The MIRG 2002 Study:

Assembly State, Thermodynamic and Kinetic Analysis of an Enzyme/Inhibitor Interaction
Model System

Carbonic Anhydrase II
(CA-II = ~30 kDa)

4-Carboxybenzenesulfonamide
(CBS = 201.2 Da)
Goals

AUC – Mass & assembly state

ITC – Affinity & Thermodynamics

SPR – Affinity & Kinetics
Analytical Ultracentrifuge
Two Types of Experiments

Velocity

55k rpm

Equilibrium

8-40k rpm

M, Kd, stoichiometry

M, D, sed coefft
AUC PARTICIPANTS

- Samples sent to 13 groups
- Results returned so far by 5 groups
AUC SAMPLES

- CA-II supplied as lyophilised powder (" 93% CA-II)
- Pre-run exhaustive dialysis against 20 mM sodium phosphate, 150 mM NaCl, pH 7.4
- Experimental conditions: 20°C (velocity) and 4°C (equilibrium)
- \( v_{\text{bar}} \) calculated from a.a. sequence based on consensus volumes (Perkins 1986)
EQUILIBRIUM

- 3 concentrations
  - (0.9, 0.3, 0.1 a.u. absorbance optics)
  - (1.0, 0.33, 0.01 mg/ml interference optics)
- 3 rotor speeds (24k, 28k, 34k rpm)
- Analyse with favourite software
- Determine molecular weight
VELOCITY

• 3 concentrations
  – (0.9, 0.3, 0.1 a.u. absorbance optics)
  – (1.0, 0.3, 0.2, 0.01 mg/ml interference optics)
• Rotor speed 50k rpm
• Analyse with favourite software
• Determine molecular weight (and sedimentation coefficient)
AUC (Equilibrium) Results

- M (kDa):
  - 1 (24k)
  - 1 (28k)
  - 1 (34k)
  - 2 (24k)
  - 2 (28k)
  - 2 (34k)
  - 3 (global)
  - 4 (global)
  - 5 (24k)
  - 5 (28k)
  - 5 (34k)

- [CA-II] (mg/ml):
  - 0.0
  - 0.5
  - 1.0

- AUC Values:
  - 25.0
  - 27.0
  - 29.0
  - 31.0
  - 33.0
  - 35.0
  - 37.0
  - 39.0
  - 41.0
  - 43.0
  - 45.0
AUC (Velocity) Results

$\text{[CA-II]} \, (\text{mg/ml})$

$s_{20,w} \, (\text{S})$

1 (Lamm)
2 (vH-W)
3 (sedfit)
3 (sved)
3 (dcdt+)
5 (sedfit sp 1)
5 (sedfit sp2)
5 (sedfit sp3)
Summary

- AUC able to verify integrity of sample
- Results sensitive to both method and analysis
- AUC community is very heterogeneous
- AUC activists are very poor at responding to deadlines
- AUC part of MIRG project is very much ongoing
Titration Calorimetry
Summary: ITC Set-up

Number of participants: 12

Instruments used:  
  MicroCal VP-ITC  (9 submissions)  
  MicroCal MCS-ITC  (2 submissions)  
  CSC 4200  (1 submission)

