Foundation for Inorganic Chemistry Lectures - 2007

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1. Jack bean urease: the nickel metalloenzyme
2. Metallohydrolase models
3. Exonucleolytic proofreading in DNA replication
4. The proofreading exonuclease: engineering, specificity and lanthanides
5. Binuclear metallohydrolases: how do they really work?
6. Structure and mechanism of urease: a binuclear metallohydrolase

USyd Chem 17-04-07
Jack Bean Urease: the Nickel Metalloenzyme

\[ \text{H}_2\text{NCONH}_2 \rightarrow (\text{NH}_4^+)_2\text{CO}_3^{2-} \]

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USyd Chem 17-04-07
Canavalia ensiformis

Jack bean
Sword bean
James B. Sumner (1887-1955)

Nobel Laureate in Chemistry (1946)

Studied biochemistry with Otto Folin at Harvard Medical School.

Although Folin advised him to take up Law, since he thought that a one-armed man could never make a success of chemistry, Sumner persisted and obtained his Ph.D. degree in June, 1914.

Assistant professor, Cornell Medical School (1914)

(Nobel Lectures, Chemistry 1942–1962, Elsevier, Amsterdam, 1964)
However, upon observing a drop of the liquid under the microscope it was seen to contain many tiny crystals. These were of a shape that I had never observed previously. I centrifuged off some of the crystals and observed that they dissolved readily in water. I then tested this water solution. It gave tests for protein and possessed a very high urease activity. I then telephoned to my wife, "I have crystallized the first enzyme".
Urease efficiently hydrolyses are very stable substrate

\[
\text{H}_2\text{N-CO-NH}_2 \rightarrow \text{H}_2\text{NCOOH} + \text{NH}_3 \rightarrow \text{CO}_2 + 2\text{NH}_3
\]

\[k_{\text{cat}} = 3000 \text{ s}^{-1}\]

\[k_{\text{cat}} \approx 4 \times 10^{-12} \text{ s}^{-1} \text{ (pH 7, 25°C)}\]

\[
\text{H}_2\text{N-CO-NH}_2 \rightarrow \text{HN=C=O} + \text{NH}_3 \rightarrow \text{CO}_2 + 2\text{NH}_3
\]

\[k \approx 5 \times 10^{-10} \text{ s}^{-1} \text{ (pH 7, 25°C)}\]

Zerner group (University of Queensland)  
*Biochemistry* (1969)

Jack Bean Urease (EC 3.5.1.5). A New Purification and Reliable Rate Assay*

Robert L. Blakeley,† Edwin C. Webb, and Burt Zerner

- Purification by crystallization + chromatography
- pH stat assay

Jack Bean Urease (EC 3.5.1.5). Demonstration of a Carbamoyl-Transfer Reaction and Inhibition by Hydroxamic Acids*

Robert L. Blakeley,† John A. Hinds,† Hugo E. Kunze, Edwin C. Webb, and Burt Zerner

\[
\text{H}_2\text{N-CO-NH}_2 \quad \rightarrow \quad \text{H}_2\text{NCOOH} + \text{NH}_3 \quad \rightarrow \quad \text{CO}_2 + 2\text{NH}_3
\]

Hydroxamates as slow, tight binding inhibitors: \( \text{R-CO-NHOH} \)

- Metal ion analysis
Assay of urease

Jack Bean Urease (EC 3.5.1.5). A New Purification and Reliable Rate Assay*

Robert L. Blakeley,† Edwin C. Webb, and Bart Zerner

- pH stat assay - measures uptake of acid at constant pH

\[ \text{Na}_2\text{B}_4\text{O}_7 \text{ (borax)} + \text{methyl red / bromocresol green} \]
Crystals of urease are apple green

Jack Bean Urease (EC 3.5.1.5). A New Purification and Reliable Rate Assay*

Robert L. Blakeley,† Edwin C. Webb, and Burt Zerner

- Purification by crystallization + chromatography
Equivalent weight of urease

Urease + $[^{14}\text{C}]-\text{acetohydroxamic acid}$ or $[^{32}\text{P}]-\text{phosphoramidate}$

gel filtration

Measure $^{14}\text{C}/^{32}\text{P}$ and activity

Equivalent weight 97 kDa
amino acid sequence: 840 $\alpha\alpha$, 91 kDa
Hexamer of 97 kDa subunits
(protein plus bound sugars)
Electronic absorption spectrum of urease

Urease, pH 7.0

Urease, pH 7.0 + 5 mM β-mercaptoethanol
Electronic absorption spectrum of urease

Titration of β-mercaptoethanol
$K_D = 0.95 \text{ mM}$
(note the wavelength scale cf. previous slide)

Thiols bind to the chromophore to produce charge transfer bands.

