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Varying the Directionality of Protein Catalysts for Aldol and Retro-aldol Reactions

Toshifumi Fujioka,[‡,†,[a]] Nobutaka Numoto,[‡,[b]] Hiroyuki Akama,[‡,†,[c]] Kola Shilpa,[‡,[c]] Michiko Oka,[[a]] Prodip K. Roy,[[c]] Yarkali Krishna,[[c]] Nobutoshi Ito,[[b]] David Baker,[[d]] Masayuki Oda,*[,[a]] and Fujie Tanaka*[[c]]

[a] T. Fujioka, M. Oka, Prof. Dr. M. Oda
Graduate School of Life and Environmental Sciences
Kyoto Prefectural University
1-5 Hangi-cho, Shimogamo, Sakyo-ku, Kyoto, Kyoto 606-8522, Japan
E-mail: oda@kpu.ac.jp

[b] Prof. Dr. N. Numoto, Prof. Dr. N. Ito
Medical Research Institute
Tokyo Medical and Dental University (TMDU)
1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510, Japan

[c] Dr. H. Akama, K. Shilpa, Dr. P. K. Roy, Dr. Y. Krishna, Prof. Dr. F. Tanaka
Chemistry and Chemical Bioengineering Unit
Okinawa Institute of Science and Technology Graduate University
1919-1 Tancha, Onna, Okinawa 904-0495, Japan
E-mail: ftanaka@oist.jp

[d] Prof. Dr. D. Baker
Department of Biochemistry, Institute for Protein Design, and Howard Hughes Medical Institute
University of Washington
Seattle, WA 98195, USA

‡ (Fujioka, T.; Numoto, N.; Akama, H.; Shilpa, K.) These authors contributed equally.

† Fujioka, T.; current address: Division of Biological Science, Nara Institute of Science and Technology, Ikoma, Nara 630-0192, Japan; Akama, H.; current address: Research Center for Infection Control, Omura Satoshi Memorial Institute, Kitasato University, Shirokane, Tokyo 108-8641, Japan

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Abstract: Natural aldolase enzymes and created retro-aldolase protein catalysts often catalyze both aldol and retro-aldol reactions depending on the concentrations of the reactants and the products. Here, we report that the directionality of protein catalysts can be altered by replacing one amino acid. The protein catalyst derived from a scaffold of a previously reported retro-aldolase catalyst catalyzed aldol reactions more efficiently than the previously reported retro-aldolase catalyst. The retro-aldolase catalyst efficiently catalyzed the retro-aldol reaction but was less efficient in catalyzing the aldol reaction. The results indicated that protein catalysts with varying levels of directionality in usually reversibly catalyzed aldol and retro-aldol reactions can be generated from the same protein scaffold.

Introduction

Aldolase enzymes and designer (retro)aldolase protein catalysts often reversibly catalyze C-C bond forming aldol and C-C bond cleaving retro-aldol reactions, which are the most fundamental reactions in biology and chemistry, depending on the concentrations of substrates and/or products.[1-7] For example, D-2-deoxyribose-5-phosphate aldolase (DERA) catalyzes the aldol reaction to form D-2-deoxyribose-5-phosphate and the retro-aldol reaction of the same compound to decompose it into D-2-glyceraldehyde-3-phosphate and acetaldehyde.[1] *N*-Acetylneuraminic acid lyase catalyzes reversible reactions of pyruvate with *N*-acetyl-D-mannosamine to yield *N*-acetylneuraminic acid.[2] Aldolase antibodies generated against 1,3-diketone derivatives also catalyze both aldol and retro-aldol reactions.[3-5] Some designer (retro)aldolase proteins generated by computational designs with directed evolution also catalyze both aldol and retro-aldol reactions.[6,7] For these, the improved catalysts were often identified based on the activity for catalyzing retro-aldol reactions, and the best retro-aldolase catalysts among those derived from a structural scaffold were also efficient catalysts for the aldol reactions.[6,7] Naturally occurring enzymes may catalyze both forward and reverse reactions to maintain homeostasis. However, depending on the use of the catalysts, it may not be necessary for designer catalysts to reversibly catalyze the reactions. A question exists in whether the directionality of designer protein catalysts can be varied. Enabling the creation of protein catalysts that favor one direction in commonly reversibly catalyzed reactions would expand the uses of protein catalysts and provide an understanding of catalysis. Here we report that the directionality of protein catalysts can be altered by replacing one amino acid.

