Crystal Structure of Rat Short Chain Acyl-CoA Dehydrogenase Complexed with Acetoacetyl-CoA

COMPARISON WITH OTHER ACYL-CoA DEHYDROGENASES*

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The acyl-CoA dehydrogenases are a family of flavin adenine dinucleotide-containing enzymes that catalyze the first step in the ß-oxidation of fatty acids and catabolism of some amino acids. They exhibit high sequence identity and yet are quite specific in their substrate binding. Short chain acyl-CoA dehydrogenase has maximal activity toward butyryl-CoA and negligible activity toward substrates longer than octanoyl-CoA. The crystal structure of rat short chain acyl-CoA dehydrogenase complexed with the inhibitor acetoacetyl-CoA has been determined at 2.25 Å resolution. Short chain acyl-CoA dehydrogenase is a homotetramer with a subunit mass of 43 kDa and crystallizes in the space group P321 with a = 143.61 Å and c = 77.46 Å. There are two monomers in the asymmetric unit. The overall structure of short chain acyl-CoA dehydrogenase is very similar to those of medium chain acyl-CoA dehydrogenase, isovaleryl-CoA dehydrogenase, and bacterial short chain acyl-CoA dehydrogenase with a three-domain structure composed of N- and C-terminal a-helical domains separated by a ß-sheet domain. Comparison to other acyl-CoA dehydrogenases has provided additional insight into the basis of substrate specificity and the nature of the oxidase activity in this enzyme family. Ten reported pathogenic human mutations and two polymorphisms have been mapped onto the structure of short chain acyl-CoA dehydrogenase. None of the mutations directly affect the binding cavity or intersubunit interactions.

The mammalian acyl-CoA dehydrogenases (ACD)1 are a family of homologous flavoproteins that are involved in mitochondrially fatty acid and amino acid metabolism. Current members include SCAD, MCAD, LCAD, VLDCA, IVD, SBCAD, IBD, and GCAD. A bacterial SCAD (bSCAD) has also been described in the Gram-positive anaerobe Megasphaera elsdenii. S-, M-, and L- are all involved in the oxidation of straight chain fatty acids, whereas IVD, SBCAD, IBD, and GCAD are involved in the catabolism of leucine, isoleucine, valine, and lysine, respectively (1–6). All of these enzymes are soluble homotetramers with a subunit mass of ~43 kDa and are components of the mitochondrial matrix, with the exception of VLCAD, which is a homodimer of subunit mass 73 kDa and is bound to the matrix face of the inner mitochondrial membrane (7, 8). The ACDs catalyze the ß-dehydrogenation of acyl-CoA thioesters, in which an ß-hydrogen is abstracted as a proton from the acyl-CoA thioester substrate with a ß-hydrogen transferred as a hydride ion to the N(5) position of the enzyme-bound FAD (9–11). The reduced ACD is then reoxidized by electron transfer flavoprotein in a series of two one-electron transfers (12).

The three-dimensional structures of MCAD, bSCAD, and IVD have already been reported (13–16). These data confirmed the homotetrameric nature of the enzyme and the identity of the catalytic glutamate that abstracts an ß-hydrogen as a proton. Amino acid sequence alignments identified a common glutamate in SCAD and bSCAD at the same position. It was expected that the analogous Glu-368 residue in SCAD would be in position to act as the catalytic residue, whereas the corresponding values are 9 and 1.1 mol respectively in the wild-type enzyme, whereas the corresponding values are 9 and 1.1 mol in the E368G mutant. On the other hand, the analogous mutant in MCAD dramatically changed the specificity of MCAD from an optimal substrate of octanoyl-CoA to dodecanoyl-CoA. Although mutagenesis thus far has focused on alanine at position 368, it was anticipated that other residues at surrounding positions would converge to catalyses. This work was supported by Grants GM29076 (to J.-P. K.) and DK54936 (to J. V.) from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The atomic coordinates and structure factors (code 1JQI) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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on the catalytic residue with the most success, clearly the amino acid residues in the binding cavity are of equal, if not greater, importance, as the minimal effect seen in the SCAD mutant study demonstrates.

