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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 chemospecifically. 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, eth-

anol), 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

¹ Abbreviations used: ACC, 1-aminocyclopropane-1-carboxylic acid; ACCO, ACC oxidase; DTT, dl-dithiothreitol; Fe(NH₄)₂(SO₄)₂·6H₂O, 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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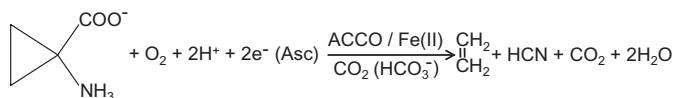


Fig.1. Reaction catalyzed by ACCO.

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* DH5 α 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 $\mu\text{g ml}^{-1}$ 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 $\mu\text{g ml}^{-1}$), and protease inhibitor Complete EDTA-Free (Roche). Three ultrasonic pulses were used to lyse 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 ($\lambda_{593} = 34,500 \text{ L cm}^{-1} \text{ mol}^{-1}$) using a Thermo Scientific spectrophotometer.

ACCO activity assays

ACCO activity was measured using ACC, ammonium iron(II) sulfate hexahydrate $[\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}]$, 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 $[\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}]$, 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

were performed by varying the concentration of ACC while maintaining the concentration of the other substrates. Nonlinear curve fitting to the Michaelis–Menten equation was performed using SigmaPlot 11.0 to determine kinetic parameters. Gas chromatography measurements were performed with an Agilent 6890N gas chromatograph equipped with a fused silica capillary column Gas-Pro (J & W, 30 m × 0.32 mm) and a flame ionization detector. The following conditions were used: He (99.999%) as a carrier gas (1.4 ml min⁻¹), in split mode (20:1), $T_{\text{injector}} = 250$, $T_{\text{oven}} = 90$, and $T_{\text{detector}} = 260$. Ethylene was quantified versus an external standard of ethylene (99.99%) from Sigma.

Oxygen consumption was measured using an Oxygraph device (Hansatech Instrument, King's Lyan, UK) equipped with an oxygen electrode, a gas-tight cap, and a Teflon-coated stirrer bar. The Oxygraph was controlled by and configured from a personal computer, via custom software supplied by the manufacturer, which also recorded oxygen concentration in the chamber over time. Instrument calibration was performed according to the manufacturer's instructions using the same buffer for the enzymatic reactions and 99.5% nitrogen. Temperature was maintained at 19 °C. Standard reaction mixture (1 ml) contained 100 mM Hepes (pH 7.2), 205.6 µg of ACCO, 18 mM NaHCO₃, 8 mM L-ascorbic acid, and 5 mM ACC. Reconstituted ACCO with 0.74 Fe(NH₄)₂(SO₄)₂·6H₂O molar excess was used to start the reactions. All rates were calculated subtracting background oxygen consumption [19]. Measurements were performed by varying the concentration of ACC while maintaining the concentration of the other substrates. Nonlinear curve fitting to the Michaelis–Menten equation was performed using SigmaPlot 11.0 to determine kinetic parameters.

Cyanide was quantified after derivatization by a modification of the method of Driesener and coworkers [28], in which the cyanide anion reacts with naphthalene-2,3-dicarboxyaldehyde (NDA) and a primary amine, generating the fluorescent 1-cyanobenz[*f*]isoindole (CBI) derivative (Fig. 2) [29]. Standard reaction mixture (100 µl) contained 100 mM Hepes (pH 7.2), 1.29 µg of ACCO, 18 mM NaHCO₃, 8 mM L-ascorbic acid, 80 µM Fe(NH₄)₂(SO₄)₂·6H₂O, and 5 mM ACC. Initial velocities were measured at 18 °C. Reactions were ended with the addition of 50 mM ethylenediaminetetraacetic acid (EDTA, pH 8.0). All rates were calculated subtracting background cyanide production. For derivatization, 3.44 mM NDA in methanol and 1.9 mM ACC in buffer (100 mM Hepes, pH 7.2) were added to the ACCO reaction. Derivatization proceeded for 5 min at 95 °C. After derivatization, fluorescence was measured by

