Treponema denticola cystalysin catalyzes β-desulfination of L-cysteine sulfinic acid and β-decarboxylation of L-aspartate and oxalacetate

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Abstract Pyridoxal 5′-phosphate-dependent cystalysin from *Treponema denticola* catalyzes the β-displacement of the β-substituent from both L-aspartate and L-cysteine sulfinic acid. The steady-state kinetic parameters for β-desulfination of L-cysteine sulfinic acid, k_{cat} and K_m, are 89 ± 7 s^{-1} and 49 ± 9 mM, respectively, whereas those for β-decarboxylation of L-aspartate are 0.8 ± 0.1 s^{-1} and 280 ± 70 mM. Moreover, cystalysin in the pyridoxamine 5′-phosphate form has also been found to catalyze β-decarboxylation of oxalacetate as shown by consumption of oxalacetate and a concomitant production of pyruvate. The k_{cat} and K_m of this reaction are 0.15 ± 0.01 s^{-1} and 13 ± 2 mM, respectively. Possible mechanistic and physiological implications are discussed.

Key words: Cystalysin; Pyridoxal 5′-phosphate; β-Decarboxylation; β-Desulfination

1. Introduction

Cystalysin from the oral pathogen *Treponema denticola* is a pyridoxal 5′-phosphate (PLP)-containing enzyme. The crystal structure of the recombinant enzyme was solved at 1.9 Å and revealed that its tertiary fold and quaternary arrangement are similar to those of aminotransferases [1]. Its main catalytic activity is the α,β-elimination of L-cysteine to produce pyruvate, ammonia and H_2S [2]. In addition to L-cysteine, L-cystine, L-djenkolic acid, L-cystathionine, β-chloro-L-alanine and L-serine act as substrates in the α,β-elimination [3]. Recently, kinetic evidence has been provided for an alanine racemase reaction catalyzed by cystalysin with turnover times measured in seconds. It has also been shown that cystalysin is able to catalyze the overall transamination of both alanine enantiomers [4].

A thorough characterization of the reactions of L-cysteine sulfinic acid and L-aspartate catalyzed by cystalysin revealed that the enzyme is able to catalyze the β-desulfination of L-cysteine sulfinic acid and the β-decarboxylation of L-aspartate. Moreover, we report results showing that cystalysin in the pyridoxamine 5′-phosphate (PMP) form catalyzes the conversion of oxalacetate into pyruvate, i.e. catalyzes the β-decarboxylation of oxalacetate.

2. Materials and methods

2.1. Materials

PLP, PMP, L- and D-alanine, L-cysteine sulfinic acid, L-aspartate, oxalacetate, oxidized nicotinamide adenine dinucleotide (NAD^+), reduced NAD (NADH), pyruvate, rabbit muscle L-lactic dehydrogenase, malate dehydrogenase, bovine liver L-glutamic dehydrogenase, alanine dehydrogenase in 50% glycerol, D-amino acid oxidase, and isopropyl β-D-thiogalactoside were from Sigma. L-cysteine and L-alanine were from Fluka.

2.2. Enzyme preparation

Cystalysin from *T. denticola* was expressed in *Escherichia coli* and purified as described [3]. The enzyme concentration was determined by using an ε_M of 12.77×10^4 M^{-1} cm^{-1} at 281 nm. The PLP content of holocystalysin was determined by releasing the coenzyme in 0.1 M NaOH and by using $\varepsilon_M = 6600$ M^{-1} cm^{-1} at 388 nm.

2.3. Enzyme assays

Ammonia production by the reaction of cystalysin with L-cysteine sulfinic acid or L-aspartate was determined by a spectroscopic assay using glutamate dehydrogenase, which forms glutamate from α-ketoglutarate and ammonia with concomitant conversion of NADH to NAD^+. Pyruvate or oxalacetate were determined with the NADH-dependent lactate dehydrogenase [3] or malate dehydrogenase, respectively. In the experiments designed to determine the kinetic parameters of β-decarboxylation of oxalacetate, production of pyruvate was determined by an assay based on measuring the dinitrophenyl hydrazine derivative of pyruvate by high-performance liquid chromatography (HPLC) essentially as described by Neidle and Dunlop [5]. L- and D-alanine produced during the reaction of cystalysin with L-cysteine sulfinic acid or L-aspartate were determined by the racemase assays described previously [3]. Sulfite production was measured with fuchsin [6]. The detection and quantification of PLP and PMP during the reactions were performed with the HPLC procedure described previously [7]. The rate constant of increase in PMP was obtained by fitting the coenzyme concentration versus time curve to the appropriate first-order kinetic equation. Apocystalysin was prepared as described in [3]. Absorption measurements were made with a Jasco V-550 spectrophotometer.