For aldol and retro-aldol reactions catalyzed by a protein catalyst, both directions of the catalyzed reactions may occur with equilibrium in the same reversible pathway.[1-7] Therefore, catalyst selection based on the activity of the retro-aldol reaction may be a good way to find catalysts catalyzing both retro-aldol and aldol reactions.[6,7] Previously reported designer (retro)aldolase protein catalysts that catalyze aldol and retro-aldol reactions often use a lysine amino group as the key catalytic residue, which forms iminium ions and/or enamines.[3,4,6,7] The reversible feature of the reactions may be an intrinsic feature of the catalyst based on the enamine mechanism that uses a lysine side chain amino group as an enamine-forming group.[8] However, even when a catalyst catalyzes both aldol and retro-aldol reactions and the same amino acid residues are involved in the catalysis, the pathway of catalyzed aldol reaction is not necessarily the reverse of the

catalyzed retro-aldol reaction or vice versa. The free energy required to form the transition state of the aldol reaction direction can be different from that of the retro-aldol reaction direction.[9] That is, a good catalyst catalyzing a retro-aldol reaction may not necessary be a catalyst for the aldol reaction and vice versa. In actual examples, it has not been demonstrated whether designer protein catalysts that efficiently catalyze one direction over the other direction for aldol and retro-aldol reactions can be obtained. As a first step toward the design and creation of direction-controlled catalysts, we report protein catalysts derived from a structural scaffold that have different directionalities for aldol and retro-aldol reactions.

Results and Discussion

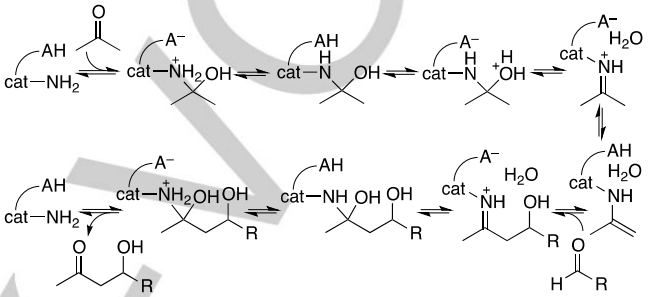
Working Hypothesis

We hypothesized that the creation of protein catalysts favorably catalyzing either aldol reactions or retro-aldol reactions from the same scaffold would be possible. We reasoned that directing the catalysis for either aldol or retro-aldol reactions would come from small structural differences. This is because in catalysis by small organic molecules, small changes in their structures often alter the catalytic efficiency, substrate scope, and stereoselectivities.[10-12] Small organic molecules that catalyze aldol reactions often do not efficiently catalyze the reverse retro-aldol reactions of the products,[13-16] although retro-aldol reactions are often observed as side reactions[14-16] and retro-aldol reactions can be incorporated within the designed reactions.[17] In the catalysis of aldol reactions by small organic molecules, interactions of a catalyst with an aldol product often cause isomerization of the product but do not lead to the retro-aldol reaction of the product.[14,18] In addition, for protein catalysts, it has been reported that a single mutation can alter the substrate scope and the reaction stereoselectivity.[19]

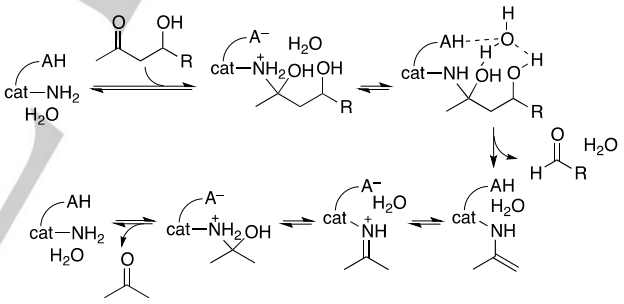
Varied directionalities may originate from differences in a type of substrate specificity. For example, the degree of differences in *K*m values and/or binding affinities between the starting materials and product (i.e., the aldehyde used for the aldol reaction and the aldol product) may result in differences in the apparent rates at certain concentrations.

