

## PhD course Jyväskylä 2017

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**Where are the protons? Measuring  
and modelling proton equilibria in  
complex macromolecular systems.**



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Lecture 3  
Application of NMR spectroscopy  
to study electrostatics



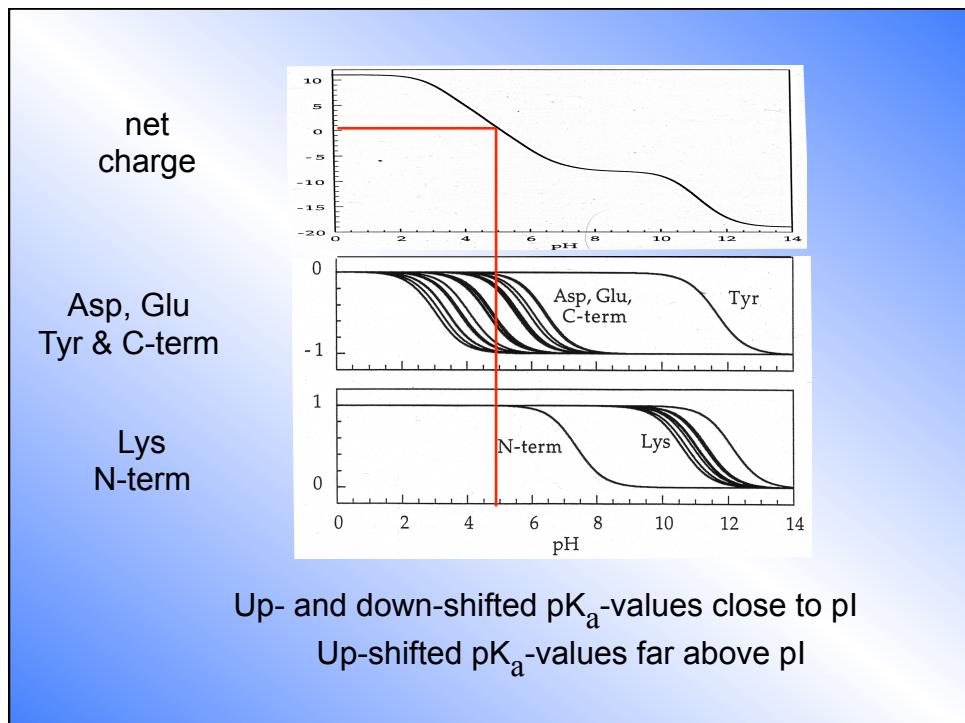
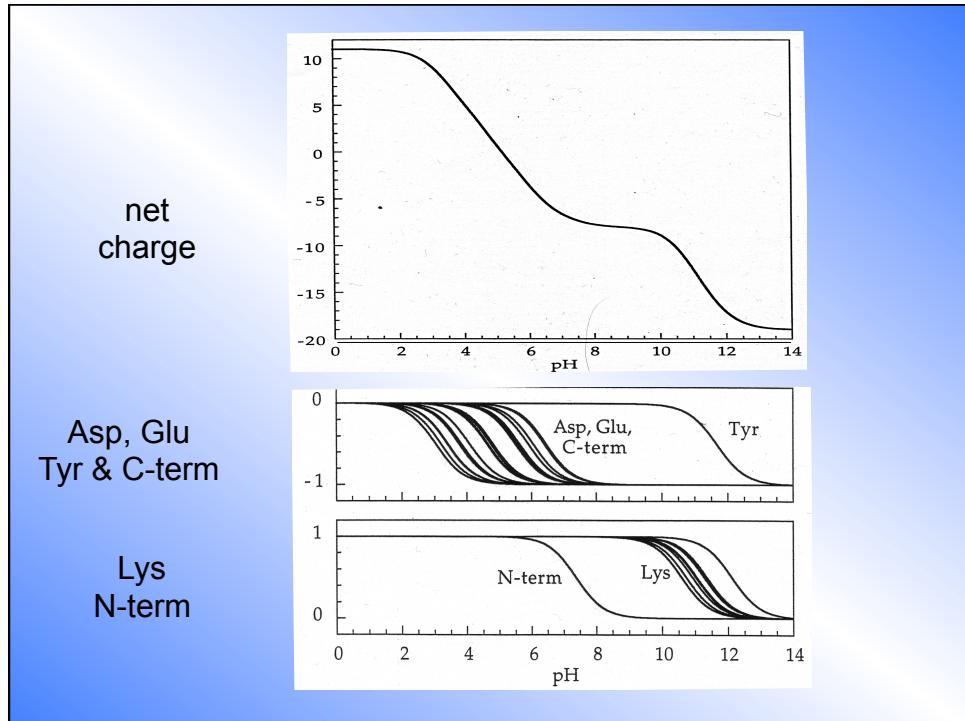
## Acknowledgement



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Biophysical Chemistry, Lund University

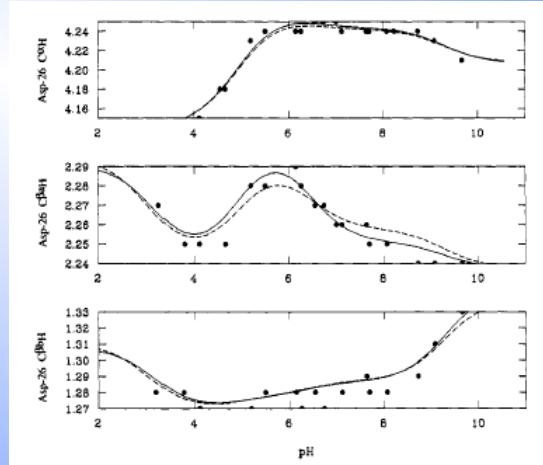
$pK_a$  values of ionizable groups in proteins are of interest as electrostatic interactions play an important role in functional aspects of proteins.

