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SHORT COMMUNICATION

Coenzyme preference of *Streptococcus pyogenes* δ^1-pyrroline-5-carboxylate reductase: evidence supporting NADPH as the physiological electron donor

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Abstract The streptococcal enzyme that catalyzes the last step in proline biosynthesis was heterologously expressed and the recombinant protein was purified to electrophoretic homogeneity and characterized thoroughly. As for δ^1-pyrroline-5-carboxylate reductases from other sources, it was able to use either NADH or NADPH as the electron donor in vitro. However, with NADH the activity was markedly inhibited by physiological levels of $NADP^+$. Results also strengthen the possibility that an unusual ordered substrate binding occurs, in which the dinucleotide binds last.

Keywords Electron donor · Proline biosynthesis · Substrate ambiguity · Substrate binding

Introduction

Proline plays a remarkable role in protein composition, uniquely contributing to protein folding, structure and stability (Phang et al. 2010). Furthermore, increasing evidence suggests that not only the intracellular accumulation of free proline is involved in stress tolerance and osmoregulation (Kavi Kishor et al. 2005), but also the free interconversion of glutamate and proline greatly contributes to control the redox status of the cell (Krishnan et al. 2008), to modulate the NADP(H) to NAD(H) ratio (Szabados and Savoure 2010), and to trigger apoptosis (Hu et al. 2007).

In both the proline biosynthetic pathways from glutamate and ornithine, a δ^1-pyrroline-5-carboxylate reductase (P5CR) [EC 1.5.1.2] catalyzes the final conversion of P5C to proline (Fig. S1), with the concomitant oxidation of NAD(P)H to $NAD(P)^+$ (Aral and Kamoun 1997). As it occurs at the converging point of two routes, P5CR may be subjected to fine modulation, even if not controlling the rate-limiting step. Its expression was indeed found to be regulated at both the transcriptional and the translational level (Hua et al. 2001). Moreover, the occurrence of multiple enzyme forms showing differential properties has been reported (Murahama et al. 2001).

In the last decade, some information about the three-dimensional structure of P5CR has been made available. The human enzyme is composed by a unique polypeptide chain that forms homodimers, which further assemble into a decamer. It showed a higher affinity for NAD(H), a feature assessed by measuring an artificial thioproline dehydrogenase reaction (Meng et al. 2006). A similar structure was found also for the enzyme from a human pathogen, *Streptococcus pyogenes*. A preference for NADP(H), as well as the occurrence of product inhibition, was hypothesized on the basis of a preliminary evaluation. However, once again the enzyme was assayed by measuring non-physiological reactions, i.e., the reverse oxidation of proline and its analogs thioproline and dehydroproline (Nocek et al. 2005).

An exhaustive elucidation of the functional properties of P5CR would be valuable, also because this enzyme has recently been identified as a potential new target for biologically active compounds. Some phosphonate inhibitors were indeed found to exert both phytotoxic (Forlani et al. 2007, 2008) and antibiotic (Forlani et al. 2011) effects. Here we report the results of a thorough biochemical

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characterization of P5CR from *S. pyogenes*, shedding more light on its substrate preference and reaction mechanism.

Materials and methods

The pMCSG7 vector (Nocek et al. 2005) was used to overexpress the M1 GAS *S. pyogenes* P5CR gene in *E. coli* BL21(DE3) pLysS cells. Transformation, induction and affinity purification of the enzyme were as described previously (Forlani et al. 2011). Protein concentration was determined by the method of Bradford (1976), using bovine serum albumin as the standard. The actual concentration of purified P5CR in final preparations was estimated on the basis of the predicted molar absorption coefficient (Pace et al. 1995).

P5CR activity was measured by following the P5C-dependent oxidation of NAD(P)H (Forlani et al. 2011). For kinetic evaluations, curve fitting by non-linear regression analysis was performed with Prism 5 (GraphPad Software). K_I value for $NADP^+$ in the case of P5C (uncompetitive inhibition) was estimated from Lineweaver–Burk plots of activity. In the case of NADH, because of a competitive type of inhibition, K_I values were estimated from Dixon plots by evaluating the effect of various inhibitor levels in the presence of four substrate concentrations. Reported data are means ± SEM over results obtained with different inhibitor or substrate concentrations, respectively. The dissociation constant of EIS complex was calculated according to Cornish-Bowden (1974).

