Commentary

Imaging of metabolites by using a fusion protein between a periplasmic binding protein and GFP derivatives: From a chimera to a view of reality

Mark Stitt*

Max Planck Institute of Molecular Plant Physiology, Am Mühlenberg 1, 14476 Golm, Germany

Biological systems contain an immense number of individual components, which undergo dynamic and highly interactive responses in time and space. Analysis of these responses will provide a key to unlock the information encrypted in the genome sequences that are accumulating around the world. This task is being driven by powerful methods that allow comprehensive of gene expression, protein localization and protein–protein interactions. Emerging technologies that allow a comprehensive analysis of metabolites (1) will also make a vital contribution, by uncovering many of the phenotypic changes that result from alterations of the genotype, or that accompany changes in gene expression. High throughput profiling technologies suffer, however, from a serious blind spot. Understanding of biological function also requires spatial resolution, at the cellular and subcellular level. For several decades, this sort of information was obtained in a painstaking way, for example, by isolating cell types or organelles and investigating what proteins, enzyme activities, and metabolites they contained (2), or by producing tissue sections for *in situ* hybridization or immunolocalization of transcripts and proteins. Molecular cell biology now uses generic methods to investigate the tissue and cell-specific localization of transcripts and the cellular and subcellular distribution of proteins, for example reporter genes or GFP fusion proteins. Analogous techniques are urgently needed to measure metabolite levels *in situ*. Metabolites change even more dynamically than transcripts or proteins, but only a minute fraction of metabolites possess spectral properties that allow them to be directly visualized and imaging by NMR is restricted to metabolites that are present at relatively high concentrations (3). The development of generic techniques to monitor *in situ* metabolite levels is an enormous technical challenge, because of their immense chemical heterogeneity.

Metabolites change even more dynamically than transcripts or proteins.

In this issue of PNAS, Fehr *et al.* (4) take an important step toward this goal. By using a strategy that can be adapted for the *in vivo* analysis of the levels of a large number of different metabolites and nutrients (see below), they have created a nanosensor that allows maltose to be monitored *in vivo*. The immediate urge to develop this nanosensor came from their investigations into the functional genomics of plant sugar, amino acid, and nucleobase transporters. There are large numbers of these transporters, encoded by multigene families whose members show complex developmental and cell-specific expression patterns (5, 6). Plants contain an enormous range of metabolites, and have complicated long- and short-distance transport pathways that differ fundamentally from the systems found in animals. Understanding the role of the individual transporters will deliver vital insights into the way that plants transport, sense, and allocate the resources that they require for growth and storage. In many cases, however, knockout mutants are proving to be too blunt a tool to precisely define their function, because the mutations are lethal or the phenotype is either highly plieotropic or too subtle to detect. To allow direct determination of their function, Fehr *et al.* (4) decided to develop methods that would allow them to measure the concentrations of the substrates of transporters *in vivo* at a high cellular and even subcellular resolution.

Their approach exploits the widely applied tool of fluorescence resonance energy transfer (FRET) (7, 8). The principle underlying this approach is that when two chromophores with overlapping but slightly different absorbance and fluorescence spectra are brought close enough together in the appropriate orientation, part of the light absorbed by the partner with the shorter (higher energy) absorbance range will be transferred to the other partner, leading to a decrease in the intensity of the fluorescence emitted by the short-wavelength range partner, and an increase in the intensity of the fluorescence emitted by the longer wavelength-range partner. This can be exploited by expressing a pair of chromophoric proteins with suitable spectral properties as fusion proteins, either attaching them to two different proteins to investigate whether and when these two proteins come into a close interaction *in vivo*, or attaching both to the same protein to investigate conformational changes that are induced in it by, for example, ligand binding. Most applications of FRET use green fluorescent protein (GFP) as the chromophore (7, 8). This jellyfish protein has three key properties: the chromophore is formed by folding of the protein itself and this occurs readily even in heterologous hosts, it absorbs and fluoresces strongly, and it has been possible to engineer a series of modified forms with altered spectral properties (9). FRET has been widely applied to monitor protein–protein interactions (10), analyze conformational changes in molecular motors (11), monitor protein kinase activities (12) and the subcellular targeting and regulation of protein phosphatases by regulatory subunits (13), and to monitor changes in cellular free Ca^{2+} (14, 15), pH (16), protein kinase activator (17) cGMP (18), and diacylglycerol and phosphoinositides (19).

