〈Original Article〉

Structural comparison of creatinases for investigating substrate binding

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Summary For further understanding the substrate binding of creatinase, the kinetic parameters of seven known enzymes and the tertiary structures of more significant ones of those were compared. Used structures were constructed by homology modeling based on the X-ray structure of the enzyme from *Pseudomonas putida*. The structural comparison of these creatinases showed that the specific residues in the N-terminal domain, rather than the C-terminal catalytic domain, had influenced on in the substrate affinity. This result would be assistance in the improvement of creatinase for creatinine and creatine determinations.

Key words: Creatinase, Tertiary structure, Homology modeling, Substrate affinity, Creatinine assay

1. Introduction

Creatinase (creatine amidinohydrolase, EC 3.5.3.3) catalyses the hydrolyzation of creatine to sarcosine and urea. It is involved in the bacterial metabolism of creatinine with the related enzymes[1]. The enzyme is commercially used in the diagnostic analysis of creatinine and creatine with coupling of related enzymes[2, 3]. Several creatinases have been found, produced, and proved useful in the enzymatic assays[4-10]. However, the enzymatic properties of these enzymes are not yet discussed from the viewpoint of tertiary-structural comparison, although the X-ray structures of the enzymes from *Pseudomonas putida* and *Actinobacillus* sp. have already been solved[11-13]. With respect to the enzymes for creatinine determination, creatininase and sarcosine oxidase have been already studied and improved by using protein engineering techniques on the basis of tertiary-structural analysis[14-16]. In contrast, the structure-based improvement of creatinase still has not progressed.

This report shows the tertiary structures of creatinases constructed by homology modeling. Comparison of these structures indicated that the N-terminal domain, rather than the C-terminal catalytic domain, was important for the substrate affinity. This provides a reasonable starting point for analyzing the structure-function relationships and improving the enzyme properties.

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2. Materials and methods

Homology search analysis of the amino acid sequences of several creatinases was performed using the software GENETYX (Software Development Co., Ltd., Tokyo, Japan). The sequences used in this study are in the DDBJ database under accession numbers E17219, D00656, D14463, E16405, and BD342394, and in the UniProt ID: P38488. Homology modeling was used to build the models of creatinases. The three-dimensional protein models were generated by the software MODELLER[17], based on the structure of *P. putida* (PDB ID: 1chm). The program Pymol was used for molecular visualization.

3. Results and discussion

In previous studies, several creatinases from seven different genera have been reported with their enzymatic properties[4-10]. The enzymes from *Arthrobacter, Flavobacterium, Bacillus, Actinobacillus, Alcaligenes, Erwinia*, and *Pseudomonas* were designated CreA, CreF, CreB, CreC, CreL, CreE, and CreP, respectively. The kinetic parameters and assay conditions of these creatinases are compared in Table 1. The parameters of CreP and CreE could not be compared

Table 1 Comparison of kinetic parameters and assay conditions

Creatinase	K_m (mmol/L)	V_{max} (U/mg)	Assay codition	Reference
CreA	46	21	37℃ pH 7.6	4
CreF	40	11.3	37℃ pH 7.7	5
CreB	NF	10.3	37℃ pH 7.5	6
CreC	19	18.1	37℃ pH 7.5	7
CreL	15.2	19.5	37℃ pH 7.6	8
CreP	14.3	15.0	25℃ pH 7.8	9
CreE	4.2	7.8	25℃ pH 7.8	10

CreA, CreF, CreB, CreC, CreL, CreP, and CreE are from *Arthrobacter*, *Flavobacterium*, *Bacillus*, *Actinobacillus*, *Alcaligenes*, *Pseudomonas*, and *Erwinia*, respectively.
NF; Data not found.

		Total sequence						
		CreA	**CreF**	**CreB**	**CreC**	**CreL**	**CreP**	**CreE**
N-Terminal domain	**CreA**		63	78	-	62	63	62
	CreF	52		66	-	64	**95**	65
	CreB	73	52		-	64	66	65
	CreC	-	-	-		-	-	-
	CreL	49	50	52	-		63	**94**
	CreP	51	**95**	51	-	50		66
	CerE	49	52	52	-	**91**	52	

Fig. 1 Homology search of amino acid sequences of creatinases. Total sequences and N-terminal domains were compared, and each identity was indicated by a percentage. The CreC sequence was not compared because it was incomplete.

with those of others because they were assayed at the different reaction temperature. Generally, K_m and V_{max} values are decreased at low temperatures. The K_m value of CreL was smaller than those of CreA, CreF, and CreC, i. e., its binding affinity was higher for creatine. On the other hand, the K_m of CreE was much smaller than that of CreP. Of all known creatinases, CreL and CreE are practical for diagnostic use because of their superior substrate-binding abilities. The different substrate affinities appeared from the important viewpoint of tertiary-structural comparison, although the V_{max} values also differed from each other (Table 1).