Results and discussion

When dealing with proteins from pathogenic bacteria, heterologous expression allows minimizing possible biohazard risks connected with their manipulation. In order to isolate and characterize P5CR from *S. pyogenes*, the streptococcal gene was expressed in *E. coli*. High expression levels were obtained, with concentrations corresponding to about 9.5% of soluble proteins. The presence of such high quantities did not apparently affect cell viability. At low temperatures (20–24°C) the heterologous enzyme was not sequestered in inclusion bodies. The subsequent use of a stepwise elution protocol yielded homogeneous enzyme preparations in a single step (Fig. S2). Starting from 1 g (wet weight) induced cell pellet, 11 mg pure protein was obtained, corresponding to about 38 mg protein L^{-1} bacterial culture.

With this material, the physiological forward reaction of *S. pyogenes* P5CR was characterized. If a remarkable amount of information has become available concerning either the transcriptional regulation (Kavi Kishor et al. 2005) or the structural biology (Tanner 2008) of proline-metabolizing enzymes, much less is known about their modulation at the post-translational level. This lack of information is even more pronounced for P5CR, since in most instances biochemical characterization has been carried out to date using artificial substrates and non-physiological conditions, such as highest pH values (Deutch et al. 2001). Results herein obtained are summarized in Table 1. Maximal activity was found in a narrow range of pH values around 7.5, conditions under which the reverse reaction was negligible. A highest rate was evident, with a turnover number of 340–620 catalytic events s^{-1} depending on the substrates, and a catalytic efficiency ($k_{cat}/K_M = 2.8 \times 10^7$ M^{-1} s^{-1}) significantly higher than those reported for other enzymes in amino acid metabolism (Miller and Wolfenden 2002). For instance, the catalytic efficiency of *E. coli* γ-glutamyl kinase, the enzyme that catalyses the first step in proline biosynthesis, is 4.2×10^4 M^{-1} s^{-1} (Pérez-Arellano et al. 2006), whereas that of P5C dehydrogenase, the enzyme that oxidizes P5C back to glutamate, ranges from 3.4 to 4.2×10^5 M^{-1} s^{-1} in rat (Small and Jones 1990) and potato (Forlani et al. 1997), respectively. As a term of comparison, the human P5CR catalyses the oxidation of the proline analog thioproline with an efficiency as low as 4.4×10^4 M^{-1} s^{-1} (Meng et al. 2006). The addition of physiological concentrations of proline (up to 10 mM) to the reaction mixture was found ineffective. At higher levels, a different behavior was evident at varying the electron donor. With NADPH, no effect was found up to 400 mM proline. With NADH, a slight inhibition took place, with a 25% reduction of the catalytic rate at 200 mM (Fig. S7). These results ruled out the possibility that, similarly to the enzyme for most other sources (Merrill et al. 1989), *S. pyogenes* P5CR may be subjected to feedback inhibition mechanisms.

Both NADH and NADPH served as the electron donor. Consistently with previous speculation (Nocek et al. 2005), a higher affinity was evident for the latter, yet with NADPH a lower V_{max} was achieved (Table 1). A possible regulative role of the intracellular pyridine nucleotide pools was investigated by including increasing concentrations of the oxidized forms into the reaction mixture. In the presence of NAD^+, no effect was evident. The same result was obtained when $NADP^+$ was added to the NADPH-containing assay mixture. On the contrary, a striking effect was exerted upon the NADH-driven reaction by micromolar concentrations of $NADP^+$ (Fig. 1a). The inhibition was of competitive type, with a K_I as low as 10 μM (Fig. 1b). Under aerobic conditions, comparable concentrations of NAD(H) and NADP(H) are present, ranging from 0.5 to 1 mM, with a NAD(P)H/$NAD(P)^+$ ratio of about 0.2 (Iwami and Yamada 1999; Shi et al. 2009). This implies that NADH cannot serve as electron donor in vivo, since

Table 1 Properties of *Streptococcus pyogenes* P5CR

pH optimum[a]	7.2 ↔ 7.8
$V_{max\ (NADH)}$[b]	21.2 ± 0.5 μkat (mg protein)$^{-1}$
$V_{max\ (NADPH)}$[b]	11.6 ± 0.3 μkat (mg protein)$^{-1}$
$k_{cat\ (NADH)}$[c]	620 s^{-1}
$k_{cat\ (NADPH)}$[c]	340 s^{-1}
$K_{M(app)}$ for L-P5C $_{(NADH)}$[d]	0.349 ± 0.029 mM
$K_{M(app)}$ for L-P5C $_{(NADPH)}$[e]	0.184 ± 0.019 mM
$K_{M(app)}$ for NADH[f]	0.176 ± 0.007 mM
$K_{M(app)}$ for NADPH[g]	0.012 ± 0.002 mM
Inhibition by EDTA (IC_{50})[h]	29 ± 9 mM

