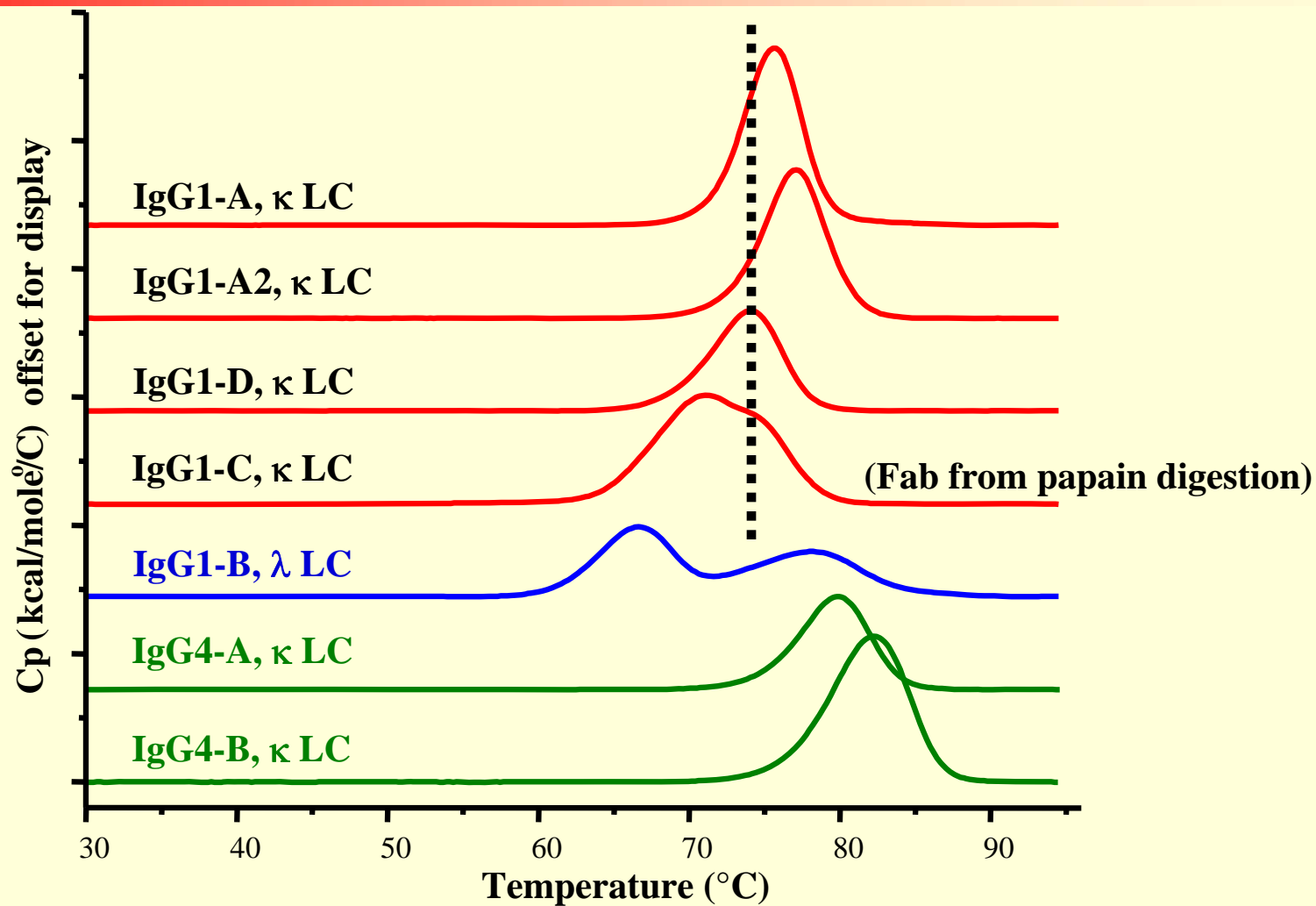


+

IgG1-C Fc



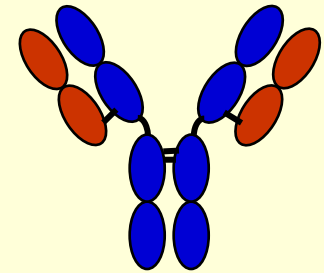
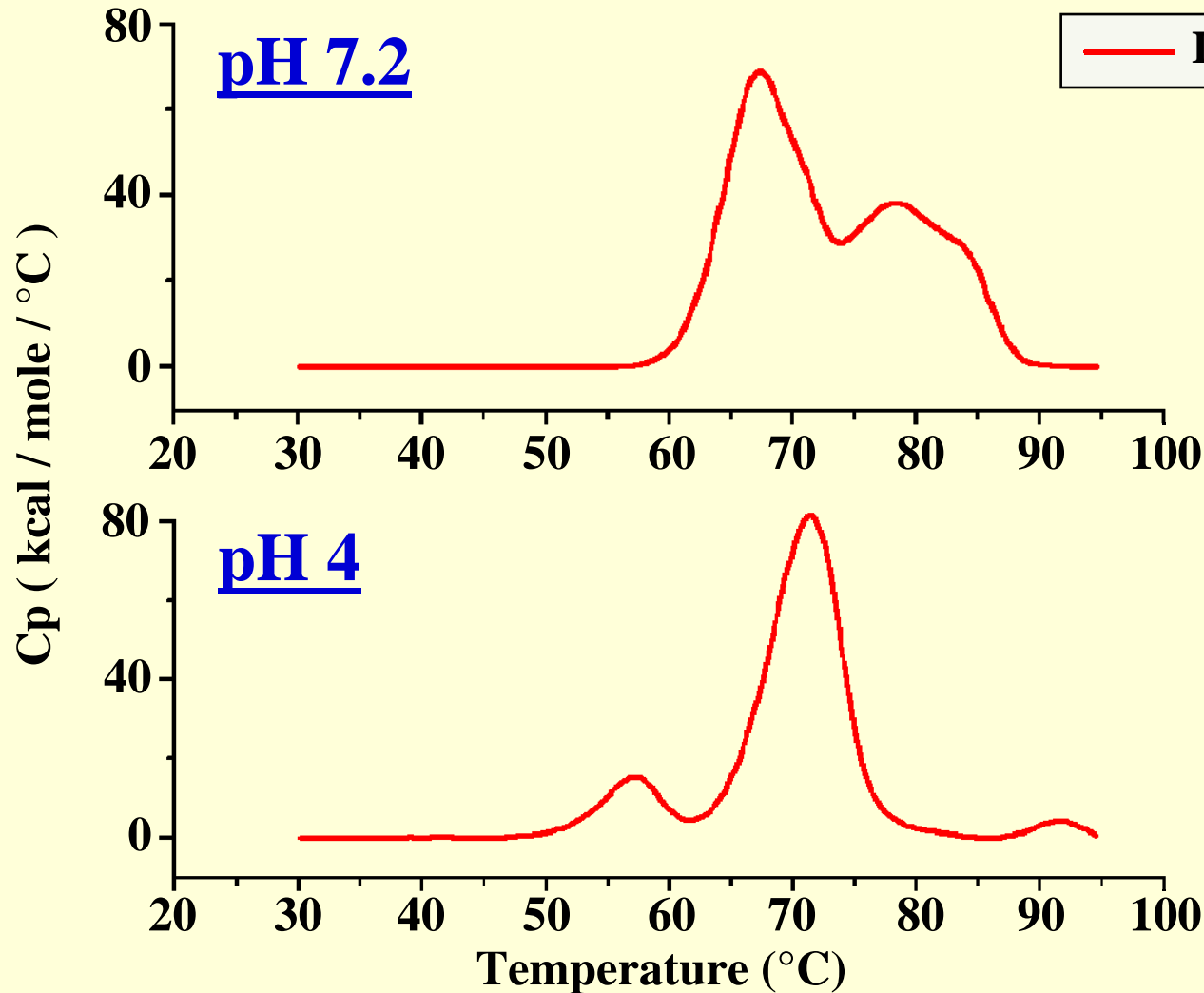
Thermograms of Antibody F(ab')₂ Fragments in PBS, pH 7.2



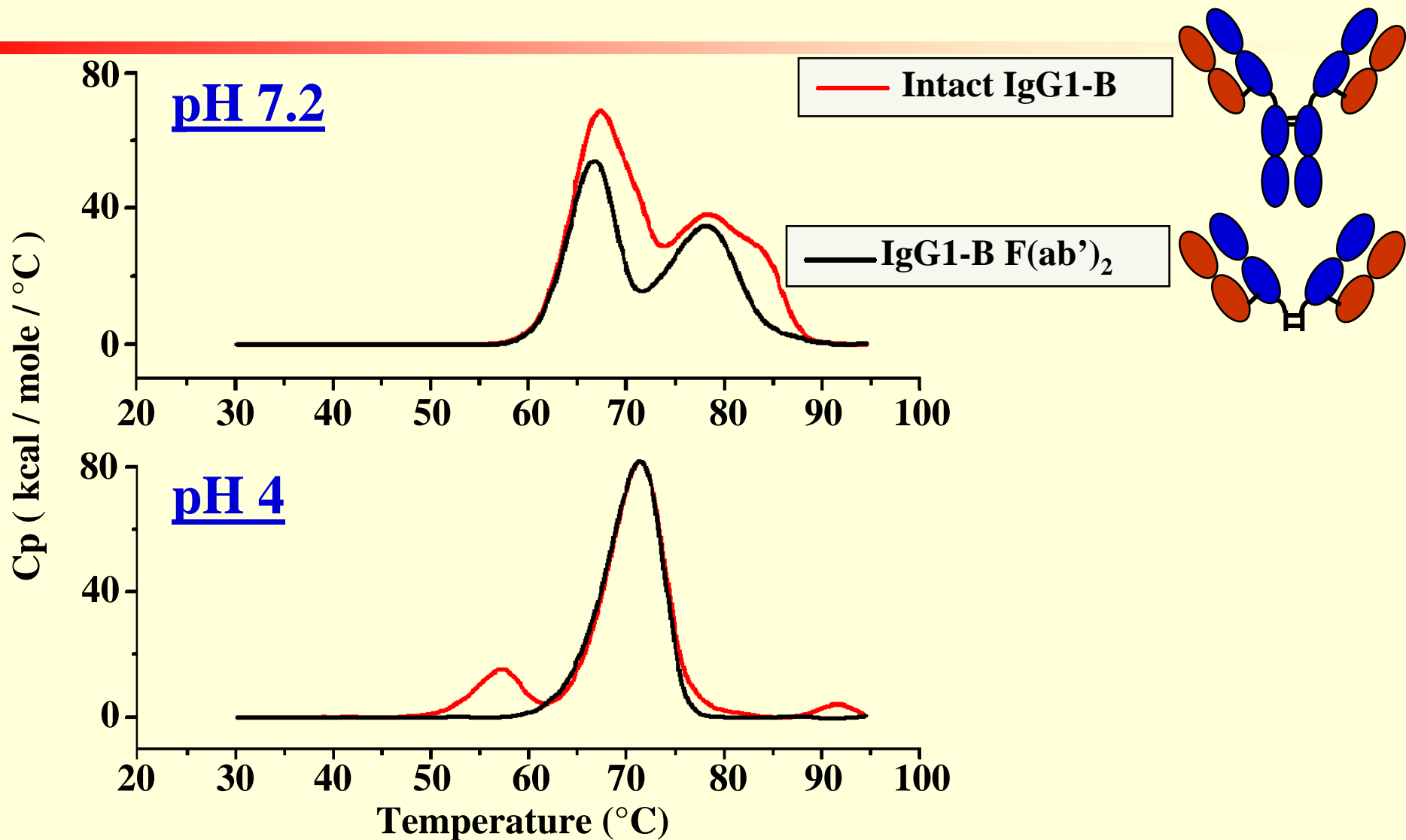
Assessment of Antibody Stability at Low pH

- **Protein A chromatography is commonly used as an efficient (affinity) method in the purification of IgG molecules or Fc-containing fusion proteins.**
- **Antibody (or Fc-protein) elution from a Protein-A column requires the use of a low-pH elution buffer, typically between 3-4. In process, proteins may be held for prolonged periods of time under these low-pH conditions to facilitate viral inactivation.**
- *What, if any, structural effects does low-pH exposure have on the antibodies themselves?*

