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Purification of recombinant mandelate racemase: Improved catalytic activity ☆

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ABSTRACT

Mandelate racemase (MR, E.C. 5.1.2.2) from *Pseudomonas putida* catalyzes the Mg^{2+}-dependent 1,1-proton transfer that interconverts the enantiomers of mandelate and has been studied extensively as a model for understanding how enzymes catalyze the deprotonation of carbon acid substrates with relatively high pK_a values. Purification of recombinant MR as a fusion protein with an N-terminal hexahistidine tag using immobilized-nickel ion affinity chromatography and elution with a linear gradient of EDTA revealed three enzyme species (mrI, mrII, and mrIII). While mrIII was catalytically inactive, both mrI and mrII catalyzed the racemization of (*S*)-mandelate with turnover numbers (k_{cat}) of 190 ± 22 and 940 ± 24 s^{-1}, respectively. Circular dichroism analysis suggested that mrIII was a partially unfolded or misfolded form of the enzyme. Replacement of the N-terminal hexahistidine tag by a StrepII-tag appeared to ameliorate the folding problem yielding a single enzyme species with a turnover number of 1124 ± 43 s^{-1}. The MR fusion protein bearing an N-terminal StrepII-tag and a C-terminal decahistidine tag also exhibited reduced turnover (k_{cat} = 472 ± 37 s^{-1}). These results highlight a potential problem that may be encountered when producing fusion enzymes bearing a polyhistidine tag: soluble, active enzyme may be obtained but care must be taken to ensure that it is free of minor misfolded forms that can alter the apparent activity of the enzyme.

Affinity tags are often used for the purification of recombinant proteins because high purity is achieved in a single step with often only one additional chromatographic step being required to obtain proteins purified to homogeneity [1–7]. In addition to affinity tags such as glutathione *S*-transferase (GST) [8], maltose-binding protein (MBP) [9], and streptavidin [10,11], one of the most commonly employed affinity tags is the polyhistidine tag (e.g., hexahistidine (His_6) or decahistidine (His_{10})) [12]. Despite their widespread use, in some cases polyhistidine tags have been reported to have significant effects on the properties of fusion proteins relative to their wild-type counterparts including effects on enzymatic activity [13–18], interaction with metal ions [19], oligomerization state [20–23], disulfide structure [24], folding kinetics [17], and biological function [25–30]. In addition, the location [31,32] and the length [17] of the His-tag can affect the aggregation state and solubility of fusion proteins. Consequently, many of the recombinant tagged-fusion proteins contain a protease-specific cleavage site for removal of the affinity tag [1,2,23,33].

If an enzyme bearing an affinity tag exhibits reduced catalytic activity (i.e., decreased k_{cat}) relative to the wild-type enzyme, it is possible that either all of the fusion protein is functioning at this reduced level or there are two or more distinct populations of enzyme with differing activities which may arise, presumably, from misfolded or partially folded conformations. For example, Halliwell et al. [34] showed that fusion of a His_6-tag to the N-terminus of lactate dehydrogenase had no affect on enzymatic activity relative to the wild-type enzyme; however, the presence of a C-terminal His_6-tag caused an approximately 1.5-fold reduction in k_{cat}. Using affinity chromatography on 5′-AMP-Sepharose, these authors showed that the C-terminally tagged enzyme could be separated into a bound fraction with wild-type catalytic activity and an unbound fraction which was inactive. While it is well known that large affinity tags (e.g., MBP and GST) can affect the conformational homogeneity of proteins [35], the impact of small affinity tags on enzyme activity has often been overlooked, probably because affinity-based protein purification protocols do not usually permit separation of proteins with different conformations (e.g., folded vs. misfolded species) [36].

Herein we describe an improved purification protocol for mandelate racemase (5.1.2.2; MR) that yields enzyme with an optimal turnover number due to removal of misfolded protein. MR catalyzes the Mg^{2+}-dependent 1,1-proton transfer that interconverts the enantiomers of mandelate and has been studied extensively

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as a model for understanding how enzymes catalyze the deprotonation of carbon acid substrates with relatively high pK_a values. Like other members of the enolase superfamily of enzymes, MR has a two-domain structure which includes a core $(\beta/\alpha)_7\beta$-barrel fold and an N-terminal $\alpha + \beta$ capping domain (Fig. 1). Recombinant MR from *Pseudomonas putida* (ATCC 12633) was first expressed in *Escherichia coli* [37] and later in *Pseudomonas aeruginosa* ATCC 15692 [38] without affinity tags. More recent studies have utilized MR bearing an N-terminal His_6-tag [39–46] and removal of the tag was shown not to significantly affect the activity of the enzyme [39]. Recombinant, His_6-tagged MR typically exhibits turnover numbers (k_{cat}) of 447 ± 12 and $514 \pm 48\ s^{-1}$ in the $S \rightarrow R$ and $R \rightarrow S$ reaction directions, respectively [45]. Interestingly, k_{cat} values ranging from ~100–300 s^{-1} [37,47] to 700 s^{-1} [48] have been reported for wild-type MR not bearing a His_6-tag, although most studies generally report k_{cat} values on the order of 400–500 s^{-1} [49–51]. Using immobilized-metal ion affinity chromatography (IMAC), we show that MR purified as a fusion protein bearing a His_6-tag exhibits an approximately 30–50% reduction in the turnover number of the enzyme which arises from the presence of alternatively folded forms of the tagged recombinant protein. Full catalytic activity can be achieved by appropriate fractionation using IMAC or through the use of an alternative affinity tag.