Differences in directionalities may be observed as variances in the equilibrium position of the forward and backward reactions (i.e., aldol and retro-aldol reactions), and the differences may originate from differences in the equilibrium position in the steps of the reactions. For example, for aldol reactions catalyzed via an enamine/imine/iminium ion formation, the steps include addition of an amino group to the carbonyl group to form a hemiaminal, formation of an iminium intermediate, formation of an enamine, formation of a C-C bond, and hydrolysis of the imine/iminium ion (Scheme 1);[1,2,8] the retro-aldol reaction may occur through the reverse pathway. Changes of the equilibrium position in one or more steps may affect the reaction rates.

Varied directionalities may also originate from differences in reaction mechanisms and/or in interactions that stabilize transition states. Differences in catalytic active site residues or structures may stabilize distinct transition states of the forward and backward reactions (i.e., aldol and retro-aldol reactions). The pathway of the bond forming aldol reaction may or may not be the reverse of the bond cleaving retro-aldol reaction. The mechanism of the aldol reaction may be different from the reverse of the mechanism of the retro-aldol reaction. For example, in the retro-aldol reaction, C-C bond cleaving step may occur before the formation of an iminium ion of the aldol[20] (Scheme 2); this mechanism is not the exact reverse of aldol reaction. In many aldol and retro-aldol reactions, the C-C bond forming step and the C-C bond cleaving step are the rate-limiting steps, respectively.[8,20] Interactions favoring a C-C bond forming transition state in an aldol reaction over the C-C bond cleaving transition state in the retro-aldol reaction could result in a shift that favors the aldol reaction, although altered interactions can also stabilize or destabilize transition states of both forward and backward reactions.



Scheme 1. A possible mechanism of amine-catalyzed aldol and retro-aldol reactions that are involved in enamine/iminium intermediate formation (catalyst may provide additional interactions to those shown here)



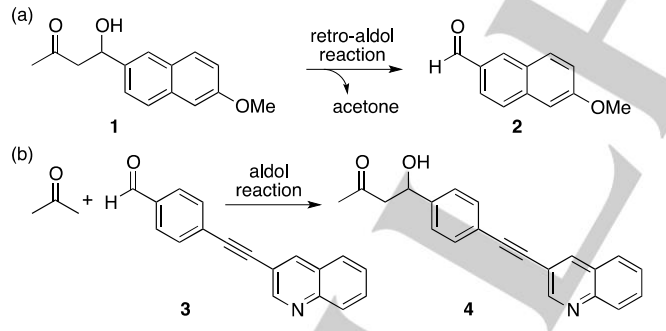
Scheme 2. A possible mechanism of amine-catalyzed retro-aldol reaction (catalyst may provide additional interactions to those shown here)

In an aldol reaction, faster enamine formation may accelerate the formation of the aldol product by increasing the concentration of the enamine used for the C-C bond formation step or by increasing the frequency of the formation of the C-C bond forming transition state that leads to the formation of the aldol product. However, the enamine formation rate is not necessarily correlated with the aldol reaction rate.[21] When the C-C bond formation step is the rate-limiting step in an aldol reaction, a faster enamine formation rate may not affect the rate of the formation of the aldol product,[21] even though the enamine formation step is essential for the formation of the aldol product. Thus, for alteration of directionalities, differentiating the transition states between the aldol and retro-aldol reactions and selective stabilization of either the transition state could be necessary. These possible origins and causes for the varied directionalities could be related to each other or could be inseparable. Small

structural changes (such as one or a few mutations) may result in the alteration of the directionality.