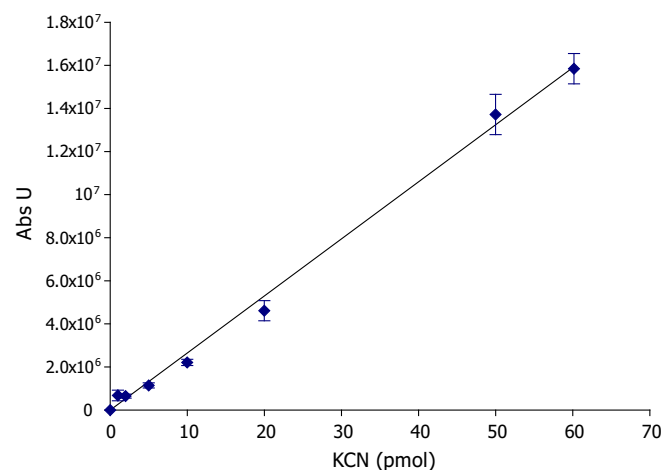


Fig. 3. Calibration plot of KCN derivatized with NDA and ACC. Lower and higher dots on plot are detection and quantification limits for CBI under working conditions.

high-performance liquid chromatography (HPLC). Chromatographic separation was achieved by injecting 20 µl of derivatized supernatant into a Hypersil C18 ZORBAX Eclipse reverse phase column (4.6 × 100 mm). The chromatography buffers were 2 mM ammonium formate (pH 3.0) (solvent A) and methanol (solvent B), and the initial mobile phase contained 20% solvent B for 5 min, followed by a gradient to 65% solvent B over 20 min and then 100% solvent B over 2 min, where it was held isocratically for 10 min. The CBI derivative eluted with a retention time of approximately 27 min and was detected fluorometrically by the signal emitted at $\lambda_{\text{ex}} = 418$ nm and $\lambda_{\text{em}} = 454$ nm. To establish a cyanide calibration plot, potassium cyanide (KCN) standards (1–99 pmol) in 100 mM Hepes (pH 7.2) were prepared and subjected to the same conditions as ACCO assays (Fig. 3).

Results and discussion

The tomato ACCO gene codified in pET-21a has been widely used for the heterologous expression of the enzyme in *E. coli* [12,17,24,30]; however, the resulting ACCO was lower than expected based on the literature and on our experience [31]. To increase the yield, the tomato ACCO gene was subcloned into pET32a(+), acquiring the expected ability to produce three times more protein than with the previous construction, which is crucial for an optimal purification methodology. The current protocol used for ACCO purification generates enough material for protein characterization; however, we identified two weaknesses: (i) the preparation obtained was not high purity, and this will certainly affect the determination of the catalytic parameters, and (ii) the Fe naturally present on the active site of the enzyme is removed as a result of the EDTA addition at the protein extraction stage of the purification [25]. Because ACCO catalysis is Fe dependent, this ion must be provided again in the reaction medium, with the unavoidable generation of reactive oxygen species.

To relieve these restrictions, we developed an EDTA-free protein purification protocol that allowed fast purification from large culture volumes, important to avoid excessive handling procedures that can damage the protein [19], and with higher yields of protein (10 mg/L of ~98% relative purity) (Fig. 4). We obtained a highly active protein preparation with 16.5 mol ethylene/mol enzyme/min [24], which is in the activity range described by using the methodology previously reported by Thrower and coworkers [19]. The natural Fe content of 35% in ACCO was conserved because purification was performed without EDTA. High protein yield with

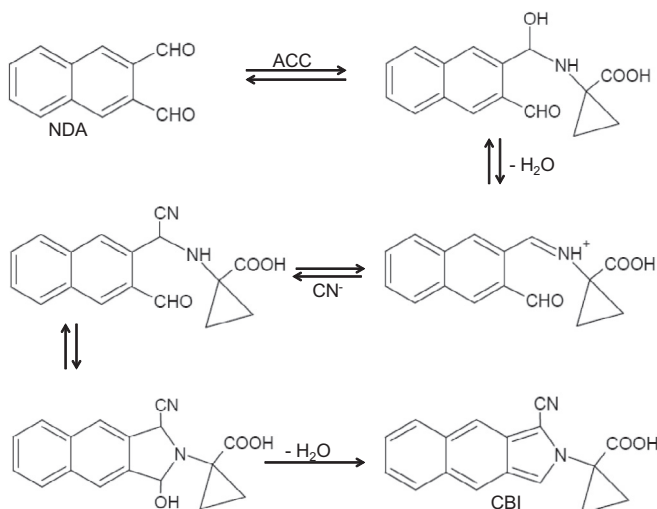


Fig. 2. Scheme of cyanide derivatization with NDA and ACC. (Modified from Carlson et al. [29].)

