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Validation of a general method for activity estimation of cyanide evolving oxidoreductases

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ABSTRACT

Ethylene is a key molecule in organic synthesis currently produced by steam cracking of fossil hydrocarbons. In nature, ethylene is produced in higher plants by 1-aminocyclopropane-1-carboxylic acid oxidase (ACCO). Biocatalytic alternatives for ethylene production are still far from being competitive with traditional production plants. Furthermore, data dispersion shown in the literature adds uncertainty to the introduction of ACCO as a biocatalyst, especially when larger numbers of isoforms or mutants are to be compared. Here we propose a new method for measuring ACCO activity based on cyanide detection. Data provided here indicate that cyanide detection is more precise, more responsive, and much more stable than any other method tested for ACCO activity estimation so far. Briefly, enzymatically produced cyanide can be detected by its derivatization with naphthalene-2,3-dicarboxyaldehyde (NDA) to generate 1-cyanobenz[f]isoindole (CBI), which is further detected by high-performance liquid chromatography (HPLC) coupled with a fluorescence detector. Cyanide can be detected in the range between 0.99 and 60.17 pmol, which is three orders of magnitude more sensitive than the currently used ethylene estimation method.

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The transition of conventional industries toward cleaner alternatives relies on the development of suitable biocatalytic processes. Biocatalysis excels over chemical counterparts due to its inherent ability to perform reactions stereo-, enantio-, and chemo-specifically. There are a number of biocatalytic reactions that have been successfully incorporated into industrial processes with the concomitant reduction of required water and energy and also in the generation of byproducts [1,2]. One of the many fields of the chemical industry that may be displaced by biocatalytic processes is the production of a group of small molecules that are important feedstock for the chemical industry: methanol, ammonia, aromatics, and olefins [3–5].

Ethylene, the simplest olefin, is a key molecule in organic synthesis [6]. Global ethylene production in 2011 was close to 142 million metric tons, and it is forecasted to reach 165 million metric tons by 2015, surpassing all other organic petrochemicals in production and in the amount sold [1,7]. Ethylene is used as a raw material for a great number of commercial synthetic organic chemical products (e.g., polyethylene, acetaldehyde, ethylene oxide, ethanol), being mainly produced by steam cracking of hydrocarbons in the petrochemical industry, a process that requires high pressure and high temperature. Although biocatalytic alternatives for ethylene production are still far from competitive with traditional production plants, they offer new opportunities in accordance with the principles of green chemistry [2,7,8]. Ethylene, an essential hormone for many aspects of plant life such as root development, germination, senescence, fruit ripening, and defense mechanisms [9], is naturally produced by higher plants from 1-aminocyclopropane-1-carboxylic acid (ACC),[1] a metabolite of methionine [10], and the last step of its biosynthesis is catalyzed by ACC oxidase (ACCO) (Fig. 1) [11].

There is a significant amount of information regarding the sequence, structure, and catalytic mechanism of ACCO [12–17], and this enzyme arises as a natural target for further catalytic improvement by protein engineering methods. ACCO activity is usually measured following Mitchell and coworkers, where the

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[1] *Abbreviations used:* ACC, 1-aminocyclopropane-1-carboxylic acid; ACCO, ACC oxidase; DTT, dl-dithiothreitol; $Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$, ammonium iron(II) sulfate hexahydrate; NDA, naphthalene-2,3-dicarboxyaldehyde; CBI, 1-cyanobenz[f]isoindole; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; KCN, potassium cyanide.

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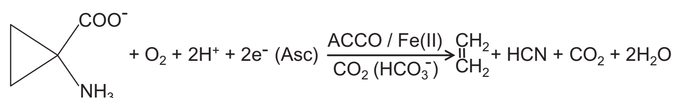

Fig.1. Reaction catalyzed by ACCO.

production of ethylene formed is determined on a gas chromatograph attached to a flame ionization detector [18]. Seldom has the determination of oxygen consumption using a Clark electrode been reported as well [19]. By using these methodologies, catalytic parameters of native enzymes from different sources have been reported with K_m values for ACCO ranging from 0.032 to 2.87 mM [20–23]. The two orders of magnitude variation among these data may arise from differences in the purification protocol, in the reaction conditions, or in the detection uncertainty. Strong variation on the catalytic parameters is shown even for the same isoform expressed in *Escherichia coli* with K_m values ranging from 0.03 to 0.130 mM, whereas apparent k_{cat} values ranged from 6.1 to 354 min^{-1} [24,25]. Data dispersion shown in the literature adds uncertainty to the introduction of ACCO as a biocatalyst, especially when larger numbers of isoforms or mutants are to be compared. Therefore, it is necessary to obtain more robust and sensitive methodologies for the screening of ACCO in a protein engineering scheme.

Here we propose a new method for measuring ACCO activity based on cyanide detection. Indeed, data provided here indicate that cyanide detection is more precise, more responsive, and much more stable than any other method tested for ACCO activity estimation so far.