The ability to assign a  $pK_a$  to a specific ionizable group in a protein (based on its chemical shift) can aid in the mechanistic description of binding, catalysis, and other behaviour



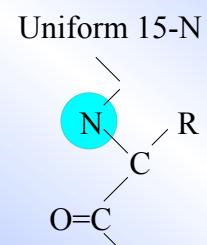
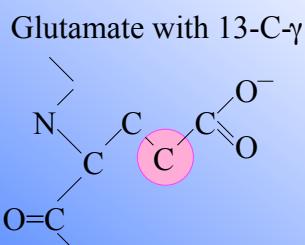
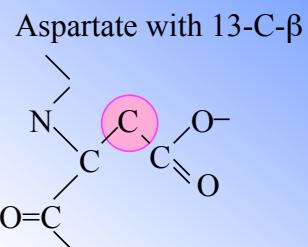
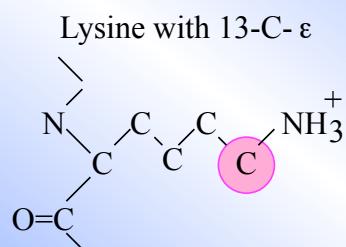
Traditional NMR approaches to pKa determination in proteins:

${}^1\text{H}$  1D  
 ${}^1\text{H}$  2D  
TOCSY

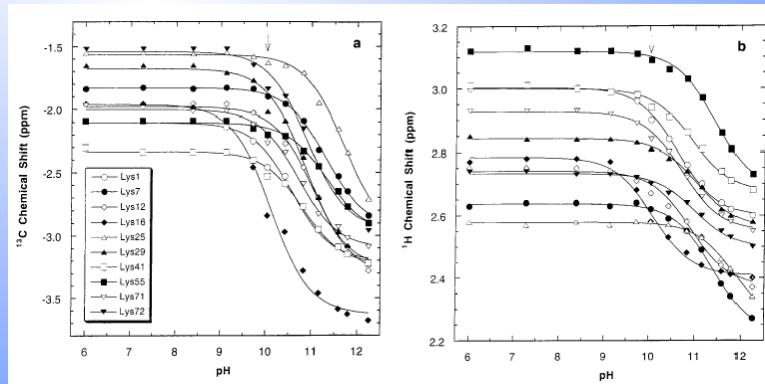


Forman-Kay et al. (1992) *Biochemistry* **31**, 3442-52

Isotope labeling for pK<sub>a</sub> values by NMR



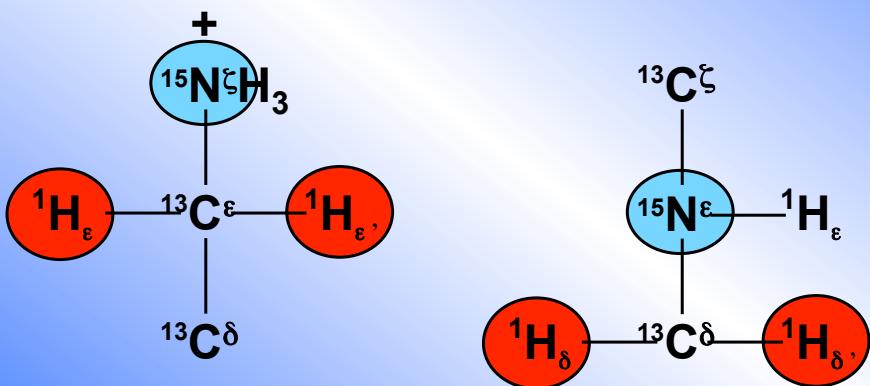
## pK<sub>a</sub> values of lysines using <sup>13</sup>C and <sup>1</sup>H



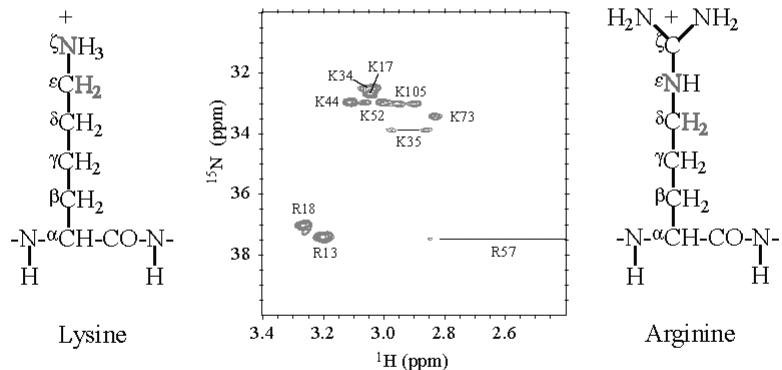
0.5 mM apo Calbindin D<sub>9k</sub> (low salt)  
 [Kesavatera et al. (1996), *J Mol Biol* **259**, 828]