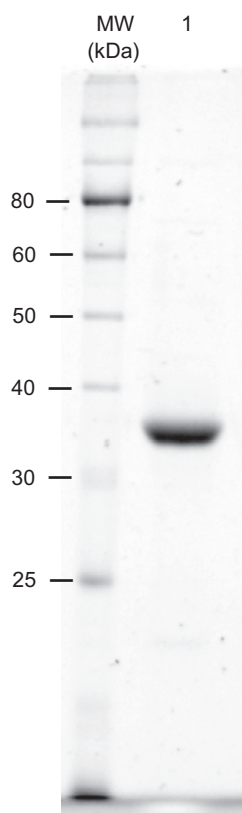


Fig. 4. Denaturing polyacrylamide gel electrophoresis (PAGE) revealed by 2,2,2-trichloroethanol molecular weight ladder (MW) and ACCO preparation after EDTA-free purification protocol (lane 1).

good specific activity indicated that no deactivation took place during purification and, therefore, that no EDTA addition is required throughout the process.

The enzyme preparation was characterized using the following methods: production of ethylene, oxygen consumption, and cyanide production. Ethylene production was measured as reported elsewhere, varying ACC while keeping all other substrate concentrations fixed at saturation [25]. Ethylene was quantified using a calibration plot ranging from 0.81 to 801 nmol that fits into a linear equation. Steady-state kinetics of ethylene production was determined using velocity rates for ethylene production under initial velocity conditions. Ethylene production rates were analyzed using the Michaelis–Menten model, and kinetic parameters obtained were $K_m = 0.17 \pm 0.07$ mM and $k_{cat} = 17.45 \pm 1.41$ min⁻¹ ($R^2 = 0.85$).

For oxygen consumption, we followed the methodology described by Thrower and coworkers [19], and the enzymatic reaction was started with protein previously charged with 0.74 molar excess of ferrous iron to avoid oxygen consumption in the electrode due to free iron in the reaction medium. The reaction was followed for several minutes, and activity rates were measured in accordance with steady-state kinetics. Oxygen consumption rates were subjected to the same treatment as ethylene production data, and kinetic parameters obtained were $K_m = 0.60 \pm 0.23$ mM and $k_{cat} = 3.98 \pm 0.46$ min⁻¹. Although these data sets presented significant dispersion, they satisfactorily fulfilled the Michaelis–Menten model ($R^2 = 0.84$).

All of our results but k_{cat} obtained with oxygen consumption approached those found in the literature for the same ACCO isoform, where K_m values ranged from 0.03 to 0.15 mM and apparent k_{cat} ranged from 11.3 to 354 min⁻¹ [24,25]. It is important to note that there is at least one order of magnitude difference between

reports. When we compare the catalytic parameters reported here, we find that K_m is nearly 4 times larger and k_{cat} and approximately 4 times smaller between oxygen consumption and ethylene production. These differences may arise from physical or chemical differences between methods. For instance, ethylene detection is dependent on accumulation of the product in vials, on saturation of the liquid phase of the reaction, and on further manipulation of the sample until its detection in the gas chromatograph [32,33]. Therefore, this assay does not necessarily provide a direct value for concentration of ethylene but does so for the rate at which the ethylene equilibrates between the reaction mixture and the enclosing air phase [34]. In addition, the sensitivity of this method is lower, with a confidence interval between 210 and 420 pmol ethylene ml⁻¹. On the other side, precision of oxygen measurements using Clark electrode deviations have been reported to depend on different aspects such as membrane damage [35], deterioration of the electrode by deposition of AgCl in the silver anode [36], and the occurrence of nonenzymatic oxygen consumption exacerbated by ascorbic acid [19,37–40]. In addition, oxygen detection is highly dependent on the temperature and is a time-consuming method not suitable for the analysis of a large number of samples. Finally, it is not a sensitive method (0.01×10^6 to 8.4×10^6 pmol oxygen min⁻¹) [41], demanding large amounts of ACCO to detect a significant signal.