The creatinase molecule forms a dimer of identical subunits. Each subunit is characterized by two clearly separated domains which are the smaller N-terminal domain with approximately 160 amino acid residues and the C-terminal catalytic domain with approximately 240 residues. Many active site residues exist on the C-terminal domain, including His232 of CreP that is the general acid and base involved in the hydrolysis of the guanidinium group of creatine. All active site residues were confirmed to be perfectly conserved in all enzymes (data not shown).

Homology search of total and N-terminal amino acid sequences are summarized in Figure 1. It shows that these amino acid sequences are highly similar though belonging to different genera. The total sequence homologies were extremely higher (62-95% identities). In contrast, the homologies of N-terminal domains indicated relatively lower tendencies (49-94% identities). Two practical enzymes, CreL and CreE, had similar sequences, and the total and N-terminal sequence identities were 94 and 91%, respectively (Fig. 1). On the other hand, CreF and CreP also had similar sequences, and the total and N-terminal sequence identities were 95% (Fig. 1). These results suggest that the structural comparison between CreL and CreF or that between CreE and CreP would be helpful for investigating the substrate binding of creatinase.

Therefore, three-dimensional structure models of the dimer molecules of CreF, CreL, and CreE, were built by computer analysis based on the CreP sequence and its X-ray structure[11]. Overall subunit structures of all four enzymes could be well superimposed with root mean square deviation (RMSD) of atomic Cα positions of 0.14-0.34 Å. Creatinase molecule is composed of the two identical subunits, which are designated subunit A and B. Two active sites are formed from the C-terminal catalytic domain of subunit A (or subunit B) and the N-terminal domain of subunit B (or subunit A). For the purpose of discussing the interaction of creatinase with substrate, the active sites of CreL and CreE were structurally compared with those of CreF and CreP, respectively.

After superimpositions of CreL-CreF and CreE-CreP, the selected amino acid residues that are within 6 Å of the substrate analog, N-carbamoylsarcosine, are shown in Figure 2. N-Carbamoylsarcosine is a competitive inhibitor that binds to the active site of creatinase. Each active site of the structure model including the substrate analog was obtained by superposing the coordinates on that of CreP[11].

In the case of structural comparison between CreL and CreF, the residues belonging to the C-terminal domains were highly conserved (Fig. 2A). However, the residues belonging to the N-terminal domains could recognize some different states between both enzymes. In particular, an aspartate residue in the N-terminal domain shifted to quite different positions between CreL and CreF. The side chain of D101 in CreF was located at a distance of 4.8 Å from the carboxyl group of the substrate analog, whereas that of D102 in CreL was 6.3 Å away. The carboxyl group of creatine can interact with R64 of subunit B and R335 of subunit A in CreF, or R66 of subunit B and R336 of subunit A in CreL. The substrate binding would be likely to be disturbed by the negative charge of D101 in CreF.

In the case of structural comparison between CreE and CreP, the residues belonging to the C-terminal domains were slightly altered, although those belonging to the N-terminal domains showed clear differences (Fig. 2B). As shown in Table 1, the K_m, V_{max}, and catalytic efficiency (V_{max}/K_m) of CreE were 0.29, 0.52, and 1.8 times those of CreP, respectively. In contrast, the K_m, V_{max}, and V_{max}/K_m of CreL were 0.38, 1.7, and 4.5 times those of CreF, respectively. The different situation of the active sites may reflect the different V_{max} values.

