Benchmark

Direct Detection of Protein Thiol Derivatization by PAGE

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Derivatization of a protein thiol with a chemical reporter can be monitored directly for reporters possessing a suitable marker such as fluorescence or radioactivity. Thiol-reactive fluorescent markers might include thioflo (5), cpm (6,8), or a thiol-reactive Europium-EDTA chelate (3). Thiol-reactive radioactive markers might include ^{125}I -labeled AET (2), or ^{55}Fe -EDTA or ^{63}Ni -EDTA chelates (3,4). With the exception of the EDTA chelates, derivatization with the above compounds has been monitored by PAGE. However, for the radioisotopically labeled chelates, protein derivatization was monitored by scintillation counting (3,4).

While PAGE and scintillation counting (above) provide direct assays for thiol derivatization, thiol derivatization can also be monitored indirectly for chemical reporters that cannot be assayed directly. This is done by assaying the unreacted thiols before and after derivatization with the primary reagent using a detectable (fluorescent) second reagent. Such methodology is customarily used to assay thiol derivatization with the "chemical nuclease" FeBABE [Iron (S)-1-(p-bromoacetamidobenzyl)ethylene-diaminetetraacetate (6)]. FeBABE is a chemical reporter similar to the above chelates, designed for assaying protein-nucleic acid proximity within protein-nucleic acid complexes. A disadvantage would be that unreacted thiols may

have become chemically modified during derivatization (e.g., thiol oxidation by the Fe^{3+} moiety of FeBABE when using the FeBABE reporter). Moreover, indirect measurement requires the use of a second reagent that reacts efficiently with thiols.

Mass spectrometry (MS) provides an opportunity to simultaneously detect both reacted and unreacted thiols, provided the sample can be ionized. However, MS would be quantitative only to the extent that ionization is unaffected by covalent attachment of the reporter. Neither the "indirect", MS-based, nor scintillation assays would indicate, directly, whether derivatization with the primary reagent has inhibited the ability of the protein to form non-covalent (protein-protein or protein-nucleic acid) complexes. With the indirect assay, for example, the propensity of the

second reagent to inhibit complex formation may be unrelated to that of the primary one, and with MS, maintaining the integrity of complexes during ionization can be challenging (if not impossible) to do. The direct detection of PAGE-fractionated complexes would seem preferable.

Therefore, we have extended the applicability of direct ^{55}Fe detection by implementing a PAGE ("in-gel") detection assay for protein derivatization with FeBABE. The protein to be assayed is first derivatized with the BABE portion of FeBABE, and then the mixture is incubated with $^{55}\text{FeCl}_3$. ^{55}Fe BABE-labeled proteins are subjected to SDS-PAGE to examine the extent of derivatization; in parallel, an electrophoretic mobility shift assay (EMSA) gel can be run to assess complex formation by the derivatized pro-

Figure 1. Thiol-FeBABE derivatization of protein samples. Main panel: demonstration of protein labeling with ^{55}Fe . The storage phosphorgrams of SDS polyacrylamide gels show the derivatization of each of 16 VP39 single-cysteine proteins with ^{55}Fe BABE, along with wild-type VP39 (two cysteines, positions 178 and 272) and cysteine-minus VP39 control. Q183C preparations were dominated by two proteolytic products, as noted elsewhere (7). ^{55}Fe binding to VP39 was not detected in the absence of prior BABE coupling (data not shown). Right panel: To show clearly the absence of signal for cysteine-minus protein, wild-type and cysteine-minus controls from another experiment (in which the amount of ^{55}Fe was equal to that used for the mutants in the main panel) are shown. Arrowhead indicates VP39- ^{55}Fe BABE.