Taking into account the drawbacks of the two methodologies available for ACCO activity assays, we propose a methodology based on the method of Driesener and coworkers [28,42]. Briefly, enzymatically produced cyanide can be detected by its derivatization with NDA to generate CBI, which is further detected by HPLC coupled with a fluorescence detector. Modifications needed to be developed for the use of ACC as primary amine instead of taurine. When the enzymatic activity was tested in the presence of taurine and ACC, the chromatogram presented a complex pattern due to different CBI isoforms, one for each R-amine group present. In the chromatograms with and without taurine (Fig. 5A and B, respectively), compound identification was assigned by systematic exclusion of one or more reactants. The retention time for CBI formed with the R-amine group of ACC presents a retention time close to 26 min and is clearly distinguishable from that formed with taurine that appears at 22 min. The retention time shift observed among chromatograms may be due to the different viscosity of individual samples. ACC was added to the reaction in excess, as were the substrates for ACCO activity reaction and for HCN detection. Besides, controls where all reagents present at the ACCO reaction mixture were added individually or in different combinations were run to ensure that none of them interfered with cyanide measurement. It is important to note that complete derivatization required 5 min at 95 °C and pH control with Hepes (100 mM, pH 7.2) [43,44]. Once the derivatization methodology was settled, a calibration plot was established with a KCN standard in the range between 0.99 and 60.17 pmol that fitted into a linear equation, making this method three orders of magnitude more sensitive than ethylene estimation [42] and all other colorimetric cyanide determination methodologies reported to date [45,46]. A highly sensitive methodology allows a better quantification of the released product; the estimation is performed at the liquid phase, striving against the ethylene assay weaknesses described previously.

Different ACCO concentrations were tested to confirm the linear character of the cyanide production rate (Fig. 6). To obtain steady-state kinetic values for cyanide production, experiments were performed varying ACC content while keeping all other substrates at saturation levels. For each ACC concentration, reactions were allowed to proceed for 2 min at 19 °C, samples were taken every 30 s for derivatization, and initial velocity was determined by feeding data into a linear equation fitting (Fig. 7). Nonlinear curve

