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Carbon dioxide fixation by reversible pyrrole-2-carboxylate decarboxylase and its application

Marco Wieser, Toyokazu Yoshida, Toru Nagasawa *

Department of Biomolecular Science, Faculty of Engineering, Gifu University, Gifu-Yanagido, 501-1193 Japan

Abstract

Inducible pyrrole-2-carboxylate decarboxylase from *Bacillus megaterium* PYR2910 catalyzes the decarboxylation of pyrrole-2-carboxylate to stoichiometric amounts of pyrrole and CO_2. A unique feature of the homodimeric enzyme is its requirement for an organic acid such as acetate, propionate, butyrate or pimelate. A catalytic mechanism including a cofactor function of the organic acid was proposed. Due to an equilibrium constant of 0.3–0.4 M, the enzyme also catalyzes the reverse carboxylation of pyrrole after the addition of bicarbonate. For the synthesis of pyrrole-2-carboxylate, the reverse reaction was optimized and the equilibrium shifted towards the carboxylate. The product yield was 230 mM (25.5 g/l) pyrrole-2-carboxylate from 300 mM pyrrole in a batch reaction and 325 mM (36.1 g/l) from 400 mM pyrrole in a fed batch reaction, using both whole cells and the purified enzyme in a pH 8.0 reaction mixture with bicarbonate saturation of 1.9 M.

Keywords: Non-oxidative decarboxylation; Pyrrole-2-carboxylate; Reaction equilibrium; Reversibility; Organic acid; Carbon dioxide fixation; *Bacillus megaterium*

1. Introduction

Due to the abundance of CO_2 as the major greenhouse gas and C—C bond forming properties, CO_2 fixing enzymes are of high interest. Since carboxylations are thermodynamically difficult, they are usually coupled to thermodynamically favorable reactions, such as in the case of the well-known CO_2 fixing enzymes ribulose-1,5-bisphosphate carboxylase, which catalyzes the central CO_2 fixation in photosynthetic organisms [1] or PEP carboxylase. So far, the number of reports on enzymatic carboxylations of aromatic moieties in microorganisms is limited to the anaerobic degradation of phenolic compounds via aromatic acids [2–11] and the purine biosynthesis [12,13]. Furthermore, reversibility among aromatic acid decarboxylases has only been reported from three enzymes [13–15].

We have isolated pyrrole-2-carboxylate decarboxylase from *Bacillus megaterium* PYR2910 converting pyrrole-2-carboxylate to pyrrole and CO_2 [16]. This enzyme was also found to catalyze the reverse carboxylation of pyrrole at an appreciable rate [17,18]. An overview on this new type of reversible CO_2 fixing enzyme is given here.

* Corresponding author. Tel.: +81-58-293-2647; fax: +81-58-293-2647.

E-mail address: tonagasa@biomol.gifu-u.ac.jp (T. Nagasawa).

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2. Results and discussion

2.1. Pyrrole-2-carboxylate decarboxylation — a novel enzyme reaction

Previous studies on the enzymatic metabolism of pyrrole-2-carboxylate were confined to ring hydroxylations [19,20]. The enzymatic decarboxylation of pyrrole-2-carboxylate and the reverse carboxylation of pyrrole have not been described before. Decarboxylation and reverse carboxylation catalyzed by pyrrole-2-carboxylate decarboxylase from *B. megaterium* PYR2910 were confirmed by isolation and identification of the respective reaction products [16,18].

2.2. Enzyme induction and assays

The strain PYR2910 was isolated from soil for its ability to grow on pyrrole-2-carboxylate as the sole source of carbon and energy [16]. Thiophene-2-carboxylate and L-thioproline (each 0.2%, w/v), which are analogous compounds of the substrate pyrrole-2-carboxylate but not converted by the enzyme, were found to be the most effective enzyme inducers leading to a 3-fold higher specific enzyme activity than the substrate-inducer pyrrole-2-carboxylate [17].

The standard decarboxylation was carried out at 30°C in 2 ml of 100 mM potassium phosphate buffer (pH 7.0), 140 mM ammonium acetate, 100 mM pyrrole-2-carboxylate and an appropriate amount of enzyme. The reverse reaction was performed at 20°C in a tightly closed reaction vessel containing 2 ml of 100 mM potassium phosphate buffer (pH 5.5), 140 mM ammonium acetate, 100 mM pyrrole, 20 mM dithiothreitol, 30 units/ml enzyme and 3 M $KHCO_3$. A pH 5.5 buffer was used to counteract the pH increase by the HCO_3^- addition. One unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of 1 μmol pyrrole/min in the decarboxylation assay.

