"Use of Fluorescent Probes to Study Structural and Functional Aspects of Enzymes"

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Much of the field of biochemistry is devoted to the study of the structure and function of enzymes. Advances in biotechnology in the past few decades have allowed for cloning and expression of large quantities of enzymes, but more physical techniques are required for characterization of the structure and function of enzymes. Fluorescence spectroscopy is a sensitive and useful tool for such studies and its utility is discussed here. Examples of data from fluorescence experimentation are provided.

ENZYMES AND THEIR SIGNIFICANCE

Most enzymes consist of proteins, which are polymers of amino acids, except a few known cases where RNA functions as an enzyme ("ribozymes"). Enzymes are catalysts which stabilize the transition state of a reaction. Thus, a larger number of molecules will have the energy necessary to attain the transition state. The reaction will then reach equilibrium faster.

Reactions catalyzed by enzymes proceed fast enough to allow physiological processes, indeed life as we know it, to be possible. Enzymes are highly efficient and very specific, important features in the complex environment of a cell or organism. Enzymes have the ability to be regulated by various means, allowing for physiological control or responses to signals.

Some of the physiologically important functions enzymes perform are conversion of the chemical energy of ATP into mechanical force in muscles, control of osmotic balance across cell membranes, replication of the genetic material of cells, transmission of impulses in nerves and conversion of nutrients into useable energy, among many others. There are literally thousands of enzymes at work in an organism, some of which are highly conserved from single-cell organisms to humans.

Genetically inherited disorders are caused by defective enzymes. For example, genetic defects in the enzymes which degrade lipids lead to lipid storage diseases, Tay-Sachs disease being the best known. This disease is caused by a defective enzyme called β -N-acetylhexosaminidase A, which does not break down the ganglioside GM₂ as the normal enzyme does. This lipid accumulates in the brain, which is fatal to the individual at an early age. Many examples of defective enzymes leading to disease states exist. The more that is known about enzymes and their function, the more possible it becomes to treat diseases.

FLUORESCENCE SPECTROSCOPY

There are many ways of studying enzymes and it is beyond the scope of this article to review all the methods. The method this article will focus on is fluorescence spectroscopy. Excellent reviews of fluorescence may be found in the literature (1) and volumes of fluorescence methods also exist (2,3). Fluorescence is

a physical process in which a molecule absorbs light and becomes excited to a higher electronic state. The excited molecule is generally short-lived, only in the range of a few nanoseconds (10^{-9} seconds). When the excited molecule decays back to the ground state and ejects a photon, the process is termed fluorescence. Decay only occurs from the lowest vibrational level of the S₁ or singlet state.

Examples of fluorescent molecules are shown in Figure 1, and examples of their applications are described below. Hundreds of fluorophores are commercially available from companies such as Molecular Probes, Inc. of Eugene, Oregon, USA. Tryptophan is a naturally occurring amino acid, and is the most efficient fluorescent amino acid. Tyrosine and phenylalanine are also fluorescent, but are less efficient than tryptophan. The presence of conjugated double bonds is an essential feature of all fluorescent molecules. Indeed, it is the π bonding electrons which get excited to higher electronic levels, a π to π^* transition, which is quantum mechanically allowed.

Fig. 1 - Structures of fluorescent biomolecules; A) tryptophan and B) 3'-O-(N-methylanthraniloyl) adenosine-5'-triphosphate (mantATP).

Examples of the kinds of spectra obtained from fluorescence are shown in Figure 2. Fluorescence instruments require two monochromators and hence two kinds of spectra are possible: excitation and emission. The excitation spectrum, when corrected, should be the same as the absorbance spectrum of that molecule. An excitation spectrum fixes the emission wavelength and scans across the excitation wavelengths. An emission spectrum is the reverse, where an excitation wavelength is fixed and the emission wavelengths are scanned.