The deficiencies of all the ACDs combined represent an im-

portant group of metabolic disorders; however, only a few patients have been described with SCAD deficiency (24–26). SCAD deficiency can present in infants with an acute acidosis and myopathy or in adults with a chronic myopathy. Presentation typically involves muscle weakness, lethargy, free carnitine depletion with elevated plasma butyryl-carnitine, and ethylmalonic aciduria. Metabolic stress can result in the excretion of methylsuccinate. Several patients with SCAD deficiency have been reported as well as mutations thereof (27–30). As the amino acid sequence identity between rat and human SCAD is 92%, we have mapped 10 reported clinical mutations and two polymorphisms onto the SCAD structure reported herein in an effort to gain insight into the mechanism of pathogenesis of SCAD deficiency. Here we report the three-dimensional structure of rat SCAD at 2.25Å resolution.

EXPERIMENTAL PROCEDURES

Purification and Crystallization of Rat SCAD—Expression and purification of recombinant SCAD has been described elsewhere (17). Briefly, the mature coding region from the cDNA of SCAD was cloned into the isopropyl-1-thio-

-H9252-D-galactopyranoside-inducible expression vector pKK223-3 with the addition of an N-terminal methionine. After induction, Escherichia coli cultures were harvested by centrifugation and lysed by sonication. Recombinant SCAD was purified by successive chromatography steps including DE52 (Whatman), hydroxyapatite, and S-Sepharose (Amersham Biosciences). The enzyme was crystallized using the sitting drop vapor diffusion method (31). Trigonal crystals

![Fig. 1. The overall polypeptide folding of the SCAD monomer. A, ribbon diagram of the SCAD monomer. Helices are labeled A–K and β-strands 1–7. FAD is rendered in yellow and 3-acetoacetyl-CoA in green. B, stereo view of overlay of SCAD (blue), MCAD (red), bSCAD (green), and IVD (gray). FAD is rendered in yellow and 3-acetoacetyl-CoA in indigo. Orientation of the monomer is the same as in A. The figure was generated using Molscript (44) and Raster3D (45).](image-url)
with an orange tint grew from drops of 8.8 mg/ml SCAD in the presence of 1.2 molar equivalents of acetoacetyl-CoA/monomer of enzyme in 85 mM Tris acetate, pH 7.0, and 270 mM ammonium sulfate equilibrated against a solution containing 85 mM Tris acetate, pH 7.0, and 3.78 M ammonium sulfate. The crystals belong to a trigonal space group with unit cell dimensions of \( a = 143.61 \text{ Å} \) and \( c = 77.46 \text{ Å} \).

**Data Collection and Structure Determination**—Diffraction data were collected using a Rigaku RU-200 rotating anode x-ray generator operating at 50 kV and 100 mA with a 0.3-Å collimator coupled to an RAXIS-II detector system. Data reduction was carried out with the Denzo/Scalepack software package (32). Data collection and structure refinement statistics are given in Table I. The structure of SCAD was solved by the molecular replacement method using the software package TURBO (35). Water molecules were added in areas of electron density in difference Fourier maps that were generated by manually replacing amino acid residues in MCAD to match the SCAD sequence. The highest peak from the cross-rotation function between the Cα traces of one monomer each of SCAD, MCAD, IVD, and bSCAD to 1.2 Å was used in the subsequent translation search. Two trigonal space groups, P321 and P312, were consistent with the systematic absence of extinction reflections. X-PLOR rigid body refinement based on the highest peak on the P321 translation search using reflections from 15 to 4 Å lowered the R-value from 43.7 to 36.1%. Several cycles of refinement with 6993 reflections with intensities of \( -4 \sigma \) (15–4 Å) yielded an R-value of 24.2%. At this point, all data with \( I/\sigma > 8 \) were used for the subsequent refinement using X-PLOR and later CNS (34), and manual adjustments were performed with the molecular graphics package TURBO (35). Water molecules were added in areas of electron density in difference Fourier maps that were \(<3.3 \text{ Å} \) from a hydrogen bond partner. The final model containing 6070 non-hydrogen, non-solvent atoms and 260 water molecules yielded a final \( R_{	ext{cryst}} \) of 16.0% (\( R_{	ext{free}} = 20.6\% \) (Protein Data Bank accession code 1JQI)).