2.3. Characteristics of the purified enzyme

Pyrrole-2-carboxylate decarboxylase was purified from *B. megaterium* PYR2910 [16]. It has a molecular mass of 98 kDa and consists of two identical subunits. The V_{max} and K_m values for the decarboxylation were determined to 989 units/mg and 24 mM, respectively. Under reverse reaction conditions, the K_m values for pyrrole and $KHCO_3$ were 61 and 560 mM, respectively, and the v_{max} of the carboxylation was 47.2 μmol/mg min [17]. The lower V_{max} of the carboxylation might be due to the thermodynamically more difficult C—C bond formation compared to the C—C bond cleavage.

Both decarboxylation and CO_2 fixation depended on anaerobic or microaerobic conditions with activities falling from 100% in the standard, non-shaken reaction to 17–25% in a shaken reaction (120 strokes/minute). Presumably in accordance with this preference for microaerobic conditions, reducing agents effectively supported enzyme activity and stability. During purification and storage of the enzyme, 1 mM dithiothreitol was found to stabilize the enzyme [16]. Furthermore, in long-term reverse reactions, reducing agents in higher concentration of 20 mM were a prerequisite for high CO_2 fixation turnovers with dithiothreitol enhancing the carboxylation 16.2-fold compared to a control without reducing agent, followed by ascorbate (15.5-fold). The temperature and pH optima of decarboxylation and CO_2 fixation were 45°C and pH 7.0, respectively. Among a number of tested compounds for decarboxylation and carboxylation, only pyrrole-2-carboxylate and pyrrole, respectively, were converted by this highly specific enzyme [16,17]. Identical pH and temperature optima and correspondent substrate specificities with similar affinities for pyrrole and pyrrole-2-carboxylate suggested one catalytic enzyme center for decarboxylation and carboxylation.

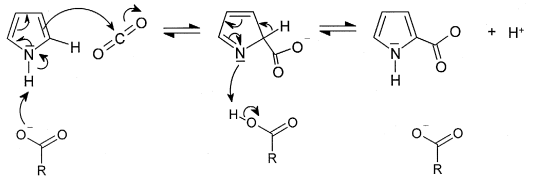
2.4. Organic acid cofactor

Decarboxylases have been reported to either depend on pyridoxal 5′-phosphate or thiamine pyrophosphate, as in the case of amino acid and α-keto acid decarboxylases, respectively [21–23], or to have no cofactor requirement such as non-oxidative, aromatic acid decarboxylases [14,15,24–36]. We found that the purified pyrrole-2-carboxylate decarboxylase from *B. megaterium* PYR2910 depends on the addi-

tion of an organic acid, which was not consumed during the reaction. Organic acids have only previously been described to stabilize enzymes [37] but not to activate them. A number of organic acids were tested as activators of pyrrole-2-carboxylate decarboxylase [16]. The enzyme activity increased with the number of carbons of the organic acid rising from formate to butyrate, and decreased above four carbons. Highest activity was found with pimelate (1750 μmol/min mg, K_m 1.8 mM), followed by butyrate (76% relative activity, 45 mM), propionate (74%, 42 mM) and acetate (56%, 43 mM). The structural resemblance of pimelate to propionate plus a butyrate is remarkable, and it might be concluded that these acids reflect the steric dimensions of the enzyme site, where the organic acid affects the catalysis.

2.5. Possible reaction mechanism

We have proposed an enzyme mechanism including a cofactor role of the organic acid. Analogous to the decarboxylation of pyrrole-2-carboxylate by heat [38,39], an electrophilic substitution at C2 pyrrole with a C2 protonated intermediate is probable (Scheme 1). The negatively charged organic acid might attack the only positive ring position at N1 attracting its proton. This eases the electron delocalization in the ring, thus increasing the electron density at C2. The C2 proton, therefore, can be substituted by the electrophilic carbon of CO_2 (rather than by the less electrophilic carbon of HCO_3^-). The electrophilic substitution also allows the reverse decarboxylation with a protonated intermediate stabilized by the organic acid. Support for the presumed catalytic function of the pyrrole N1-proton might be deduced from the fact that *N*-methylpyrrole is not converted by the enzyme.



Scheme 1. Possible reaction mechanism.