Design and Identification of Catalysts for Aldol Reactions

To generate aldol and retro-aldol catalysts with an altered level of directionality, we began with the previously reported, computationally designed protein catalyst M48K variant of RA61.[20] Here, we refer to this variant as the "wild-type" catalyst. This catalyst was generated to bind to a transition state model of the retro-aldol reaction of **1** to form aldehyde **2** (Scheme 3a) via an amine-based catalysis mechanism, of which K176 is the catalytic lysine and catalyzes the retro-aldol reaction.[20] The transition state model used for the generation of RA61 included a hemiaminal structure[20] like that shown in Scheme 2. To alter this catalyst to catalysts catalyzing aldol reactions, we prepared libraries at selected amino acid positions. The positions used for the preparation of the libraries included His46, Ile72, and Arg121, and are located in the cavity of the active site or at the edge of the cavity. We expected that these libraries would provide mutants having amino acid residues that would interact with the catalytic residues or that would tune the catalytic active site shape for catalysis of aldol reactions. We also expected that amino acid replacement could widen the active site cavity to accelerate the movement of substrates and the products and to accept various substrates. During the evaluation of produced proteins generated from the libraries using the aldol reaction of acetone with fluorogenic aldehyde **3** to generate fluorescent aldol product **4**[22] (Scheme 3b), mutant I72D was identified as a catalyst for accelerating the aldol reaction to form **4**, whereas the wild-type protein did not catalyze the aldol reaction of acetone and aldehyde **3** to form **4**. Thus, we decided to evaluate catalysis by this mutant RA61 M48K/I72D (mutant I72D) and by the original catalyst RA61 M48K (wild-type).

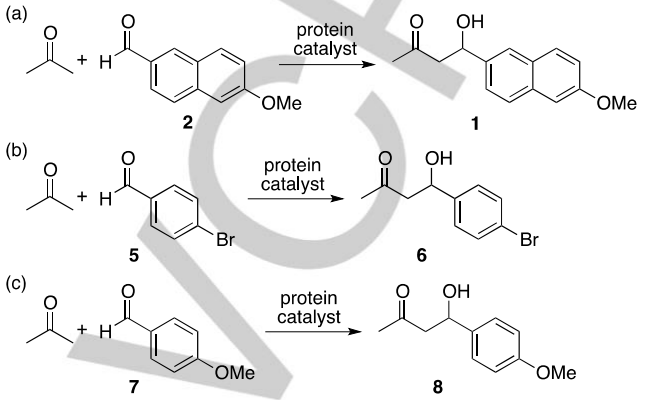


Scheme 3. Aldol and retro-aldol reactions of fluorogenic substrates used for the evaluation in the development of protein catalysts

Catalysis in Aldol Reactions

Mutant I72D protein and the wild-type protein were evaluated in aldol reactions of acetone with aldehydes to form the aldol products (in reactions with aldehyde **2** to form aldol **1**, with aldehyde **5** to form aldol **6**, and with aldehyde **7** to form **8**; Table 1). The reactions were performed using purified protein in 5% DMSO in phosphate buffer (pH 7.5) at 25 °C. The formation of the aldol product was monitored by HPLC analysis, and the initial rates were determined. Mutant I72D catalyzed all three aldol reactions, and the mutant I72D-catalyzed reactions showed saturation kinetics described by the Michaelis-Menten equation. On the other hand, for the wild-type-catalyzed aldol reactions, only the reaction to form **6** was able to be characterized. The wild-type protein showed little or no rate acceleration of the aldol reactions of acetone with **2** or with **7**.

Table 1. Kinetic parameters of the aldol reaction catalyzed by mutant I72D and by the wild-type[a]



entry	product (reaction)		kinetic parameters: mutant I72D	kinetic parameters: wild-type
1	**1**	k_{cat} (min^{-1})	3.4×10^{-3}	–[b]
	(reaction a)	K_m (μM)	110	–[b]
		k_{cat}/k_{uncat}	1.4×10^{3}	–[b]
2	**6**	k_{cat} (min^{-1})	1.6×10^{-2}	4.1×10^{-3}
	(reaction b)	K_m (μM)	420	170
		k_{cat}/k_{uncat}	2.6×10^{3}	6.7×10^{2}
3	**8**	k_{cat} (min^{-1})	2.9×10^{-3}	–[b]
	(reaction c)	K_m (μM)	200	–[b]
		k_{cat}/k_{uncat}	1.5×10^{3}	–[b]

[a] Reaction conditions: [protein catalyst] 10 μM, [acetone] 5% (v/v) (680 mM), [aldehyde substrate **2**, **5**, or **7**] 50~800 μM, 5% DMSO in 25 mM potassium phosphate, pH 7.5, 100 mM NaCl, at 25 °C; mutant I72D = RA61 M48K/I72D, wild-type = RA61 M48K. The first-order kinetic constant of the background reaction (k_{uncat}) was 2.3×10^{-6} min^{-1} for the formation of **1**, 6.1×10^{-6} min^{-1} for the formation of **6**, and 2.0×10^{-6} min^{-1} for the formation of **8**. [b] Not determined because of low activity or no catalysis.

