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When Is Weaker Better? Design of an ADP Sensor with Weak ADP Affinity, but Still Selective against ATP

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ABSTRACT A simple ADP biosensor would be of broad usefulness in monitoring the large number of metabolic processes that produce ADP. Several new systems have been recently described including one in the current issue of *ACS Chemical Biology* that provides a simple readout of the ADP concentration without significant interference by ATP.

ADP plays a central role as the product of many metabolic reactions. Detection of ADP could therefore be used to monitor the progress of numerous reactions including ATPases that produce ADP and P_i and kinases that produce ADP and a phosphorylated product. Although a range of methods are available for detection of ADP, including the well-known coupled enzyme system of pyruvate kinase and lactate dehydrogenase, a simple real time assay for ADP would be of broad use to quantify the reactions of these enzymes. A biosensor for ADP could provide such a simple, single reagent approach that would avoid the multiple points for possible interference in a coupled assay. One problem with the development of sensors for ADP is that this application requires that they are able to respond strongly to ADP, while not responding strongly to the very similar ATP that is often in large excess in enzyme assays. A recent article in *ACS Chemical Biology* by Kunzelmann and Webb (*1*) describes the development of a new fluorescent biosensor for ADP with both a large fluorescence increase on ADP binding (15-fold) and strong discrimination against ATP (>100-fold).

This is actually the third ADP biosensor that Webb and colleagues have developed, each with improved properties for specific applications. The first was based on nucleotide diphosphate kinase and involved addition of a coumarin fluorophore group that responds to the phosphorylation of the enzyme by ATP and its dephosphorylation by ADP (*2*). Because this reaction is reversible, it effectively measures the ADP:ATP ratio rather than the absolute concentrations. Additionally, it has limited selectivity as it also responds to GTP/GDP. Their second design was based on ParM, a bacterial protein that is related to actin. ParM might seem an unusual choice because it is an ATPase, rather than just an ADP binding protein. Additionally, ParM has a K_d value for the fluorescent ATP analogue ε-ATP that is almost 60-fold tighter than for ε-ADP (*3*). Despite this higher affinity for ε-ATP over ε-ADP, a coumarin-tagged version was found that had a large fluorescence increase on binding ADP and that bound ADP more tightly than ATP by >400-fold (*4*), a striking reversal of the pattern for ε-nucleotides with the wild-type enzyme. Mutations were also introduced that greatly reduced the rate of ATP hydrolysis and coupled formation of filaments. In fact, for many applications the chief disadvantage of this biosensor was that its K_d of 0.5 μM for ADP was too tight. For assays at concentrations of ADP above the K_d, the biosensor must be in excess of the ADP in order to produce a linear response. A requirement for a high concentration of biosensor would be a disadvantage in high-throughput screening or other large-scale applications.

The third sensor developed by Webb and colleagues also used ParM, but now with rhodamine dyes attached at two sites.

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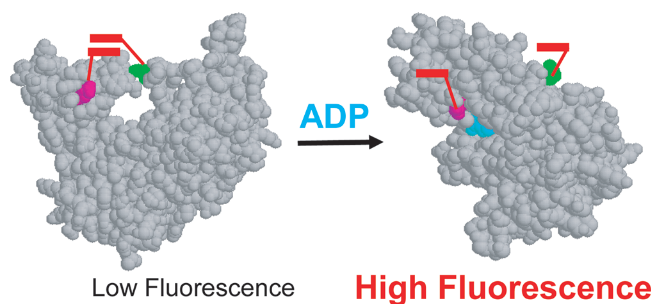

Figure 1. Conformational change on ADP binding to ParM. Views based on crystal structures of ParM in the apo (1MWK) or apd (1MWM) form (*8*). The two aspartic acids that were converted to cysteines and labeled with tetramethyl rhodamine are shown in green and magenta. The bound ADP is colored cyan. The two positions in the apo form are close enough for a rhodamine dimer to form but are far apart in the ADP bound form.

Rhodamine dyes are highly fluorescent but become quenched if they can approach close enough to dimerize. This approach has been productive in a number of previous sensor applications (*5–7*). Sites were chosen on ParM for labeling with rhodamine that were expected to be close together in the apo form (and thus more likely dimerized and quenched) but much further apart in the ADP bound form (based on the X-ray structure of the apo and ADP bound forms of ParM (*8*)). The best observed pair as indicated in Figure 1 produced a large increase in fluorescence on ADP binding and was highly selective for ADP over ATP. Furthermore the K_d for ADP of 30 μM is now much weaker. The higher K_d means that the fluorescence of a substoichiometric level of biosensor will still show an increasing response to ADP into the mid-micromolar range. In this case, weaker is better.

A limitation of this ADP biosensor for some applications will be its slow kinetics of binding and release of ADP. Thus it will be of limited use for following rapid reactions by single turnover methods in the stopped flow. The earlier coumarin based ADP biosensor is faster but still comparatively slow. By contrast, a dirhodamine biosensor that was developed by Webb and colleagues for P_i (*7*) has very fast binding kinetics and has found extensive use in single turnover experiments.

A wide range of other approaches have also been recently applied to the development of biosensors for ADP and ATP. These include assays utilizing competitive displacement of fluorescent ADP analogues from an ADP-specific antibody (*9, 10*), use of an ADP-specific aptomer (*11*), or use of nonprotein ATP binding sensors (*12*). Each approach has its relative advantages in terms of sensitivity and selectivity. An additional exciting recent development is that of genetically encoded reporters for ATP and ADP (*13, 14*) that can provide information about cellular metabolic energy levels in real time and with subcellular localization.

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