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# A Chemical Proteomic Probe for Detecting Dehydrogenases: *Catechol Rhodanine*

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**Summary:** The inherent complexity of the proteome often demands that it be studied as manageable subsets, termed subproteomes. A subproteome can be defined in a number of ways, although a pragmatic approach is to define it based on common features in an active site that lead to binding of a common small molecule ligand (ex. a cofactor or a cross-reactive drug lead). The subproteome, so defined, can be purified using that common ligand tethered to a resin, with affinity chromatography. Affinity purification of a subproteome is described in the next chapter. That subproteome can then be analyzed using a common ligand probe, such as a fluorescent common ligand that can be used to stain members of the subproteome in a native gel. Here, we describe such a fluorescent probe, based on a catechol rhodanine acetic acid (CRAA) ligand that binds to dehydrogenases. The CRAA ligand is fluorescent and binds to dehydrogenases at  $pH > 7$ , and hence can be used effectively to stain dehydrogenases in native gels to identify what subset of proteins in a mixture are dehydrogenases. Furthermore, if one is designing inhibitors to target one or more of these dehydrogenases, the CRAA staining can be performed in a competitive assay format, with or without inhibitor, to assess

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the selectivity of the inhibitor for the targeted dehydrogenase. Finally, the CRAA probe is a privileged scaffold for dehydrogenases, and hence can easily be modified to increase affinity for a given dehydrogenase.

**Keywords:** Dehydrogenase, oxidoreductase, catechol rhodanine, chemical proteomics, subproteome, staining

#### **1. Introduction**

Chemical proteomics is the study of families of proteins related by their active site pockets, typically by labeling the proteins in gels [\(1,](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4121126/#R1) [2\)](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4121126/#R2). This requires a chemical probe that has a detectable group (e.g., a fluorescent label) tethered to an active site ligand [\(3,](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4121126/#R3) [4\)](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4121126/#R4). The active site ligand might covalently react with a conserved active site residue [\(5\)](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4121126/#R5), in which case the probe is called an activity-based probe [\(6\)](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4121126/#R6). Such probes have been developed for serine hydrolases and cysteine proteases [\(7,](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4121126/#R7) [8\)](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4121126/#R8). In contrast, affinity-based probes bind noncovalently, such as ATP tethered to a fluorophor to detect kinases [\(9,](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4121126/#R9) [10\)](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4121126/#R10). One problematic design constraint for affinity-based probes is that the ligand must be tethered to a detectable group, and the addition of the linker/fluor pair can disrupt binding interactions [\(11\)](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4121126/#R11). This is especially problematic since enzyme affinity for cofactors is typically not strong  $(K_d > 10 \mu M)$ . An improved affinity-based probe would have a high affinity family-specific ligand that is itself fluorescent, so that a linker/fluor does not need to be added [\(12\)](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4121126/#R12). There are few examples of such probes that can be used in displacement assays, in a native gel [\(13\)](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4121126/#R13). The catechol rhodanine acetic acid (CRAA) probe presented herein binds in the NAD(P) cofactor site of dehydrogenases, and its ability to target dehydrogenases broadly has recently been demonstrated [\(14,](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4121126/#R14) [15\)](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4121126/#R15).

The catechol rhodanine scaffold was also used as a template in a focused combinatorial library, yielding potent (50–200 nM) biligand inhibitors for multiple dehydrogenases [\(14\)](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4121126/#R14). Therefore, it serves the dual role of being a fluorescent probe, and a scaffold for a focused library targeting dehydrogenases. It can be used as a stain for dehydrogenases [\(15\)](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4121126/#R15), either in its current form, or as modified in a focused library. The fluorescent CRAA probe was initially used to inhibit dihydrodipicolinate reductase (DHPR), an anti-infective drug target. It also shows in-gel binding to two lactate dehydrogenase (LDH)

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isozymes and to 1-deoxy-d-xylulose-5-phosphate reductoisomerase (DXPR), making it a generally useful staining reagent for dehydrogenases. Because binding is noncovalent, it can also be used in a displacement assay performed in a native gel, by monitoring a decrease in fluorescent band intensity as the NAD(P) pocket is occupied by a competing ligand. Finally, the CRAA probe can be used as a stain for mixtures of proteins (ex. tissue extracts), to profile the mixture in terms of proteins that are likely to be dehydrogenases.