Total number of titrations: 27

C-value range: 6 to 134

$\chi^2$ range: 1114 to 117140
<table>
<thead>
<tr>
<th>ID#</th>
<th>Make</th>
<th>[CAII] (µM)</th>
<th>[CBS] (µM)</th>
<th>Inj. vol. (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>MC MCS-ITC</td>
<td>16</td>
<td>390</td>
<td>15 x 10</td>
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<tr>
<td>22</td>
<td>CSC 4200</td>
<td>70.8</td>
<td>556</td>
<td>21 x 10</td>
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<td>24</td>
<td>MC VP-ITC</td>
<td>42</td>
<td>400</td>
<td>2 + 31 x 8</td>
</tr>
<tr>
<td>26</td>
<td>MC VP-ITC</td>
<td>22.65 or 22.9</td>
<td>870 or 642</td>
<td>0.1 + 60 x 4.5</td>
</tr>
<tr>
<td>28</td>
<td>MC VP-ITC</td>
<td>23.4 or 39</td>
<td>665</td>
<td>1 + 25 x 10</td>
</tr>
<tr>
<td>30</td>
<td>MC VP-ITC</td>
<td>35 or 37</td>
<td>780 or 380</td>
<td>1 + 25 x 10</td>
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<tr>
<td>36</td>
<td>MC VP-ITC</td>
<td>30.5 or 56.1</td>
<td>1144</td>
<td>2 + (24 or 29) x 5</td>
</tr>
<tr>
<td>37</td>
<td>MC VP-ITC</td>
<td>31.5</td>
<td>384</td>
<td>14 x 17 or 30 x 8</td>
</tr>
<tr>
<td>42</td>
<td>MC VP-ITC</td>
<td>8.61</td>
<td>100</td>
<td>2 + 28 x 10</td>
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<tr>
<td>43</td>
<td>MC VP-ITC</td>
<td>28</td>
<td>896</td>
<td>3 +39 x 3</td>
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<tr>
<td>44</td>
<td>MC VP-ITC</td>
<td>11.4</td>
<td>114</td>
<td>1.5 +15 x 18.7</td>
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<tr>
<td>48</td>
<td>MC MCS-ITC</td>
<td>21.4 to 23.4</td>
<td>300</td>
<td>2 + 19 x 10</td>
</tr>
</tbody>
</table>
ID 36_2
MicroCal VP-ITC
1144 μM CBS
56.1 μM CAII
C-value = 51
2 + 29 x 5 μL inj.
210 second intervals
310 rpm stir speed

Reported average values:

N = 0.87 (±0.03)
K = 1.11 (±0.05) x 10^6 M^-1
ΔH = -11.54 (±0.007) kcal/mol
### Binding Parameters from ITC Data Submissions

<table>
<thead>
<tr>
<th>ID#</th>
<th>Stoichiometry</th>
<th>$K_a$ (M$^{-1}$x10$^{-6}$)</th>
<th>$\Delta H$ (kcal/mol)</th>
</tr>
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<tbody>
<tr>
<td>9</td>
<td>1.01</td>
<td>1.23</td>
<td>-8.93</td>
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<td>22</td>
<td>0.95</td>
<td>1.44</td>
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<td>0.81</td>
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<td>0.93</td>
<td>0.84</td>
<td>-10.8</td>
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<td>28</td>
<td>0.987</td>
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<td>-10.2</td>
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<td>30</td>
<td>0.921</td>
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<td>37</td>
<td>0.961</td>
<td>0.92</td>
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<td>43</td>
<td>1.000</td>
<td>1.01</td>
<td>-10.51</td>
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<tr>
<td>44</td>
<td>0.9</td>
<td>1.1</td>
<td>-13</td>
</tr>
<tr>
<td>48</td>
<td>0.52</td>
<td>1.3</td>
<td>-12</td>
</tr>
<tr>
<td>Average</td>
<td>0.93</td>
<td>1.1</td>
<td>-10.7</td>
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<tr>
<td>Stand. Dev.</td>
<td>0.17</td>
<td>0.2</td>
<td>1.6</td>
</tr>
</tbody>
</table>
ITC Summary

ITC Set-up

Number of participants: 12

Instruments used:
- MicroCal VP-ITC (9 submissions)
- MicroCal MCS-ITC (2 submissions)
- CSC 4200 (1 submission)

Total number of titrations: 27
C-value range: 6 to 134
Chi² range: 1114 to 117140
CBS molar extinction coefficient range: 1110 to 1430 M⁻¹ cm⁻¹
ITC Thermodynamic Parameters

Average of reported thermodynamic parameters:
n = 0.92 (±0.17)
Ka = 1.1 (±0.2) x 10^6 M\(^{-1}\)
\(\Delta H = -10.7 (±1.6)\) kcal/mol

Average of reported thermodynamic parameters after removing anomalous data set:
n = 0.96 (±0.11)
Ka = 1.1 (±0.2) x 10^6 M\(^{-1}\)
\(\Delta H = -10.6 (±1.6)\) kcal/mol

Average of partially optimized* thermodynamic parameters:
n = 0.94 (±0.10)
Ka = 1.1 (±0.3) x 10^6 M\(^{-1}\)
\(\Delta H = -10.5 (±1.0)\) kcal/mol