## The H(C)N experiment

correlates amino <sup>15</sup>N chemical shift with H<sup>ε</sup> (Lys)  
 correlates amino <sup>15</sup>N chemical shift with H<sup>δ</sup> (Arg)

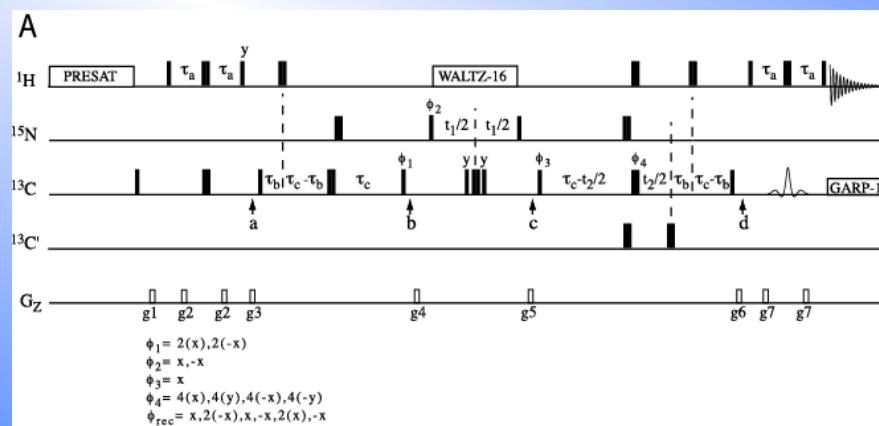


# The H(C)N experiment

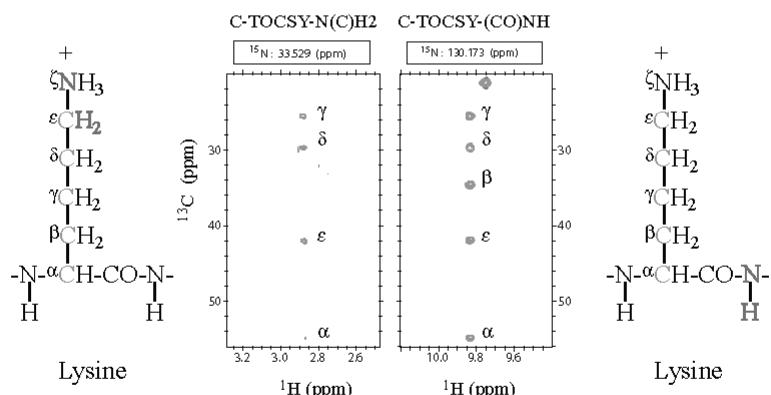


FKBP12, pH 7.0 [André, et al. *JACS* (2007).]