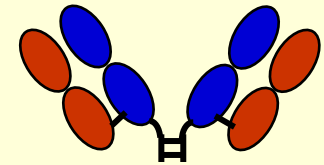
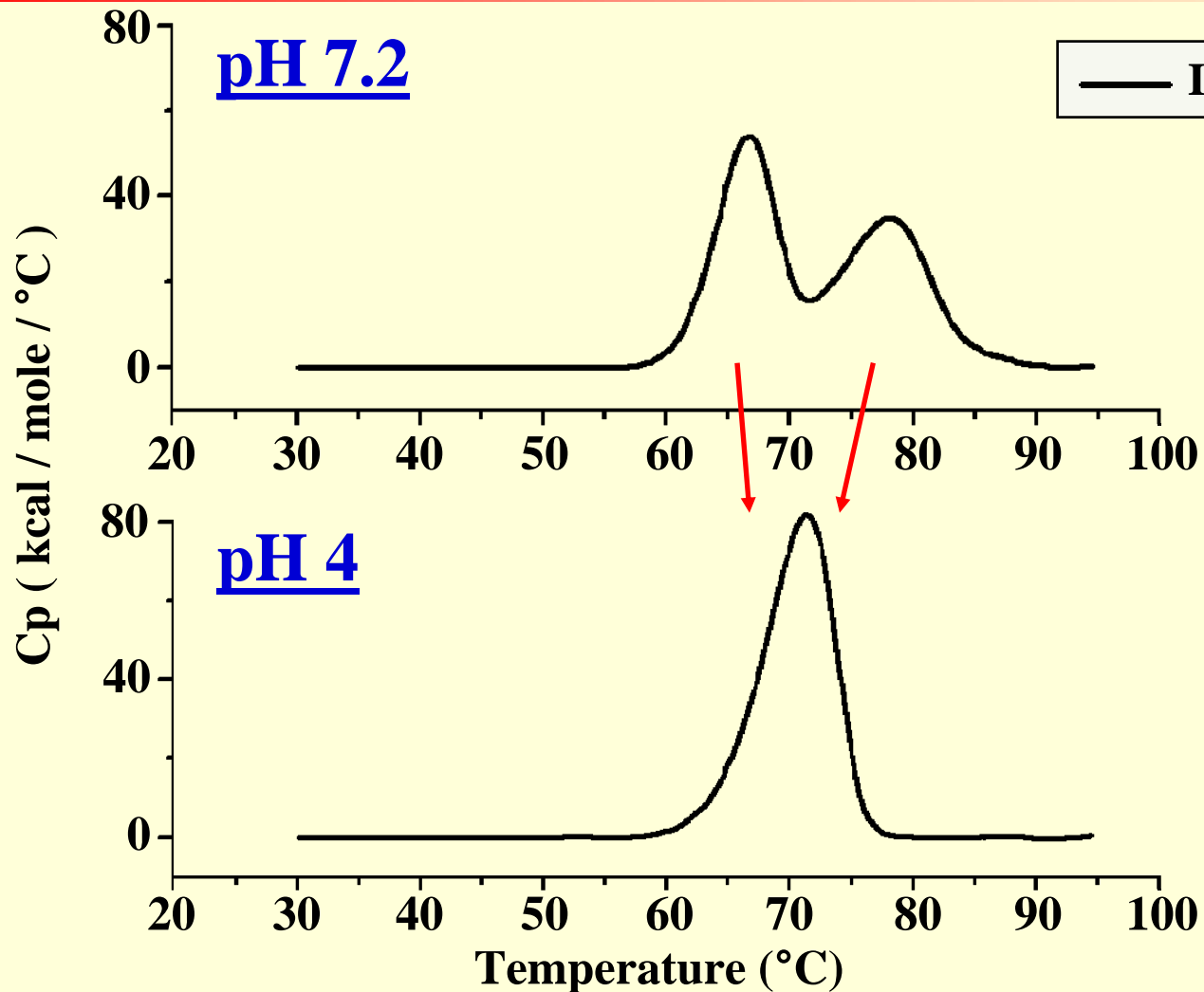
pH-Dependant T_m Shifts of IgG Domains



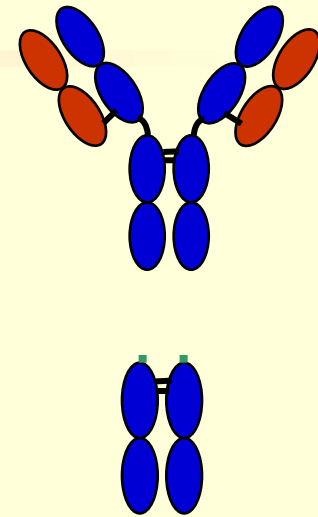
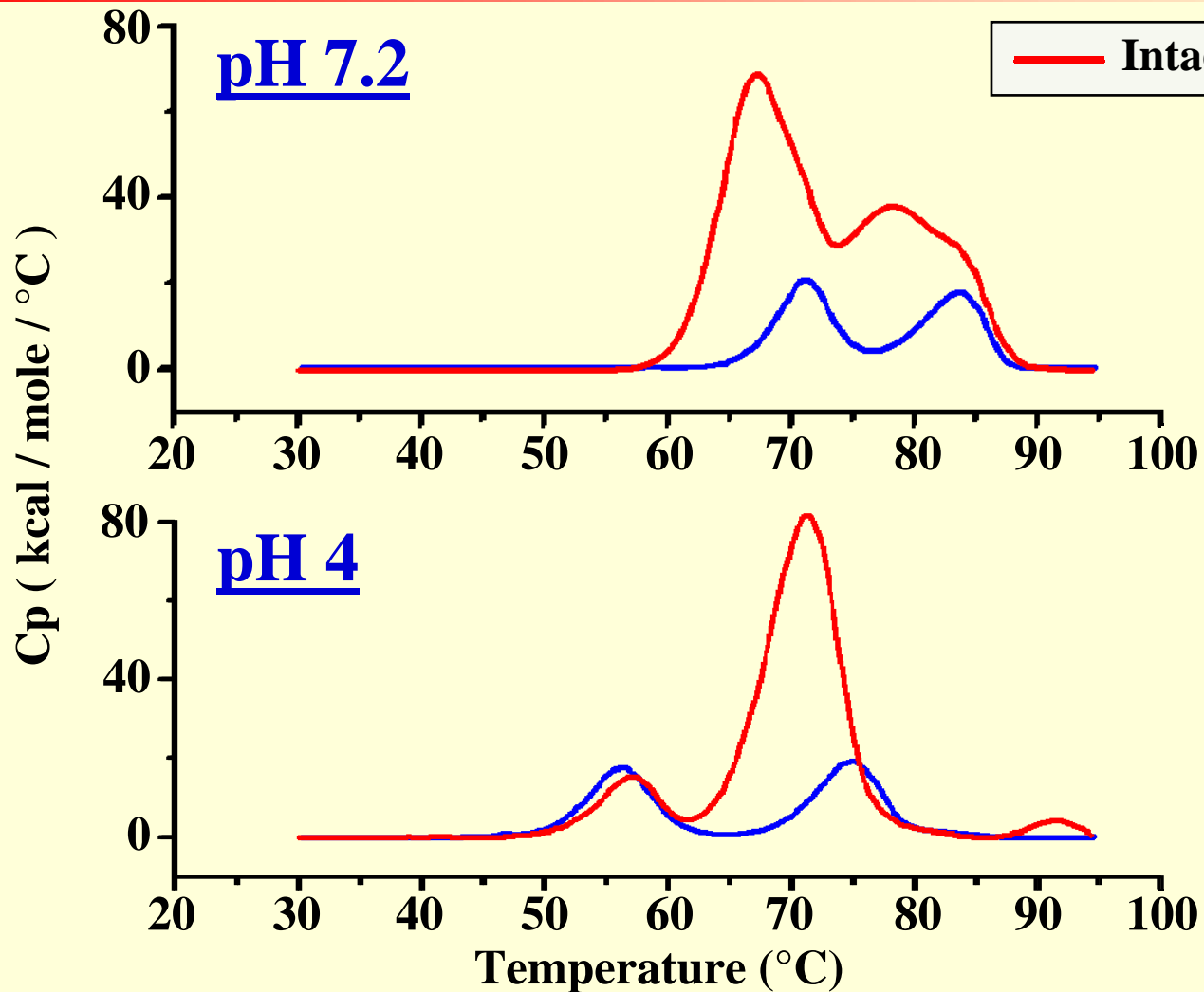
Intact IgG1-B and F(ab')₂ at pH 4 and pH 7.2



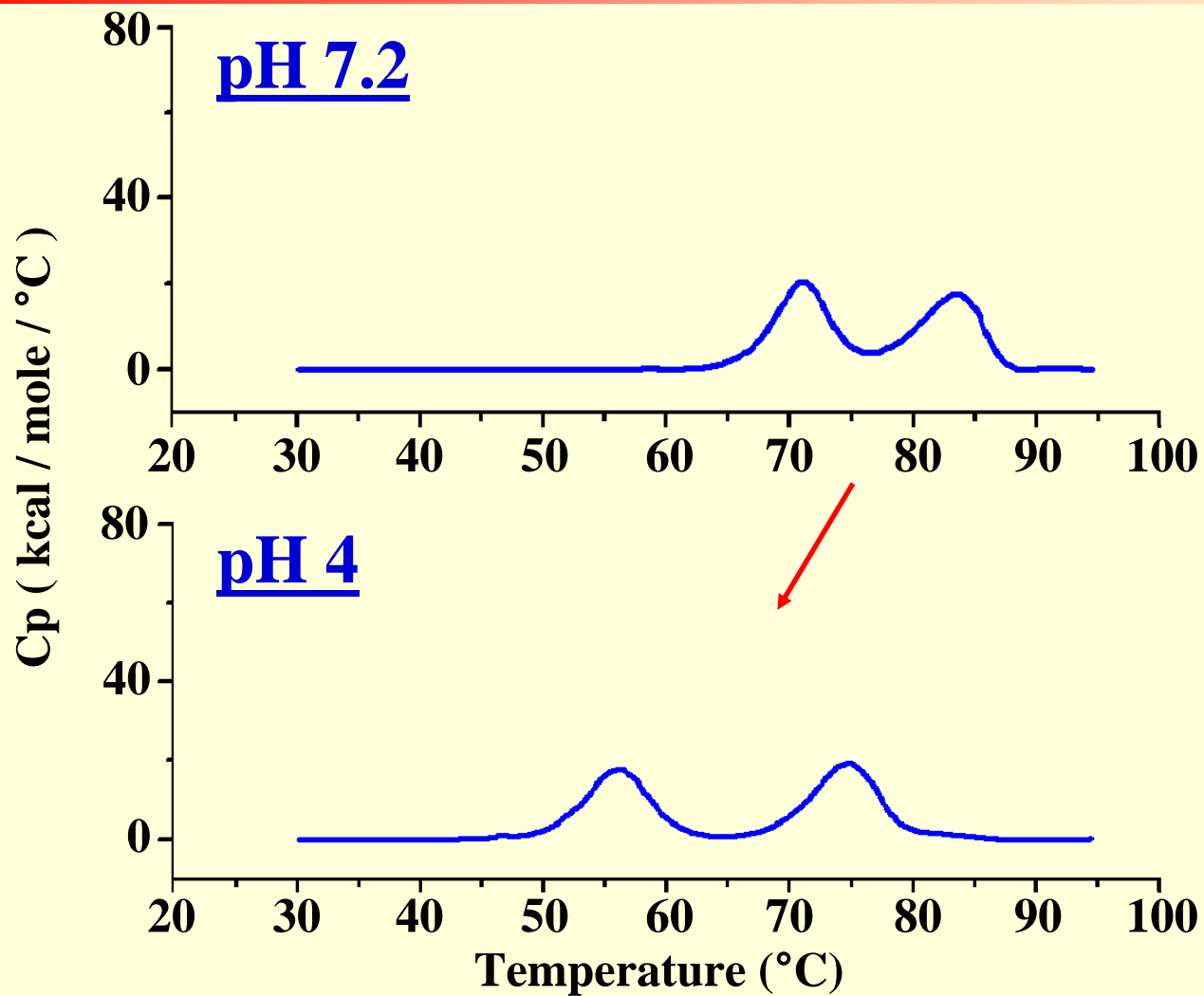
At lower pH, IgG1-B Fab2 transitions are no longer resolved.