3. Results and discussion

When cystalysin was incubated with L-cysteine sulfinic acid or L-aspartate in the presence of glutamate dehydrogenase no appreciable production of ammonia could be detected. Moreover, reaction of cystalysin with L-cysteine sulfinic acid or L-aspartate in the presence of lactate dehydrogenase or malate dehydrogenase, respectively, does not produce detectable amounts of pyruvate or oxaloacetate. These results indicate that the enzyme is not able to catalyze the α,β-elimination of

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Abbreviations: PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate

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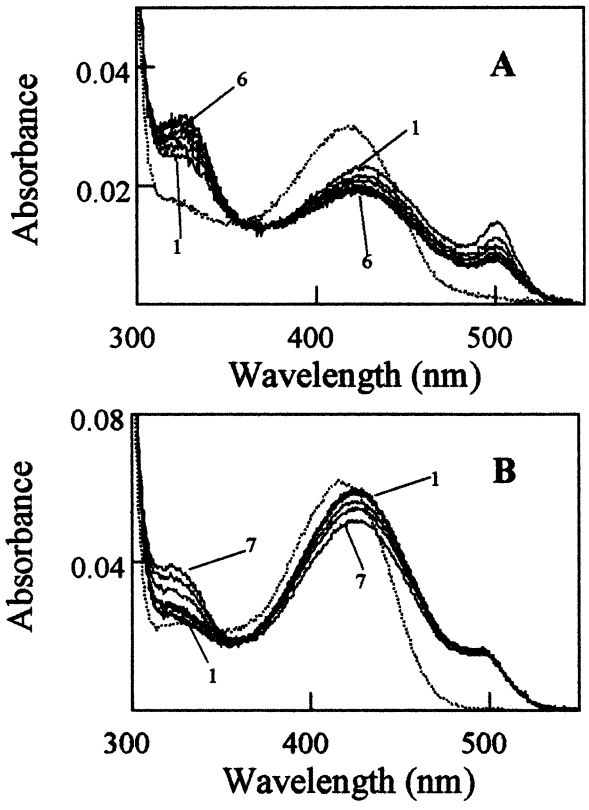


Fig. 1. Spectral changes occurring on the addition of L-cysteine sulfinic acid or L-aspartate to cystalysin. A: Absorption spectra of 2.6 μM cystalysin (dotted line) in 20 mM potassium phosphate buffer, pH 7.4, and of the enzyme plus 0.2 M L-cysteine sulfinic acid (solid line) at 2.5 (line 1), 3.5, 5.5, 7.5, 8.5, and 10.5 min (line 6). B: Absorption spectra of 4.8 μM cystalysin (dotted line) in 20 mM potassium phosphate buffer, pH 7.4, and of the enzyme plus 0.5 M L-aspartic acid (solid line) at 1.15, 5, 10, 17, 35, 48, and 63 min (line 7).