Besides spectra, one can also measure the polarization or anisotropy of a fluorescent molecule. These quantities are a measure of the mobility of the molecule. That is, if a molecule moves significantly during its excited state lifetime, its polarization or anisotropy will be low, close to 0, suggesting isotropic motion. If it does not move significantly during its excited state lifetime, its polarization or anisotropy will be relatively high. The theoretical positive limit for a fluorophore's polarization is 0.5 and 0.4 for anisotropy, while the theoretical lower limit is -0.33 for polarization and -0.2 for anisotropy. The actual limiting value for each molecule is different and can also be different across exciting wavelengths as different transitions are excited preferentially. This forms the basis of the excitation polarization spectra below (Figure 3).

Polarization and anisotropy are ratios of intensities with polarizers placed in the light path. (For reviews of the theories and applications of polarization see Weber (4) and Jameson and Sawyer (5).) With a polarizer in the path of the exciting light selecting for electric vectors oriented straight up and down, or parallel (||), the emission intensity (I) is collected through a polarizer which is successively moved to two positions, parallel (||) and perpendicular (⊥). Polarization and anisotropy are then defined as follows.

$$\text{Polarization} = \frac{I_{||} - I_{\perp}}{I_{||} + I_{\perp}}$$

$$\text{Anisotropy} = \frac{I_{||} - I_{\perp}}{I_{||} + 2 \cdot I_{\perp}}$$

The denominator in anisotropy is actually the total intensity. This makes anisotropy an additive quantity, which will be useful in the experimental data presented below.

Structural details can be obtained from fluorescence resonance energy transfer. The theory of FRET was originally described by Perrin (6) and Förster (7,8). The dependence of transfer efficiency upon the distance between donor and acceptor molecules and upon the overlap integral between the donor's emissi-

Fig. 2 - Uncorrected excitation (left) and emission (right) spectra of mantATP in buffer at pH 7.4. The excitation spectrum was taken with the emission monochromator set at 445 nm, and the emission spectrum was taken with the excitation monochromator set at 355 nm. Spectra were taken with a parallel polarizer in the emission path to eliminate polarization artifacts from the monochromator, e.g., the Wood's anomaly.

Fig. 3 - Excitation polarization spectrum of tryptophan mutants of fructose-6-P, 2-kinase:fructose-2,6-bisphosphatase. Emission was collected through a Hoya UV-32 and a Corning 7-54 filter to isolate emission above 320 nm from exciting wavelengths. Spectra for the single trp-containing mutants, trp299 (•) and trp320 (■), are higher than that for the double trp-containing mutant, trp299,320 (▲).

on and acceptor's absorbance were verified by Stryer and Haugland (9). Excellent reviews on fluorescence resonance energy transfer have appeared in the literature recently (10,11). Briefly, two molecules are required, a donor of energy and an acceptor. If the emission of the donor overlaps with the absorbance of the acceptor, energy can be transferred via a dipole-induced dipole nonradiative mechanism after the donor has been excited. Besides spectral overlap, relative proximity and proper orientation are required. Based on the efficiency of energy transfer (E), it is possible to calculate the distance between the donor and acceptor molecules based on the following equation:

$$E = R_0^6 / (R_0^6 + R^6)$$

where R_0 is the critical transfer distance for a given donor-acceptor pair, or distance at which transfer efficiency is 50%, and R is the distance between the donor and acceptor.

It is possible for the same molecules to undergo homotransfer as the excitation and emission spectra can have overlap. Tryptophan has been shown by Weber to undergo homotransfer (12). A feature of energy transfer is that it is relieved or inhibited when exciting at the red edge of excitation, Weber's red edge effect (12,13).

FLUORESCENCE STUDIES OF ENZYMES

Fluorescence probes may be readily applied to the study of enzyme structure, function and dynamics. Indeed, fluorescence has some advantages over other techniques in that it is highly sensitive (samples in the micromolar range are routine, and now single molecule detection is possible with two-photon excitation), non-invasive and non-destructive, and is performed in solution under equilibrium conditions.

