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Abstract

The conversion of 5-aminoimidazole ribonucleotide (AIR) to 4-carboxy-AIR (CAIR) represents an unusual divergence in purine biosynthesis: microbes and nonmetazoan eukaryotes use class I PurEs while animals use class II PurEs. Class I PurEs are therefore a potential antimicrobial target; however, no enzyme activity assay is suitable for high throughput screening (HTS). Here we report a simple chemical quench that fixes the PurE substrate/product ratio for 24 h, as assessed by the Bratton-Marshall assay (BMA) for diazotizable amines. The $ZnSO_4$ stopping reagent is proposed to chelate CAIR, enabling delayed analysis of this acid-labile product by BMA or other HTS methods.

Keywords: purine biosynthesis, aminoimidazole, substrate depletion, chelation

1 De novo purine biosynthesis harbors an unusual divergence in a ubiq-

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uitous biosynthetic pathway [1]. In most microbes and nonmetazoan eukaryotes, a class I PurE [PurE1, N^5-carboxyaminoimidazole ribonucleotide (NCAIR) mutase, EC 5.4.99.18] reversibly converts NCAIR to 4-carboxy-5-aminoimidazole ribonucleotide (CAIR) (Fig. 1) [2]. Animals produce CAIR by a different route, using a class II PurE (PurE2, 5-aminoimidazole ribonucleotide (AIR) carboxylase, EC 4.1.1.21) [3, 4, 5]. Microbial pathogenesis relies upon purine biosynthesis [6], particularly *purE1*, which is therefore a promising but unexploited antibiotic target [7, 8]. Inhibitors selective for PurE1 could have antibacterial, antifungal, or herbicidal activity [9]. In this work, we describe a functional assay for PurE that is compatible with inhibitor identification by high-throughput screening (HTS) methods.

Currently available PurE functional assays are not amenable to HTS methods. A continuous ultraviolet (UV) assay that monitors the absorbance decrease due to CAIR decarboxylation [10, 11, 2] is subject to spectral interference by many candidate inhibitors. CAIR synthesis assays are complicated by the acid lability of the NCAIR substrate [12, 13, 2], the need for multiple coupling enzymes [14, 9], and, for discontinuous assays detecting inorganic phosphate production, handling of malachite green reagent [15] and a background due to formation of phosphate by the coupling enzymes. The Bratton-Marshall assay (BMA) [16] was used in early studies of PurE2 [3], but seldom thereafter. The BMA yields intensely colored AIR and CAIR derivatives, AIR-BM and CAIR-BM (Fig. 1), that possess characteristic visible absorbance spectra [17], are relatively stable, and might be suitable for HTS methods. The BMA, however, requires multiple timed reagent additions that would be difficult to implement during parallel screens. An alternative

would be to stop the PurE reaction prior to BMA workup using a reagent that does not alter product yields.

The first task was to determine an extinction coefficient for CAIR-BM at 500 nm, a goal that requires pure CAIR. A new CAIR synthesis, which uses LiOH to saponify aminoimidazole-4-carboxamide ribonucleotide (AICAR) [18] and thereby facilitates salt removal (see Experimental Procedures in the Supplementary material), furnished CAIR in high yield (85%) and purity (99%). No signal corresponding to AIR H2 was observed in ^{1}H NMR spectra (Fig. S1 in the Supplementary material). Mass spectrometry (MS) analysis of BMA reaction mixtures revealed only the expected molecular ions for single BM adducts and ions present in control mixtures (Table S1 in the Supplementary material). HPLC analysis of CAIR subjected to BMA workup showed 97% CAIR-BM / 3% AIR-BM (Fig. S2 in the Supplementary material); a small amount of AIR evidently forms during the BMA acid quench step. CAIR-BM appears to consist of a mixture of species that cannot be resolved by HPLC but that possess the same molecular formula. An isobaric mixture might also account for the complex shape of the CAIR-BM visible spectrum (Fig. S3 in the Supplementary material). Aliquots of a solution containing pure CAIR were (1) used to determine an accurate CAIR concentration, by complete enzymatic conversion to AIR followed by BMA analysis, and (2) used to obtain a spectrum of CAIR-BM, which gave $\epsilon_{500}^{\text{CAIR-BM}} = 11\,200$ M^{-1} cm^{-1} (for comparison, $\epsilon_{500}^{\text{AIR-BM}} = 24\,800$ M^{-1} cm^{-1}[17]).

The second task was to identify an enzyme quenching method that is compatible with BMA workup and product analysis. CAIR and analogues form stable complexes in which aminoimidazole N3 and O7 atoms (Fig. 1)

coordinate a transition metal ion [19]. $(CAIR)_n$-metal ($n = 2$ or 3) complexes (Fig. S4 in the Supplementary material) do not bind to or inhibit chicken PurE2 in the presence of excess free CAIR [20, 21, 22]. Mg^{2+} inhibits PurE2-mediated CAIR decarboxylation but not AIR carboxylation, consistent with the formation of a low-affinity metal-CAIR complex [10, 4]. To determine if transition metals can rapidly and reversibly form CAIR-metal complexes, a ten-fold molar excess (relative to the initial CAIR concentration) of $NiSO_4$ or $ZnSO_4$ was added to UV-monitored CAIR decarboxylation reaction mixtures. CAIR decarboxylation stopped immediately, but could be restarted by the addition of EDTA (Fig. S5 in the Supplementary material). EDTA had no effect on BMA workup; the initial acidification step apparently dissociates CAIR complexes formed with either Ni^{2+} or Zn^{2+}.