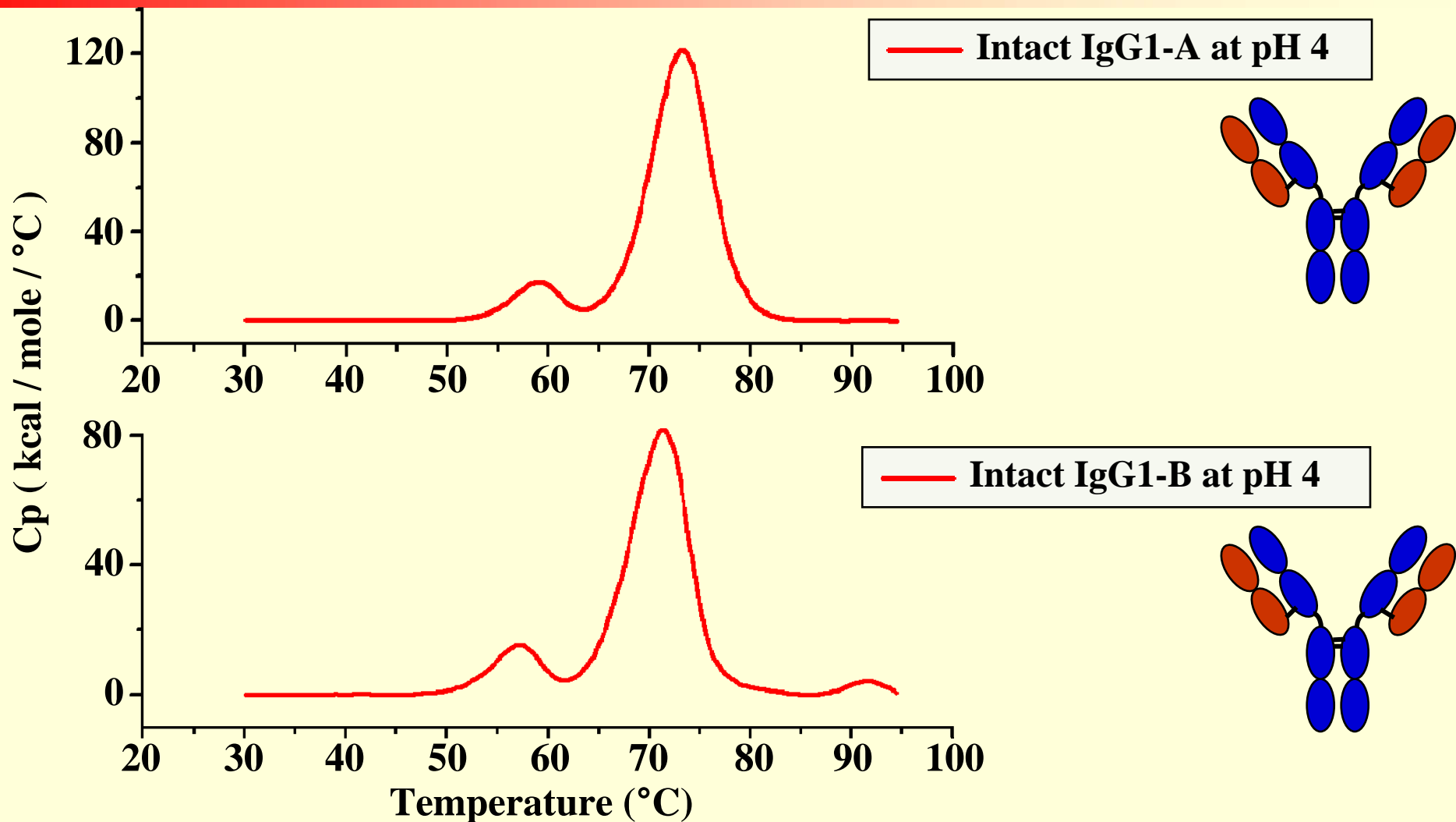
Intact IgG1-B and Fc at pH 4 and pH 7.2



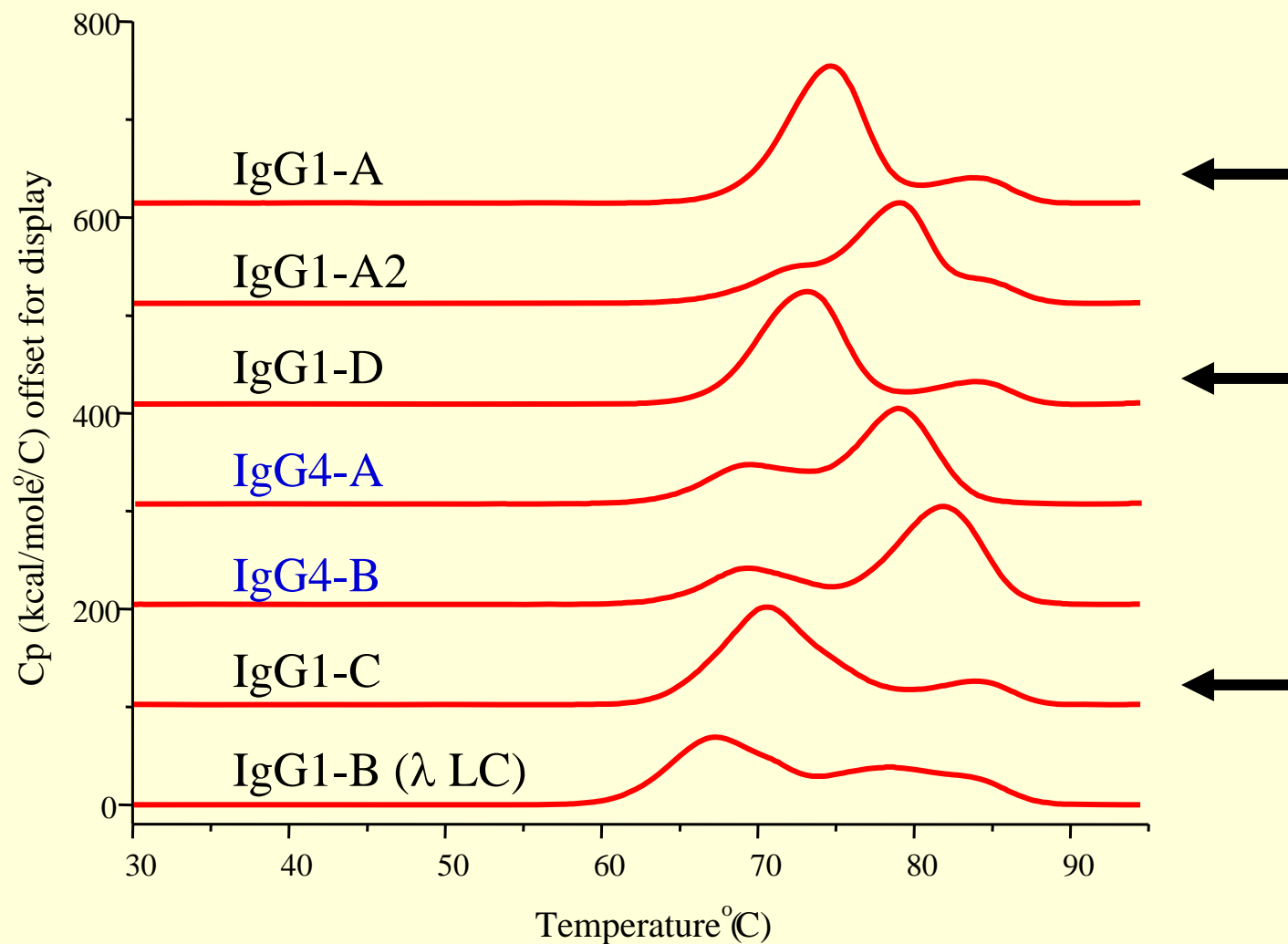
Fc at pH 4 and pH 7.2



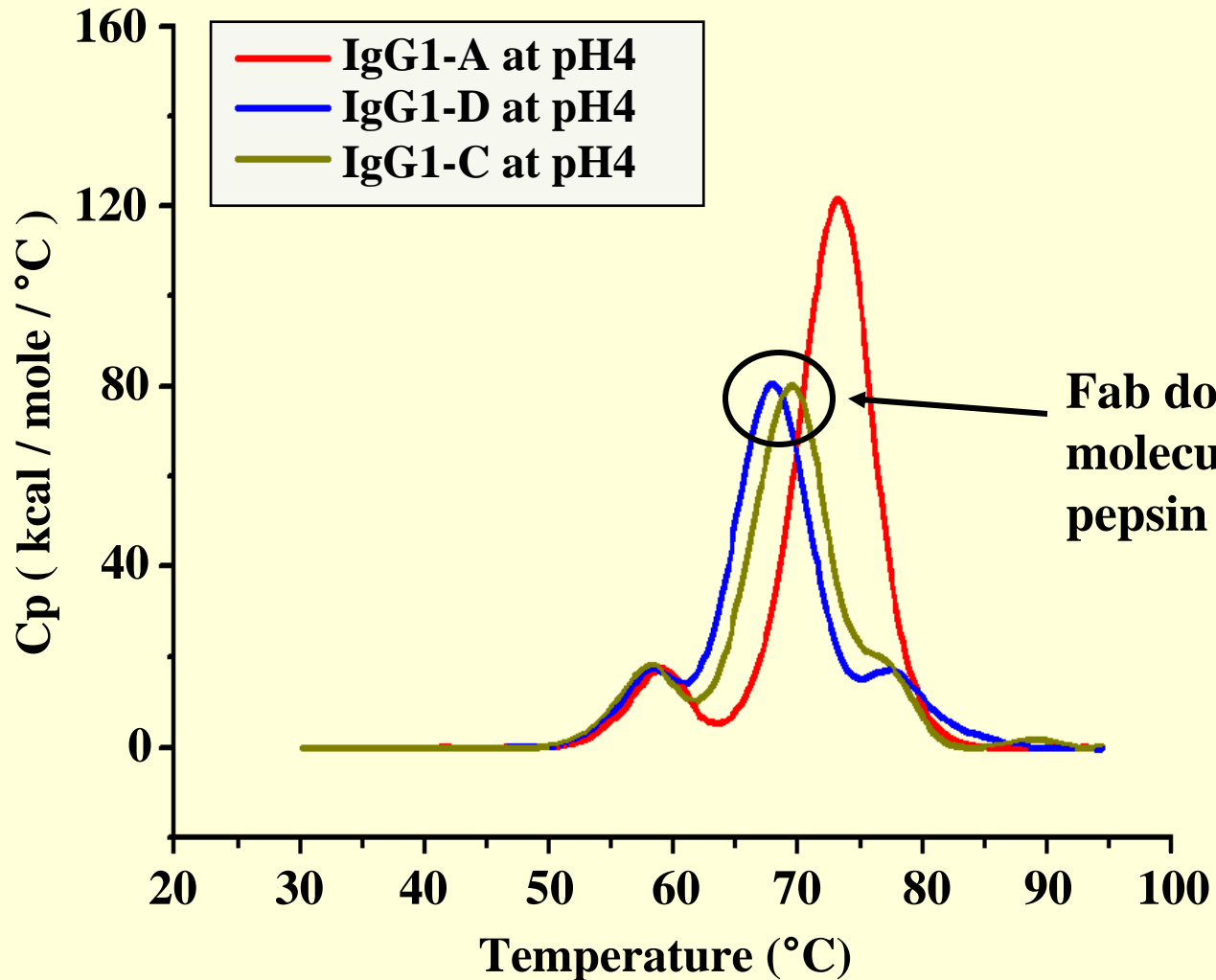
Intact IgG1-A (κ LC) and IgG1-B (λ LC) are not as Distinct at pH 4