The key step in Fehr *et al.* (4) is to combine FRET with the metabolite recognition capacity of a bacterial periplasmic binding protein (PBP). There are a very large number of different PBP's located in the extracellular periplasmic space of Gram-negative bacteria. Each binds a specific compound or group of compounds with high affinity. Some sub-

See companion article on page 9846.

*E-mail: mstitt@mpimp-golm.mpg.de.

www.pnas.org/cgi/doi/10.1073/pnas.162375899

sequently interact with transport proteins and release the metabolite to allow it to be imported, whereas others act as chemosensors. Although the primary structure of PBP's differs considerably, the spatial structure is conserved, consisting of two ellipsoidal lobes and a hinge region. Binding of substrate leads to closing and a slight rotation of the lobes (20, 21).

Fehr *et al.* (4) attached two different GFP variants to the N and C termini of a PBP, which binds maltose (the maltose binding protein, MBP). Native MBP binds maltose in an effectively irreversible mode, and release requiring an interaction with the maltose transport protein. MBP therefore had to be modified to optimize it for this new role as the sensing component in a nanosensor. Redesign was aided by detailed information about the three-dimensional structure of the free and bound forms of PBP's (20–22). To achieve reversible maltose binding and a maltose-dependent FRET signal, a slightly truncated form of MBP was used. Further site-directed changes in individual amino acids in the maltose-binding site generated two further nanosensors with a progressively lower affinity for maltose. The end result was a set of three nanosensors, which together covered a wide concentration range from 0.3–2000 μM maltose. In addition, an engineered form was produced with a very low affinity for maltose, which acted as a control that other effectors (e.g., protons) were not leading to artifactual signals (see below).

This set of nanosensors was used to assay maltose in an ELISA-plate-based test, to measure maltose *in vitro* in complex mixtures, and for a semiquantitative measurement of the changes of maltose *in vivo* in yeast after adding external maltose. This approach allows direct monitoring of transport and the consequences for the concentration of maltose in the yeast cell and even within the various subcellular compartments. It has several possible applications to understand the role of maltose in plant metabolism, in particular during starch metabolism, where maltose plays a key role as a major product during starch degradation, being probably the major form in which carbon is exported from the plastid to the cytosol (23).

The work of Fehr *et al.* (4) builds on pioneering developments by the group of Tsien, which has already led to the development of nanosensors for free Ca^{2+}, protons, membrane potential, and cGMP (see above). However, use of a PBP provides, to my knowledge, the first *in vivo* nanosensor for a metabolite as opposed to a signaling component. More crucially, this approach has great potential for expansion. As there are numerous periplasmic binding proteins, which bind a wide range of sugars, amino acids, nutrients, and other metabolites (http://www.biology.ucsd. edu/~ipaulsen/transport.), it opens the perspective of a set of nanosensors that can be used to monitor the levels of many key metabolites *in vivo*, by using a detection system that will allow such measurements to be interfaced with the *in vivo* detection of signaling and cellular events (see above).

The potential of the PBP family has also been realized in studies in which hybrid devices consisting of PBP plus a nonproteinaceous transducer element were developed. In one study (24) the C terminus of PBP was attached covalently to an electrode surface and a Ru(II) redox reporter group, then introduced via a modified cysteine onto the side that faced the electrode. After binding of a ligand, the resulting hinge-bending motion alters the electronic coupling between the Ru(II) redox reporter group and the electrode and generates an electrochemical signal. In this way, electrical sensors for maltose, glucose, and glutamine were created. In an alternative approach, a probe whose fluorescent signal depends on its immediate environment was attached via a modified cysteine to a sulfate-sensing PBP (25). When sulfate binds, the conformation around the probe is altered, resulting in a change in fluorescence. Such nonenzymic nanosensors have enormous potential for analysis of samples *ex situ*, because they do not require the addition of further assay components and detection does not depend on diffusible components. They might also be usable after microinjection or in microelectrodes to determine metabolite levels *in vivo*.

Despite the large number of PBP's, there are many important metabolites that they do not bind. The approach taken by Fehr *et al.* (4) opens up the possibility of extending the range, by starting out from a fusion with an existing PBP, carrying out mutagenesis, and employing high throughput screening to identify candidates that bind new substrates. A complementary strategy would be to design completely new proteins. For use as a FRET-based biosensor, it is crucial that ligand binding leads to a change of conformation that modifies the energy transfer between the two GFP derivatives. This requires them to be far enough apart and/or for the angle between them to be changed. The development of Ca^{2+}-sensing chameleons provides a paradigm (14). After binding of Ca^2, calmodulin binds and alters the orientation of a 26-aa residue of light chain myosin. Calmodulin was fused with this 26-aa sequence to produce a 2-lobed fusion protein, which alters its conformation when Ca^{2+} binds. This fusion protein was further modified to create a biosensor by adding a GFP derivative onto each lobe. This approach benefited from the fact that the Ca-promoted conformation change occurs naturally. With increased knowledge of protein structure and the way it is affected by ligand binding, it may be possible to use analogous approaches for other metabolites too.