Zn^{2+} appeared to form a CAIR complex with higher kinetic stability than Ni^{2+}, and to pose fewer concerns with regard to toxicity, spectral overlap with (C)AIR-BM, and redox chemistry. A CAIR decarboxylation reaction mixture incubated at 30 °C was stopped at ∼30% completion by the addition of ∼3 molar equivalents of $ZnSO_4$ (Fig. 2). Equivalent yields of CAIR-BM were obtained if the BMA workup was delayed by up to 4 h. In contrast, Ni^{2+}-stopped reactions lost CAIR at the rate of about 10% per h at 30 °C (data not shown). A saturation curve employing $ZnSO_4$ quenching gave kinetic constants similar to the standard continuous UV assay (Fig. S6 in the Supplementary material). Indistinguishable velocities were obtained at 40 μM CAIR for a quenched reaction worked up promptly or after a 24 h delay. Therefore $ZnSO_4$ is a suitable stopping reagent for PurE reaction mixtures.

Metal stopping reagents are proposed to work by rapid substrate depletion, an uncommon mode of enzyme inhibition (e.g., vancomycin [24]) [25]. Immobilized metal affinity chromatography (IMAC) was used to test if CAIR binds to Ni^{2+}, immobilized on a nitrilotriacetic acid (NTA) column. An equimolar mixture of CAIR and AIR was applied to the NiNTA column. The first column fraction contained pure AIR, consistent with selective retention of CAIR on the column as a CAIR-Ni^{2+} complex (Figs. S7 and S8 in the Supplementary material). Subsequent fractions contained CAIR, indicating that the Ni^{2+}-CAIR interaction was reversible (data not shown). While these results do not exclude the possibility that PurE is inhibited by direct protein-metal interactions, they indicate that the rapid formation of a stable CAIR-transition metal complex is sufficient to account for $ZnSO_4$-mediated arrest of CAIR decarboxylation.

In summary, we have identified a PurE reaction quencher that facilitates HTS using the BMA as a detection method. PurE-mediated CAIR decarboxylation stopped by a single liquid addition, of a $ZnSO_4$ solution, allows delayed BMA workup. The BMA is compatible with candidate inhibitors that absorb UV light, a common practical concern for detecting initial hits, which often have low potency and therefore require high compound concentrations that cause significant spectral interference. BMA-detected PurE screens require no other enzymes and can be used to screen candidate inhibitors that do not contain diazotizable amines or strong metal chelators. The metal stopping reagent could be adapted for use with other detection methods or in assays of 5-aminoimidazole-4-*N*-succinylcarboxamide ribonucleotide (SAICAR) synthetase (PurC, EC 6.3.2.6), the other enzyme known

to use CAIR as a substrate. SAICAR has recently been shown to activate cancer metabolism via pyruvate kinase isoform M2 [26].

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Figure Captions

Figure 1: PurE1 reaction and CAIR/AIR detection by Bratton-Marshall assay (BMA). NCAIR decarboxylates to AIR (half-life of 0.9 min at pH 7.8 and 30 °C) during the enzyme assay [2], an acid-dependent process that goes to completion during the BMA. R5P = ribosyl 5′-phosphate, R = H (AIR-BM) or COO^- (CAIR-BM). CAIR aminoimidazole atoms are numbered as in PDB residue type C2R. CAIR atoms proposed to coordinate transition metals are indicated with unfilled arrows.

Figure 2: Zn^{2+} ions stop CAIR decarboxylation. Reaction mixtures (1.26 mL final volume) at 30 °C contained 50 mM Tris•HCl, pH 8.0, 42 μM CAIR, and 0.13 μg *Acetobacter aceti* PurE1 (0.005 units) [23]. At 5 min, $ZnSO_4$ (0.1 mM) was added to one reaction. Aliquots (0.2 mL) were removed prior to the addition of enzyme (zero time point) and at indicated intervals, and immediately added to a 1.7 mL centrifuge tube containing a freshly prepared mixture of 20% (w/v) trichloroacetic acid / 1.33 M potassium phosphate, pH 1.4, (0.1 mL) and 0.1% (w/v) sodium nitrite (0.1 mL). After 3 min, 0.5% (w/v) ammonium sulfamate (0.1 mL) was added to destroy excess nitrite. After an additional min, Bratton-Marshall reagent [0.1 mL of 0.1% (w/v) *N*-(1-naphthyl)ethylenediamine dihydrochloride] was added. After 10 min, the solution (0.6 mL) was centrifuged (16 000g, 30 s) to eliminate bubbles and the absorbance at 500 nm (A_{500}) was recorded.

CAIR consumption was quantitated using a differential extinction coefficient for CAIR to AIR conversion, $\Delta\epsilon_{500} = \epsilon_{500}^{\text{AIR-BM}} - \epsilon_{500}^{\text{CAIR-BM}} = 13\,600 \pm 1600$ M^{-1} cm^{-1}. [CAIR] values at time t were determined using ($A_{500}^{\text{AIR-BM}}$ –

$A^t_{500})/\Delta\epsilon_{500}$, where $A^{\text{AIR-BM}}_{500}$ was from a control reaction in which CAIR was completely converted to AIR.

The symbols and error bars represent the mean and standard deviation, respectively, for at least three independent replicates of $ZnSO_4$-quenched (filled circles) and -unquenched control (open circles) reactions. Concentrations determined at $t > 5$ min were significantly different in quenched and control reaction mixtures [one-tailed t-test, P-value < 0.01 (**) or < 0.001 (***)]. The slope of the line connecting the first two points corresponds to 0.003 units of enzyme activity, comparable to the 0.004 units expected under the reaction conditions [23].