Representative Thermograms for Intact Antibodies in PBS



mAb Structural Differences at pH 4



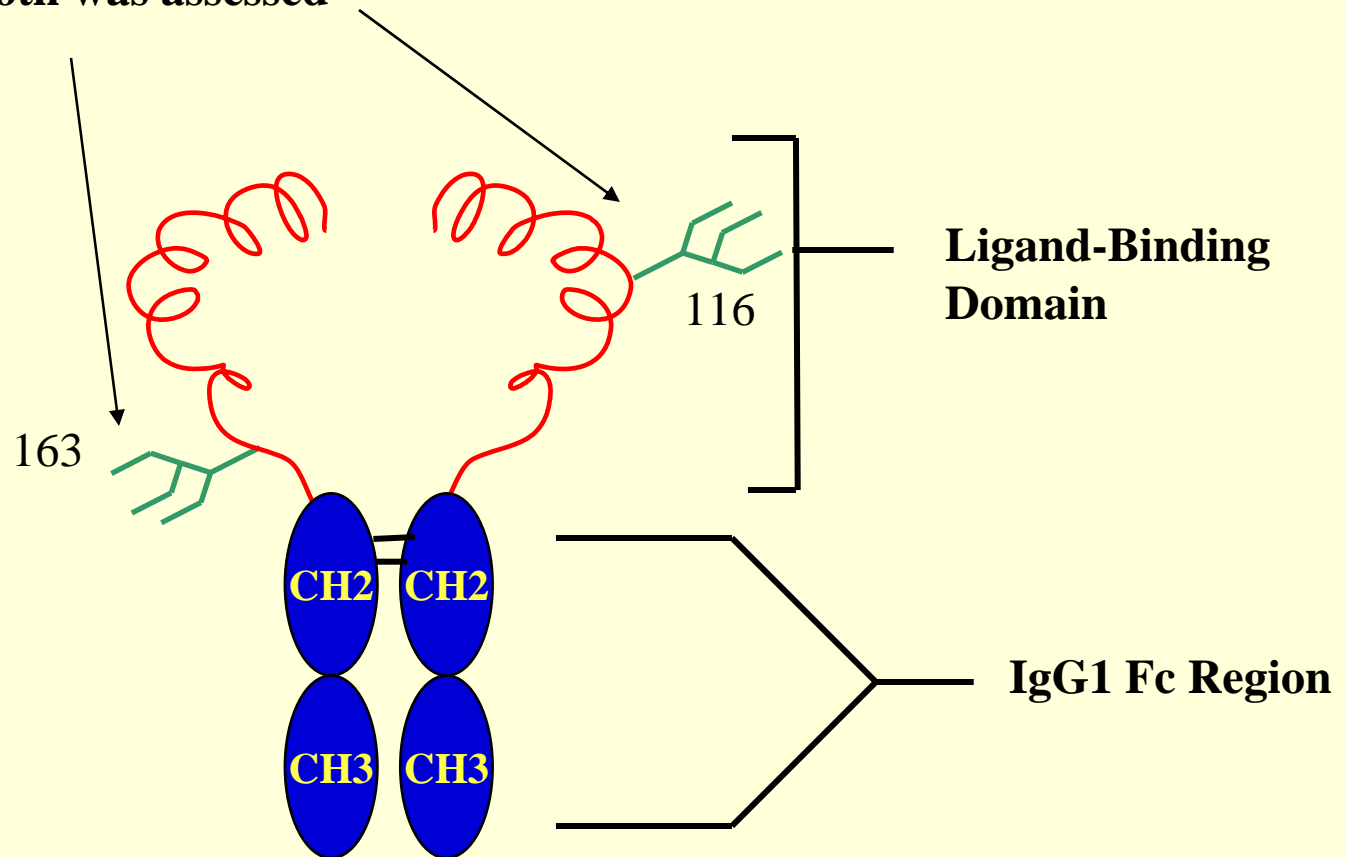
DSC Analysis of Antibody Fragments

- **IgG thermogram transitions were unambiguously assigned to specific antibody domains.**
- **There are not significant, stability-altering interactions between domains (Fab and Fc) in the intact antibodies tested.**
- **There are profound antibody-to-antibody differences in DSC profiles; these differences can be attributed to LC type, subclass and variable Fab regions.**
- **Antibody domain stabilities are highly pH-dependent. Pepsin lability of some IgG1 Fabs indicates structural disparity at pH 4.**
- *Knowledge obtained can be applied toward understanding the behavior of other Fc-containing (fusion) proteins.*

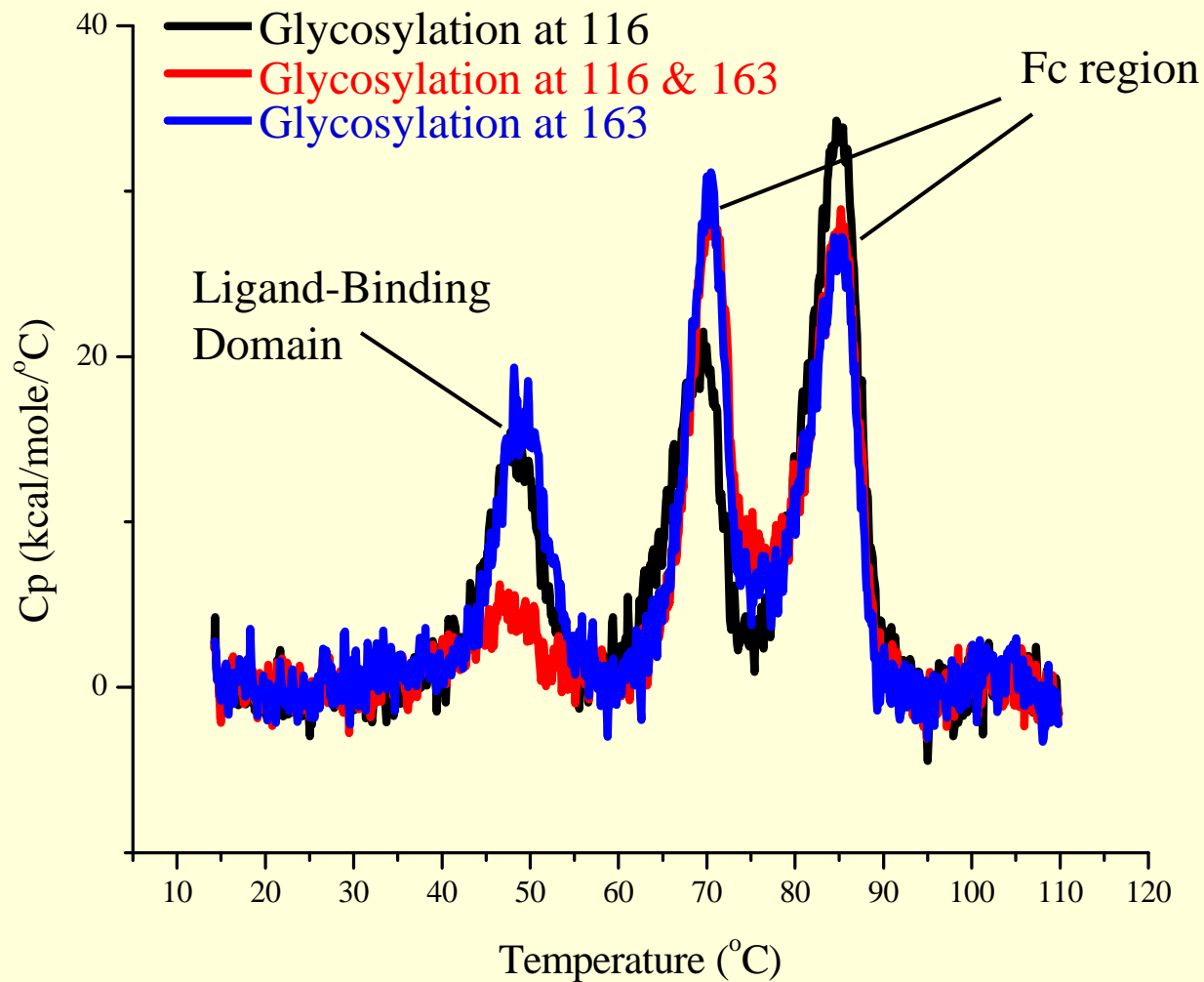
Structural Analysis of an Fc-fusion Protein

