Enzyme Kinetics of Recombinant Dihydroorotase from *Methanococcus jannaschii*

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ABSTRACT. Dihydroorotase (DHOase) catalyzes the reversible cyclization of N-carbamoyl-L aspartate (CA) to L-dihydroorotate (DHO) in the third step of *de novo* pyrimidine biosynthesis. The reaction is pH dependent; at low pH the biosynthetic reaction is favored (CA to DHO) and at high pH the degradative reaction is favored (DHO to CA). Even though DHOases share a common catalytic mechanism, they form a very diverse family of proteins. *Methanococcus jannaschii* is a hyperthermophilic and barophilic archaeon and its DHOase (*Mj* DHOase) is the first archaeal one that is being studied. A previously conducted physicochemical characterization of *Mj* DHOase gave information into its similarities and differences from the other known DHOases. In that study, enzyme kinetics were only analyzed in the degradative direction. The purpose of the current work is to further characterize *Mj* DHOase by studying the kinetics in the biosynthetic direction and the dependence of the reaction on pH in both directions. The properties obtained are compared with other known DHOases. The specific activity of *Mj* DHOase in the biosynthetic direction is approximately half the specific activity in the degradative direction, similar to human DHOase. *Mj* DHOase exhibits the characteristic pH dependence of the reaction.

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INTRODUCTION

Dihydroorotase (DHOase) catalyzes the third reaction of *de novo* pyrimidine biosynthesis, the conversion of N-carbamoyl-L aspartate (CA) to L-dihydroorotate (DHO) (Fig. 1). This is a reversible reaction with the biosynthetic direction (CA → DHO) favored at low pH and the degradative direction (DHO → CA) favored at high pH. DHOase is a Zn metalloenzyme and member of the amidohydrolase superfamily of proteins.

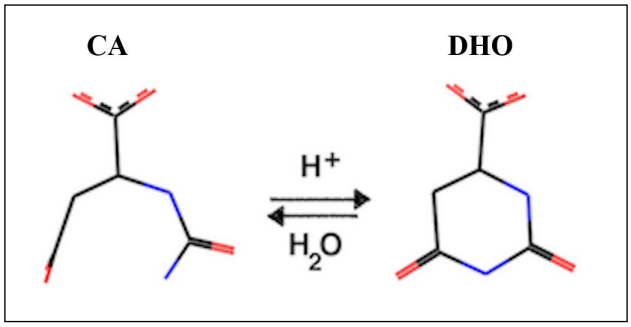

FIGURE 1. Reaction catalyzed by dihydroorotase. Figure drawn with the program PyMOL (The PyMOL Molecular Graphics System, version 2.4, Schrödinger, LLC).

DHOases were initially classified into 2 types: long and short. Long are more ancient and larger with a molecular mass of approximately 45,000 Da, while short are more recent with a molecular mass of approximately 38,000 Da (Fields et al. 1999). A later phylogenetic analysis indicated that long DHOases are subdivided into archaeal, bacterial type I, bacterial type III, human CAD (a multifunctional protein that combines glutamine-dependent carbamyl phosphate synthetase, aspartate transcarbamylase, and dihydroorotase activities), and inactive CAD in fungi; conversely, short DHOases subdivide into bacterial type II, plant, and active DHOases in fungi (Grande-Garcia et al. 2014). Representatives from several of these subtypes have been studied (Grande-Garcia et al. 2014). From the phylogenetic analysis, archaeal type appear to be closely related to bacterial type I and have been studied very little. *Methanococcus jannaschii* is a hyperthermophilic and barophilic archaeon. The DHOase from *M. jannaschii* (*Mj* DHOase) is the first archaeal one that is being studied.