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Figure 2. Chelation before coupling and coupling before chelation. The storage phosphogram shows an SDS polyacrylamide gel of the ^{55}Fe -labeled single cysteine mutant P71C. Lane 1 depicts ^{55}Fe labeling exactly as described in Figure 1. In lane 2, the order of steps has been changed such that ^{55}Fe is first incubated with BABE, after which the single cysteine of the protein is derivatized with the ^{55}Fe BABE complex. Other than the modified order of steps, all experimental conditions were identical. The lower level of labeling for experiments in which the chelate is formed before derivatization was observed for each of several single cysteine mutants tested (data not shown). Arrowhead indicates VP39- ^{55}Fe BABE.

teins. Being an Auger electron emitter, ^{55}Fe is readily detectable in the dried gels using either X-ray film (neither intensifying screen nor fluorography being necessary) or a standard storage-phosphor screen and electronic imager (such as the Bio-Rad FX; Bio-Rad Laboratories, Hercules, CA, USA).

Figure 1 shows SDS-PAGE data for a panel of 16 single-cysteine mutants of the 333 amino acid vaccinia virus protein VP39. The following experimental conditions were used in Figure 1. Before reaction with BABE, proteins were at a concentration of 1 μM in 10 mM HEPES, pH 8.0, 25 mM NaCl, 0.1 mM EDTA, 10% glycerol, 0.01% NonidetTM P-40. Seventeen picomoles of protein F13C or 35.5 pmol each of the remaining VP39 proteins (7,9) were mixed with 1 μL BABE (Dojindo Molecular Technologies, Gaithersburg MD, USA; 17.75 mM in DMSO), and the mixtures were incubated at room temperature for

4 h. $^{55}\text{FeCl}_3$ [(18.7 μL) 20 mCi/mL in 0.5 M HCl; PE-NEN] was dried under vacuum and then re-dissolved in DMSO to a final concentration of 1.43 mM. One microliter of this (sufficient to render approximately 10% saturation of BABE with $^{55}\text{Fe}^{3+}$) was added to each of the protein-BABE conjugation mixtures, except for the cys-minus protein. A greater amount (5 μL) was added to the latter in an attempt to check for possible background labeling of the cysteine-minus control, resulting in a high background throughout this gel lane. After 60 min incubation at room temperature, 18 μL were withdrawn from each reaction mixture, mixed with 6 μL 4 $\times$ SDS-PAGE loading buffer, and loaded on a 10% SDS polyacrylamide gel. After electrophoresis (3 h at 25 mA), the gel was dried and imaged on storage phosphor using a Bio-Rad FX Imager.

Although a signal could be detected

for each mutant (even for mutant F13C and for the full-length band in the preparation of Q183C after prolonged exposure), significant variability in signal strength was observed among the 16 mutants, indicating variability between the thiols in coupling efficiency. Changing the order of steps such that the chelate is formed before thiol derivatization yielded protein that was ^{55}Fe -labeled almost as abundantly as that in which coupling preceded chelation (Figure 2).

Upon subjecting aliquots of the 16 derivatized proteins shown in Figure 1 to the EMSA (1) by incubation of the derivatized proteins with vaccinia protein VP55 plus an appropriate oligonucleotide followed by nondenaturing gel electrophoresis (excluding EDTA from the EMSA gels), the ^{55}Fe BABE-coupled proteins were observed to be excluded from EMSA complexes (data not shown). In control experiments in which aliquots of the ^{55}Fe BABE-derivatized VP39 mutant or the oligonucleotide were ^{32}P -end-labeled immediately before the EMSA, complexes were readily detected (data not shown). Apparently, complex formation was severely impaired by prior FeBABE coupling to each mutant protein.

Although the in-gel detection assay provides a convenient way to assess the propensity of the derivatized protein to form non-covalent complexes (above), absolute quantitation of EDTA:protein stoichiometry might be problematic with respect to, say, the previously described scintillation counting methods (3,4) because of quenching by the gel slice. However, relative quantitation might be possible with the in-gel method, by co-electrophoresing a standard with rigorously known EDTA:protein stoichiometry.

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