--Effects of glycosylation

Glycosylation at position 116,
163 or both was assessed

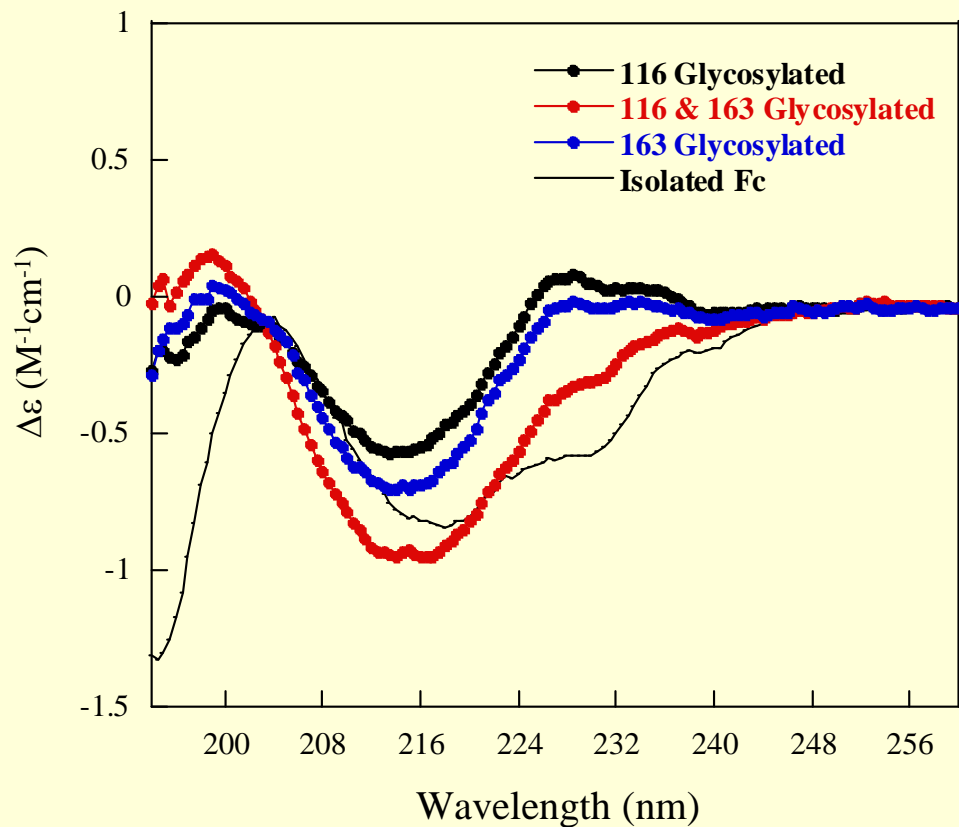


DSC Comparison of Fc-fusion Glycosylation Variants

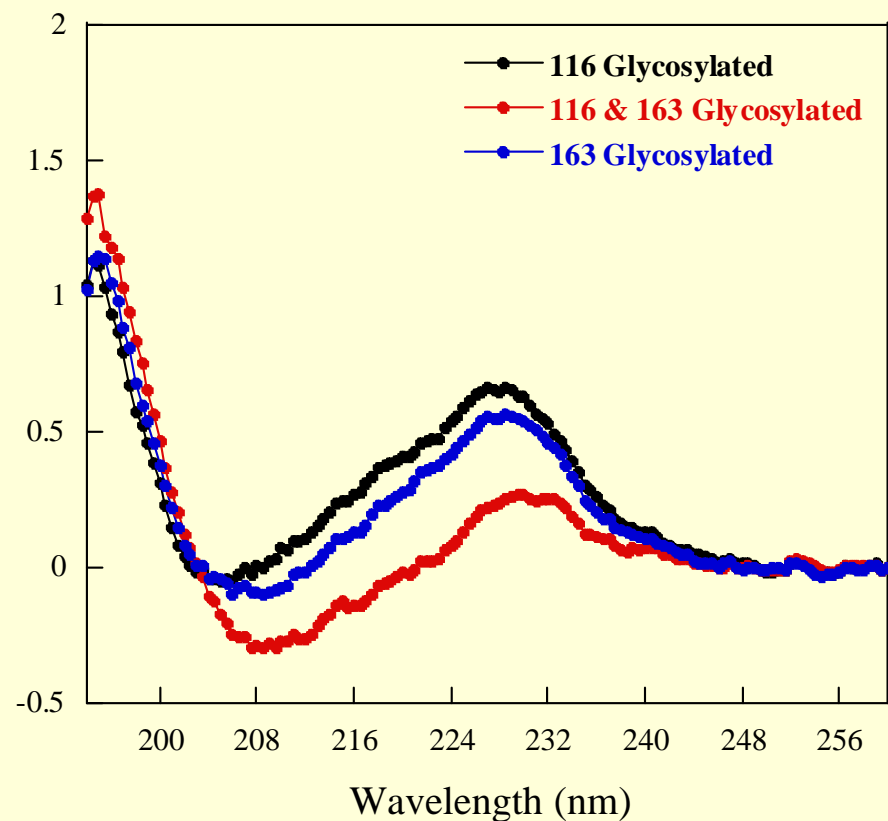


Far-UV CD of Fc-fusion Protein Glycosylation Variants

Intact Fusion Proteins



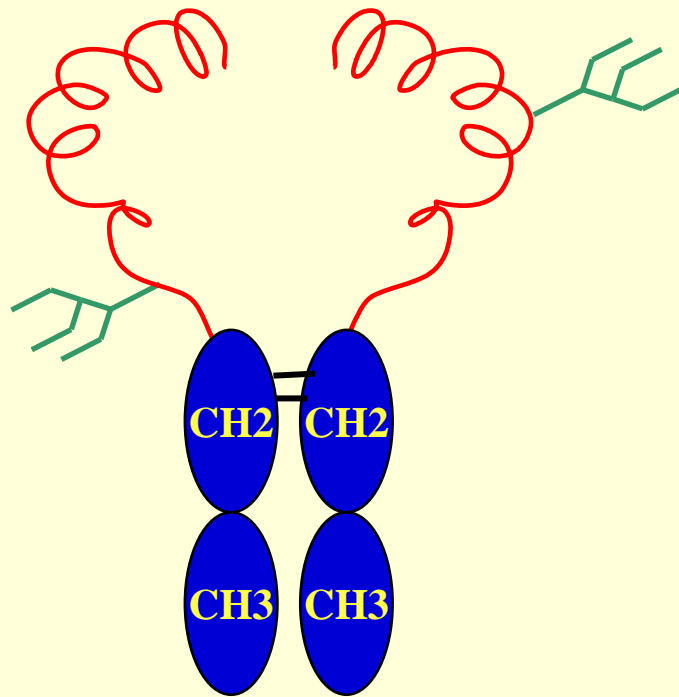
Spectral Contribution of Fc has Been Subtracted



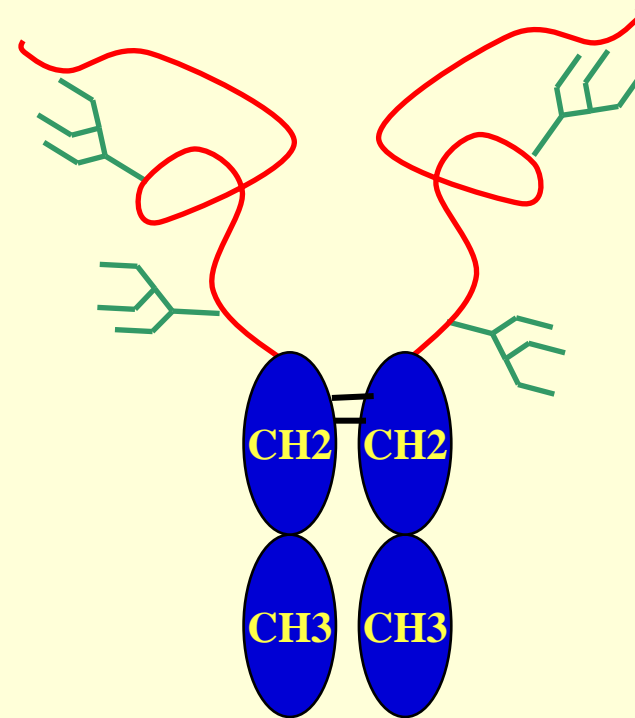
Proposed Model:

Over glycosylation prevents folding of binding domain

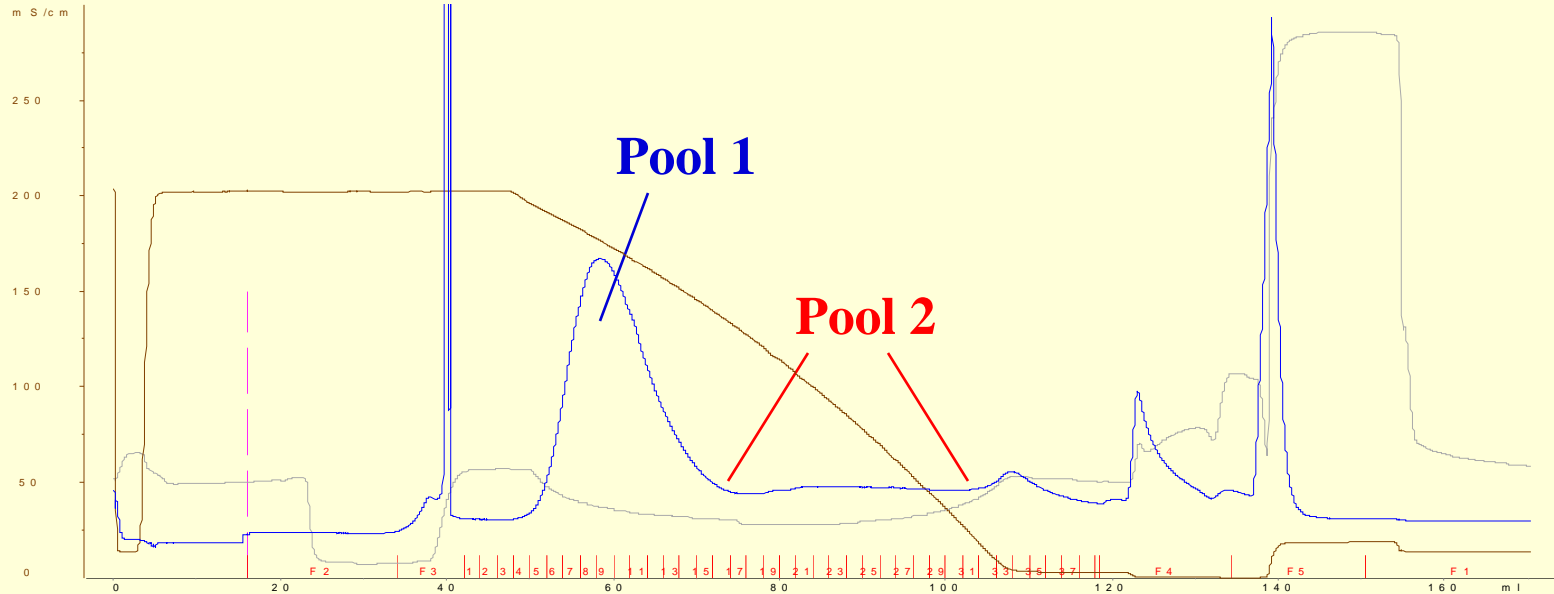
Single glycan occupancy
at 116 or 163



Double glycan occupancy
at both 116 and 163



Structural Analysis of an (the same) Fc-fusion Protein --In-process chromatographic pools

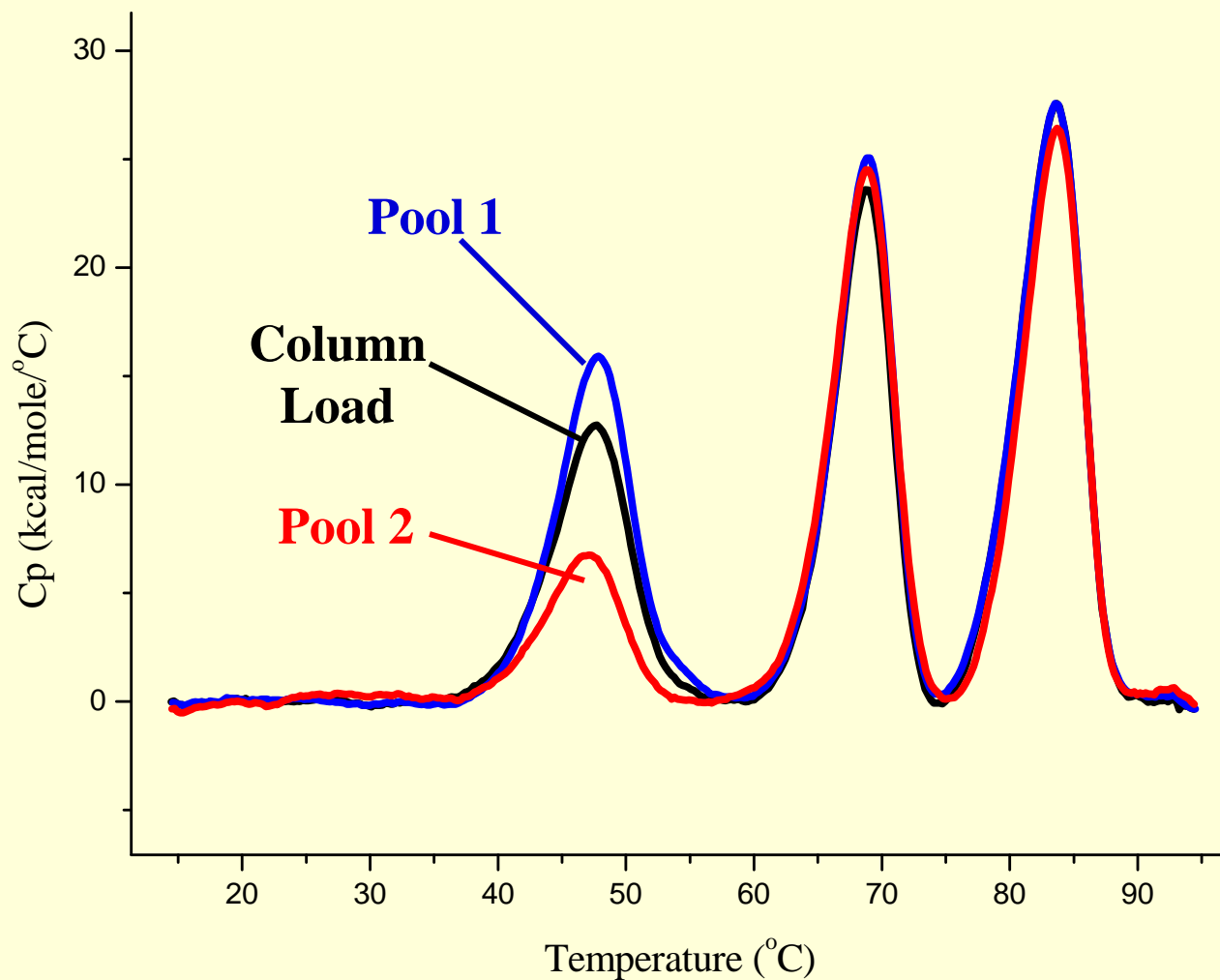


Chromatographic polishing step of an Fc-fusion protein: unexpected fractionation during gradient.