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A previous physicochemical characterization of *Mj* DHOase gave insight into its similarities and differences from the DHOases of the other types and subtypes (Vitali et al. 2017). *Mj* DHOase is a monomer—unlike most DHOases that form dimers—and is most similar to the bacterial type I DHOase from *Bacillus anthracis* (*Ba* DHOase). *Mj* DHOase is unlikely to associate with ATCase as in the case for *Aquifex aeolicus* DHOase (*Aa* DHOase) (Zhang et al. 2009), because it associates with a regulatory subunit (Hack et al. 2000). It contains 2 Zn ions in the active site unlike *Aa* DHOase that has only one. Previously, the kinetic behavior of *Mj* DHOase was studied only in the degradative direction as typically, at physiological pH, the degradative reaction is favored (Vitali et al. 2017). The adaptation of the enzyme at high temperatures was also discussed.

The purpose of the current study is to further characterize the archaeal subtype by studying the kinetics of *Mj* DHOase in the biosynthetic direction, as well as the dependence of the reaction with pH in both directions. The kinetic parameters were determined at 25 °C and 45 °C. A comparison of the features obtained with known representatives from the other types and subtypes is made.

METHODS AND MATERIALS

The protein used was the same as in the earlier study and the preparation procedure was described in that paper (Vitali et al. 2017). The dihydroorotase activity in the biosynthetic direction was studied in 100 mM Mes pH 5.8 by measuring the dihydroorotate formed per minute at 230 nm, $\varepsilon_{230\ nm}$ = 1.17 mM^{-1} cm^{-1} (Washabaugh and Collins 1984) using substrate concentrations to 9.08 mM. The assay was carried out in a SpectraMax® Plus 384 microplate reader from Molecular Devices using the software SoftMax® Pro 6.3. The microplates used had 8×12 wells and the assay volume was 200 µl. The assay was carried out at 25 °C and 45 °C. The reaction was initiated by addition of the enzyme. The background reaction of carbamoyl aspartate to dihydroorotate in the absence of enzyme was also measured and was negligible. Protein concentration was measured with the Bradford assay using the Coomassie Plus Protein Assay reagent from Pierce™ and BSA as the standard. Kinetic parameters were calculated by fitting data to the Michaelis-Menten equation using GraphPad Prism version 8.0.0 (GraphPad Software). According to the Michaelis-Menten model, the reaction rate V (the rate of formation of product) is related to the concentration of the substrate [S] by $V = V_{max}\,[S] / (K_m + [S])$ where K_m is the Michaelis constant and V_{max} is the maximum rate achieved by the system. Specific activity is the maximum rate per mg of enzyme. The data points in the 25 °C curves are averages of 4 measurements and in the 45 °C curves are averages of 2 measurements.

The pH dependence of the biosynthetic and degradative activities were measured at 45 °C using buffers Mes-K for pH 5 to 7, Hepes-Na for pH 7 to 9, and Tris-Cl for pH 7.5 to 9. The activity for the biosynthetic reaction was measured in $A_{230\ nm}$/min with enzyme concentration of 3.69 µg/ml and substrate concentration of 6.81 mM. The measurements of the activity in the degradative direction were done with the colorimetric assay (Prescott and Jones 1969) and with readings $A_{466\ nm}$ as described previously (Vitali et al. 2017). The enzyme concentration was 0.76 µg/ml and the substrate concentration was 3.15 mM. Each data point was measured 3 times.

RESULTS

Saturation curves for the biosynthetic reaction are presented in Fig. 2 at 25 °C and 45 °C. The enzyme follows Michaelis-Menten kinetics. The specific activity and K_m values are given in the Table for both temperatures. The values of the degradative reaction (Vitali et al. 2017) are also included in the Table as well as the kinetic parameters of selected dihydroorotases that have been studied from other types and subtypes.

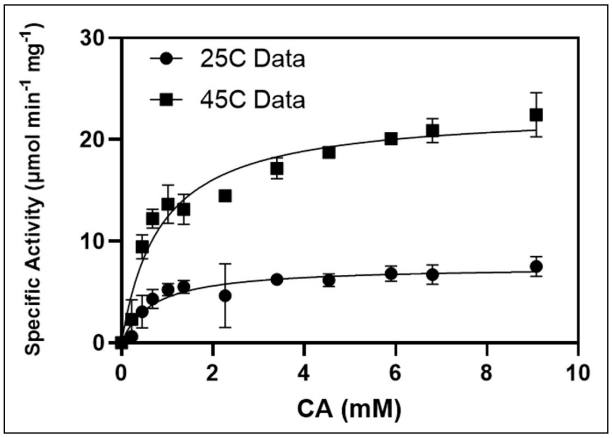

FIGURE 2. Saturation curves of *M. jannaschii* DHOase in the biosynthetic direction at pH 5.8. Data are averages of 4 measurements at 25 °C and 2 measurements at 45 °C. The curves were prepared with GraphPad Prism.