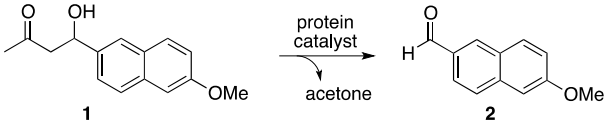
The rate acceleration (k_{cat}/k_{uncat}) with mutant I72D above background in the aldol reaction of acetone with aldehyde **5** to form **6** was 2600 and that with the wild-type was 670 (Table 1, entry 2). The k_{cat} value of the mutant I72D was approximately 4-fold that of the wild-type protein. Mutant I72D was a better catalyst than the wild-type in all aldol reactions evaluated. Whereas the wild-type catalyzed the retro-aldol reaction of **1** to generate aldehyde **2**,[20] the wild-type did not catalyze the reverse reaction (i.e., the aldol reaction of acetone and **2** to form **1**) to any meaningful extent. Thus, for these catalysts, the activity for catalyzing the retro-aldol reaction did not correlate with the ability to catalyze the aldol reaction. The results indicate that catalysts designed to efficiently catalyze the retro-aldol reaction of **1** through binding and stabilizing a transition state of the retro-

aldol reaction of **1** may not efficiently catalyze the aldol reaction to form **1**.

Catalysis in Retro-Aldol Reactions

Mutant I72D protein and the wild-type protein were evaluated in the retro-aldol reaction of **1**. The reactions were performed using 10 μM protein in 5% DMSO in phosphate buffer (pH 7.5) at 25 °C, and the formation of aldehyde **2** was detected by HPLC analysis. No saturation of the velocity was observed up to 1600 μM concentration of **1**. Thus, the data were analyzed by first-order kinetics described as $V = k_{app}[S]$, in which [S] is the concentration of **1**, at the protein catalyst concentration used in the evaluation. The k_{app} values at 10 μM concentration of the protein catalyst were 3.6 x 10^{-5} min^{-1} for mutant I72D and 4.1 x 10^{-5} min^{-1} for the wild-type (Table 2).

Table 2. The retro-aldol reaction of **1** catalyzed by mutant I72D and by the wild-type[a]



1 **2**

	mutant I72D	wild-type
k_{app} (min^{-1}) by HPLC assay[b]	3.6 x 10^{-5}	4.1 x 10^{-5}
k_{app} (min^{-1}) by fluorescence assay[c]	6.2 x 10^{-5}	9.2 x 10^{-5}

[a] Reaction conditions: 5% DMSO in 25 mM potassium phosphate, pH 7.5, 100 mM NaCl, at 25 °C; $V = k_{app}[S]$, in which [S] is the concentration of **1**; mutant I72D = RA61 M48K/I72D, wild-type = RA61 M48K. [b] [protein catalyst] 10 μM. [c] [protein catalyst] 6 μM.

The RA61-catalyzed retro-aldol reaction of **1** was previously characterized using a fluorescence assay that monitors the formation of fluorescent aldehyde **2**.[20,23] The retro-aldol reactions of **1** catalyzed by mutant I72D and by the wild-type were also analyzed by the fluorescence assay using 6 μM protein in 5% DMSO in phosphate buffer (pH 7.5) at 25 °C. The fluorescence intensity of a fluorescent compound does not have a linear relationship with the concentration of the compound and is often affected by many factors, such as the presence of other compounds. We noticed that the presence of the substrate affected the fluorescence; we used a 1.0 cm optical path length for the fluorescence assay, and because of this path length, it may be possible that the effect of the substrate became prominent compared to the plate type assays where the path length was short. Using a calibration curve that took into account the substrate concentration, the rates of the retro-aldol reactions of **1** catalyzed by mutant I72D and by the wild-type were determined. The catalyzed reactions did not show saturation kinetics. The results of the catalyzed retro-aldol reactions of **1** (up to 500 μM) analyzed by the fluorescence assay were also analyzed by first-order kinetics in the same way as those analyzed by the HPLC assay and are shown in Table 2.