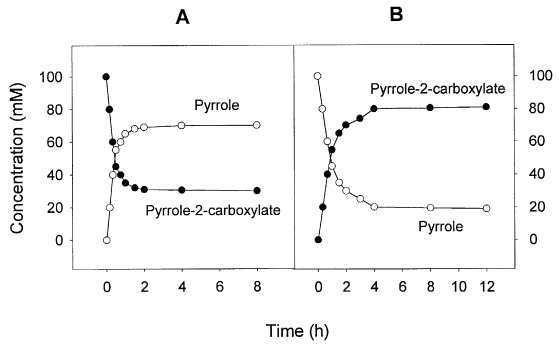


Fig. 1. Time course of the reactions catalyzed by pyrrole-2-carboxylate decarboxylase under standard decarboxylation (A) and reverse carboxylation (B) conditions. The pyrrole/pyrrole-2-carboxylate ratio after attainment of the reaction equilibrium depended on a number of reaction parameters such as temperature, pH, pressure and bicarbonate concentration. In the reverse carboxylation (B), this ratio was shifted towards pyrrole-2-carboxylate mainly by the addition of bicarbonate.

2.6. Reversibility and carboxylating agent

In contrast with the majority of enzymes, pyrrole-2-carboxylate decarboxylase attains equilibrium with the substrates only partially consumed in the course of either decarboxylation or carboxylation (Fig. 1). The decarboxylation of 100 mM pyrrole-2-carboxylate was equilibrated after 1 h resulting in an equilibrium constant of 0.3 M [17]. Due to this 'balanced' equilibrium, the enzyme also catalyzed the reverse carboxylation of pyrrole after the addition of HCO_3^-, resulting in a similar equilibrium constant of 0.4 M and a shift of the [pyrrole]/[pyrrole-2-carboxylate] ratio towards the acid.

The reverse CO_2 fixation depended on a CO_2 source (CO_2 or HCO_3^-), which was additionally the reverse reaction limiting factor. Due to high water solubilities, the best CO_2 sources were bicarbonates (HCO_3^-) with $KHCO_3$ leading to 82 mM pyrrole-2-carboxylate from 100 mM pyrrole, followed by NH_4HCO_3 (94% relative activity), $NaHCO_3$ (81%), $BaCO_3$ (17%) and $CaCO_3$ (16%). Other carbonates (CO_3^{2-}), CO_2 gas or dry ice were with low or without effect due to a low water solubility of CO_3^{2-} and CO_2 at neutral pH [40]. The reverse reaction showed a substrate saturation dependence from HCO_3^- with optimal HCO_3^- concentrations above 2.5 M.

2.7. Optimization of the CO_2 fixation

For highest carboxylation yields, saturating amounts of 3 M $KHCO_3$ were used leading to a shift of the reaction equilibrium towards the carboxylate. HCO_3^- addition was accompanied by CO_2 gas evolution resulting in an increased pressure in the tightly closed reaction vessel of 1.38 atmosphere which supported the reverse reaction productivity 2.5-fold compared to atmospheric pressure. High pressures are also known to be applied in organic–chemical carboxylations [41]. As biocatalyst, either concentrated cells with an optical density at 610 nm of 40, previously grown under inducing conditions, or the purified enzyme, both in a concentration corresponding to 100 units enzyme activity/ml, were employed. Additionally, acetate as enzyme cofactor and L-ascorbate as anti-oxidizing, enzyme protecting agent was added to the reaction mixture. For maximal CO_2 fixation rates, 300 mM pyrrole were optimal. Higher pyrrole concentrations inhibited the enzyme. In a batch reaction, 25.5 g/l (230 mM) pyrrole-2-carboxylate were formed from 20.7 g/l (300 mM) pyrrole with the mass gain resulting from the carbon and oxygen incorporation (Fig. 2A). The productivity was increased to 325 mM (36.1 g/l) by feeding 150 mM pyrrole after 3 h to a reaction with initially 250 mM pyrrole (Fig. 2B). The yield after bioconversion was 80%, limited by the equilibrium. The overall yield after isolation was 52%.

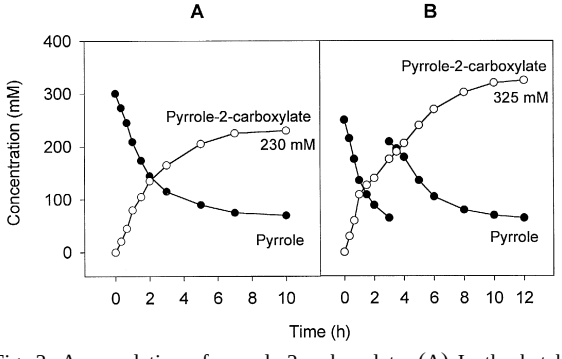


Fig. 2. Accumulation of pyrrole-2-carboxylate. (A) In the batch reaction for maximal production, initially, 300 mM pyrrole were used. (B) The fed batch reaction was started with 250 mM pyrrole, followed by a second addition of 150 mM after 3 h. The reactions were taken out in different vessels to avoid pressure losses resulting from frequent sample withdrawals.