Despite their beauty and sensitivity, the use of biosensors harbors a danger inherent in any approach that monitors a parameter by proxy, which is that the go-between may be unreliable. There are numerous potential sources of interference. One relates to the possibility that the sensor system is not specific, because other ligands bind at the binding site. The specificity of the sensor therefore has to be rigorously checked against a wide range of possible alternative substrates. In the case of the maltose sensor, it is evident that it also binds maltotriose and longer α(1–4) glucans (4). A second and even more pernicious source of artifacts is that binding of ligands elsewhere on PBP or on the GFP derivatives may alter the absorption spectrum, energy transfer, emission spectrum, or fluorescence yield. For example, changes of pH can severely interfere with Ca^{2+} chameleons, and although further modifications are decreasing this sensitivity (15), this is a problem that must be critically assessed for each new sensor. Fehr *et al.* (4) include a vital control, which is to engineer a form of the fusion protein in which substrate binding is severely impaired, to act as a control against artifacts caused by nonspecific interactions of protons or other ligands with the nanosensor. A third problem is that the binding range is inappropriate, which will lead to loss or attenuation of the signal. Fehr *et al.* map out one response to this problem, which is to create a series of biosensors with a progressive change in the binding affinity for the ligand, and to investigate which is most appropriate for a particular application. Fourth, as in any strategy based on fluorescence, quenching can frustrate FRET because other chromophores in the biological object absorb the excitation or the emitted light.

For these reasons, *in situ* measurements of metabolite levels with biosensors will require careful checks and calibration. Even these are unlikely to totally exclude the possibility of errors, so newly developed sensors would benefit from comparison with the results from conventional fractionation techniques. In plants, for example, several techniques exist based on fractionation of protoplasts that allow separation and quenching of different cell fractions within tenths of a second (2), whole plant tissues can be freeze-clamped in liquid nitrogen and subsequently resolved into their major subcellular compartments by nonaqueous density gradient centrifugation (2, 26), tissues can be microdissected by conventional tech-

niques or by emerging technologies like laser-catapulting (http://www.rwjpri.com/lajolla/research/index.htm) and histochemical assays of metabolites on tissue sections can be used to reveal the spatial distribution of metabolites, at least at the level of cell types (27) By judicious combination of biosensors with conventional biochemistry, it should be possible to provide solid verified data in set cases, and underpin the reliability of the far more detailed and dynamic information provided by biosensors in a wider range of experimental situations.

A further limitation in FRET-based studies is that there are experimental constraints on the number of parameters that can be measured simultaneously. This is partly because there are only a limited number of pairs of suitable chromophores that can be used to produce a particular nanosensor and, even more crucially, there is a limitation on the number of fusion proteins that can be simultaneously introduced into a given organism and cell. A two-pronged approach using GFP fusion proteins and FRET to measure one parameter and hybrid devices on microelectrodes to measure further parameters could allow this limitation to be circumvented, at least to an extent.

In the end, however, a challenging dilemma emerges. On the one hand, state-of-the-art technologies now support the unbiased and broad analysis of transcripts, proteins, and metabolite profiles at an organism or organ level. On the other hand, nanosensors are being developed that provide exquisite insights into the dynamic changes in time and space of individual metabolites. The next challenge is to develop techniques that will allow cells and parts of cells to be separated into samples that come from sufficiently defined types of cell or areas of the cell to provide meaningful information, but are large enough to be fed into platforms for profiling transcripts, proteins, and metabolites.

In the case of proteins, classical cellular fractionation or membrane purification techniques are already being combined with highly sensitive mass-spectroscopy-based platforms for protein identification. This is providing increasingly comprehensive information about the proteins present in different cell types, organelles, or membranes. For transcripts and especially metabolites, the technical challenge is much larger, because changes in the levels and distribution must be prevented during the fractionation process. Depending on the metabolite, for example, the half-life may be in the range of days, hours, minutes, seconds, or even milliseconds (see ref. 2). To avoid such changes, it is necessary either to separate the fractions very rapidly, or to quench the tissue and then establish and maintain conditions that arrest chemical changes while the tissue is being fractionated. Possible strategies include the upscaling and automation of microdissection, the adaptation of nonaqueous density gradient fractionation to a wider range of biological applications, or the development of techniques that allow large scale and automated particle-sorting in water-free or low temperature conditions. By combining these with an analytic platform that allows quantitatively accurate determination of 100s or 1000s of metabolites, sophisticated statistical tools and the ability to tag specific subcellular regions and structures by using GFP fusion proteins and other novel methods, it may be possible to produce a complementary window onto the multifarious goings-on in the cell.