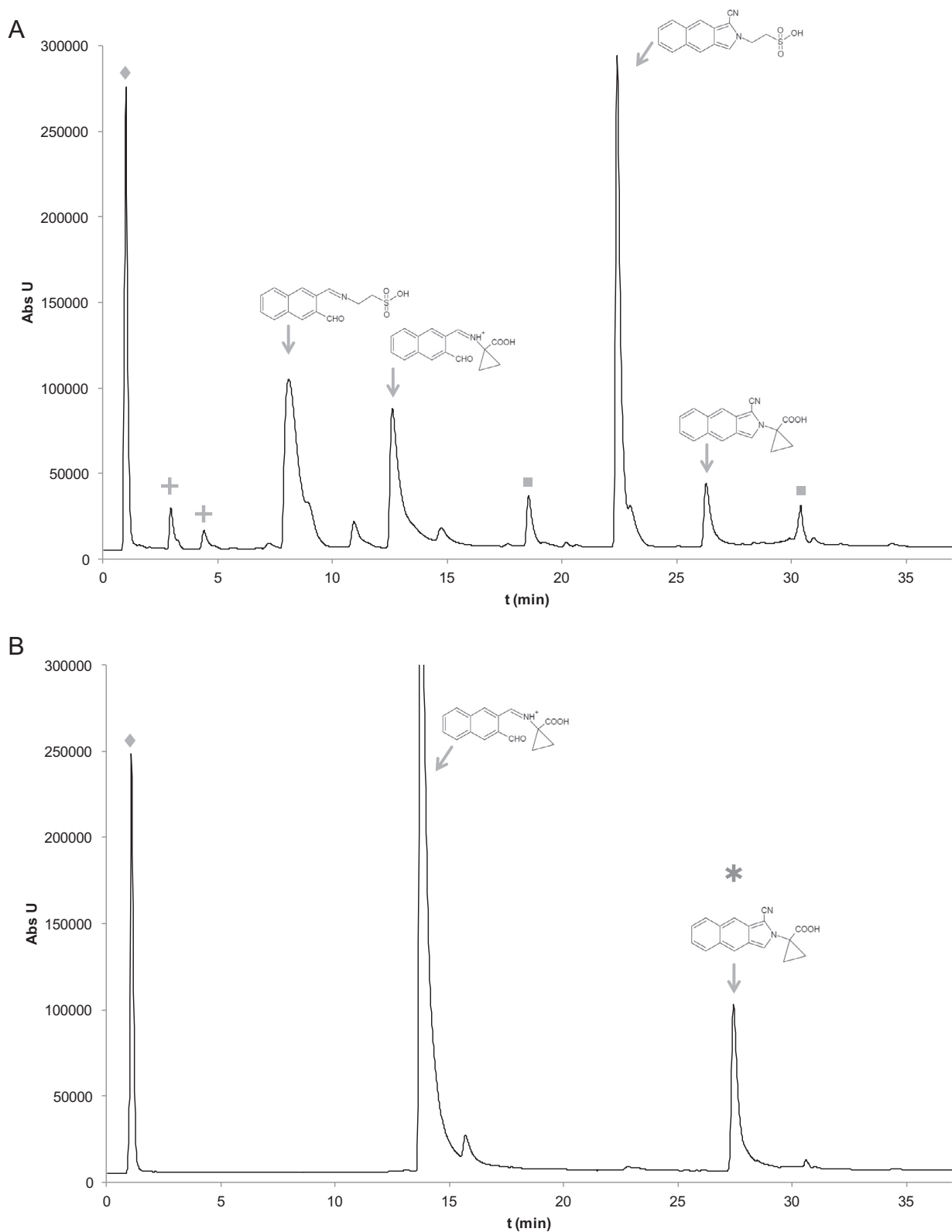


Fig.5. Fluorescent HPLC analysis of CBI from ACCO reaction supplemented with KCN derivatized with taurine and ACC (A) and derivatized with ACC (B). ◆, Adduct formed when the reaction mixture is complete; +, taurine adducts; ■, adducts formed when ACCO and taurine present in the reaction; *, CBI-ACC adduct.

fitting into the Michaelis–Menten model was performed to initial velocities using the data presented in Fig. 8, where $K_m = 0.88 \pm 0.14$ mM and $k_{cat} = 10.52 \pm 0.55$ min⁻¹ ($R^2 = 0.97$) values were obtained.

When we compared these kinetic parameters with those obtained in other experiments, we found that K_m was similar to that obtained with the oxygen consumption methodology; on the other hand, k_{cat} resembled that obtained with ethylene production

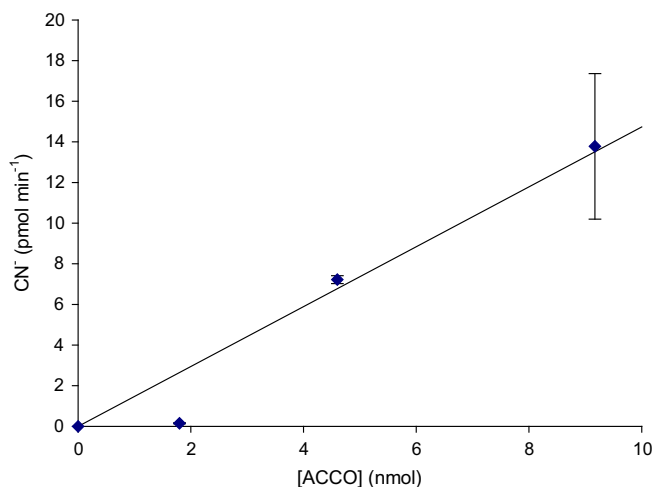


Fig. 6. Cyanide (CN⁻) production rate at different ACCO concentrations. Data were fitted to an exponential equation ($R^2 = 0.94$).