**RESULTS AND DISCUSSION**

**Overall Structure of SCAD**—As expected from their sequence similarity, the overall structure of SCAD is similar to those of MCAD, IVD, and bSCAD. Like the structures of the other ACDs, the monomeric structure of SCAD is composed of three domains: an N-terminal α-helical domain containing six helices (A–F), a medial β-sheet domain containing seven β-strands (1–7), and a C-terminal α-helical domain containing five helices (G–J in Fig. 1A). SCAD, like the other ACDs, forms a tetramer of identical monomers with 222 symmetry. The crystallographic asymmetric unit contains a dimer that forms the tetramer with a crystallographic 2-fold axis. The root-mean-square deviation for the α-carbon atoms between the two monomers in the asymmetric unit is 0.27 Å. All but five residues were identified in the electron density map, the three N-terminal residues (Leu-1, His-2, Thr-3), the initiating methionine, and Ser-388 at the C terminus. Fig. 1B shows an overlay of the Cα traces of one monomer each of SCAD, MCAD, IVD, and bSCAD. The average root-mean-square deviation between the Cα of SCAD and the other ACD structures ranges from 1.7 Å with MCAD and 1.5 Å with bSCAD to 1.2 Å with IVD. The structural alignment in Fig. 2 shows that all
homologous strands and helices in the four structures have the same number of residues with the exception of the E-helix in which SCAD, IVD, and bSCAD have 21 residues compared with 20 in MCAD. The E-helix lines the “bottom” of the substrate binding cavity. This insertion (Ser-97 in SCAD) in the E-helix has the local effect of shifting its N-terminal two turns of the helix slightly closer to the binding cavity in SCAD/IVD/ bSCAD and making the binding cavity more shallow. The length of loops is a primary difference in the structures, and yet most of these length differences do not appear to result in any significant difference in enzyme function. In the loops between β-strands 5 and 6, Pro-194 adopts a cis conformation.

Substrate Binding Cavity—The 3′-AMP moiety of the CoA is exposed to solvent, and the fatty acyl moiety is buried deeply inside the molecule near the isoalloxazine ring of the FAD. The binding cavities of SCAD, IVD, and bSCAD are more shallow than that of MCAD, which at the bottom expands away from the isoalloxazine ring of the FAD and ends near Glu-99. In the structure of MCAD without substrate, there are series of well defined water molecules in the substrate binding cavity. When the substrate binds in MCAD, water molecules are displaced, and the side chains of amino acids Glu-95, Glu-99, Leu-103, Val-259, Tyr-375, and Glu-376 move aside to accommodate the substrate (14). The movement of these amino acid side chains enlarges the cavity to accommodate substrate. Although structures of SCAD, bSCAD, and IVD without bound ligand have not been determined, it is reasonable to assume that water occupies the binding cavity in all ACDs in the absence of substrate, which is then displaced upon substrate binding.

As the CoA portion of the substrate is common for all of the ACDs, it would be expected that the regions involved in binding the CoA moiety would be very similar. The backbones for all four dehydrogenases overlay very well at the top of the binding cavity. When the substrate binds in MCAD, water molecules are displaced, and the side chains of amino acids Glu-95, Glu-99, Leu-103, Val-259, Tyr-375, and Glu-376 move aside to accommodate the substrate (14). The movement of these amino acid side chains enlarges the cavity to accommodate substrate. Although structures of SCAD, bSCAD, and IVD without bound ligand have not been determined, it is reasonable to assume that water occupies the binding cavity in all ACDs in the absence of substrate, which is then displaced upon substrate binding.

Substrate Specificity—Table II contains a list of structurally homologous residues in the ACDs that line the binding cavity for the fatty acyl portion of the CoA thioester substrate. In addition, the isoalloxazine ring of the FAD forms one wall of the binding cavity in all ACDs. Eight residues are responsible for shaping the binding cavity of SCAD; Val-90, Val-94, Ala-92, Ala-99, Ala-100, Thr-96, Tyr-375, and Tyr-376. The small but significant activity toward octanoyl-CoA (V_{max}/K_m for octanoyl-CoA is 70-fold smaller than that of butyryl-CoA) of SCAD (17), suggesting that there is a certain degree of flexibility among the residues lining the binding cavity to allow longer substrates to bind. In bSCAD as in SCAD, eight residues shape the binding cavity, all of which are in the same position as SCAD and five of which are identical to those in SCAD: Ile-88, Ala-92, Leu-96, Phe-126, Thr-162, Val-259, Glu-254, Gly-374, Ala-375.