The difference between the k_{app} values of mutant I72D and of the wild-type in the catalytic activities for the retro-aldol reaction was small (1.1~1.5-fold). The wild-type was a slightly better catalyst than mutant I72D for the retro-aldol reaction, or there were no essential differences in the catalytic activities in the retro-aldolase catalysis between the mutant and the wild-type.

For mutant I72D and the wild-type, the catalytic activities in the retro-aldol reaction did not correlate with the catalytic activities in the aldol reaction. The wild-type, which showed slightly higher catalytic activity in the retro-aldol reaction of **1** than mutant I72D, did not show higher catalytic activity in the aldol reactions. Mutant I72D, which showed higher k_{cat} values for the aldol reactions than the wild-type, showed similar or slightly less catalytic activity for the retro-aldol reaction than the wild type. The directionality of mutant I72D for the aldol and retro-aldol reactions was different from that of the wild-type.

Structures

Secondary structures of mutant I72D and the wild-type proteins were analyzed using circular dichroism spectroscopy. The spectra indicated that the secondary structures of mutant I72D and the wild-type were similar (Supporting Information).

Crystal structures of mutant I72D obtained under various conditions were analyzed. Five types of forms (named Forms I, II, III, IV, and V) were obtained under the crystallization conditions used. The overall structures of the five forms were similar to the previously reported structure of the wild-type (RA61 M48K, PDB ID 3B5L).[20] An overlay of the wild-type with mutant I72D Form I is shown in Figure 1a. The main chain structures and the residues in the catalytic active sites are in similar positions in mutant I72D and the wild-type, with the exceptions of His46 and Arg121. In mutant I72D Form I, the His46 side chain was observed in two positions.

An overlay of mutant I72D Form I with Form V is shown in Figure 1b. In mutant I72D Forms I and V, both Lys48 and Lys176 were clearly observed. In Forms II, III, and IV, the side chains of one or both of these residues were disordered. The orientations of the residues of Tyr97, Arg121, and Trp178 and the loop structures around Arg121 to Arg131 differed in Form I and Form IV, although other parts of the structures were similar. The differences in the loop structures suggest that the active site of mutant I72D is flexible.

In the wild-type, Ile72 may provide hydrophobic interactions with Tyr97 and Trp178 at an entrance of the active site cavity, and the hydrophobic interaction may stabilize the protein structure. In mutant I72D, Asp72 may interrupt hydrophobic interactions, making the active site flexible.

The distances between the side chain functional groups of the residues in the active sites of Forms I and V of mutant I72D and the previously reported wild-type are shown in Figure 2. In Form V, an imidazole, which was used in the crystallization buffer, was observed near Lys176 and Asp72.