Materials and methods

General

Deoxyribonuclease I (DNase I), (*S*)- and (*R*)-mandelic acids, buffers, and all other chemicals were purchased from Sigma–Aldrich Canada Ltd. (Mississauga, ON, Canada) unless mentioned otherwise. His·Bind resin and the pET-15b and pET-52b(+) expression systems were purchased from Novagen Inc. (Madison, WI, USA). *Strep*-Tactin Superflow resin was purchased from IBA GmbH (Göttingen, Germany). DNA oligonucleotide primers were obtained from Integrated DNA Technologies (IDT) (Coralville, IA, USA). Protein molecular weight and DNA ladder markers and AvrII restriction endonuclease were purchased from New England Biolabs (Ipswich, MA, USA), while XhoI and KpnI restriction endonucleases were purchased from Invitrogen (Burlington, ON, Canada). The DNA sequences of plasmids encoding the MR fusion proteins were verified by commercial sequencing at the Robarts Research Institute (London, ON, Canada). Plasmid isolations and PCR product purifications were conducted using kits from Qiagen Inc. (Mississauga, ON, Canada) according to the manufacturer's protocol. Protein purifications were conducted using an ÄKTA FPLC from GE Healthcare (Baie d'Urfé, PQ, Canada) with protein elution detected by monitoring the absorbance at 280 nm. Circular dichroism (CD) spectral measurements and enzyme assays were conducted using a JASCO J-810 spectropolarimeter. SDS–PAGE was conducted using standard protocols [52].

StrepII-tagged MR

The pET-15b plasmid bearing the open reading frame (ORF) encoding MR [39] was used as the template for PCR-dependent amplification [52] of the ORF using *Pfu* DNA polymerase (Bio Basic Inc., Markham, ON, Canada) and the forward and reverse primers 5′-CCGCGCGGGTAC↓CATATGAGTGAAGTACT-3′ and 5′-AGCAG-CATCGAGCT↓CTTACACCAGATATTT-3′ introducing KpnI and SacI restriction sites (indicated by arrows) at the 5′- and 3′-ends, respectively. After the double digestion of both the pET-52b(+) plasmid and the PCR product with KpnI and SacI, the PCR product was inserted into the vector using T4 DNA ligase. Correct insertion of the target ORF was verified by sequencing the DNA with the standard T7 promoter and T7 terminator primers (Novagen) and the internal primers: 5′-CCACTAGTAAAACTCCTCGG-3′ (forward) and 5′-GCTTTGAACATCTCCTCAGG-3′ (reverse). This plasmid encodes the MR fusion protein bearing an N-terminal StrepII-tag and a C-terminal His_{10}-tag. Preparation of an ORF encoding the MR fusion protein bearing only an N-terminal StrepII-tag was conducted as mentioned above except that the reverse primer 5′-GGTGGCAGCAGC↓CTAGGTTACACCAGATATTT-3′ was used thereby introducing an AvrII restriction site to permit insertion into the pET-52b(+) plasmid with elimination of the sequence encoding the His_{10}-tag.

Expression of MR in E. coli

E. coli BL21(DE3) or *E. coli* C43 (Lucigen, Middleton, WI) cells, transformed either by heat shock or electroporation [52] with the appropriate expression vectors, were used for overproduction of the recombinant tagged MR fusion proteins. Overproduction of soluble MR was not affected by varying the concentration of isopropyl-β-D-thiogalactopyranoside (IPTG) between 0.1 and 1.0 mM, hence the *E. coli* cells were grown without induction in LB medium (1000 mL) containing ampicillin (100 μg/mL) at 37 °C in 2 L flasks with shaking at 240 rpm. The cells were harvested at logarithmic phase ($OD_{600} \sim 1$), after 5 h of growth by centrifugation (3000*g*, 10 min, 4 °C).

Protein purification

For purification of His_6-MR, cell paste (5–7 g) was suspended in 5 mL of Tris–HCl buffer (20 mM, pH 7.9) containing imidazole (40 mM), NaCl (250 mM), 20 μL of DNase I (20 μg/mL), and phenylmethanesulphonylfluoride (0.001%). The cells were lysed using

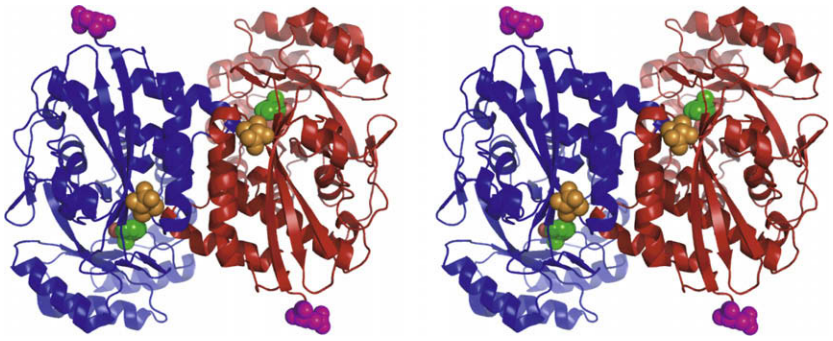

Fig. 1. Stereoview of the X-ray crystal structure of wild-type MR dimer with bound (*S*)-atrolactate (PDB entry 1MDR [74]). The secondary structures of the α-carbon backbone of the individual monomers (red and blue) are shown. N-termini (magenta) and C-termini (orange) are shown in space-filling representation. The bound substrate analogue (*S*)-atrolactate is shown in space-filling representation with carbons and oxygens colored green and red, respectively. This figure was prepared using MacPyMol [75]. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)