Bioanalytical chemistry

2. Enzymes as analytical reagents

Suggested reading: Sections 3.1 to 3.5.1.3 of Mikkelsen and Cortón, *Bioanalytical Chemistry*

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**Primary Source Material**
- Chapter 8 of Biochemistry: Berg, Jeremy M.; Tymoczko, John L.; and Stryer, Lubert (NCBI bookshelf).
- http://chem.ch.huji.ac.il/~eugeniik/index.htm
- http://chem.ch.huji.ac.il/~eugeniik/electron_mediators.htm
Enzymes used for analytical purposes: alcohol dehydrogenase oxidizes alcohols

- Alcohol dehydrogenase has proven to be one of the more useful enzymes for bioanalytical applications. When might you want to detect the presence of alcohols?
- There are many other examples of enzymes that can be used either directly or indirectly as diagnostic reagents [from Table 9.2 of Gary Walsh, Proteins: Biochemistry and Biotechnology, John Wiley & Sons; 2nd edition (2002)]. Here are a few:
  - Arginase: determination of L-arginine levels in plasma and urine
  - Cholesterol esterase: determination of serum cholesterol levels
  - Creatine kinase: diagnosis of cardiac and skeletal malfunction
  - Glycerol-3-phosphate dehydrogenase: determination of serum triglycerides
  - Uricase: determination of uric acid

  - Q: With alcohol dehydrogenase is NAD+ a co factor? And would the enzyme have a spot of the molecule and NAD+ to facilitate the formation of the transition state?
  - A: In this case, NAD+ is best described as a substrate for the enzyme. That is, the enzyme catalyzes the reaction: alcohol + NAD+ -> aldehyde + NADH. The active site of the enzyme has binding sites for the both the alcohol and the NAD+. 
Enzymes used for analytical purposes: Glucose oxidase catalyzes glucose oxidation

- Glucose oxidase catalyzes the oxidation of glucose to gluconolactone with the help of an FAD cofactor. How would you assay this reaction?
- The catalytic activity of many enzymes depends on the presence of small molecules termed **cofactors**, although the precise role varies with the cofactor and the enzyme. Such an enzyme without its cofactor is referred to as an *apoenzyme*; the complete, catalytically active enzyme is called a *holoenzyme*.
- Cofactors can be subdivided into two groups: metals and small organic molecules. The enzyme carbonic anhydrase, for example, requires Zn$^{2+}$ for its activity. Glycogen phosphorylase, which mobilizes glycogen for energy, requires the small organic molecule pyridoxal phosphate (PLP).
- Cofactors that are small organic molecules are called **coenzymes**. Often derived from vitamins, coenzymes can be either tightly or loosely bound to the enzyme. If tightly bound, they are called **prosthetic groups**. Loosely associated coenzymes are more like cosubstrates because they bind to and are released from the enzyme just as substrates and products are. The use of the same coenzyme by a variety of enzymes and their source in vitamins sets coenzymes apart from normal substrates, however. Enzymes that use the same coenzyme are usually mechanistically similar.
- As we will see, glucose oxidase has proven to be one of the most useful enzymes known. It is the basis for glucose biosensors that are used for monitoring blood glucose levels of people with diabetes.
  - **Q**: I was wondering when we talk about glucose oxidase do we assume that FAD is always there as well?
  - **A**: FAD is a cofactor that must be associated with the enzyme for it to be active. For enzymes that require cofactors, the cofactor is typically bound with high enough affinity that it will co-purify along with the protein during purification. If not, extra cofactor could be added during or after purification. When discussing the active enzyme, it is implied that the cofactor is present.
Enzymes used for analytical purposes: Horseradish peroxidase catalyzes the oxidation of a wide variety of substrates.

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\begin{align*}
\text{H}_2\text{O}_2 & + 2 \text{H}^+ \\
& + 2 \text{e}^- \\
\rightarrow & 2 \text{H}_2\text{O}
\end{align*}
\]


- Horseradish peroxidase utilizes a heme cofactor to catalyze the reduction of hydrogen peroxide with the simultaneous oxidation of some other organic molecule. For the sake of this course we will assume that 'substance X' could be practically any other organic molecule that can serve as the source of the electrons.

- This ability to oxidize a wide variety of substrates means that horseradish peroxidase is a very useful as the coupling enzyme. It can be used with enzyme reactions that result in the production of hydrogen peroxide and this makes it a very useful biotechnological tool.

- This will be further illustrated on the next slide.
Amplex Red:
a fluorogenic peroxidase substrate

In this two enzyme ‘coupled assay’, glucose oxidase is first oxidizing glucose with formation of gluconolactone and hydrogen peroxide. From an analytical point of view we still need to detect the formation of the product somehow. This detection is done by adding horseradish peroxidase and a chromogenic or fluorogenic substrate such as commercially available Amplex Red. There are a wide variety of substrates available for horseradish peroxidase.

Horseradish peroxidase reduces the hydrogen peroxide generated by glucose oxidase and simultaneously oxidizes amplex red to the brightly red fluorescent dye resorufin. The amount of resorufin generated can be quantified by absorbance or fluorescence spectroscopy.

A key reference regarding the mechanism of Amplex Red: Gorris and Walt, J. AM. CHEM. SOC. 2009, 131, 6277–6282. The authors provide evidence that the mechanism proceeds via two steps. In the first step a 1 electron oxidation of Amplex Red occurs to give the radical (centered on a phenol oxygen). In the second step there is a dismutation reaction in which two radicals react to form resorufin and regenerate one molecule of Amplex Red.
**The PiPer phosphate Assay Kit from Molecular Probes: a fluorescence-based assay for free phosphate**

- Coupled assays do need to be limited to 2 enzymes. A nice example of a 3 enzyme coupled assay is the PiPer Phosphate Assay Kit from Molecular Probes.
- In the presence of inorganic phosphate, maltose phosphorylase converts maltose to glucose-1-phosphate and glucose. Then, glucose oxidase converts the glucose to gluconolactone and $\text{H}_2\text{O}_2$.
- Horseradish peroxidase reduces the hydrogen peroxide generated by glucose oxidase and simultaneously oxidizes amplex red to the brightly red fluorescent dye resorufin.
- The resulting increase in fluorescence is proportional to the amount of Pi in the sample. All of the other components would be added in excess such that they are not limiting in terms of the overall conversion from inorganic phosphate to resorufin.
- A key reference regarding the mechanism of Amplex Red: Gorris and Walt, *J. AM. CHEM. SOC.* 2009, **131**, 6277–6282. The authors provide evidence that the mechanism proceeds via two steps. In the first step a 1 electron oxidation of Amplex Red occurs to give the radical (centered on a phenol oxygen). In the second step there is a dismutation reaction in which two radicals react to form resorufin and regenerate one molecule of Amplex Red.