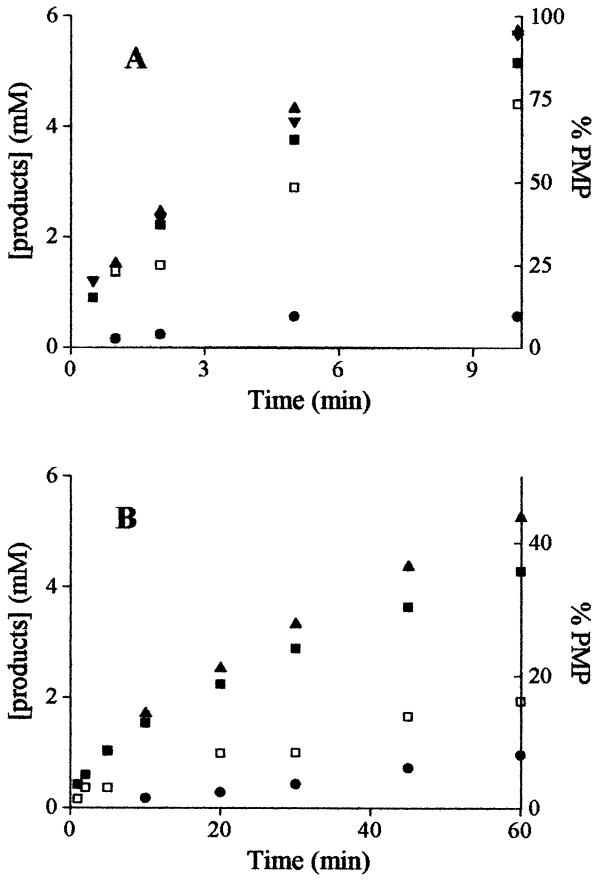


Fig. 2. Time course of the reaction of cystalysin with L-cysteine sulfinic acid or L-aspartate and PMP content. A: Cystalysin (0.3 μM) was incubated with 0.2 M L-cysteine sulfinic acid in 20 mM potassium phosphate buffer, pH 7.4. B: Cystalysin (4 μM) was incubated with 0.5 M L-aspartic acid in 20 mM potassium phosphate buffer, pH 7.4. Aliquots were removed at the indicated times and treated as reported in Section 2. Symbols: (■) L-alanine, (●) D-alanine, (▲) L-+D-alanine, (▼) sulfite, (□) PMP. Data represent the means for three independent experiments; S.E.M. in each case was less than 5%.

these amino acids, which would lead to the formation of the corresponding keto acid and ammonia. However, both L-cysteine sulfinic acid and L-aspartate induce changes in the spectra of the protein-bound coenzyme, which undergo further time-dependent changes. In fact, as shown in Fig. 1A and B, addition of L-cysteine sulfinic acid or L-aspartate to the enzyme results in the immediate appearance of absorbance bands centered at 428, ~500 and 325 nm that are probably due to the formation of an external aldimine, a quinonoidal and a C4′sp^3 species, respectively. Both the 428 and the 500 nm bands decrease with time, while the 325 nm peak increases with time. This behavior strongly suggests an active site-directed event and the occurrence of a reaction between cystalysin and each of these ligands. Unexpectedly, we found that the reaction of cystalysin with L-cysteine sulfinic acid or L-aspartate produces alanine, in L- and D-forms. Moreover, in the case of L-cysteine sulfinic acid, the amount of sulfite formed is equivalent to the amount of L- plus D-alanine produced (Fig. 2A and B). It is noteworthy that the amount of L-alanine generated far exceeds that of D-alanine; the latter enantiomer probably results from the alanine racemase activity of cystalysin [4]. These data are consistent with the β-desulfination of L-cysteine sulfinic acid and the β-decarboxylation of L-aspartate. The kinetic parameters for these reactions have been determined and their values are reported in Table 1. Considering that β-desulfination and β-decarboxylation are side reactions of cystalysin, they occur with high k_{cat} values; in fact, k_{cat} for L-cysteine sulfinic acid is about 2–3-fold higher than that of the main reaction of cystalysin at the same pH value, i.e. α,β-elimination of L-cysteine, L-cystathionine and L-cystine, and only slightly lower than that of the α,β-elimination of β-chloro-L-alanine, *O*-acetyl-L-serine and L-djenkolic acid [3]. Nevertheless, the catalytic efficiency of the β-desulfination and β-decarboxylation reactions is much lower than that determined for all substrates undergoing α,β-elimination. This is mainly due to increased K_m values. In addition, both enantiomers of alanine, substrates for racemization and transamination reactions catalyzed by cystalysin, bind to the enzyme with an affinity higher than that of L-cysteine sulfinic acid and L-aspartate [4]. This could imply that the negative

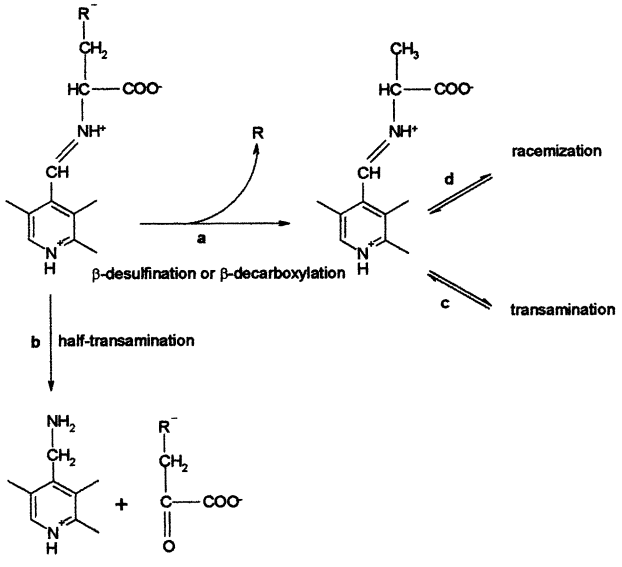
Table 1
Kinetic parameters for β-desulfination of L-cysteine sulfinic acid and β-decarboxylation of L-aspartate and oxaloacetate