### **2. Materials**

HP 8452A diode array spectrophotometer for UV-Vis measurements. All spectroscopic measurements were taken at 25°C in a 1 mL quartz cuvette.Kodak Image Station 2000MMT System for ingel fluorescence scanning (IGFS).

Canon CanoScan (D1250 U2F) scanner for visible imaging of gel bands.

#### *2.1 Proteins and Staining Reagents*

- 1. *E. coli* DHPR expressed in *E. coli* (BL21) and purified following previously described methods [\(16\)](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4121126/#R16).
- 2. L-lactic acid dehydrogenase (LDH, Bovine heart).
- 3. *E. coli* DOXPR expressed and purified from *E. coli* (provided as a generous gift by Triad Therapeutics).
- 4. CRAA was prepared and purified as described in the next chapter.
- 5. CRAA staining buffer: dissolve CRAA (2 mM) in 25 mM Tris-HCl, pH 8.5.

#### *2.2 Running the Native Gel*

- 1. Bio-Rad protein assay reagent.
- 2. NuPage™ and Novex™ products for native gel and SDS-PAGE (Invitrogen®).
- 3. Native stain: 15.5 ml of 1M Tris-HCl, pH6.8, and 2.5 ml of a 1% solution of Bromophenol blue, 7.0 ml of water, and 25 ml of glycerol.

4. Tris-Glycine running buffer: Dissolve 3.0 g of Tris base and 14.4 g of glycine in water and adjust the volume to 1 liter. pH was adjusted to 8.3.

#### *2.3 In-Gel Staining of the Dehydrogenase Subproteome using CRAA*

- 1. Phosphate buffered saline (PBS): 8 g NaCl, 0.2 g KCl, 1.44 g Na<sub>2</sub>HPO<sub>4</sub>0.24 g KH2PO<sub>4</sub> in 1.0 L, pH 7.4.
- 2. Blocking buffer: 2. 5% nonfat drug milk in PBS [\(17\)](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4121126/#R17).
- 3. CRAA staining solution: 2.0 mM CRAA in 25 mM Tris-HCl, pH 8.5.
- 4. Gel fixing solution: 50% methanol, 10% acetic acid and 40% deionized water.
- 5. Coomassie blue staining solution: 0.1% Coomassie Brilliant Blue R-250 in 50% methanol and 10% glacial acetic acid.
- 6. Destaining solution: 10% glacial acetic acid, 40% methanol and 50% deionized water.

### **3. Methods**

Catechol Rhodanine acetic acid (CRAA) is a privileged scaffold, in that it binds to many dehydrogenases. It is visibly colored and is also fluorescent under slightly basic conditions ( $pH > 7$ ). It binds to dehydrogenases with its para-phenol and carboxylic acid in their deprotonated form [\(Fig. 1\)](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4121126/figure/F1/). Since the binding is noncovalent, it is reversible so that CRAA can be displaced by a higher affinity ligand like NADH. This makes CRAA a useful reagent for detecting dehydrogenases in native Gels using both direct binding [\(Fig. 2\)](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4121126/figure/F2/) and displacement assays [\(Fig. 3\)](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4121126/figure/F3/).

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[Fig. 1](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4121126/figure/F1/) Schematic description of the process whereby a native Gel of dehydrogenase proteins is run, then stained using the CRAA probe.



**[Fig. 2](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4121126/figure/F2/)** CRAA-staining of a dehydrogenase (DHPR), and fluorescence imaging. Two native (10% Tris–glycine) gels were run, and DHPR was stained using 2.0 mM CRAA. Lane 1, NativeMark protein standard. Lanes 2–8: E. coli DHPR (10 µL) at concentrations of 0.22, 0.43, 0.86, 1.29, 1.72, 2.59, and 3.45 µg/µL. (A) Staining with CRAA at pH 6.5 with fluorescence imaging (Excitation at 465 nm, detection at 535 nm). (B) Same as in (A), but stained at pH 8.5. (C) Same as in (B), but imaged using a Canon CanoScan (D1250 U2F) scanner. Data modified from (15).