Inhibitors that bind to the catalytic site displace mercaptoethanol (next slide), so the chromophore must be at the catalytic site.
Electronic absorption spectrum of urease

Effect of acetohydroxamate (1 mM β-mercaptoethanol)

Second order rate constant for inhibition is the same as for spectral change,

... and is fully reversed on reactivation

Figure 1: Visible electronic absorption spectra of urease at 25°: (A) urease at 41.1 mg/ml in oxygen-free 0.028 M phosphate buffer, pH 7.0 (1 mM each in EDTA, β-mercaptoethanol); (A’) buffer baseline; (B) urease at 40.1 mg/ml, 10 min after the solution in A was made 10 mM in acetohydroxamic acid. Note that the spectra continue on the 0.5 to 1.0 absorbance range at low wavelength.
Electronic absorption spectrum of urease - effect of EDTA

Effect of EDTA treatment at low pH

Indicates that EDTA, a chelating agent removes the chromophore: suggests the chromophore is a metal ion.

Figure 8: Effect of EDTA on the visible spectrum of urease in oxygen-free 0.1 M acetate, pH 3.8 (1 mM in 8-mercaptoethanol). --- Urease at 11.3 mg/ml, [EDTA] = 0, spectrum recorded between 1 and 12 min after acidification. --- The same enzyme solution (11.0 mg/ml), 2.0 hr after it was made 1.0 mM in EDTA. The enzyme retained 6.1% of its original specific activity (assayed at pH 3.5). Spectra were recorded at 25.0°C, and have been corrected for variation in the solvent baseline.
# Inhibition of urease by EDTA

## Table III

Assays of urease samples prepared by partial inactivation in the presence of EDTA at low pH for specific enzymatic activity and metal ion content. 

<table>
<thead>
<tr>
<th>Specific Enzymatic Activity</th>
<th>Metal Ion Content (g-atom/97,000 g protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ni</td>
</tr>
<tr>
<td>[mkat/100 mg protein]</td>
<td></td>
</tr>
</tbody>
</table>

**Experiment I**

- 86.36<sup>c</sup>
  - 1.7<sub>d</sub>  
  - < 0.02<sup>d</sup>  
  - < 0.1<sup>d</sup>  
  - < 0.1<sup>e</sup>
- 77.0<sup>i</sup>
  - 1.8<sub>d</sub>  
  - < 0.02<sup>d</sup>  
  - < 0.1<sup>d</sup>  
  - < 0.1<sup>e</sup>
- 72.7<sup>j</sup>
  - 1.5<sub>d</sub>  
  - < 0.03<sup>d</sup>  
  - < 0.2<sup>d</sup>  
  - < 0.1<sup>e</sup>
- 45.35<sup>i</sup>
  - 1.0<sub>d</sub>  
  - < 0.03<sup>d</sup>  
  - < 0.2<sup>d</sup>  
  - < 0.1<sup>e</sup>
  - 0.8<sub>f</sub>  

**Experiment II**

- 78.45<sup>g</sup>
  - 1.8<sub>d</sub>  
  - < 0.07<sup>d</sup>
- 14.20<sup>j</sup>
  - 0.5<sub>d</sub>  
  - < 0.05<sup>d</sup>

**Experiment III**

- 82.56<sup>g</sup>
  - 1.7<sub>d</sub>
- 0<sup>k</sup>
  - 0.0<sub>f</sub>
- 0<sup>l</sup>
  - 0.06<sub>f</sub>

---

Urea + EDTA (pH 3.8)

**centrifugation**

Measure activity & metal ions (AAS)
Inhibition of urease by EDTA

Figure 7: Inhibition of urease by EDTA at low pH. Correlation of specific activity with nickel content for partially-inhibited (Δ) and native (○) enzyme samples. Conditions were as described in Table III. The line is a theoretical relationship which assumes that fully active urease (specific activity 93.0 [mkat/l.]/A₂₈₀) contains 2.0 g-atom nickel per mole of subunits (mol. wt. 97,000).
Nickel content of several urease preparations - 2 nickel ions per active site

