Already discussed:

- H2O+ (pH)
- pI
- Stability
- Salts
  - Hofmeister/Arakawa & Timasheff
- Salting out
- Substrates & inhibitors of enzymes
- MM equation
- Cooperativity
- Reversible inhibitors
- Metals and other things that bind (Scatchard)
- Denaturants

What about very Reactive Reagents?

- Some compounds or ions are intrinsically very reactive
  - Iodo compounds
  - Aldehydes
  - Anhydrides, acyl halides
- They can react with complementary groups on the protein even without binding (distinguish "binding" from "bonding")
- Extent of reaction depends on
  - Intrinsic reactivity
  - Accessibility
- Use these concepts for chemical modification of proteins

Why would you want to do it?

1. Alter Properties
   - Surface charge
   - Solubility
2. Probe accessible regions
   - Which groups are solvent-accessible?
   - Which groups become accessible under different conditions
   - Denaturation
   - Binding of effector, inhibitor, substrate, other subunits
   - Which groups are in contact - crosslinking
3. Determine which groups are responsible for properties
   - Active-site residues
   - Folding
4. Immobilization
   - Chromatography
   - Bionectors
5. Attachment of probe
   - Fluorescent dye or other reporter molecule
6. Stabilization or linking of related enzymes
7. Creation of bioconjugates for assay, delivery, etc.
8. Other???

Chemical Crosslinking

Active Site Modification
How do you do it?

1. Use intrinsic reactivity of certain sidechains.
   - nucleophiles (substitution, addition)
     - lysine
     - cysteine
     - histidine
     - serine
     - threonine
   - electrophiles (substitution, addition)
     - carbonyl groups of Asp, Glu
     - aromatics
     - oxidizable groups
     - cysteine
     - reducible groups
     - other
     - methionine

To react with a nucleophile,

the reagent must have:
1) a good leaving group
   a. halides
   b. carboxylates or other stable anions
   c. N-hydroxysuccinimide
   d. in-situ-generated leaving groups
2) a group that will permit addition reactions
   (e.g., C=O)
   a. aldehydes, ketones
   b. double bonds conjugated to carbonyls

Chemical Modification of Cysteine using its nucleophilic properties

These reagents react with other nucleophiles

- Iodoacetamide
- Iodoacetate
- N-Ethylmaleimide

Both react very little with other nucleophiles

Chemical Modification of Cysteine more sophisticated reagents

- Ellman’s Reagent (5-5’-dithiobis(2-Nitrobenzoic acid))
  yellow
- Kenyon’s Reagent (methyl methanethiosulfonate)

Chemical Modification of Lysine

- Succinic anhydride
- O-Methyl isourea

Reductive alkylation

Biotinylation of Lysine using an N-hydroxysuccinimide ester of biotin

- N-hydroxysuccinimide
- Biotinylated lysine sidechain
Crosslinking Proteins via Lysine

Dimethyl suberimidate (dihydrochloride)

\[
\text{H}_2\text{C} = \text{O} \quad \text{NH}_2 \quad \text{O} \quad \text{CH}_3
\]

Glutaraldehyde is also common ( Schiff base formation)

Chemical Modification of Lysine or a Carboxylate Group

1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (EDC)

\[
\text{H}_2\text{C} = \text{N} \quad \text{NH}_2 \quad \text{O} \quad \text{CH}_3
\]

Amide product (substituted urea by-product)

Chemical Modification of Histidine

Iodoacetic acid

(carboxymethylated product)

Diethylpyrocarbonate

(carboxymethylated product)

Ethyl carbonate

How do you do it (cont.)?

2. Use specific recognition by binding site to direct the reagent (Affinity labels, active site-directed reagents)
3. Use photoactivatable reagent that binds specifically (Photoaffinity labels, photoactivatable affinity labels)
4. Use catalytic activity to generate a reactive compound bound to the active site. (suicide reagents, mechanism-based or turnover-based reagents)

How do you know you did it?
Radioactivity, fluorescence or absorbance of labelled prote
Loss of activity (modified or denatured)?
Indicator molecule released upon modification

How do you know where it is?