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**[Fig. 3](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4121126/figure/F3/)** In-gel displacement assay. Two native gels were loaded with the same concentrations of E. coli DHPR. Lane 1: NativeMark protein standard. Lanes 2–11: E. coli DHPR (10 µL) at concentrations of 0.0072, 0.014, 0.028, 0.072, 0.143, 0.29, 0.72, 1.4, 2.2, and 4.3  $\mu$ g/ $\mu$ L, respectively. All gels were scanned as in Fig. 2C. (A) Staining with 0.5 mM NADH and 2.0 mM CRAA (pH 7.8). The gel showed no bands for CRAA bound to DHPR, due to displacement by NADH. (B) Staining as in (A), but in the absence of NADH competitor. Lowest detectable concentration of DHPR was 0.14 µg/µL. (C) The same gel stained with Coomassie blue. The lowest detectable concentration of a DHPR band was 0.072 µg/µL. Data modified from (15).

#### *3.1 Preparation of the CRAA Staining Reagent*

CRAA staining buffer was prepared immediately before use. For best staining results, it is better to use freshly prepared CRAA buffer, since CRAA can be oxidized during storage (*see* [Note 1\)](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4121126/#FN1).

#### *3.2 Running the Native Gel*

- 1. Prepare 10 μL aliquots of different concentrations of proteins, using 2.5 μL of native stain. Protein samples are always stored in an ice bath before use (*see* [Note 2\)](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4121126/#FN2).
- 2. NativeMark™ molecular weight standards from Invitrogen were also used.
- 3. The above protein samples were loaded onto a 10% Tris-Glycine native gel (Novex®), run using Tris-Glycine running buffer. The

gel was run at 125 V for  $\sim$  2.0 h, or until the Bromphenol blue front approaches the bottom of the gel (*see* [Note 3\)](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4121126/#FN3).

4. The gel is cut from the gel fastening frame, to then be used in the staining procedure.

### *3.3 In-Gel Staining of the Dehydrogenase Subproteome using CRAA*

- 1. The native Gel from the previous section was incubated with blocking buffer for 20 minutes, using gentle mixing on an orbital shaker (*see* [Note 4\)](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4121126/#FN4).
- 2. The Gel was then rinsed with PBS buffer for 20 minutes before staining.
- 3. The Gel was stained with CRAA staining solution for 20 minutes (*see* [Note 5\)](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4121126/#FN5).
- 4. The Gel was rinsed for about one hour on an orbital shaker with 25 mM Tris-HCl buffer, pH 8.5, until a clear band was seen due to binding of CRAA. The background of the Gel turns a pale yellowish color [\(Fig. 1\)](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4121126/figure/F1/). The CRAA bands can be imaged with either a fluorescence reader or simply by visible imaging using a document scanner.
- 5. The Gel was then fixed with gel fixing solution, by incubating for 15 minutes (*see* [Note 6\)](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4121126/#FN6). This completes the staining procedure.
- 6. For comparison, another Gel was stained with Coomassie blue staining solution. Gel was incubated on an orbital shaker for 1–3 hours.
- 7. The Gel was destained with a destaining solution over a period of 1 hour.
- 8. The destained gels were washed with water; then soaked in 10% glycerol for 15 minutes.
- 9. Gels were then placed in cellophane, being careful to remove any air bubbles. A small amount of 10% glycerol was added to moisten the cellophane, which is held between plastic plates that were fixed with binder clips (*see* [Note 7\)](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4121126/#FN7).
- 15.The plate with gel (vertical) was placed on a shelf at room temperature, to dry overnight.
- 16.Remove the gel from the holder. The gel can now be scanned.

