Aromatic Probes

SIGHTINGS

Aromatic disulfides such as Ellman's reagent [5,5′-dithiobis(2-nitrobenzoic acid) (DTNB)] are commonly used to measure the concentration of reduced peptides such as glutathione as well as proteins. Basically, DTNB reacts with reduced sulfhydryl groups and 5-thio-2-nitrobenzoic acid (TNB) is released. The amount of released TNB is measured by ultraviolet (UV)–visible spectroscopy and correlates with the quantity of reduced sulfhydryl groups.

A recently published report shows the use of Ellman's reagent to further characterize the active sites of the tyrosine phosphatases SHP-1 and PTB1B (*1*).

Tyrosine phosphatases have a signature sequence in their active site, with a cysteine residue that is essential for activity. The cysteine serves as the active-site nucleophile. The authors wanted to learn more about the reactivity of this active-site cysteine. They knew that the active-site cysteine of phosphatases had a pK_a that was much lower than the pK_a of other protein cysteines (4.7 to 5 versus 8 to 9.5). They reasoned that this difference in pK_a would lead to different reactivity with aromatic disulfides. Indeed, because of its low pK_a, the essential cysteine is ionized at low pH and would be expected to react rapidly with aromatic disulfides, whereas the other protein cysteine residues would react more slowly, because only a very small proportion of those would be in the reactive thiolate form.

When DTNB was mixed with the enzyme at pH 5, the release of TNB as measured by spectrophotometry had two components: an initial fast release (burst phase) and a second slower release. At pH 8, on the other hand, the authors noted the disappearance of the initial burst phase. The burst phase at pH 5 correlated with loss of enzyme activity. Moreover, the rate of the burst phase was reduced by addition of both a substrate and a competitive inhibitor of the phosphatase reaction, indicating that DTNB competes with these species for access to the active site. Taken together, these results suggest that the burst phase represents the reaction of the active-site cysteine with DTNB, while the slower second phase represents reaction with the other protein cysteine residues. Specifically, the magnitude of the burst phase would indicate the active enzyme concentration, and the rate of the burst would reflect the reactivity of the active-site cysteine. These results were reproducible with another aromatic disulfide compound, 2,2′-dipyridyl disulfide.

The protein isolated after the burst phase gave no release of TNB, whereas protein isolated after complete reaction showed release of TNB when treated with a reducing agent. These latter results are consistent with the catalysis of a disulfide bound between the active-site cysteine and a vicinal cysteine residue. Confirming this conclusion, pretreatment of the enzyme with phenylarsine oxide (known to react with vicinal dithiols) abolished the burst phase, while the slow phase was not affected. The presence of a cysteine residue vicinal to the active site was previously unsuspected. Since the study has been published, the authors have isolated and sequenced a peptide fragment corresponding to the active-site disulfide from a CNBr digest of the product (*2*), giving direct evidence for their hypothesis.

This technique has several attractive features. First, it is simple. All that is needed is a spectrophotometer and Ellman's reagent. It can be used for accurate measurement of active enzyme concentration by active-site titration. Indeed, enzyme preparations may contain a small amount of impurities or inactive enzyme, so mere determination of protein concentration may not be an accurate estimate of active enzyme concentration. Additionally, the technique

gives information on the reactivity of the active-site cysteine.

This method does have limitations. An active-site cysteine with a pK_a several orders of magnitude lower than the other cysteine residues is required. At the moment, this value of pK_a is mainly found in tyrosine phosphatases and cysteine proteases. Moreover, because the difference in reactivity between the active site and other cysteines is only about 150-fold, the method cannot be applied to crude cell extracts (with contaminating proteins), but only to purified protein that is soluble at micromolar concentrations. Finally, reducing agents (β-mercaptoethanol or dithiothreitol) should not be present, because they will interfere with the assay. So, the enzyme must be stable in the absence of such reducing agents, at least for the duration of the assay.

In this era of expensive laboratory equipment, commercial kits, and premixed buffers, it is refreshing to run into an experiment in which researchers have applied the trusty spectrophotometer and routine laboratory chemicals to test a clever hypothesis.

–Richard Peters and Robert Sikorski

References

1. M. Pregel and A. Storer, *J. Biol. Chem.* **272**, 23552 (1997).
2. M. Pregel, T. Sulea, E. O. Purisima, A. Storer, in preparation.

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"Lighting" Rods

SIGHTINGS

Clever applications of new molecular technologies seem to come along in waves. A decade later, the tsunami launched after the development of the polymerase chain reaction technique has yet to crest. Now, brace yourself for the rising tide of papers showing novel applications of the green fluorescent protein (GFP). Isolated from jellyfish, GFP is a sensitive and versatile molecular tag that can be used in many biological studies.

A new GFP-based technology was recently published in *Science* by Valdivia and Falkow (*1*). The question was relatively straightforward. The scientists were interested in knowing the identity of the genes that are responsible for the virulence of the organism *Salmonella typhimurium*. Humans infected with *Salmonella* can suffer from gastroenteritis; mice will develop murine typhoid fever. The investigators made the assumption that *Salmonella* virulence genes would be turned on during bacterial infection, specifically when the bacteria were phagocytized by macrophages, a host defense that occurs early in vivo. Once internalized, *Salmonella* can evade destruction and survive as intracellular parasites.

The investigators designed a clever and robust selection scheme using GFP as a marker to tag those genes turned on only when *Salmonella* were in an intracellular environment. A library of plasmid clones was made that linked random sequences of the *Salmonella* genome upstream of a promoterless GFP gene. By chance, some of these sequences contained bacterial promoters capable of driving GFP expression. This library was introduced into a *Salmonella* host where plasmids exist as episiomes. Next, they selected bacterial clones that expressed GFP after the bacteria were phagocytized in culture. For this they used a macrophage-like cell line called RAW 264.7 that can phagocytose *Salmonella* in vitro. Bacteria were collected after lysis of the RAW 264.7–infected cultures and the light-producing strains were collected by fluorescence-activated cell sorting (FACS). Next, they took this sorted bacterial population, grew it in culture, and counterselected against clones that could express GFP outside of the RAW 264.7 cells by FACS sorting and collection of the nonfluorescing bacteria.

The alternating positive/negative selection

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