It may be noted in the Table that the specific activity of *Mj* DHOase in the biosynthetic direction is approximately one-half the specific activity in the degradative direction at both 25 °C and 45 °C. This is similar to human DHOase (Grande-Garcia et al. 2014). Different values of the activities in the 2 directions are also observed in *E. coli* (*Ec*) but, in this case, the specific activity of the biosynthetic reaction is higher than for the degradative reaction. *B. anthracis* (*Ba*) has similar values of the specific activity in both directions. The Michaelis constant K_m is larger for the biosynthetic reaction in *Mj* DHOase and this is also the case in both human and *E. coli* DHOases. In contrast, the *Ba* DHOase has similar values for this constant in both directions.

The pH dependence of the reaction is given in Fig. 3 and is normalized to a maximum of 1 in both directions. The biosynthetic reaction is predominant at a low pH of approximately 5.5 and decreases progressively as the pH increases; the biosynthetic reaction reaches a very low rate at pH > 8.5. However, the degradative reaction is maintained as the pH decreases and is still strong even at low pH values. The 2 curves cross at a pH of approximately 6.

Table
Kinetic parameters of *Methanococcus jannaschii* DHOase and of selected DHOases for the biosynthetic and degradative reactions

		Biosynthetic		Degradative		
Organism	T (°C)	Specific activity[e] (µmol/min/mg)	K_m (mM)	Specific activity[e] (µmol/min/mg)	K_m (mM)	Type
Mj	25	7.5 ± 0.5	0.68 ± 0.17	12.2 ± 0.1[a]	0.140 ± 0.009[a]	Archaeal type
"	45	22.9 ± 0.9	0.85 ± 0.13	44.7 ± 0.6[a]	0.15 ± 0.01[a]	"
"	80	—	—	248 ± 5[a]	0.52 ± 0.03[a]	"
Ba[b]	25	2.7 ± 0.1	0.112 ± 0.024	2.4 ± 0.1	0.114 ± 0.016	Bacterial type I
Human[c]	25	4.42 ± 0.06	0.241 ± 0.054	8.20 ± 0.24	0.028 ± 0.004	CAD
Ec[d]	30	251 ± 13	1.7 ± 0.2	155 ± 2	0.080 ± 0.001	Bacterial type II

[a] Vitali et al. (2017).
[b] Rice et al. (2016), *Bacillus anthracis*.
[c] Grande-Garcia et al. (2014).
[d] Porter et al. (2004), *E. coli*.
[e] Specific activities for *Ba*, human, and *Ec* were computed from the reported K_{cat} values.