*Removed anomalous data set, normalized CBS concentration and varied some of the dilution control correction constants
Biosensor Experiments

- 200 µg CAII powder
- 100 µL of a 20 mM CBS in PBS
- Detailed protocol

⇒ Cleaning
⇒ Blank injections
⇒ Immobilize CAII
⇒ Inject CBS (20 to 0.24 µM)
⇒ Data processing and fitting
Immobilization

\[
\text{Ave} = 6,100 \pm 2000 \text{ RU (N = 36)}
\]

- Activation NHS/EDC
- Coupling CAII, pH 5.2
- Blocking ethanolamine

6540 RU
6260 RU
5610 RU
5080 RU
4860 RU
Data Processing

6a: 4960 RU CA II

CBS (20 to 0.24 µM)
Zero on the Y-Axis

6a: 4960 RU CA II
Zero on the x-Axis
Subtract Reference Surface
Subtract Blank Injections

6a: 4960 RU CA II

Response (RU) vs. Time (s) graph.
Check Reference Surface

![Graph showing the response over time with a vertical line at 0 time indicating an unmodified state.](image)
Global Data Fitting

Graphs showing data trends and fits, labeled 'CBS.txt'.

Fit parameters:
- Reaction: A + B = AB
  - k_fwd: 0.00
  - k_rev: 0.00
- Initial Conc:
  - A: Inj 1
  - B: 0.00
  - AB: 0.00
- Refractive Index

Abbreviations and units are not specified in the image.
Final Fit

Reaction
\[ A + B = AB \]
- \[ k_{fwd} = \text{26881} \]
- \[ k_{rev} = \text{0.03947} \]

Initial Conc:
- \[ A \text{ Inj: 1} \]
- \[ B = 17.93 \]
- \[ AB = 0.00 \]

Refractive Index

\[ K_D = \frac{k_{rev}}{k_{fwd}} = \text{1.4 \, \text{uM}} \]
Fit parameters:

- Reaction:
  - $A + B = AB$
  - $k_{fwd} = 43.900$
  - $k_{rev} = 0.03389$

- Initial Conc:
  - $A = 0.1$
  - $B = 2.66$
  - $AB = 0.00$

- Refractive Index

Graph showing data with $0.77 \mu M$ peak.
0.89 μM
0.83 μM
Kinetics

Association Rate

\[ k_a = 40,700 \pm 7,600 \text{ (M}^{-1} \text{s}^{-1}) \]

Dissociation Rate

\[ k_d = 0.035 \pm 0.005 \text{ (s}^{-1}) \]
$K_D = 0.90 \pm 0.24 \, \mu M$
Affinity Comparison

**ITC**

**SPR**

![Graph showing K_d (µM) comparison between ITC and SPR methods.](image)
Van’t Hoff Analysis

$\Delta H^{\text{vant}} = -10.6 \pm 1.4 \text{ kcal/mol}$
Enthalpy Comparison

ITC

SPR
## Data Summary

<table>
<thead>
<tr>
<th></th>
<th>AUC</th>
<th>ITC</th>
<th>SPR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MW (28.98 kDa)</strong></td>
<td>27.83 ± 0.75</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Stoichiometry</strong></td>
<td></td>
<td>0.94 ± 0.10</td>
<td></td>
</tr>
<tr>
<td><strong>$K_D$ (μM)</strong></td>
<td></td>
<td>0.94 ± 0.17</td>
<td>0.90 ± 0.24</td>
</tr>
<tr>
<td><strong>$\Delta H$ (kcal/mol)</strong></td>
<td>–10.8 ± 1.6</td>
<td>–10.6 ± 1.4</td>
<td></td>
</tr>
<tr>
<td><strong>$k_a$ (M$^{-1}$ s$^{-1}$)</strong></td>
<td></td>
<td></td>
<td>40,700 ± 7,600</td>
</tr>
<tr>
<td><strong>$k_d$ (s$^{-1}$)</strong></td>
<td></td>
<td></td>
<td>0.035 ± 0.005</td>
</tr>
</tbody>
</table>

20 mM Phosphate, 150 mM NaCl, pH 7.4, 25°C
Acknowledgements

Poster R1
www.abrf.org