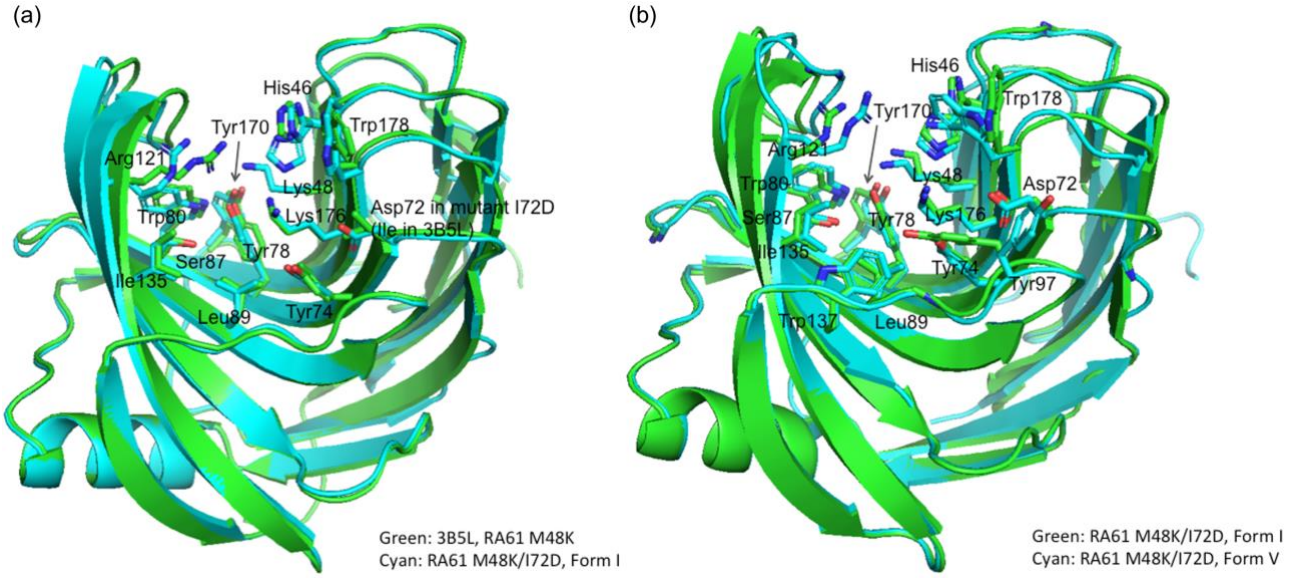


Figure 1. (a) Crystal structures of the wild-type (RA61 M48K, PDB ID 3B5L, green)[20] and Form I of mutant I72D (RA61 M48K/I72D, cyan). (b) Crystal structures of Form I (green) and Form V (cyan) of mutant I72D.

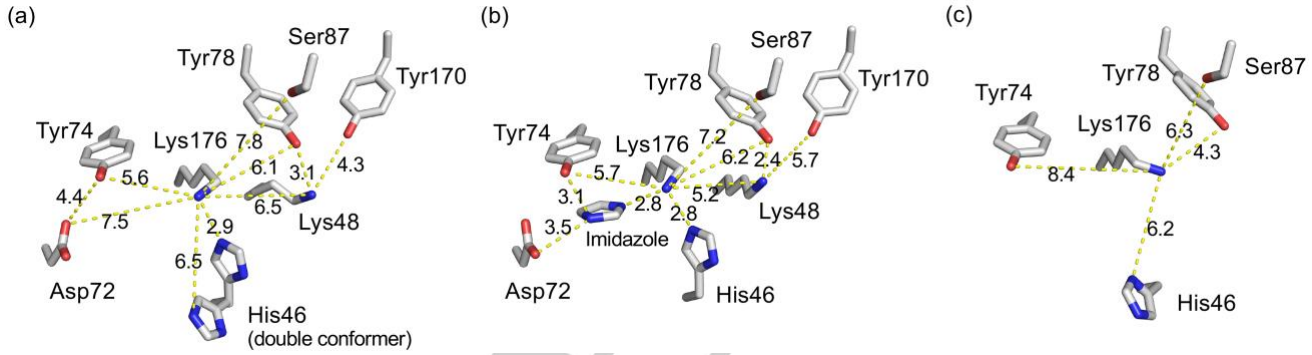


Figure 2. Distances in Å between the functional groups of the indicated side chains in the catalytic active site cavities of (a) mutant I72D (RA61 M48K/I72D) Form I, (b) mutant I72D (RA61 M48K/I72D) Form V, and (c) wild-type (RA61 M48K, PDB 3B5L).

The differences in the distances of the side chain residues between Forms I and V support our hypothesis that the catalytic active site of mutant I72D is flexible. For example, the distance between Lys176 and Lys48 is 6.5 Å in Form I and is 5.2 Å in Form V; the difference is more than 1 Å. This flexibility might be the reason that mutant I72D catalyzed the aldol reactions more efficiently than the wild-type, although the degree of the flexibility of the mutant and the wild-type cannot be compared. As RA61 was created to catalyze the retro-aldol reaction of **1**, the wild-type (i.e., RA61 M48K) catalyzes the retro-aldol reaction of **1**. When aldol **1** is produced by the aldol reaction in the active site of the wild-type, this compound may readily undergo the retro-aldol reaction before being released from the active site. On the other hand, in the active site of mutant I72D, aldol product **1** may be released before being used in the retro-aldol reaction.