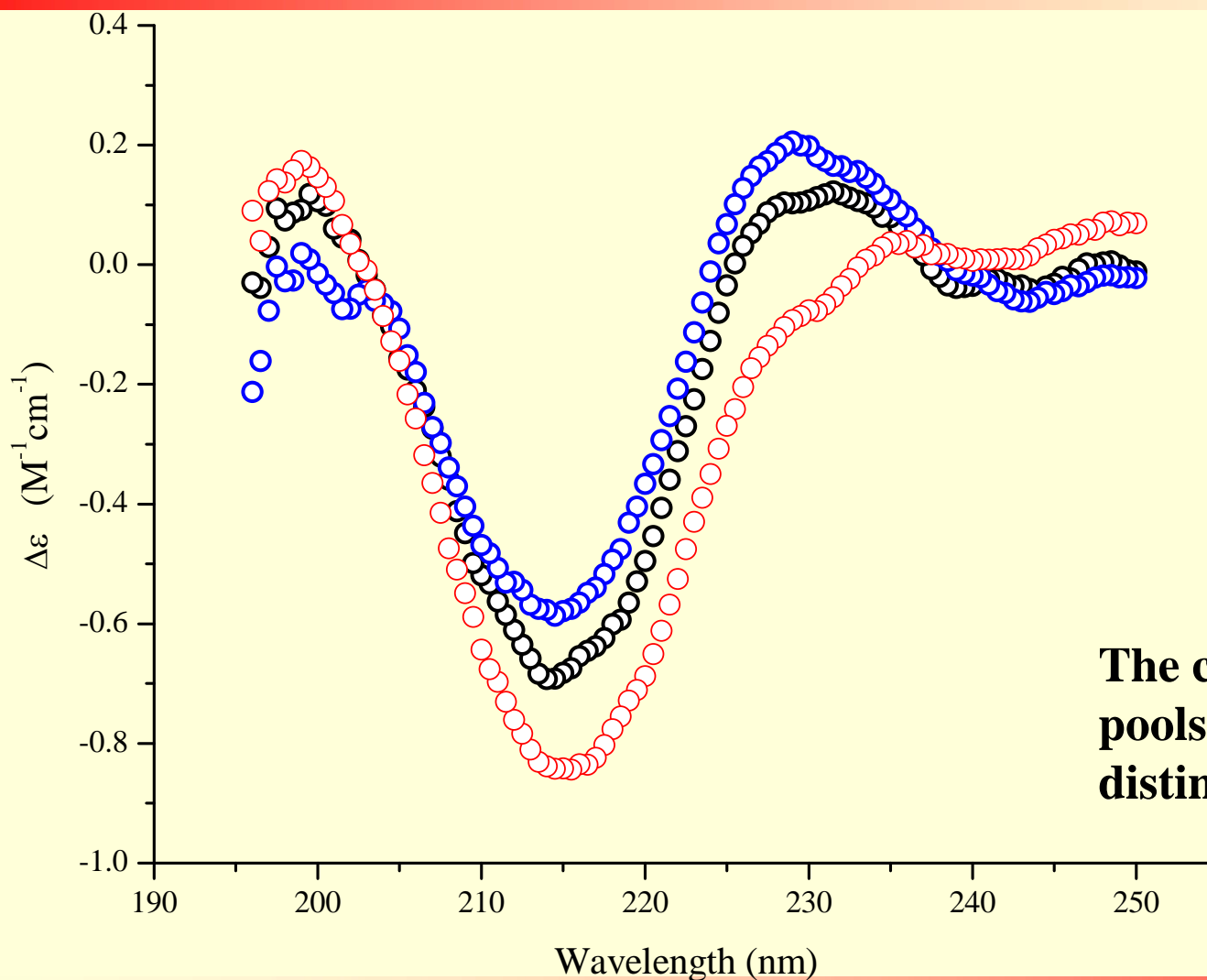
- Protein-A purification step prior to chromatographic analysis.
- The presence of HMW species (aggregates) were observed in the low-pH conditions.
- No covalent differences were detected across the chromatographic profile.

Is this fractionation due to structural differences?