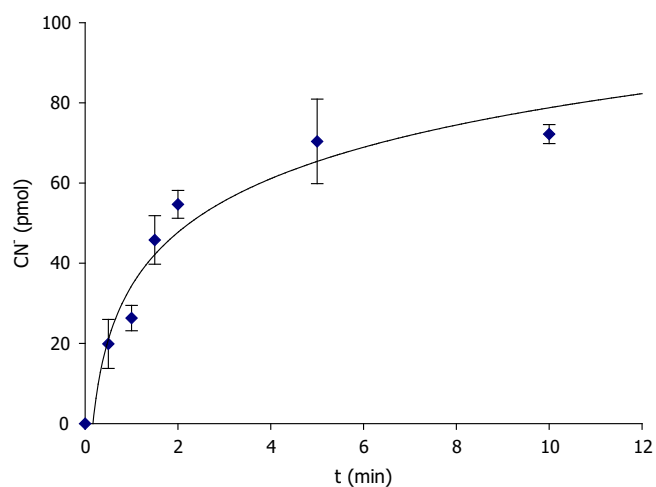


Fig. 7. Time course of the ACCO reaction at substrate excess (ACC, 5 mM). As described in the text, the linear range was established during the first 2 min of enzyme activity. Background cyanide (CN) generation in the absence of enzyme remained less than 5 pmol during all of the experiments. Data were fitted to an exponential equation ($R^2 = 0.95$).

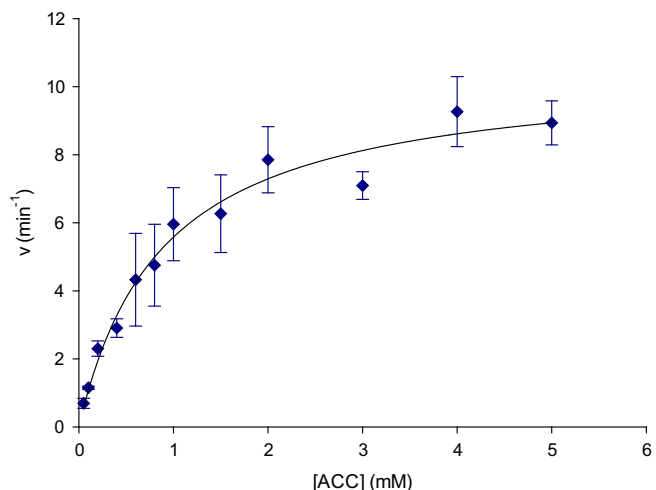


Fig. 8. Michaelis-Menten fitting for the initial velocities determined for the ACCO activity measure by cyanide production.

Table 1

Comparison of kinetic parameters among experiments.

Activity measurement	K_m (mM)	k_{cat} (min ⁻¹)	R^2
Cyanide production	0.88 ± 0.14	10.52 ± 0.55	0.97
Ethylene production	0.17 ± 0.07	17.45 ± 1.41	0.85
Oxygen consumption	0.60 ± 0.23	3.98 ± 0.46	0.84

analysis (Table 1). Regarding the disparity in K_m value, we suggest that it is due to differences in the reaction temperature for ethylene production, although perhaps not by stabilization of the enzyme–substrate complex. Regarding the lower k_{cat} value obtained by oxygen consumption, it is relevant to note that this measurement is obscured by the simultaneous occurrence of at least three different reactions in the vessel—enzymatic, Fenton, and electrode consumption—with most of the oxygen in the reaction depleted by side reactions before activity measurement.

Ethylene determination is further hampered by loss of the molecule through seals when stored in vessels and during handling. Our data suggest that the cyanide method is more precise, more responsive, and more stable than other methods tested here for estimating ACCO activity, looming as a robust methodology for activity estimation not only for ACCO but also for other cyanide evolving oxidoreductases. After the enzymatic reaction was stopped by EDTA addition, cyanide content was stable for up to 40 min of incubation at 19 °C before derivatization. Once derivatized, CBI content is stable for up to 1 h at room temperature.

Early reports of cyanide identification in ACCO reaction dealt with stoichiometric determinations [47]. In accordance, we found that similar amounts of products, independently measured, were released for the ACCO reaction: 17.44 ± 1.84 pmol cyanide/pmol ACCO/min and 16.53 ± 2.87 pmol ethylene/pmol ACCO/min were formed.

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