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
L-Cysteine sulfinic acid	49 ± 9	89 ± 7	1.8 ± 0.3
L-Aspartate	280 ± 70	0.8 ± 0.1	0.0028 ± 0.0008
Oxaloacetate	13 ± 2	0.15 ± 0.01	0.011 ± 0.002

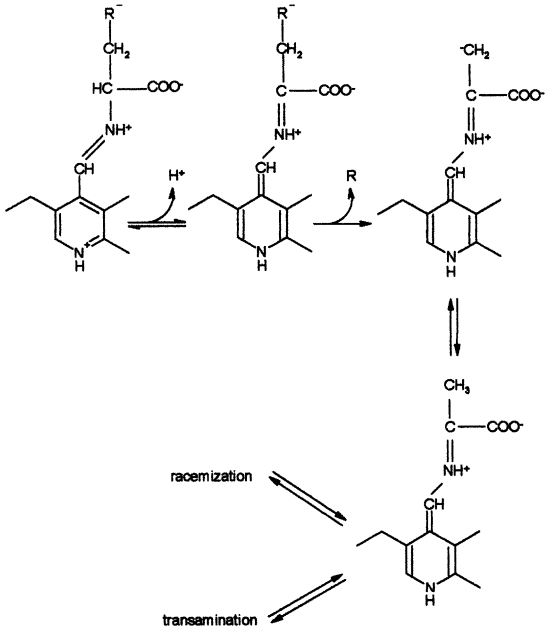
charge on the side chain of L-cysteine sulfinic acid and L-aspartate weakens their binding to the enzyme. It should be noted that the residue corresponding to R292, which in aspartate aminotransferase is involved in the distal carboxylate/sulfinate binding, is missing in cystalysin [1].

Furthermore, it should be noted that, despite the fact that substrate concentration is high, a gradual loss of β-desulfinase or β-decarboxylase activities was observed in the course of the reaction of cystalysin with L-cysteine sulfinic acid or L-aspartate, respectively (Fig. 2A and B). This time-dependent inactivation is strictly correlated to a conversion of PLP bound to PMP (as revealed either by the increase of the absorbance at 325 nm or by HPLC measurements) which in the presence of 0.2 M L-cysteine sulfinic acid takes place with a rate constant of 0.15 ± 0.02 min^{-1}, whereas in the presence of 0.5 M L-aspartic acid it occurs very slowly and is completed over a period of about 3 h.

Taken together, the results on reaction and substrate specificity indicate that cystalysin is one of the PLP enzymes able to catalyze a wide variety of reactions. The remarkable catalytic versatility of the enzyme implies that its active site, which is optimized for catalyzing α,β-elimination, also encodes structural elements required for other PLP-catalyzed reactions. It is of interest that in cystalysin PLP is bound such that K238 faces the *si* side of the cofactor and Y123 or a water molecule the *re* side [4]. Therefore, the alanine racemase activity of cystalysin has already been related to the presence of two acid-base catalysts on the opposite faces of PLP, typical of alanine racemase [8]. Moreover, cystalysin shows a higher similarity with subgroup Ib rather than with subgroup Ia to which *E. coli* aspartate aminotransferase belongs [1]. The striking difference between the two aminotransferase subgroups is the ability of aminotransferases in subgroup Ia to undergo a large conformational change from the open to the closed form upon substrate binding [9]. Such a conformational change has in fact not been observed in the enzyme–inhibitor complex of cystalysin with aminoethoxyvinylglycine [1]. β-Desulfination of L-cysteine sulfinic acid and β-decarboxylation of L-aspartate have been reported to be catalyzed by wild-type *E. coli* aspartate aminotransferase with a



Scheme I.



Scheme II.

$k_{cat} \sim 1500$-fold lower than that of cystalysin. Interestingly, mutations that prevent the enzyme from adopting a closed conformation give rise to enzymic forms displaying a more consistent β-desulfination and β-decarboxylase activity [10]. Similarly, the lack of ligand-induced conformational change closing the active site pocket of cystalysin could favor these reactions. Notwithstanding the side reactions catalyzed by cystalysin might be interesting for a better understanding of the chemistry of PLP enzymes, they do not appear to have physiological relevance in *T. denticola*, mainly because of the high K_m values for the substrates.