DSC of Fc-fusion Protein Chromatographic Pools



Far-UV CD of Fc-fusion Protein Chromatographic Pools



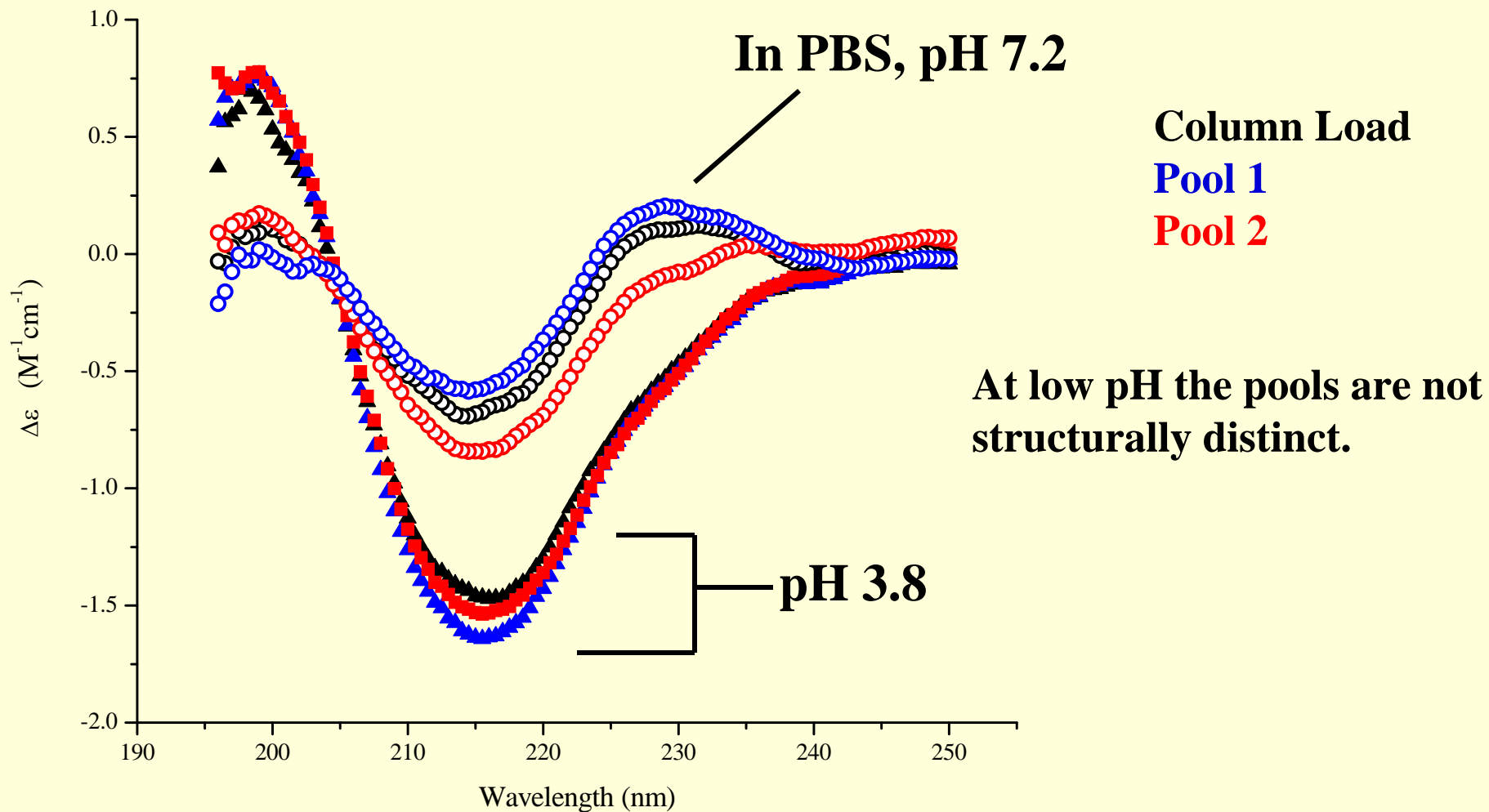
Column Load

Pool 1

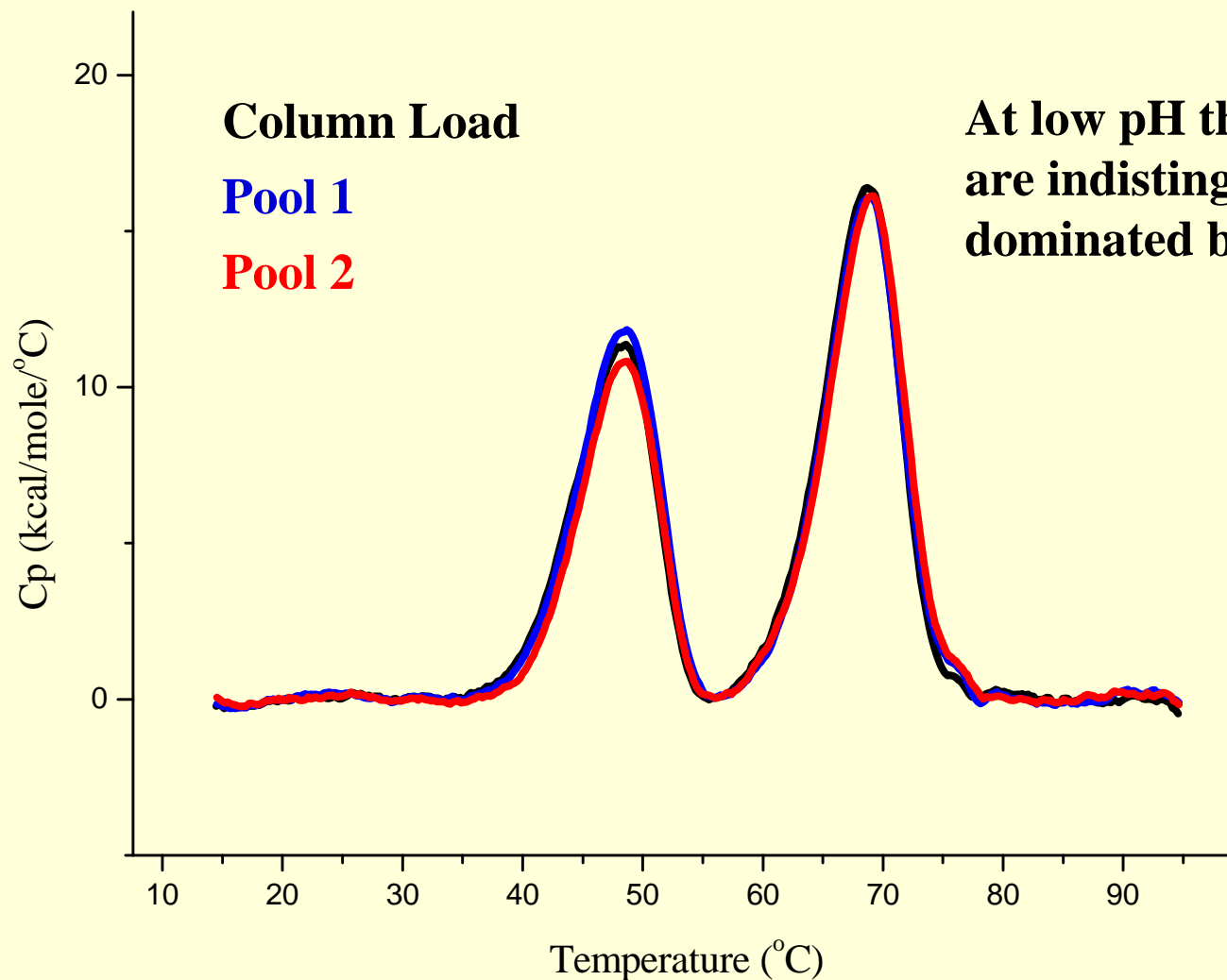
Pool 2

**The chromatographic
pools show structural
distinctions.**

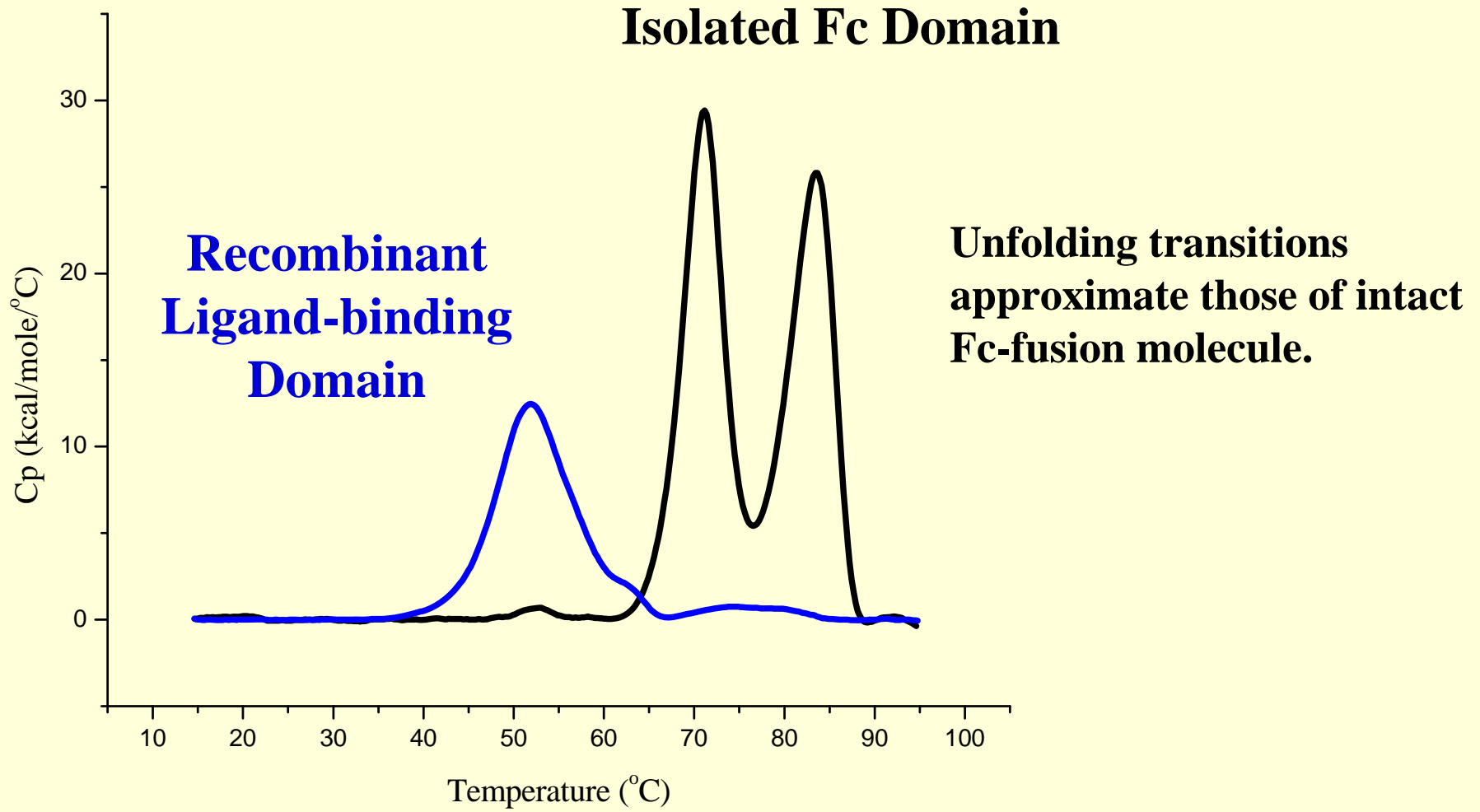
Far-UV CD of Fc-fusion Protein Chromatographic Pools



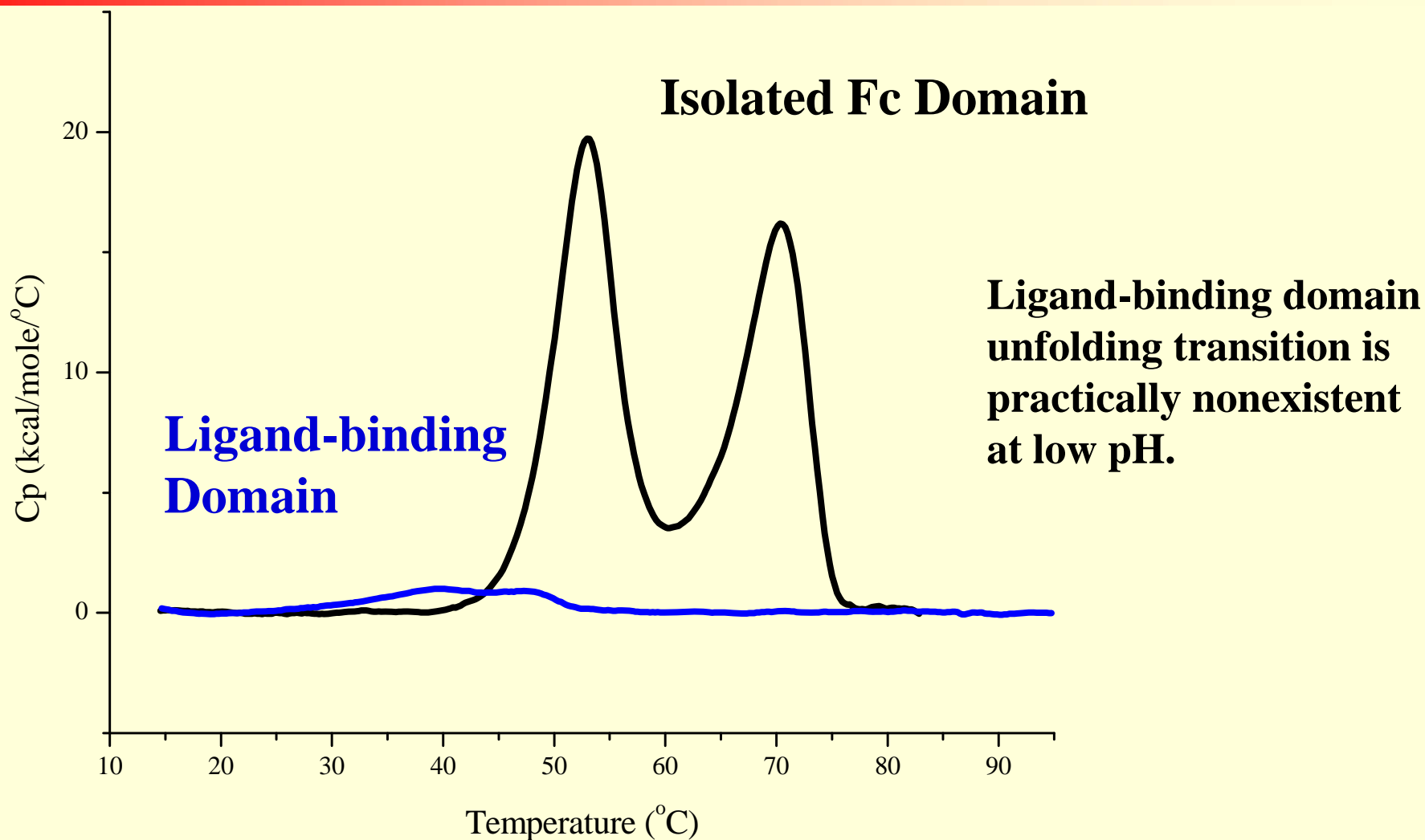
DSC of Fc-fusion Protein Chromatographic Pools at pH 3.8



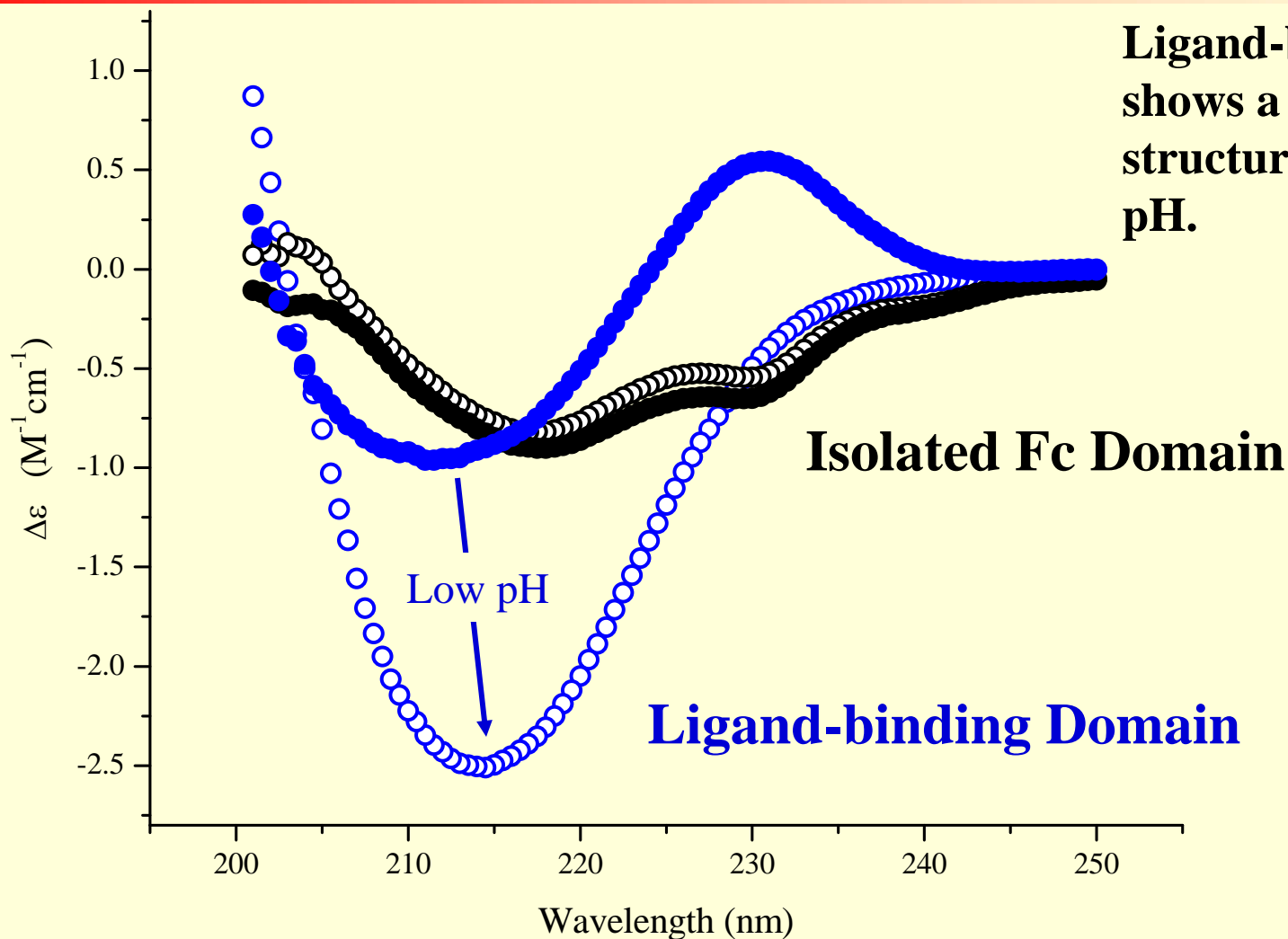
DSC of Individual Fc-fusion Protein Domains in PBS



DSC of Individual Fc-fusion Protein Domains at pH 3.8



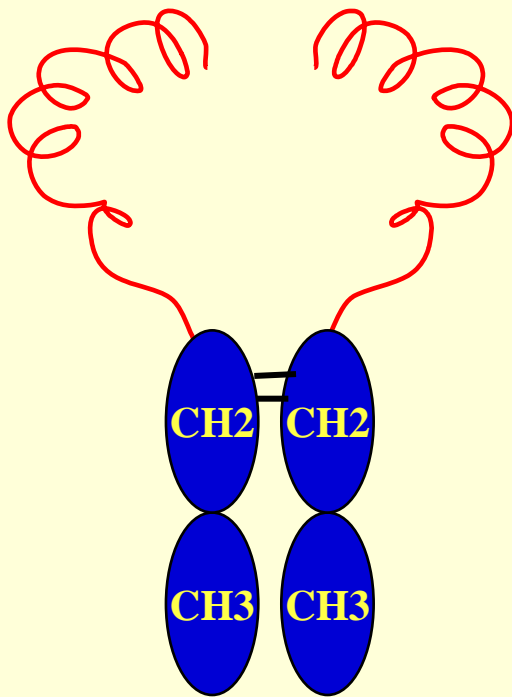
Far-UV CD of Individual Fc-fusion Protein Domains in PBS and at pH 3.8