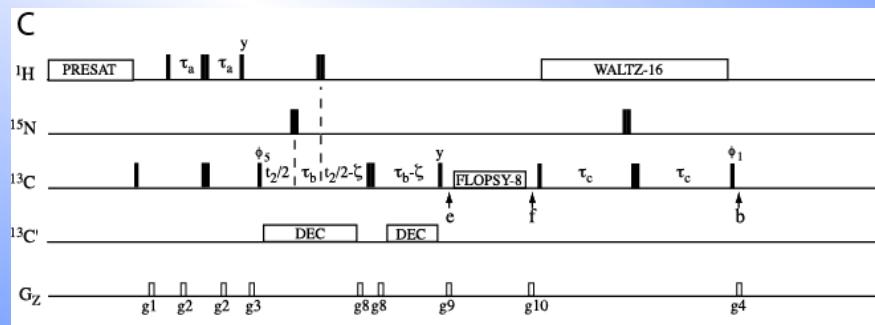
# The H(C)N experiment

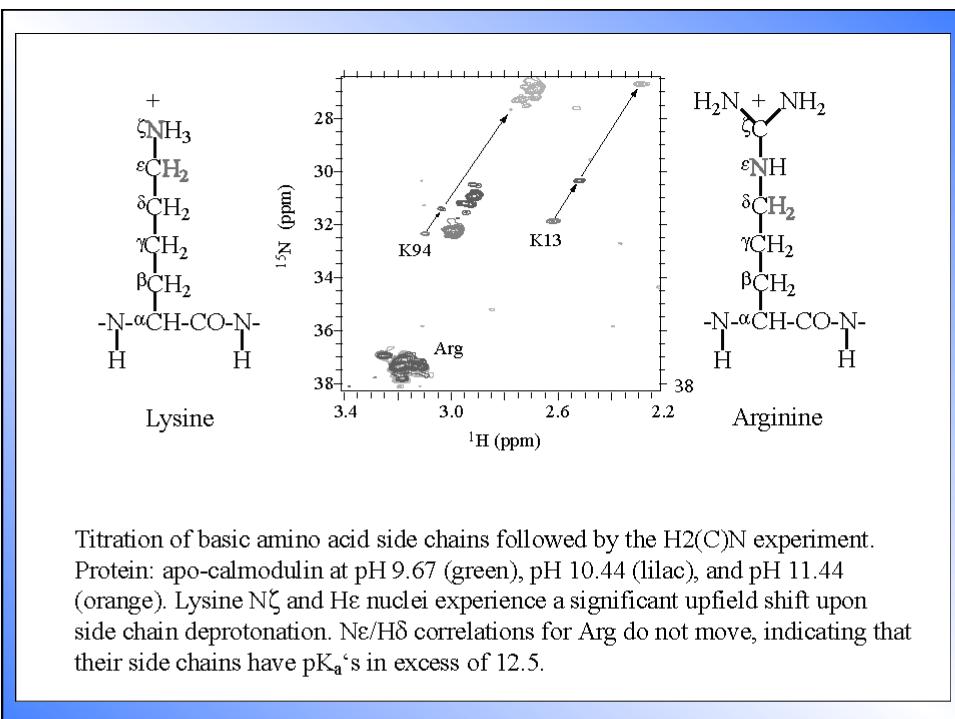


# The C-TOCSY(N)H experiment

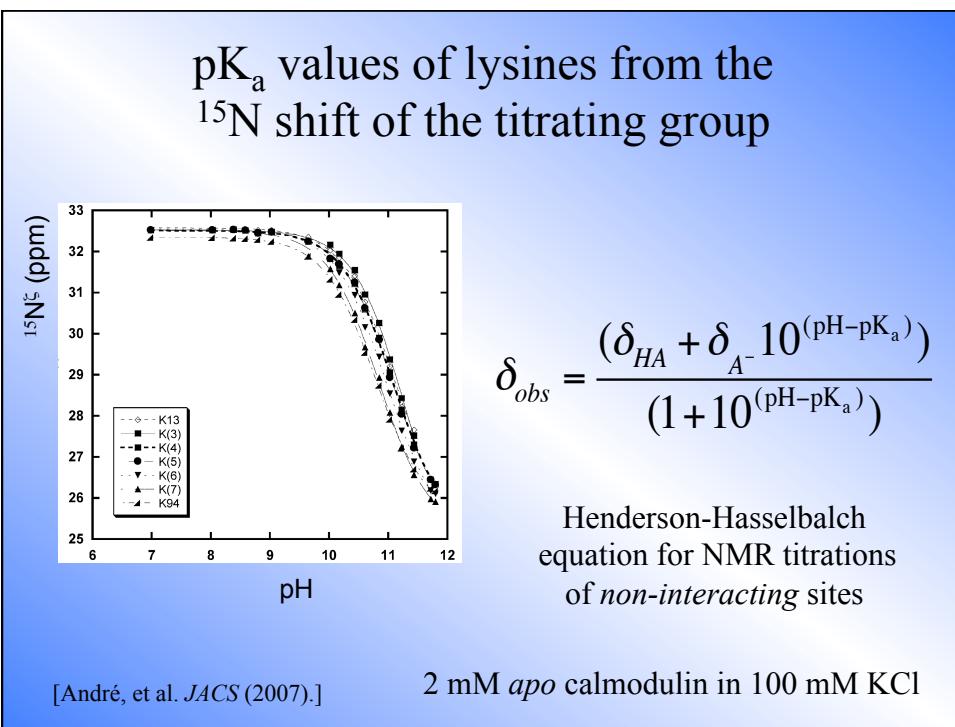


# The C-TOCSY(N)H experiment





Titration of basic amino acid side chains followed by the H<sub>2</sub>(C)N experiment.  
 Protein: apo-calmodulin at pH 9.67 (green), pH 10.44 (lilac), and pH 11.44 (orange). Lysine N $\zeta$  and H $\epsilon$  nuclei experience a significant upfield shift upon side chain deprotonation. N $\epsilon$ /H $\delta$  correlations for Arg do not move, indicating that their side chains have pK<sub>a</sub>'s in excess of 12.5.



## pK<sub>a</sub> values of lysines from the <sup>15</sup>N shift of the titrating group

**Table 2.** pK<sub>a</sub> Values of Lysine Residues in *apo* Calmodulin As Determined from N<sup>ε</sup> or H<sup>ε</sup> at 25 °C in 100 mM KCl; K(x) Indicates That the Residue Is Not Assigned

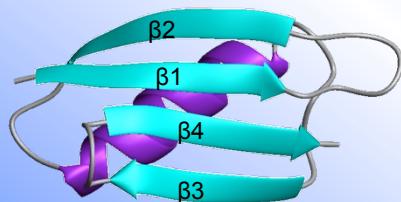
residue	N <sup>ε</sup>	H <sup>ε</sup>	N <sup>ε</sup> + H <sup>ε</sup>
K13	10.74 ± 0.02	10.72 ± 0.06	10.74 ± 0.02
K94	11.03 ± 0.03	11.21 ± 0.08	11.05 ± 0.04
K(3)	11.22 ± 0.03	11.14 ± 0.14	11.22 ± 0.05
K(4)	11.04 ± 0.02	11.10 ± 0.09	11.04 ± 0.02
K(5)	11.03 ± 0.02	11.05 ± 0.07	11.02 ± 0.02
K(6)	10.92 ± 0.01	10.81 ± 0.07	10.93 ± 0.02
K(7)	10.80 ± 0.01	10.81 ± 0.08	10.80 ± 0.02
K(8)	10.93 ± 0.02	10.95 ± 0.08	10.93 ± 0.03

remember, the model pK<sub>a</sub> = 10.4

[André, et al. *JACS* (2007).]