One example of use of fluorescence to study enzymes is the deter-

mination of association or dissociation constants of ligand binding to an enzyme. For the simple reaction of a ligand binding to an enzyme:

the association constant is defined as:

$$K_A = \frac{[P-L]}{[P][L]}$$

and the dissociation constant, the inverse of the association constant, is then:

$$K_D = \frac{[P][L]}{[P-L]}$$

Hence, determination of the constants requires determination of the molar quantities of the free and bound species. These constants can then be used in calculating the change in free energy (ΔG) according to the relation:

$$\Delta G = -RT \ln K$$

The constants are thus a direct measure of the affinity of the enzyme for its ligand. It should be noted that these constants are quite different than the kinetic constants K_M and V_{max} obtained from kinetic experiments.

To use fluorescence to measure a dissociation constant, a fluorophore which undergoes a change upon binding is required. The fluorophore can be positioned on the enzyme or the ligand. The fluorescence change can be in one of the fluorescence parameters such as intensity, anisotropy/polarization, excited state lifetime, or rotational relaxation time.

One example is a tryptophan residue in an enzyme called fructose-6-phosphate, 2-kinase:fructose-2,6-bisphosphatase, sometimes called PFK-2, which has four tryptophans (14). This enzyme has been genetically engineered to contain only one tryptophan at the #15 position (15). When the ligands fructose-

6-P and fructose-2,6-P₂ bind to the enzyme, the anisotropy of the single tryptophan at position 15 is observed to increase (16). This increase is due to a decrease in the local mobility of the tryptophan inside the enzyme. Thus, as the ligands bind, the region of the enzyme around tryptophan 15 stops moving. This fact was used to estimate the dissociation constant of each ligand, and the data are shown in Figure 4. The dissociation constants for fructose-6-P and fructose-2,6-P₂ determined by this method are $27 \pm 2 \mu M$ and $2.08 \pm 0.02 \mu M$, respectively.

This enzyme is bifunctional. In the N-terminal half of the enzyme, there is a kinase site which adds a phosphate group to fructose-6-P to form fructose-2,6-P₂. The tryptophan at the 15 position (W15) is thought to be near this site, but it is not clear from the crystal structure where the N-terminal 30 amino acids reside (17). (It should be noted that the crystal structure was solved for a mutant enzyme which contained phenylalanines in place of all four tryptophans in the wild type enzyme.) In the C-terminal half, there is a phosphatase site, which catalyzes the removal of a phosphate group from fructose-2,6-P₂ to form fructose-6-P. The tryptophan residues at positions 299 and 320 are found in the C-terminal half of the enzyme, closer to the phosphatase site. Only tryptophan 15 was sensitive to added ligands, its polarization increasing as the ligands bind. Hence, the ligands are most likely binding in the N-terminus near tryptophan 15, which is near the kinase site. The means for estimation of dissociation constants for ligand binding at the phosphatase site is not currently available.

The above studies were performed on enzyme from rat testis. In the liver, this enzyme is regulated by phosphorylation and dephosphorylation in response to hormonal signals. Future studies will examine the difference in ligand affinities when the enzyme is phosphorylated or dephosphorylated.

Fig. 4 - Titrations of the ligands fructose-6-P (A) and fructose-2,6-P₂ (B) into the single trp15 mutant of fructose-6-phosphate, 2-kinase:fructose-2,6-bisphosphatase. Solid lines are hyperbolic fits to the data (•).

Besides using tryptophan as a fluorophore, it is also possible to attach fluorophores to ligands. One example is mantATP, where the N-methylantraniloyl reporter group is attached to the ribose of ATP (see Figure 1; fluorescent nucleotides are reviewed by Jameson and Eccleston in reference 3). When this fluorescent ATP analog binds to an enzyme which uses ATP as a substrate, both the fluorescence intensity and anisotropy of the mant group increase. In a fashion similar to the experiments above with tryptophan, the dissociation constant can be calculated. In the case where the fluorescence intensity is enhanced upon binding, the data must be corrected for the enhancement. The enhancement factor (g) is the ratio of the quantum yield of the bound fluorophore to that of the free fluorophore. This

ratio is equivalent to the ratio of the excited state lifetime of the bound fluorophore to the free fluorophore.