Materials and methods

All chemical supplies were of reagent-grade purity and purchased from Sigma–Aldrich unless otherwise stated. Restriction enzymes and ligase were purchased from New England Biolabs, and pET32a(+) was purchased from Novagen.

All experiments presented in this article were performed with the same enzyme batch and are representatives of at least three rounds of experiments performed with the same number of pure samples.

Vector construction for ACCO expression

ACCO was subcloned in pET32a(+). The vector was first digested with *Nde*I and *Bam*HI and then purified with a GeneJet Gel Extraction Kit from Fermentas. The ACCO gene contained in pET-21a was restricted with the same pair of enzymes; the 945-bp fragment liberated was purified from gel. The ACCO fragment was then ligated into the previously restricted pET32a(+) and transformed into *E. coli* DH5α cells. A number of colonies were picked and screened for the ACCO gene by restriction, and the ACCO gene was further confirmed by sequencing. Expression of ACCO was placed under the control of a T7 promoter. The vector also includes the *E. coli* ampicillin resistance gene. The expression construction is referred to as pET32ACCO. This plasmid was then transformed into *E. coli* BL21 for protein expression.

ACCO expression

A 0.25-L LB broth inoculum flask (supplemented with 20 μg ml^{-1} ampicillin) was grown at 37 °C in a shaker for 12 h. The inoculum was added to a 10-L fermenter operated at 37 °C, 300 rpm, and 0.33 vvm to an A_{600} between 0.4 and 0.6, at which time isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM. Growth was continued at 28 °C for an additional 3 h, and cells and supernatant were separated by centrifugation.

Purification of ACCO

Cells were resuspended in 25 mM Hepes (pH 8.0), 50 mM NaCl, 5 mM dl-dithiothreitol (DTT), 10% glycerol, DNase I (10 U/ml), lysozyme (10 μg ml^{-1}), and protease inhibitor Complete EDTA-Free (Roche). Three ultrasonic pulses were used to lysate the resuspended cells. The lysate was centrifuged at 13,000 rpm at 4 °C for 30 min. The supernatant was precipitated with ammonium sulfate to a saturation concentration of 60%, this mixture was again centrifuged at 13,000 rpm at 4 °C, and the supernatant was collected. Ammonium sulfate was added to 80% saturation, and the mixture was centrifuged with the same conditions. The pellet was recovered and resuspended in 25 mM Hepes (pH 8.0) and 3 M ammonium sulfate and loaded onto Macro-Prep t-Butyl Resin (Bio-Rad) equilibrated with buffer (25 mM Hepes, pH 8.0) and 3 M ammonium sulfate. The column was washed with the same buffer. ACCO was eluted from the column in a linear gradient (3–0 M ammonium sulfate in buffer (25 mM Hepes).

Fractions containing ACCO were dialyzed in 25 mM Hepes (pH 8.0), 5 mM DTT, and 10% glycerol to further load onto Q-Sepharose Fast-Flow (Sigma) equilibrated with the same buffer. The column was washed with this buffer, and the retained fractions were eluted with a 0- to 0.5-M NaCl linear gradient. The fractions containing ACCO were dialyzed in 25 mM Hepes (pH 7.2) to further concentration in a Centricon 10K MWCO (molecular weight cutoff) device (Millipore). In all of the purification steps, ACCO fractions were selected as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). Gels were prestained by adding 0.5% of 2,2,2-trichloroethanol into gel mixture [26], and gel images were obtained with a Gel Doc EZ System (Bio-Rad) using the stain-free tray filter. Protein concentration was determined by Bradford assay using bovine serum albumin (BSA) as a standard.

Iron quantification

ACCO preparation iron content was determined by a modification of the Hennessy and coworkers methodology [27]. To quantify iron content in the enzymatic preparation, 250 μl of ACCO mixture was denatured by the addition of 20 μl of HCL (1 M), and this denatured solution was instantly neutralized with 20 μl of NaOH (1 M). The obtained mixture was centrifuged at 10,000 rpm for 5 min. The supernatant was reduced with 200 μl of a solution of ascorbic acid (1 M) and completed to a 1-ml final volume of buffer acetate (pH 4.5) with ferene S (final concentration of 0.638 mM). The mixture was incubated for 5 min at room temperature to allow color development. Absorbance of the sample was measured at 593 nm (λ_{593} = 34,500 L cm^{-1} mol^{-1}) using a Thermo Scientific spectrophotometer.

ACCO activity assays

ACCO activity was measured using ACC, ammonium iron(II) sulfate hexahydrate [$Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$], $NaHCO_3$, and L-ascorbic acid [25].

Ethylene determination was performed at room temperature in 1.7 ml of hermetically sealed vials. The total assay volume was 200 μl and contained 100 mM Hepes buffer at pH 7.2, 102.8 μg of ACCO, 18 mM $NaHCO_3$, 8 mM L-ascorbic acid, 80 μM $Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$, and 5 mM ACC. After 2 min of reaction, 250 μl of headspace gas was removed using a gas-tight syringe and ethylene production was quantified by gas chromatography. Measurements

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