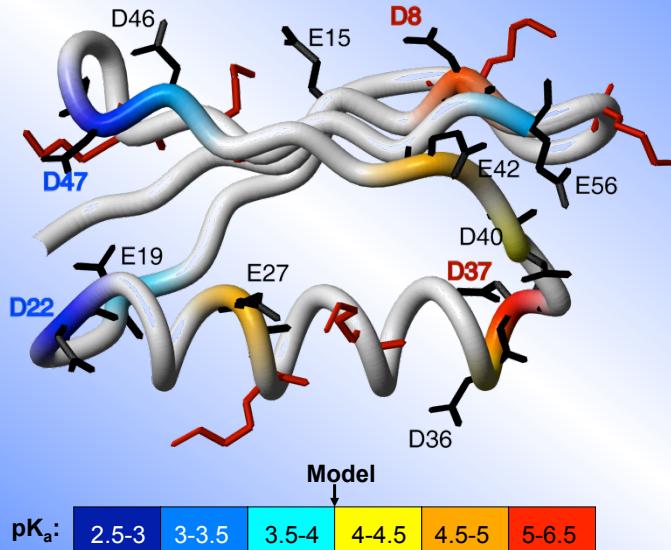
## pH-dependent stability to unfolding for the B1 domain of protein G

### PGB1-QDD



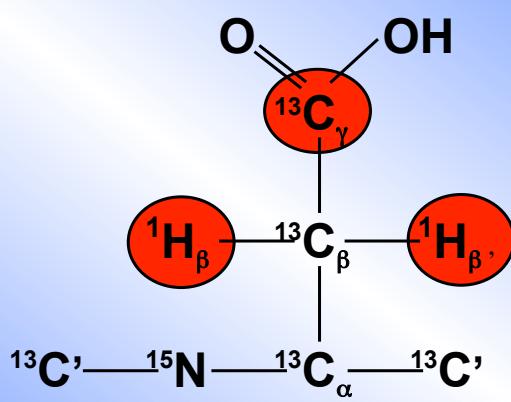
- Variant to ensure unprocessed protein
- Contains 2 extra negative charges, D8 and D37
- Total of 12 side-chain carboxyl groups

### Color coding according to $pK_a$



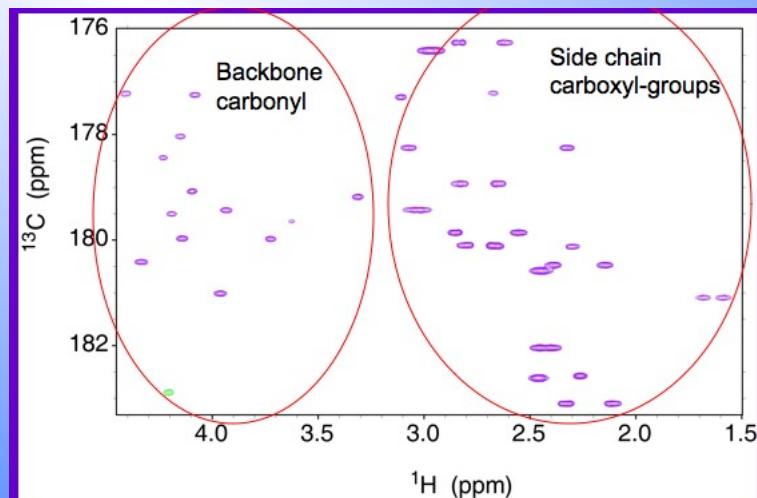
The H(C)CO experiment  
correlates carboxyl  $^{13}\text{C}$  chemical shift with  $\text{H}_\beta$  or  $\text{H}_\gamma$

For Asp:

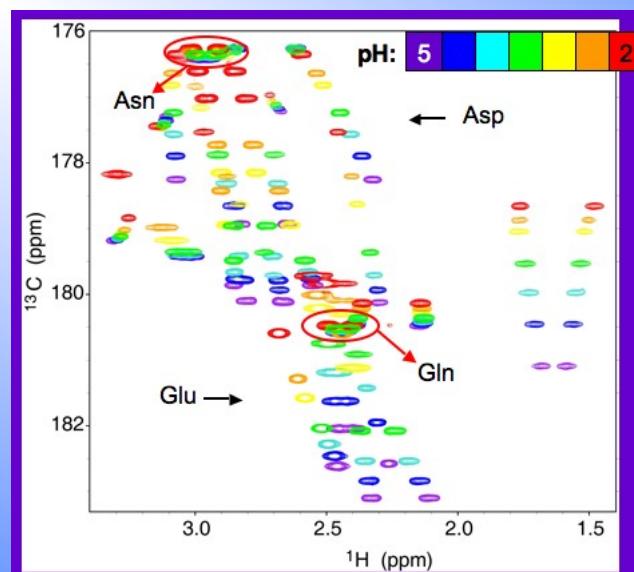


[ Oda et. al, Biochemistry 33 (1994) 5275]