1. Fiehn, O. (2002) *Plant Mol. Biol.* **48,** 155–171.
2. Stitt, M., Lilley, R. M., Gerhardt, R. & Heldt, H. W. (1989) *Methods Enzymol.* **174,** 518–552.
3. Ratcliffe, R. G. & Shachar-Hill, Y. (2001) *Curr. Opin. Plant Biol.* **4,** 191–196.
4. Fehr, M., Frommer, W. B. & Lalonde, S. (2002) *Proc. Natl. Acad. Sci. USA* **99,** 9846–9851.
5. Lalonde, S., Boles E., Hellmann H., Barker L., Patrick J. W., Frommer W. B. & Ward J. M. (1999) *Plant Cell.* **11,** 707–726.
6. Wipf, D., Ludewig, U., Tegeder, M., Rentsch., Koch W. & Frommer W. B. (2002) *Trends Biochem. Sci.* **27,** 139–147.
7. Miyawaki, A. & Tsien R. Y. (2000) *Methods Enzymol.* **327,** 472–501.
8. Hahn, K & Toutchkine A. (2002) *Curr. Opin. Cell Biol.* **14,** 167–172.
9. Heim, R. (1999) *Methods Enzymol.* **302,** 408–423.
10. De Angelis, A. A. (2000) *Physiol. Genom.* **1,** 93–99.
11. Shih, W. M., Gryczynski, Z., Lakowicz, J. R. & Spudich, J. A. (2000) *Cell* **102,** 683–694.
12. Ting, A. Y. Kain, K. H., Klemke, R. L. & Tsien, R. Y. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 15003–15008.
13. Trinkle-Mulcahy. L., Sleeman, J. E. & Lamond, A. I. (2001) *J. Cell Sci.* **114,** 4219–4428.
14. Miyawaki, M., Llopis, J., Heim, R., Michael, M. J., Adams, J. A., Ikura, M. & Tsien, R. Y. (1997) *Nature (London)* **388,** 882–227.
15. Atsushi, M., Griesback, O., Heim, R. & Tsien, R. Y. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 2135–2140.
16. Wu, M. M., Grabe, M., Adams, S., Tsien, R. Y., Moore, H, -P. H. & Machen, T. E. (2001) *J. Biol. Chem.* **276,** 33027–33035.
17. Ting, A. Y., Kain, K. H., Klemke, R. L. & Tsien, R. Y. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 15003–15008.
18. Honda, A., Adams, S. R., Sawyer, C. L., Lev-Ram V., Tsien, R. Y. & Dostmann, W. R. G. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 2437–2442.
19. Hurley, J. H. & Meyer, T. (2001) *Curr. Opin. Cell Biol.* **13,** 146–152.
20. Magnusson, U., Chaudhuri, B. N., Ko, J., Park, C., Jones, T. A. & Mowbray, S. L. (2002) *J. Biol. Chem.* **277,** 14077–14084.
21. Spurlino, L. C., Lu, G.-Y. & Quicho, F. A. (1991) *J. Biol. Chem.* **266,** 5202–5219.
22. Martineau, P. Szmelcman, S., Spurlino, J. C., Quiocho, F. A. & Hofnung, M. (1990) *J. Mol. Biol.* **214,** 337–352.
23. Stitt, M. & Rees, T. A. (1980) *Biochim. Biophys. Acta* **627,** 131–143.
24. Benson, D. E., Conrad, D. W., Lorimer, R. M. de, Trammel, S. A. & Helinga, H. W. (2002) *Science* **293,** 1641–1649.
25. Shrestha, S., Salins, L. L. E., Ensor. C, M. & Daunert, S. (2002) *Biotech. Bioeng.* **78,** 517–526.
26. Tiessen, A., Hendricks, J. H. M., Stitt, M. Branscheid, A., Gibon, Y., Farre, E. & Geigenberger, P. (2002) *Plant Cell*, in press.
27. Borisjuk, L., Walenta, S., Weber, H., Müller-Kliesen, W. & Wobus, U. (1998) *Plant J.* **15,** 583–591.