It is possible to measure both the quantum yields and lifetimes of the fluorophore, but measurement of the lifetime and data analysis allow for presence of both free and bound lifetimes. Thus, lifetimes were measured of free and bound fluorophores. Then, fraction bound is given by the following relation:

$$\text{Fraction bound} = \frac{(r_{\text{obs}} - r_{\text{free}})}{(r_{\text{bound}} - r_{\text{free}}) + (g - 1)(r_{\text{bound}} - r_{\text{obs}})}$$

where r_{obs} is the observed anisotropy, r_{free} is the anisotropy of the free mantATP and r_{bound} is that of the bound mantATP. The fraction bound is then plotted as a function of free protein, which in this experiment is being titrated in to saturate the fluorophore. The data are then fit with a hyperbolic function and the K_D extracted along with the number of binding sites (p) from the following equation after Winzor and Sawyer (18).

$$\text{Fraction bound} = \frac{p \cdot [\text{Ligand}]_{\text{free}}}{K_D + [\text{Ligand}]_{\text{free}}}$$

where p is the number of binding sites, $[\text{Ligand}]_{\text{free}}$ is the concentration of free ligand, and K_D is the dissociation constant.

An example of data with the MAP kinase Erk-2 (N152G mutant) is shown in Figure 5. It is noted that

Fig. 5 - Titration of the N152G mutant of Erk-2 into mantATP. Solid lines are hyperbolic fits to the data (•). The binding curve is incomplete due to the solubility limit of the protein and the relatively weak binding.

the magnitude of the dissociation constant and the solubility limits of the protein will determine how much of the binding curve is experimentally accessible. Hence, the curve in Figure 5 only approaches 25% saturation and the curve obviously does not approach the limiting value.

The same procedure can also be used for enzymes which have more than one binding site, such as the case with dynein, a motor protein from flagella. Based on the sequence of dynein, it was proposed to have four binding sites for ATP (19). Radiolabelled ATP was used in phase partition separation to estimate the association constants of the four binding sites for ATP (20). Mant ATP was also used to estimate the association constants, but only the first two sites were elucidated due to the low affinity of the third and fourth sites and the relatively low concentrations of dynein obtainable in solution. The association constants of mant ATP and ATP were found to be similar, which agrees with data on other enzymes (21). In the case where there are multiple binding sites, the enhancement factor must be assumed to be the same for binding at each site as it is not possible to determine each separately.

We have found with tryptophan mutants of PFK-2 that two tryptophan residues undergo homotransfer. In the case when the enzyme has tryptophans at positions 299 and 320, they transfer energy between one another. The critical transfer distance, or distance at which the transfer efficiency is 50%, between tryptophan residues was reportedly 12 Å (22). Since it is difficult to estimate the transfer efficiency, it is difficult to estimate the distance. However, the distance between W299 and W320 must be less than 15 Å. Evidence of transfer comes from the excitation polarization spectrum, which shows the classic relief of transfer at the red edge of excitation (see Figure 3). The ratio of polarizations at 310 nm (at the red-edge of excitation) to that at 295 nm may be

used as a qualitative means of detecting the presence or absence of energy transfer between tryptophans. That is, ratios of 2 or more suggest the presence of energy transfer. A ratio of almost 4 was observed for poly-L-tryptophan (unpublished results).

SUMMARY

In summary, fluorescence spectroscopy is a sensitive and powerful tool for studying structural and functional properties of enzymes. As fluorescence microscopy techniques develop, it will become possible to perform fluorescence studies in live cells or tissues, as well as on single molecules.

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