## Spectrum at pH 5 good dispersion and sensitivity



## Spectra pH 2-5

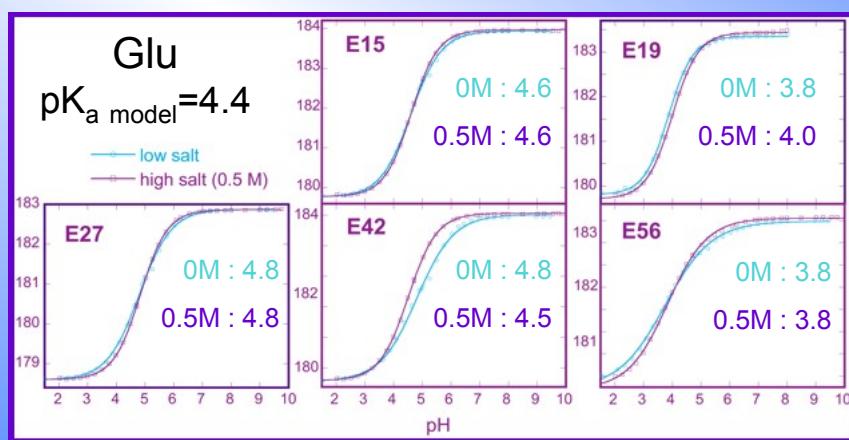


Henderson-Hasselbalch equation:  
a Hill parameter is now necessary to  
fit the data

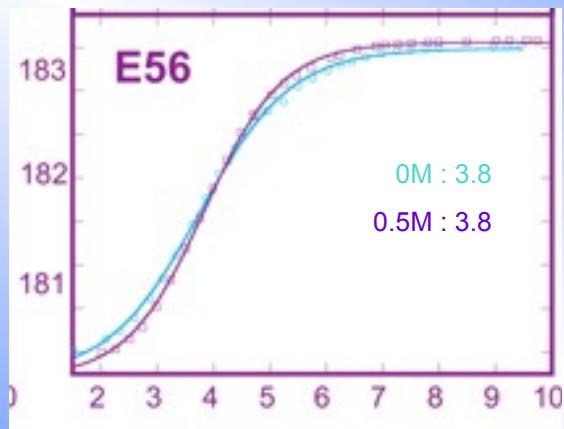
$$\delta_{obs} = \frac{(\delta_{HA} + \delta_{A^-}) 10^{n_h(pH - pK_a)}}{(1 + 10^{n_h(pH - pK_a)})}$$

Hill parameter  
to account for  
cooperativity

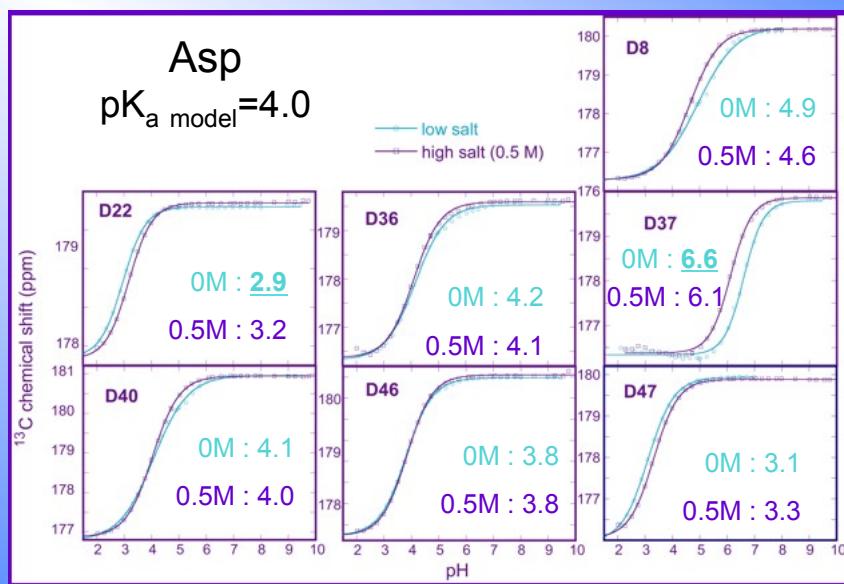
### pK<sub>a</sub> values of side-chain carboxyl groups at 0 M and 0.5 M NaCl