The wild-type protein catalyst appears to stabilize the transition state of the retro-aldol reaction of **1** as it was designed to do. However, the wild-type catalyst does not efficiently stabilize the transition state of the aldol reaction to form **1**, which is different from the transition state of the retro-aldol reaction. In contrast, mutant I72D may have the flexibility necessary to bind to and to stabilize the transition state of the aldol reactions. Mutant I72D retained the capability to catalyze the retro-aldol reaction of **1**, although the retro-aldolase activity was lower than that of the wild-type catalyst.

It is also possible that Asp72 in mutant I72D is involved in the catalysis. For RA61, in the design, Lys176, Ser87, Tyr78, and a water molecule present between Lys176 and Ser87 are the essential residues, and the water directly binds the transition state of the catalyzed retro-aldol reaction of **1**, in which Lys176 forms a hemiaminal with **1**.[20] In mutant I72D, Asp72 interacts with Tyr74, which is present within an interacting distance of the catalytic lysine residue Lys176 either directly or through a water molecule (Figure 2a,b). As the interactions provided by Asp72 can differently affect the aldol reaction and the retro-aldol reaction, the directionality provided by mutant I72D can differ from that of the wild-type.

For natural enzymes, such as fructose 1,6-bisphosphate aldolase/phosphatase, which catalyzes two different reactions, an alteration of the conformation of the enzyme protein has been suggested for the alteration of the catalyzing reactions.[24] For mutant I72D, it may be possible that different conformations of

the protein structure provide different degrees of catalysis for aldol and retro-aldol reactions. Since Asp72 in mutant I72D may be involved in the catalysis, micromechanisms of the aldol and the retro-aldol reactions may differ from those of the wild-type. As a result, the degree of the stabilization of the transition states likely differ, causing the contrasting directionalities. Although detailed mechanisms of catalysis by mutant I72D must be investigated further to accurately determine the molecular details of the catalysis, we obtained a protein catalyst with different directionality from the parent retro-aldolase protein catalyst by replacing one amino acid.

Conclusion

We have demonstrated that the catalytic active sites of protein catalysts derived from a protein scaffold can vary the direction of catalysis for aldol and retro-aldol reactions. We have demonstrated that protein catalysts with varied levels of the directionality in commonly reversibly catalyzed reactions can be created. A protein catalyst efficiently catalyzing a bond breaking retro-aldol reaction is not necessary an efficient catalyst for the forward, bond forming aldol reaction and vice versa. One amino acid alteration was sufficient to alter the directionality of protein catalysts. Our results indicate that aldol reaction-favoring protein catalysts and retro-aldol reaction-favoring catalysts can be separately developed from a single protein scaffold.

Experimental Section

The Supporting Information is available: Protein production of mutant I72D and the wild-type protein, catalytic assays and kinetic measurements using HPLC analyses and fluorescence assays, crystallizations of the proteins, and X-ray crystal structural analyses (PDF)

The atomic coordinates and experimental data of mutant I72D, Forms I to V (code 7F1H, 7F1I, 7F1J, 7F1K, and 7F1L), have been deposited in the Protein Data Bank (www.wwpdb.org).

Acknowledgements

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Keywords: designer catalysts • biocatalysis • directionality • aldol reaction • retro-aldol reaction

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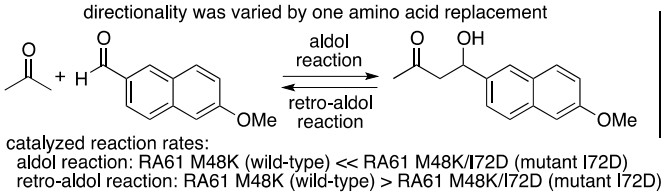
Table of Contents Graphic



Protein catalysts with varied levels of directionality in usually reversibly catalyzed aldol and retro-aldol reactions were generated using the same protein scaffold.