<table>
<thead>
<tr>
<th>Specific Activity [mkat/l./A_{280}]</th>
<th>Nickel content (g-atom/97,000 g protein)</th>
<th>Method of analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dry weight samples</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>88.07</td>
<td>2.13</td>
<td>A</td>
</tr>
<tr>
<td>87.20</td>
<td>1.89</td>
<td>A</td>
</tr>
<tr>
<td><strong>Other samples</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>86.36</td>
<td>1.7</td>
<td>B</td>
</tr>
<tr>
<td>84.51</td>
<td>1.76</td>
<td>A</td>
</tr>
<tr>
<td>82.56</td>
<td>1.88, 1.90</td>
<td>A</td>
</tr>
<tr>
<td>81.8</td>
<td>1.8</td>
<td>B</td>
</tr>
<tr>
<td>1.6</td>
<td></td>
<td>C</td>
</tr>
<tr>
<td>77.45</td>
<td>1.85</td>
<td>A</td>
</tr>
<tr>
<td>76.73</td>
<td>1.79</td>
<td>A</td>
</tr>
<tr>
<td>73.55</td>
<td>1.78</td>
<td>A</td>
</tr>
<tr>
<td>72.2</td>
<td>2.02</td>
<td>A</td>
</tr>
<tr>
<td>67.86</td>
<td>1.93</td>
<td>A</td>
</tr>
</tbody>
</table>
Jack Bean Urease (EC 3.5.1.5). A Metalloenzyme. A Simple Biological Role for Nickel?

Sir:

In 1926, Sumner isolated from jack beans (Canavalia ensiformis) the first crystalline enzyme, urease, and defined the proposition that enzymes could be proteins devoid of organic coenzymes and metal ions.¹ It is therefore with some sadness that in this communication we adduce evidence which strongly indicates that urease is a nickel metalloenzyme.

Burt Zerner,

in Dixon et al., J. Am. Chem. Soc., 1975
Nickel content of jack beans

The commercial jack bean meal which we had been using suffered a decline in quality and we could obtain no urease crystals from it. Accordingly attempts were made to obtain satisfactory meal, or satisfactory jack beans. We grew jack beans in one of the Cornell greenhouses. The beans grew ... We obtained jack beans from Texas, Guatemala and Honduras, but these were low in urease.... Later on, we were able to obtain excellent jack beans from an Arkansas farmer. He has supplied us with jack beans ever since.

(J.B. Sumner, Nobel Lecture, 1946)

<table>
<thead>
<tr>
<th>Metal Ions (μg/g of seeds)</th>
<th>Parent seeds</th>
<th>Nickel-deficient seeds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nickel</td>
<td>7.96</td>
<td>0.48</td>
</tr>
<tr>
<td>Manganese</td>
<td>22.58</td>
<td>15.91</td>
</tr>
<tr>
<td>Cobalt</td>
<td>0.56</td>
<td>0.16</td>
</tr>
<tr>
<td>Iron</td>
<td>16.65</td>
<td>20.85</td>
</tr>
</tbody>
</table>

[Urease activity] (μkat/g of seeds)  

| powder | 69.12 | 7.102 |
| seeds  | ν62   | ν6.4  |

[Urease Activity]/[Nickel](kat/g)  

| ν7.8   | ν13.3 |

Proportion of total Ni in active urease (%)  

| ν16.4  | ν27.9 |
Urease: the first proposal for a mechanism involving a binuclear metal centre

Inhibition by phosphoramidate
Attempts to remove nickel from urease - laboratory demonstration of the Donnan equilibrium

\[
\text{urease} \rightarrow \text{urease} + (\text{NH}_4^+)_2\text{CO}_3^{2-}
\]

\[\text{5 M urea} \quad \text{O/N}\]
Urease crystals: X-ray diffraction

50 mM citrate, pH 6.4
Space group: cubic $F_{4132}$
Unit cell: $a = 358 \text{ Å}$

$\sim 37\% \text{ H}_2\text{O}$
Asymmetric unit: $\sim 200 \text{ kDa}$
(P.M. Colman)

Too difficult at the time, but now possible to determine the structure of urease (lecture 6)