## pK<sub>a</sub> values of side-chain carboxyl groups at 0 M and 0.5 M NaCl: zoom in

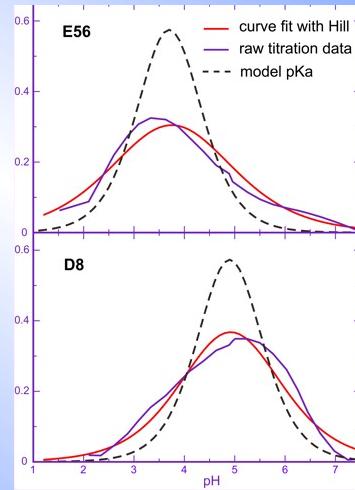


## pK<sub>a</sub> values continued

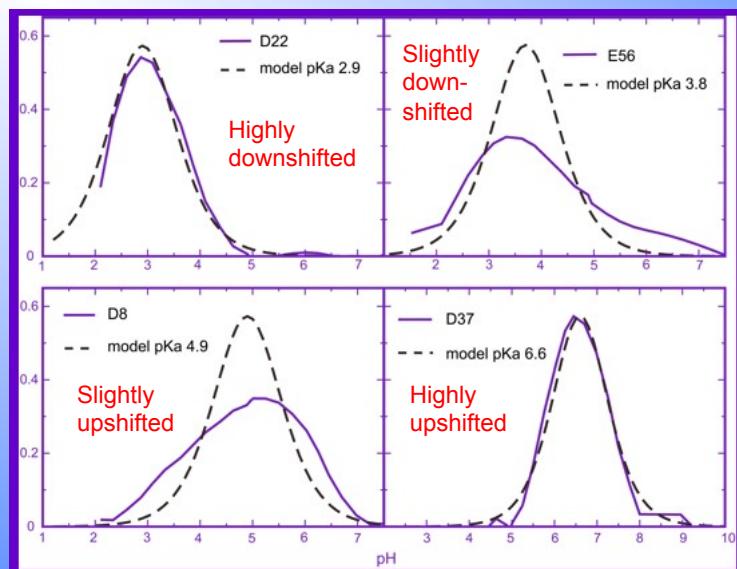


## Proton Binding Capacitance (PBC)

- PBC = first derivative of the titration curve with respect to pH
- Curve fitting with Hill-parameter cannot account for asymmetric titration curves

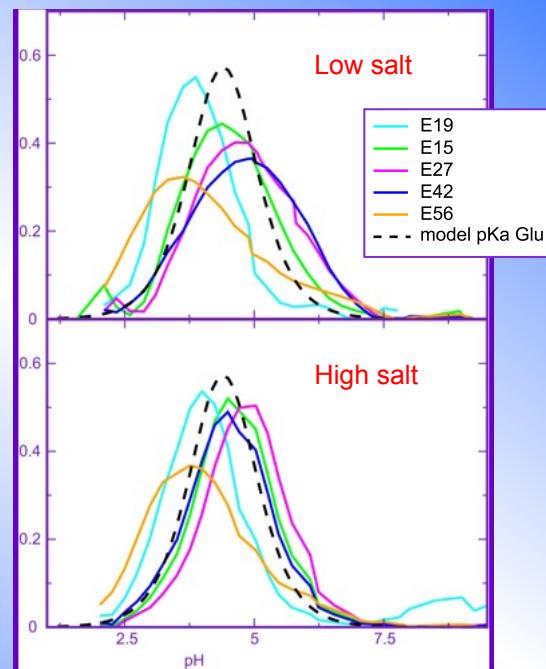


## PBC and titration asymmetry



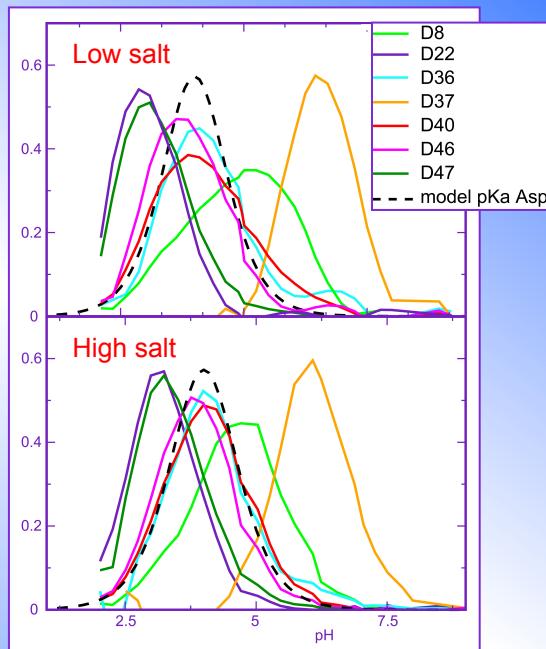
## Salt dependence of Glu pK<sub>a</sub>

- Derivative of titration curve explains cooperativity and negative-cooperativity
- Titration behavior approaches model with addition of salt

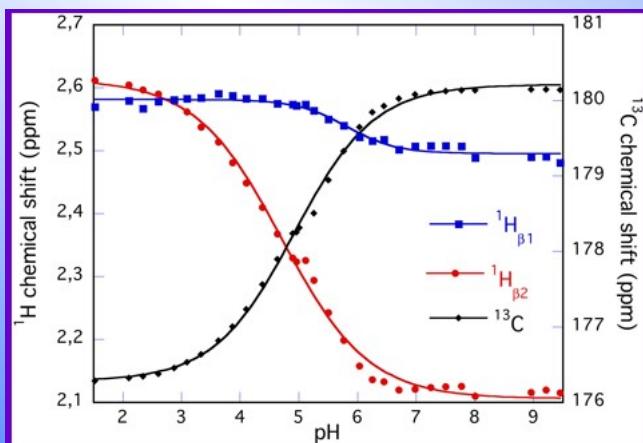


## Cooperativity of Asp

- Derivative of titration curves explain cooperativity and negative-cooperativity
- Titration behavior approaches model with addition of salt

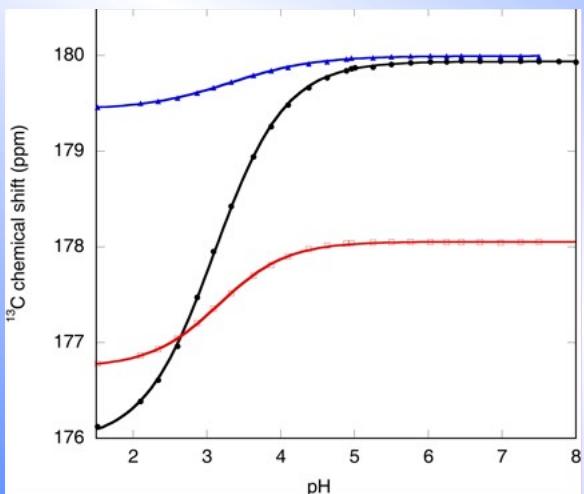


$pK_a$  values for Asp8 from  $^{13}\text{C}$  and  $^1\text{H}$  chemical shifts  
 Major differences between the two protons!