[a] The pH dependence of P5CR activity was evaluated by assaying the purified enzyme for 5 min at 37°C in the presence of 100 mM HEPES buffer, brought to the desired pH value (from 6.5 to 9.0) with KOH; results are presented in Fig. S3

[b] Maximal catalytic rate was estimated from the plots obtained at varying NADH or NADPH concentration at a saturating P5C level, and vice versa. Concentrations for the invariable substrate were 1 mM for L-P5C, 0.4 mM for NADH, and 0.2 mM for NADPH. For each couple (i.e., P5C + NADH, or P5C + NADPH), reported values are means ± SD of the results obtained with either compound as the variable substrate

[c] Catalytic constant was calculated from the maximal catalytic rate in the presence of saturating concentrations of P5C and either NADH or NADPH on the basis of a molecular weight value of 27,306, estimated from the cloned gene sequence, taking into account the 6-His-tail

[d] Apparent affinity for P5C was estimated from the activity plots obtained at varying substrate concentrations in the presence of a saturating level of NADH (0.4 mM). Concentration for the variable substrate ranged from 0.1 to 0.5 mM. No less than eight different concentrations were tested, each one in quadruplicate. Data were combined, and K_M value ± SEM was calculated

[e] Apparent affinity for P5C with NADPH (0.2 mM) as the invariable substrate. No less than 7 different concentrations were tested, each one in quadruplicate

[f] K_M value ± SEM for NADH was estimated from the activity plots obtained at varying substrate concentrations in the presence of a saturating level of P5C (1 mM L-isomer); results are shown in Fig. S4

[g] K_M value ± SEM for NAPDH was calculated from the activity plots obtained at varying substrate concentrations in the presence of a saturating level of P5C (1 mM L-isomer); results are presented in Fig. S5

[h] P5C-dependent NADH oxidation was measured in the presence of increasing levels of EDTA, added to the reaction mixture to a final dose in the 10^{-3}–10^{-1} M range. At least three replications were run for each dose, and six for the untreated control; results are shown in Fig. S6

$NADP^+$ levels are high enough to completely inhibit the reaction. Being NADPH present at saturating levels, the activity of P5CR in vivo seems limited only by P5C availability. Under micro-aerobic conditions, the NAD(P)H/$NAD(P)^+$ ratio increases to 0.7–0.8, but the amount of NADP(H) drops (Grose et al. 2006). As a consequence, NADPH may become limiting, but $NADP^+$ concentrations (15–20 μM) seem still high enough to hamper NADH utilization.

In the case of a P5CR isozyme specifically expressed in human erythrocytes, the lack of allosteric inhibition and regulation by pyridine nucleotide pool led to hypothesize that it could not serve primarily for proline synthesis, but for $NADP^+$ generation (Merrill et al. 1989). In *S. pyogenes* a second P5CR is not present (Ferretti et al. 2001), but the occurrence of a single gene coding for an enzyme devoted to a function different from the production of proline for protein synthesis might be a consequence of a genetic erosion occurring in pathogenic organisms that can obtain amino acid supply for growth from the environment. A significant loss of information concerning amino acid metabolism is indeed evident in *S. pyogenes*, whose genome sequencing provided evidence for a general auxotrophy for lysine, threonine, aromatic and branched-chain amino acids (http://kegg.jp/).

The inhibition brought about by $NADP^+$ when NADH is the electron donor most likely depends upon a higher efficiency of the phosphorylated dinucleotide for active site binding (Nocek et al. 2005). Interestingly, patterns in Lineweaver–Burk (Fig. 1c), Dixon (not shown) and Cornish-Bowden plot (Fig. 1d) accounted for an inhibition of uncompetitive type with respect to P5C. This is consistent with results obtained with phosphonate inhibitors of P5CR (Forlani et al. 2008, 2011), and provides experimental evidence supporting an ordered substrate binding, previously hypothesized only on the basis of the crystal structure of the enzyme. A surface rendering of the active center of *S. pyogenes* P5CR with $NADP^+$ showed that, upon binding of the coenzyme, the entrance to the active center cavity is effectively blocked, leaving only a small opening insufficient to allow P5C to pass by (Nocek et al. 2005). Present results strengthen the possibility that P5C may bind before NADPH. Since in the case of most NAD(P)H-dependent reductases the coenzyme binds before the substrate (Sanli et al. 2003), this is an unusual feature that might be a