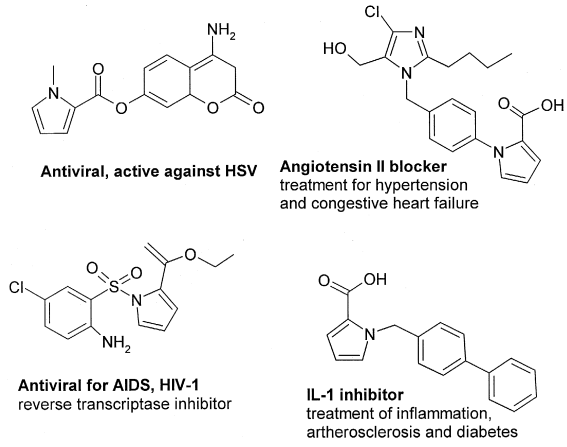


Fig. 3. Useful pyrrole-2-carboxylate derivatives.

2.8. Application

Pyrrole-2-carboxylate is employed in the synthesis of various pharmaceuticals [42,43] and a potential herbicide [44] (Fig. 3). A number of organic syntheses have been described [45–48]. However, they require multiple steps and result in low yields. Furthermore, the chemical carbonation of pyrrole with K_2CO_3 depends on high pressure and temperature [49]. The one-step bioconversion described here has advantages with regard to regiospecificity, yield, and mild reaction conditions.

The enzymatic carboxylation at high rates as shown here with pyrrole-2-carboxylate decarboxylase is a promising new tool for the regiospecific introduction of carboxy groups in precursors of pharmaceuticals and agrochemicals in order to functionalize them and thus, alter biological activities. Unfortunately, pyrrole-2-carboxylate decarboxylase is highly specific for pyrrole, and a number of pyrrole analogs were not carboxylated. This is in accordance to the highly specific decarboxylation of this enzyme with pyrrole-2-carboxylate as the only substrate and similar reversible decarboxylases with a high substrate specificity [14,15]. Since there are various other valuable targets for regiospecific carboxylations, the finding of novel CO_2 fixing enzymes with a wider substrate spectrum is desirable. We are therefore currently screening for other reversible decarboxylases, and first results show that such en-

zymes with the potential for regiospecific carboxylation are not confined to *Bacillus* species.

2.9. Other reversible decarboxylases

Among aromatic acid decarboxylases [14,15,24–36], only three other reversible enzymes have been described. Two of them are hydroxybenzoate decarboxylases from anaerobic *Clostridium hydroxybenzoicum* [14,15], which, however, catalyze only a weak CO_2 fixation due to an equilibrium far on the decarboxylation side [50]. The stronger reverse reaction of pyrrole-2-carboxylate decarboxylase results from a more balanced reaction equilibrium, the shifting of the equilibrium towards the carboxylation by optimizing the parameters bicarbonate, pressure, reducing agent, pH and temperature and the use of a less oxygen-sensitive enzyme from an aerobic source with a higher V_{max} value. Furthermore, an aminoimidazole ribonucleotide carboxylase involved in the purine biosynthesis resembles our enzyme regarding reversibility, *N*-heterocyclic substrate structure and the requirement of high amounts of HCO_3^- with a high K_m. However, at low HCO_3^- concentrations the carboxylation of the enzyme required ATP, Mg^{2+} and a second enzyme component, and the decarboxylation depended on ADP, phosphate and Mg^{2+} [13], which both could not be observed in our enzyme.

2.10. Physiological role of reversible decarboxylases

The physiological roles of microbial aromatic acid decarboxylases include the degradation of aromatic acids [28,34,35] and providing CO_2 under CO_2 limiting conditions [29]. Aryl carboxylases, on the other hand, initiate the anaerobic degradation of phenols via benzoic acids [2–11] or are involved in the de novo purine biosynthesis [12,13]. The reversible *Clostridium* decarboxylases were proposed to catalyze in vivo the anaerobic conversion of phenols to hydroxybenzoates, even if the equilibrium is on the decarboxylation side [14,15], and the hydroxybenzoates were supposed to be further metabolized by other microorganisms [50]. Despite a strong reverse reaction, we assume reversible pyrrole-2-carboxylate decarboxylase to take part in the catabolism of pyrrole-2-carboxylate due to a slow decrease of the decarboxylation product pyrrole under resting cells conditions and the non-physiologically high K_m for HCO_3^-, which is substantially above the concentrations of HCO_3^- that probably occur in vivo [51].

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