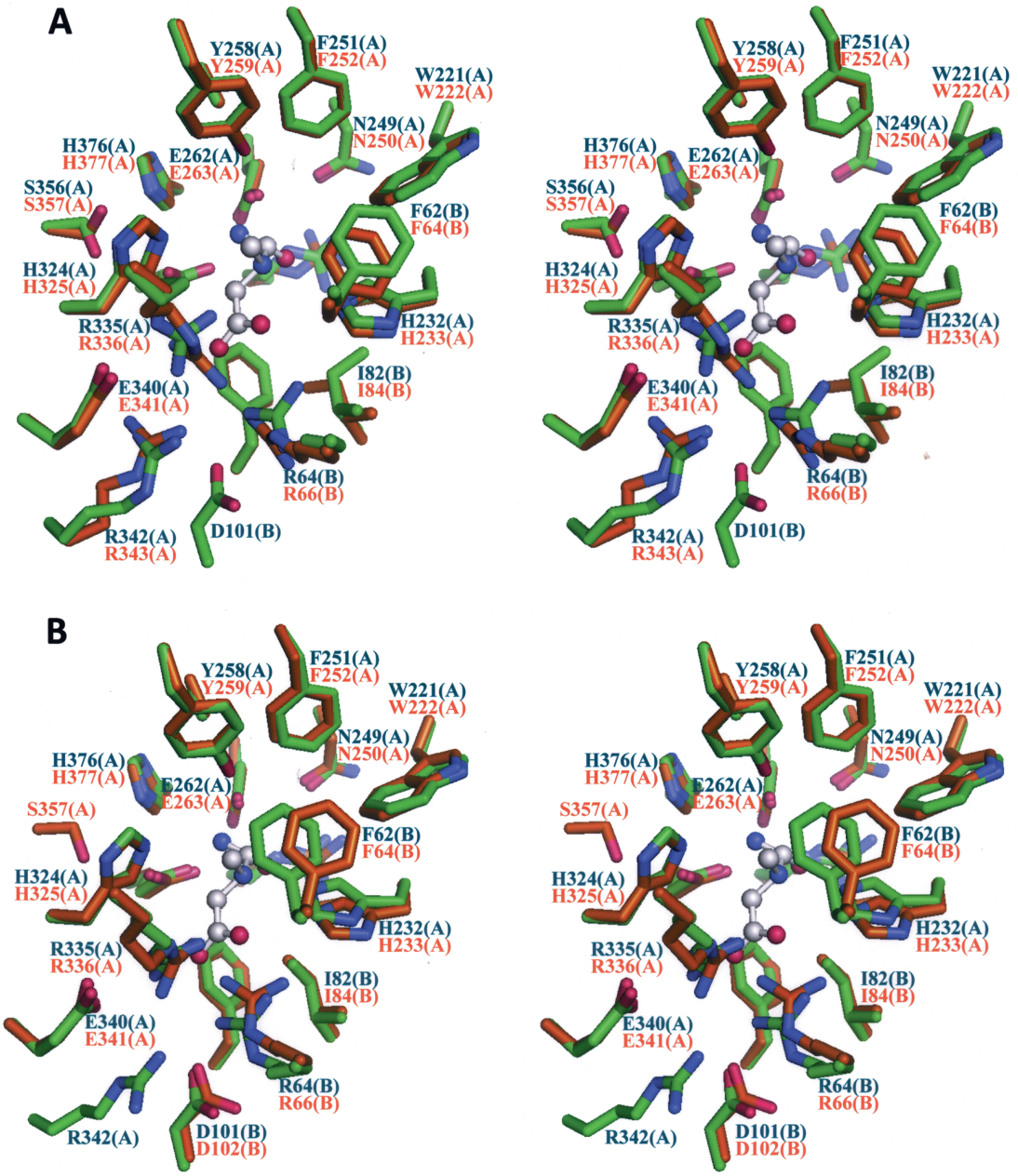

Fig. 2 Close-up stereo view of the active site regions. These are one of two active sites in the creatinase structures, that are composed of the C-terminal catalytic domain from the subunit A and the N-terminal domain from the subunit B. The side chains of amino acid residues within 6 Å of the substrate analog, N-carbamoylsarcosine, are labeled and shown by stick drawings. The subunit to which each residue belongs (A or B) are also labeled. N-Carbamoylsarcosine is represented by ball and sticks. (A) Superimposition of CreL model (orange) over CreF (green). (B) Superimposition of CreE model (orange) over CreP (green).

These results would be of assistance in the improvement of the enzyme using protein engineering techniques. In comparison with the C-terminal catalytic domain, changes in amino acid residues of the N-terminal domain are expected to improve the substrate binding with a smaller influence on the enzyme activity. The creatinase structures of the present study provide a reliable basis to predict mutations for desired effects on the substrate binding.

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