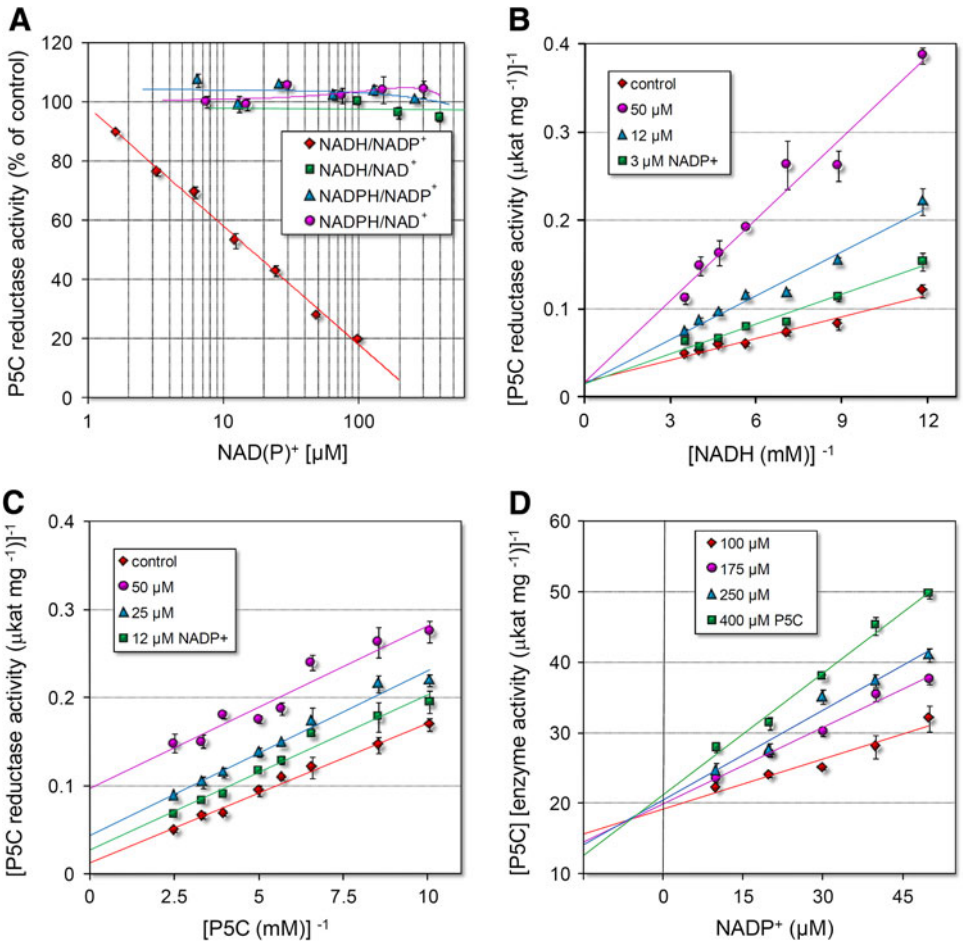

Fig. 1 Inhibition of *S. pyogenes* P5CR by $NADP^+$. When NADH was the electron donor, even lowest concentrations of $NADP^+$ were inhibitory, whereas in all other possible combinations activity was unaffected by either oxidized pyridine nucleotides (**a**). $NADP^+$ inhibited the enzyme with a mechanism of competitive type with respect to NADH (**b**), and uncompetitive with respect to P5C (**c**), with K_I values of 9.3 ± 2.5 and 7.4 ± 0.7 μM, respectively. Plotting $[S]/V$ against inhibitor concentration at varying P5C level (**d**) allowed determining also the dissociation constant of the EIS complex ($K_I' = 4.7 \pm 1.3$ μM)

consequence of the cyclic structure of both substrate and product. Overall, results emphasize the need of proper assay conditions for P5CR, whose activity seems to respond to various effectors. In particular, the use of NADPH as the electron donor seems mandatory. Because the steric hindrance is different, a search for specific inhibitors of the enzyme by using NADH as the cofactor could lead to artifactual results.

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Conflict of interest The authors declare that they have no conflict of interest.

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