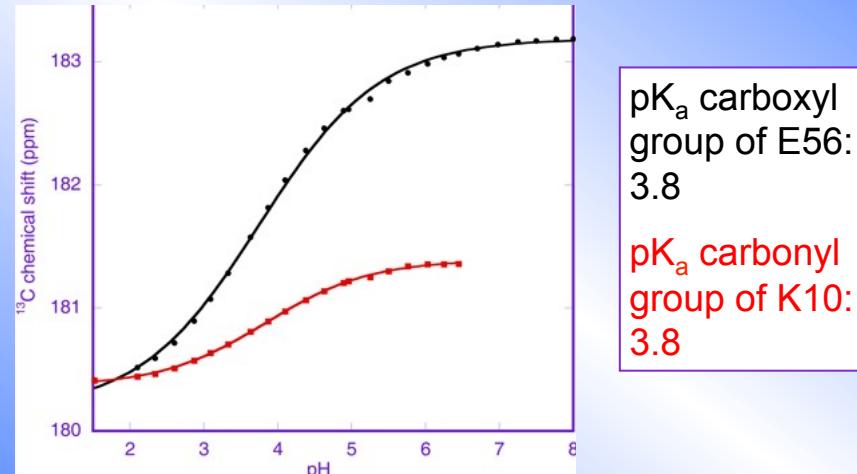
$pK_a$  CO: 4.9  
 $pK_a$   $\text{H}_{\beta 1}$ : 5.8  
 $pK_a$   $\text{H}_{\beta 2}$ : 4.7

Carbonyl  $^{13}\text{C}$  shifts for Asp47 and Ala48 report  $pK_a$  value of Asp47 carboxyl group



$pK_a$  carboxyl group of D47: 3.1  
 $pK_a$  carbonyl group of D47: 3.2  
 $pK_a$  carbonyl group of A48: 3.3

## Carbonyl shifts report on hydrogen bonds



pK<sub>a</sub> carboxyl group of E56: 3.8  
pK<sub>a</sub> carbonyl group of K10: 3.8

## How to calculate pH-dependent stability based on pK<sub>a</sub> values

$$\delta\Delta G_U(pH) = \sum_i \int 2.3RT(q_U^i - q_F^i)\delta pH$$

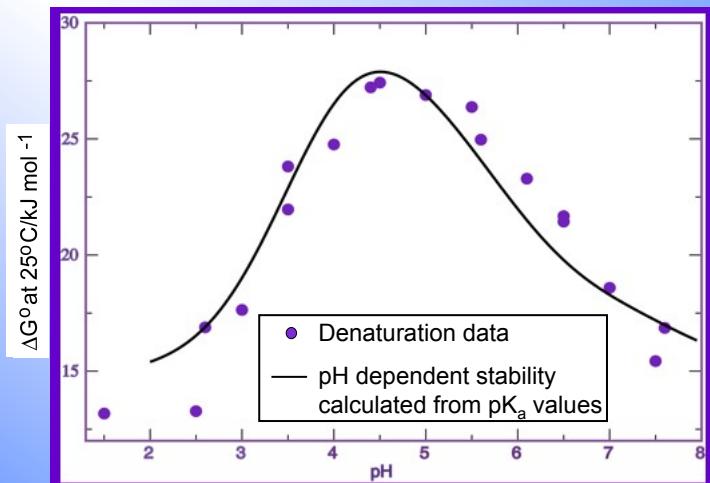
$$q^i = \frac{1}{1 + 10^{n_h(pH - pK_a^i)}}$$

$$q^i = \frac{-1}{1 + 10^{n_h(pK_a^i - pH)}}$$

- Experimental pKa values for native state

- Denatured state treated as random coil and pK<sub>a</sub> values were calculated using a Gaussian chain model [ Zhou, PNAS 99 (2002), 3569]

## $pK_a$ values explain pH-dependent stability



[ Lindman et. al, Biophys J. (2010) 99:3365-73]

## Conclusions -1

- chemical shifts of <sup>13</sup>C and <sup>15</sup>N in protein are specific and accurate reporters of side chain protonation states
- carefully designed NMR experiments can monitor the heteronuclear chemical shifts in 2D spectra with high sensitivity and good resolution
- site-specific protonation constants can be determined

## Conclusions -2

- pK<sub>a</sub> values show that pH-dependent stability can be explained purely by electrostatics
- Electrostatic contributions to protein stability are small (but not to kinetics ...!)
- There is clear evidence of local electrostatic coupling between acidic side chains in PGB1, but not for basic side chains in *apo* CaM
- We directly detect - for the first time - asymmetry in the pH-dependent protonation of individual acidic groups in proteins as a result of the change in protein electrostatic energies with pH