All together, these results could be interpreted according to Scheme I. The reaction involving the Cβ-R$^-$ cleavage (pathway a) would occur like that described by Meister et al. [11] for a specific aspartate β-decarboxylase, and by Jones et al. [12] for a side reaction of D-amino acid transaminase converting D-aspartate into D-alanine. Therefore, the electrophilic displacement of the negatively charged substituent at Cβ is not on the main pathway of α,β-elimination catalyzed by cystalysin. It is not easy to envisage why the negatively charged side chains of L-cysteine sulfinic acid and L-aspartate can be eliminated without deprotonation of Cα. As shown in Scheme II, Cα deprotonation and subsequent R$^-$ elimination would lead to a quinonoid intermediate which could be either reprotonated at Cα giving a racemic mixture of alanine or reprotonated at C4′ giving PMP and the corresponding keto acid [4].

The finding that during β-desulfination and β-decarboxylation the alanine formed is mainly the L-isomer rather than its racemic mixture argues against this mechanism. Indeed, it is consistent with the mechanism depicted in Scheme I, where the L-alanine aldimine complex is formed (pathway a). The alanine racemase activity of cystalysin is responsible for the conversion of L- to D-alanine (pathway d). PMP formation observed during these reactions could be due to a half-transamination possibly occurring from either the substrate–aldimine (pathway b) or the alanine–aldimine (pathway c) intermediates. The rates of transamination of L-cysteine sulfinic acid and L-aspartate compared with the transamination rate

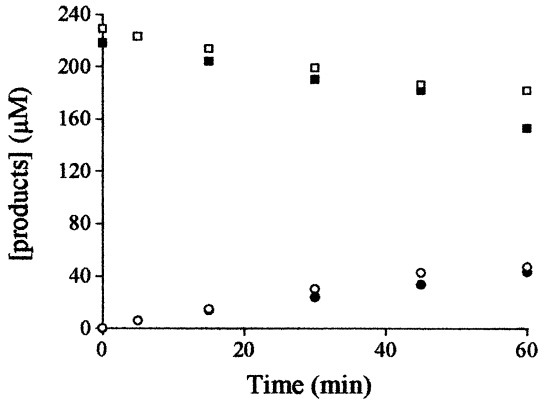
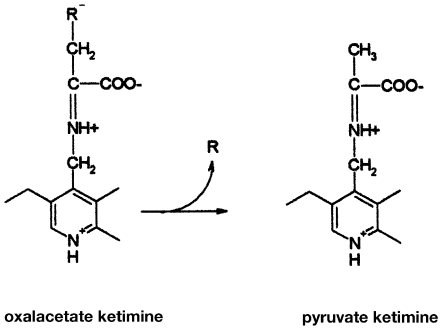


Fig. 3. Time course of the reaction of cystalysin with oxalacetate in the presence of PMP. Apocystalysin (5 μM) was incubated with 230 μM oxalacetate in the presence of 10 μM PMP in 20 mM potassium phosphate buffer, pH 7.4. Aliquots were removed at the indicated times and treated as reported in Section 2. Pyruvate formation was measured either by the coupled lactate dehydrogenase assay (●) or by HPLC (○), oxalacetate consumption was measured either by the coupled malate dehydrogenase assay (■) or by HPLC (□). Data represent the means for three independent experiments; S.E.M. in each case was less than 5%.

of alanine [4] are evidence that while PMP is formed by direct transamination of L-cysteine sulfinic acid, most of the PMP formed in the presence of L-aspartate could be due to transamination of the alanine formed by decarboxylation of L-aspartate. Nevertheless, cystalysin preferentially catalyzes cleavage of the β-substituent of both L-amino acids rather than transamination. Interestingly, in addition to aspartate β-decarboxylase [10] and kynureninase [13], some Cβ-Sγ lyases such as NifS and NifS-like proteins catalyze the electrophilic displacement of the substituent (sulfur, selenium or sulfite) at Cβ of L-cysteine, L-selenocysteine or L-cysteine sulfinic acid to yield L-alanine [14–16]. Cystalysin represents the first example of a desulfhydrase endowed with a desulfinase activity. Among Cβ-Sγ lyases, three NifS homologs from *E. coli* have been reported to catalyze an abortive transamination of L-selenocysteine and L-cysteine sulfinic acid as side reaction, and the ketimine–alanine intermediate in the catalytic pathway of these enzymes has been suggested to be partitioned between electrophilic displacement at Cβ and transamination [17].