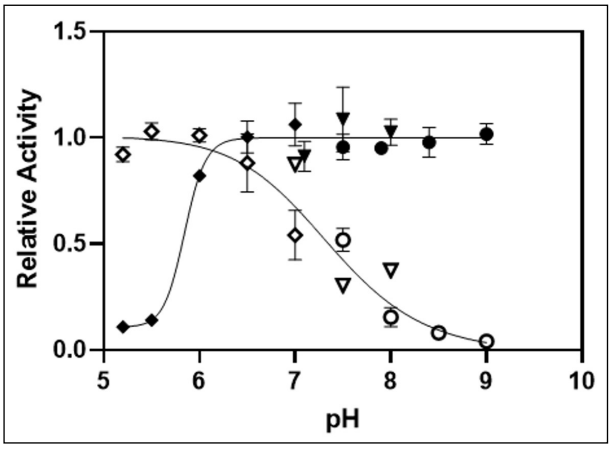

FIGURE 3. pH dependence of both biosynthetic and degradative reactions in *Mj* DHOase. The measurements were made at 45 °C. Empty symbols correspond to the biosynthetic reaction and solid symbols correspond to the degradative reaction. The activity values have been normalized to 1 in both directions. As can be seen in the Table, at saturation, the specific activity of the biosynthetic reaction at pH 5.8 is 22.9 ± 0.9 µmol/min/mg. The corresponding value of the degradative reaction at pH 8.3 is approximately twice this value, 44.7 ± 0.6 µmol/min/mg. Buffers used were Mes-K for pH 5 to 7 (diamonds), Hepes-Na for pH 7 to 9 (triangles), and Tris-Cl for pH 7.5 to 9 (circles). The curve was prepared with GraphPad Prism.

DISCUSSION

Mj DHOase follows Michaelis-Menten kinetics for both the biosynthetic reaction (current study) and the degradative reaction (Vitali et al. 2017). This result is similar to most DHOases (for example Grande-Garcia et al. 2014; Rice et al. 2016). However, Lee et al. (2005) showed that *E. coli* (*Ec*) DHOase has positive cooperativity in the degradative direction, suggesting communication between the subunits in the *E. coli* DHOase dimer. The Table shows differences in the kinetic behavior among the dihydroorotases of the different subtypes even though their active sites are similar. It may be noted that the residues involved are invariant in all and/or within subtypes, and the key interactions are mostly conserved. The differences in the kinetic parameters among the different systems in the Table probably reflect subtle differences in the vicinity of their active sites.

There is an increase in specific activity of *Mj* DHOase with temperature in the biosynthetic direction (Table), as was observed in the degradative direction (Vitali et al. 2017). This is a common feature for all enzymes and is due to the fact that as the temperature increases more enzyme molecules have enough energy to undergo the necessary catalytic conformational changes (Fields 2001; Feller 2010; Elias et al. 2014). There is also an increase in K_m with temperature in the biosynthetic direction (Table), as in the degradative reaction. This is observed in most proteins and indicates an increase in the molecular flexibility of the active site (Fields 2001). The increase in specific activity with temperature is also similar to the related aspartate transcarbamoylase enzyme from *M. jannaschii* (Hack et al. 2000). The fold-increase in turnover rate every 10 °C (Q10) in the biosynthetic reaction was calculated to be 1.75 between 25 °C and 45 °C. This value is comparable to the value in the degradative reaction (Vitali et al. 2017) and is within the range of 1.2 to 2.3 observed for thermophilic enzymes based on specific activity (Elias et al. 2014).

Even though *M. jannaschii* is a hyperthermophilic organism, it may be noted that the specific activity of its DHOase in the biosynthetic direction at 25 °C is higher than *B. anthracis* (Table), as is also the case for the degradative direction (Vitali et al. 2017). As discussed by Elias et al. (2014), the rigidity of the fold of an enzyme does not necessarily affect the flexibility of the active site and its activity.

Mj DHOase exhibits the characteristic pH dependence of the reaction with the curves in both directions crossing at a pH of approximately 6 (Fig. 3). However, there is some variability in the pH behavior of different systems. In contrast to the trend in *Mj* DHOase, the biosynthetic reaction in human DHOase maintains activity even at high pH values while the degradative reaction decreases to very small values at pH 5.5 (Grande-Garcia et al. 2014). The degradative reaction for the DHOase of the parasite *Toxoplasma gondii* maintains almost half its full activity at pH 5.5 while the direct reaction decreases to zero at a pH of approximately 8 (Robles Lopez et al. 2006).

The study of kinetic parameters is essential to understand enzyme behavior and correlate it with its structure. It would be of interest, in future studies, to correlate the pH curves with X-ray structural data of complexes of the *Mj* DHOase with the 2 substrates at different pH values. Such studies would allow observation of the changes in the conformation of the protein in the presence of the substrate or product as well as the reaction itself at different pH values.

Conclusions

The current study further extends the characterization of the archaeal subtype of DHOases and how they differ from the other types and subtypes. Clearly, more data are needed for other archaeal DHOases to generalize the similarities and differences between the archaeal and the other subtypes.

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