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#### *3.4 In-Gel Staining in a Competition Study*

- 1. For the displacement assay, two native gels were run at the same time, and pretreated with blocking buffer (5% milk in pH 7.4 PBS buffer).
- 2. One Gel was incubated in a solution containing the competition buffer: 0.5 mM NADH and 2.0 mM CRAA in 25 mM pH 8.5 Tris buffer.
- 3. The other Gel was stained with the 2.0 mM CRAA buffer but without NADH present.
- 4. The Gels were rinsed for about 1 hour until the clear red CRAAbound bands were observed, compared to the pale yellow background (*see* [Note 8\)](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4121126/#FN8).
- 5. The Gel images were recorded.
- 6. After staining with CRAA and recording those Gel images, Gels could be further stained using Coomassie blue to identify all proteins, and their positions relative to the molecular weight standards (which did not stain with CRAA).

#### *3.5 Imaging the Gel*

- 1. The Gel can be scanned with a document scanner; the visible red band from CRAA binding to dehydrogenases can be recorded using any common document scanning device.
- 2. The Gel can also be fluorescently imaged; for example, using a Kodak Image Station 2000MM system. CRAA binding to dehydrogenases shows fluorescent bands.
- 3. When the gel is recorded using Fluorescent imaging, the gels are irradiated at  $\sim$ 465 nm with detection at  $\sim$ 535 nm, using the filters on the Kodak Image Station.
- 4. Coomassie blue stained Gels were recorded using a Canon document scanner.

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**[Fig. 4](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4121126/figure/F4/)** CRAA staining of multiple dehydrogenases: competition +/− NADH. Lane 1: NativeMark protein standard. Lanes 2 and 3: LDH at concentrations of 5.4 and 10.8  $\mu q/\mu L$ . Lanes 4 and 5: E. coli DHPR at concentrations of 2.2 and 4.4  $\mu q/\mu L$ . Lanes 6 and 7: E. coli DOXPR at concentrations of 0.625 and 1.25 µg/µL. The gels were stained with 2.0 mM CRAA (pH 7.8) in the (A) presence of NADH (0.5 mM) or (B) absence of NADH. (C) The same gel was also stained with Coomassie blue. Data modified from (15).

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#### **Footnotes**

- <sup>1</sup>CRAA has low solubility in water, but it can be dissolved to  $\sim$ 4 mM at pH 7.8. CRAA can be oxidized when in solution. For this reason, it is best to prepare CRAA staining buffer fresh, as needed. 2 mM CRAA was chosen to stain dehydrogenases in native Gels, to lessen the CRAA yellowish background on the Gel.
- $2$ Only native loading buffer (i.e. no SDS) can be used to prepare protein samples. If SDS-PAGE gel loading buffer is erroneously used, protein could be denatured and will therefore not bind to CRAA when staining.
- <sup>3</sup>The 10% Tris-Glycine Novex<sup>®</sup> native gel does not have easily visualized wells when the comb is removed. It is helpful to label each well with a marker, for correct loading of protein samples in the right position.
- <sup>4</sup>Gels were soaked in 5% milk in pH 7.4 PBS buffer for 20 minutes to prevent nonspecific binding of CRAA to the Gel itself. If Gels were stained with

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CRAA buffer directly, it usually showed a reddish background and takes much longer to rinse the CRAA background away. But, too long of a washing time could also wash away stained dehydrogenases as well.

- <sup>5</sup>After staining with CRAA for about 20 minutes, the Gel needs to be washed with 25 mM Tris buffer for about 1 hour to achieve the best contrast. If CRAA staining was too long, it will take a longer time to wash away the background CRAA on the Gel.
- <sup>6</sup>The native Gels should be fixed first before staining to avoid any loss of protein from the native Gel.
- <sup>7</sup>When drying the Gel with Gel drying cellophane film, make sure there are no air bubbles between the Gel and films, or else the Gel will be distorted when dried.
- <sup>8</sup>If CRAA is used in the competition binding with NADH or other NADHcompetitive inhibitors, NADH (or the inhibitor of interest) needs to be present in both the staining and washing process, because CRAA can competitively bind to the dehydrogenases on the native gel at any stage of the process (ex. CRAA washing out of the background, can then bind to dehydrogenases in the Gel).
- <sup>9</sup>The CRAA probe has been shown, using affinity chromatography of proteome samples (human liver and *M. tuberculosis* extracts) followed by tandem mass spectrometry, to bind cross reactively with various dehydrogenases, and yet have some selectivity for the dehydrogenase family [\(18\)](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4121126/#R18).

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