An additional, and more unexpected, finding is that cystalysin in the PMP form catalyzes the β-decarboxylation of oxalacetate. Upon mixing oxalacetate with apocystalysin in the presence of PMP, a time-dependent appearance of an absorbance band at 418 nm could be observed which is strictly correlated with the conversion of PMP into PLP and the recovery of desulfhydrase activity (data not shown). These results would imply that cystalysin is able to catalyze a half-transamination converting PMP into PLP and concomitantly oxalacetate into aspartate, similar to that previously observed following the reaction of apocystalysin in the PMP form with pyruvate [4]. Surprisingly, no formation of aspartate could be observed during the reaction of cystalysin with oxalacetate in the presence of PMP, either by HPLC analysis after derivatization with 2-fluoro-2,4-dinitrophenyl-5-L-alanineamide [18] or by analysis of the reaction mixture using an amino acid analyzer. On the contrary, as shown in Fig. 3, reaction of apocystalysin with PMP and oxalacetate causes the production of pyruvate and the concomitant consumption of oxalacetate, as revealed either by coupled assay systems using lactate dehydrogenase and malate dehydrogenase, respectively, or by HPLC analysis. This finding is in line with the observation that no conversion of PMP into PLP takes place if the reaction of apocystalysin with PMP and oxalacetate is carried out in the presence of lactate dehydrogenase and NADH. Thus, the PMP to PLP conversion is possibly due to pyruvate generated by decarboxylation of oxalacetate and not by the half-transamination of oxaloacetate. The initial velocity of the β-decarboxylation of oxalacetate is directly dependent on enzyme concentration, and dependent on oxalacetate concentration according to saturation kinetics (data not shown). The K_m for oxalacetate was determined to be 13 ± 2 mM, with a k_{cat} of 0.15 ± 0.01 s^{-1} (Table 1). These results demonstrate that oxalacetate, instead of being transaminated, undergoes a PMP-assisted reaction consisting in a β-decarboxylation. According to Scheme III, it can expected that the oxalacetate ketimine is potentially susceptible to decarboxylation because the imine is β to a carboxylate. This is analogous to previous proposal to explain β-decarboxylation of oxaloacetate displayed by the active site lysine mutant of aspartate aminotransferase. This activity has not been detected with wild-type aspartate aminotransferase. In the mutant substitution of Lys-258 with Ala could virtually abolish the enzyme-catalyzed 1,3-prototrophic shift, which is central for transamination, thus stabilizing the ketimine intermediate formed from α-keto acid and E-PMP [19].

As for β-decarboxylation of oxalacetate, one could speculate on its possible importance in the metabolism of *T. denticola* taking into account a recent study on the role of glutathione metabolism in this anaerobic bacterium. Evidence has been provided that while one of the metabolites of glutathione, H_2S, is toxic to host cells, another product, pyruvate, promotes bacterial growth [20]. It should also be considered that some bacteria can grow anaerobically on saturated dicarboxylic acids using the decarboxylation of these molecules as the sole energy source [21]. Biochemical studies on different strains of fermenting bacteria have revealed two different mechanisms for adenosine triphosphate (ATP) synthesis: the decarboxylation energy is either converted directly into an energy-rich electrochemical gradient of Na^+ ions across the membrane or an electrochemical H^+ gradient is created by an electrogenic dicarboxylate/monocarboxylate antiporter together with a soluble decarboxylase. Oxalacetate decarboxylase of *Klebsiella pneumonia* is a particularly well-characterized member of the membrane-bound biotin-containing Na^+ pump decarboxylase family of enzymes, which also includes methylma-



Scheme III.

lonyl-CoA decarboxylase, malonate decarboxylase and gluconyl-CoA decarboxylase from various anaerobic bacteria [21–24]. On the other hand, the water-soluble decarboxylases, like oxalate and malate decarboxylases, require thiamine pyrophosphate as cofactor [21]. To our knowledge, the β-decarboxylase activity displayed by *T. denticola* cystalysin toward oxalacetate is the first example of a decarboxylase of a dicarboxylic acid utilizing PMP as cofactor. At present, it is difficult to assess if or which one of the possible roles could be played by β-decarboxylation of oxalacetate in *T. denticola*. Nevertheless, considering the low k_{cat}/K_m value of this reaction, it cannot be excluded that this activity could be a mere corollary of the chemical properties of the enzyme.

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