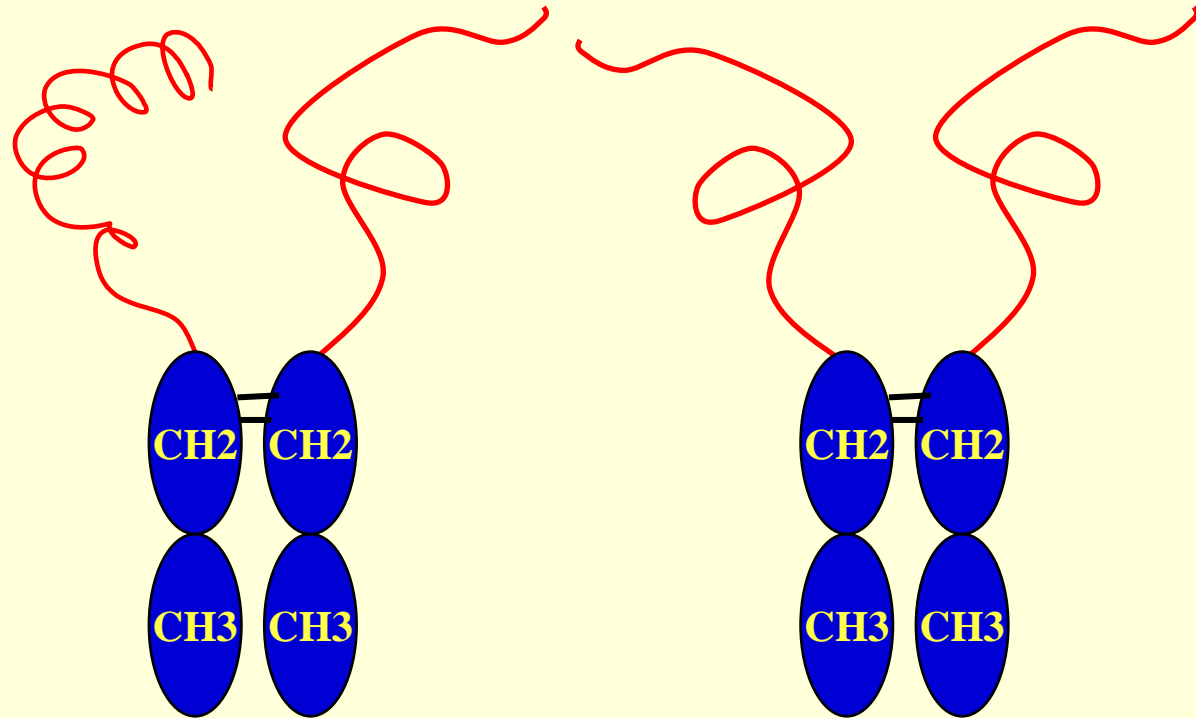
Proposed Model:

Low-pH perturbs the folding of Ligand-binding domain

**Folded Protein
in Pool 1**

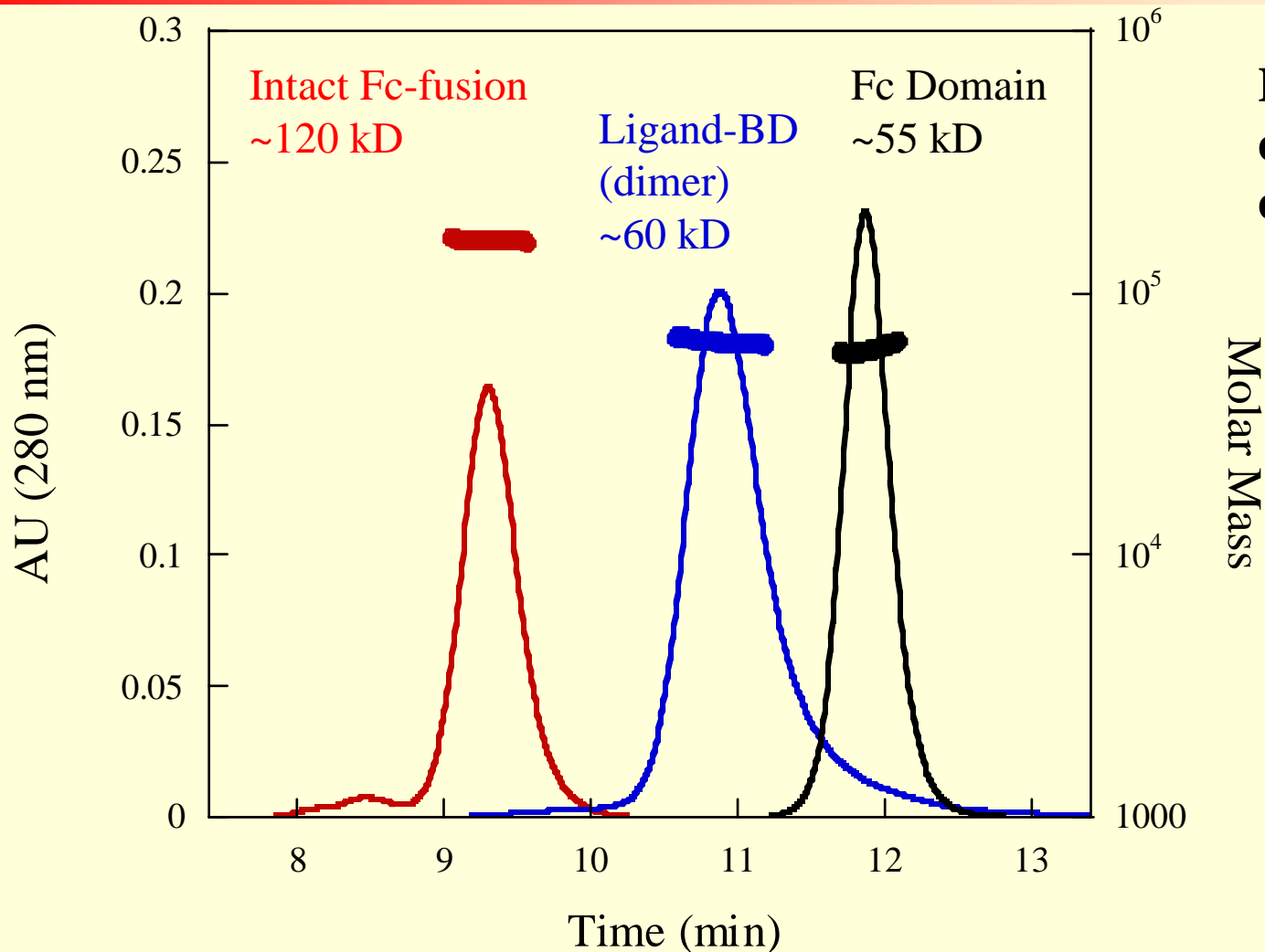


**Partially Folded Proteins
in Pool 2**



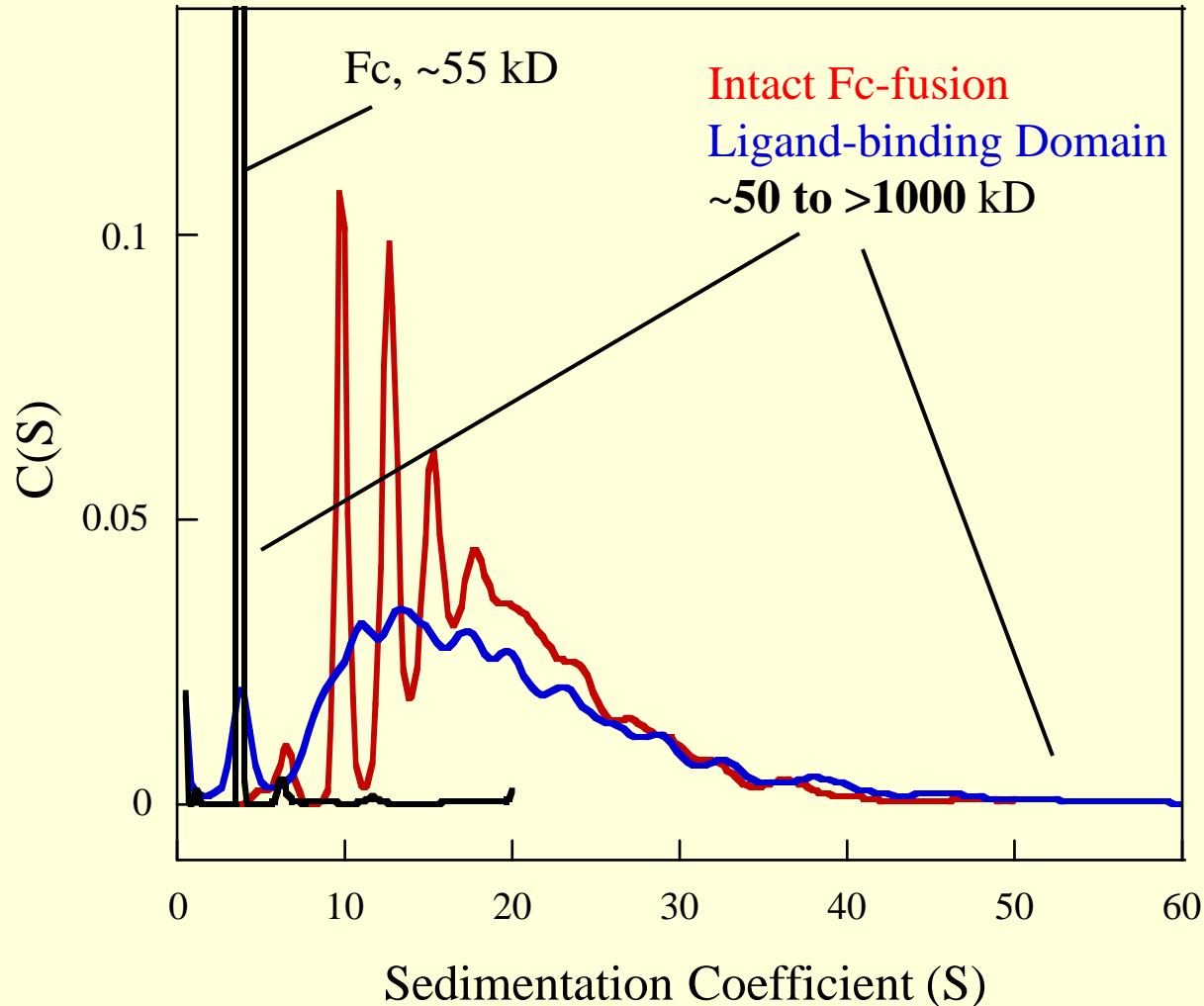
➤ *Not all molecules refold properly upon neutralization, following the Protein-A elution.*

SEC-Light Scattering of Individual Fc-fusion Protein Domains in PBS



Ligand-binding domain exists as a dimer at pH 7.2.

Sedimentation Velocity of Individual Fc-fusion Protein Domains at pH 3.8



The Ligand-binding domain, as well as the intact fusion protein, form very large oligomers at low pH.

Summary

Antibodies, and other Fc-containing proteins, can be analyzed by individual fragments in order to simplify the study of these complex molecules.

- **The assignment of specific protein domains to distinct spectral features or unfolding transitions enables us to understand, in detail, the structural effects of protein modification and changes in solution conditions.**
- **A comprehensive DSC analysis of all Wyeth antibodies and antibody derivatives (under various solution conditions) will improve our understanding of the proteins' domain stability, and may elucidate general aspects of aggregation and other degradation processes.**

Acknowledgments

Yasuko Mabuchi

Kyle Wang

Dave Sek

Donna Luisi

Erin Wiswall

Denise Kwok

Colleen Steinmeyer

Stephane Olland

John Steckert

Zhaojiang Lu

Lucy Liu

Yin Luo

Marta Czupryn

Hubie Scoble

Steve Spotts, and all at MicroCal!