2. Specific recognition
- Affinity labels, active site-directed reagents

Properties of Affinity Reagents

Chemical reactivity
(e.g., good leaving group)
Specificity
-active site recognizes it
Saturation
-kinetics not first order in reagent
Competitive with substrate
-substrate should protect against inactivation
Examples of Affinity Reagents

<table>
<thead>
<tr>
<th>Affinity label</th>
<th>Enzyme</th>
<th>Sidechain</th>
<th>Modified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tosylphenylalanine chloromethylketone (TPCK)</td>
<td>serine proteases</td>
<td>active-site serine</td>
<td></td>
</tr>
<tr>
<td>Glycidol phosphate</td>
<td>triosephosphate isomerase, emolase</td>
<td>labels nucleophiles or general base in active site</td>
<td></td>
</tr>
<tr>
<td>S-(4-bromo-2,3-dioxybutyl)coenzyme A</td>
<td>citrate synthase</td>
<td>Lys-CH₂-NH₂ or Glu-COO⁻</td>
<td></td>
</tr>
<tr>
<td>3-chloropropionyl CoA</td>
<td>fatty acyl synthase</td>
<td>labels Lys-CH₂-NH₂ in the presence of a reducing agent</td>
<td></td>
</tr>
<tr>
<td>3'-O-(4-benzoylbenzoyl)adenosine</td>
<td>several dehydrogenases, e.g., glutamate dehydrogenase</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Example: Fattyacyl Synthase

Photoaffinity labels

No chemical reactivity until irradiated
- typically have an azide group

Specificity
- active site recognizes it
- may be improved by radical traps

Competitive with substrate
- substrate should protect against inactivation

Photoactivatable reagent

(Photoaffinity labels) (Photoactivatable affinity labels)

<table>
<thead>
<tr>
<th>Photoaffinity label</th>
<th>Enzyme</th>
<th>Group Modified</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-azido-ATP</td>
<td>a. F1-ATPase</td>
<td>b. tyrosine</td>
</tr>
<tr>
<td>8-azido-cAMP</td>
<td>bovine pancreatic ribonuclease A</td>
<td>threonine</td>
</tr>
<tr>
<td>5-azido-UTP</td>
<td>UTP-glucose pyrophosphorylase</td>
<td></td>
</tr>
<tr>
<td>3'-O-(4-benzoylbenzoyl)ATP</td>
<td>F1-ATPase</td>
<td></td>
</tr>
</tbody>
</table>

3. Photoactivatable reagent

4. Catalytic activity

suicide reagents

<table>
<thead>
<tr>
<th>Suicide reagent</th>
<th>Enzyme</th>
<th>Group Modified</th>
</tr>
</thead>
<tbody>
<tr>
<td>iodosedehydride</td>
<td>chymotrypsin</td>
<td>active-site serine</td>
</tr>
<tr>
<td>β-D-galactosidase</td>
<td>methanolase</td>
<td></td>
</tr>
<tr>
<td>nitrophenyl</td>
<td>methanolase</td>
<td></td>
</tr>
<tr>
<td>xanthine</td>
<td>adenine</td>
<td></td>
</tr>
<tr>
<td>adenosine 2',3'-cyclic</td>
<td>DNA polymerase I</td>
<td></td>
</tr>
<tr>
<td>unknown</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Azides

azide ion

an organic azide

8-azido ATP
Chemical modification has become partially supplanted by site-directed mutagenesis. The ability to change a sidechain at will, completely, and with absolute specificity, gives the researcher great power. But molecular genetics and chemical modification can be used in concert. Chemical modification is limited by the presence of modifiable sidechains. Now, you can put modifiable sidechains wherever you want, then use them to attach reporter molecules such as fluorescent probes.

**Enzymatic Modification**

- Hydrolysis - discussed already
- Dephosphorylation, deglycosylation (hard)
- Transpeptidation - the Plastein Reaction*
  - Use enzymatic hydrolyzate, concentrate
  - Add protease plus free amino acids or esters
  - Obtain small, reshuffled protein-lets (3 kD) with new amino acids incorporated
  - Actual composition depends on the mix and the specificity of the protease used.
- Transesterification* - glycosidases can shuffle sugars
  - Lactase is specific for beta-galactosides
  - Invertase is specific for beta-fructosides
  - Neither cares very much what the other reagent is.

*(catalysts catalyze both forward and reverse reactions)