| rSCAD | bSCAD | IVD | pMCAD |
|-------|-------|-----|-------|
| Val-90 | Ile-88 | Leu-95 | Glu-95 |
| Val-94 | Ala-92 | Ala-99 | Ala-100 |
| Leu-98 | Leu-96 | Leu-103 | Leu-103 |
| Phe-128 | Phe-126 | Leu-133 | Tyr-133 |
| Thr-163 | Thr-162 | Thr-168 | Thr-168 |
| Glu-368 | Glu-367 | Val-259 | Val-259 |
| Tyr-367 | Tyr-366 | Tyr-371 | Tyr-372 |
| Glu-368 | Glu-367 | Tyr-375 | Tyr-375 |
| Val-259 | Val-259 | Glu-368 | Glu-368 |

The shape of the cavity opening and the pantothene-binding portion of the cavity is uniformly shaped in all of the structures. A minor difference is in the opening of MCAD, which is slightly wider and extended in the direction of the adenine moiety.
Fig. 4. Stereo diagrams of the surfaces of the substrate binding cavities of SCAD, bSCAD, IVD, and MCAD. A, SCAD; B, SCAD; C, IVD; D, MCAD. A–C are in an identical orientation to each other and differ from D to better illustrate the side extension in the IVD binding cavity and the extended binding cavity of MCAD. The images are all cropped at the same point, at the distal carbonyl in the pantothenic acid moiety of the CoA. The residues in the binding cavity for all enzymes are listed in Table II (Gly-374 in IVD was eliminated for clarity). Amino acids and the FAD are rendered as thin lines. In A and B, hexanoyl-CoA was modeled in the binding cavity of SCAD and bSCAD and is rendered as a ball-and-stick with carbons 5 and 6 of the substrate rendered as smaller balls. In C, hexanoyl-CoA is modeled in two conformations with smaller balls after the branching point at carbon 3. The larger balls essentially model the structure of an isovaleryl moiety. In D, dodecanoyl-CoA has been modeled in the binding cavity to show two different binding modes with smaller balls after the branching point at carbon 8. The molecular surface was generated with a 1.4-Å probe using the program Grasp (46). Molscript (44) and Raster3D (45) were used to render the image.
250, Tyr-366, and the catalytic residue Glu-367 (Table II, Fig. 4). In bSCAD, Ile-88 impinges on the binding cavity from the opposite side as Ile-251 does in SCAD. The homologous residue of Ile-251 in bSCAD is Val-250, which does not restrict the binding cavity. This is due to the direct approach of the Ile-88 side chain into the binding cavity (15) and the shorter side chain of the valine. As was the case with bSCAD, IVD also shares many similar residues lining the binding cavity with SCAD. The primary difference here is that the binding cavity in IVD is expanded laterally to accommodate branched chain substrates because of a glycine at position 374 (16), which is a leucine in IBD and a tyrosine in other ACDs. Residues shaping the IVD binding cavity are Leu-95, Ala-99, Leu-103, Leu-133, Thr-168, Leu-258, Leu-370, Tyr-371, Gly-374, Ala-375, and the catalytic residue Glu-254 (Table II, Fig. 4). Although bSCAD shared five identical residues with SCAD, IVD shares two. IVD has two residues in position to block the bottom of the binding cavity to longer chain substrates, Leu-258 (Ile-251 in SCAD) and Leu-95 (Val-90 in bSCAD) (16). In MCAD, because of a larger binding cavity, more residues are involved in forming the binding cavity. These residues are Gln-95, Thr-96, Glu-99, Ala-100, Thr-102, Leu-103, Tyr-133, Thr-168, Thr-255, Pro-258, Val-259, Tyr-372, Tyr-375, and the catalytic residue Glu-376 (Table II, Fig. 4). The increased size of the binding cavity of MCAD is due in part to the shorter amino acid side chains in MCAD of residues homologous to the blocking residues in SCAD/bSCAD/IVD and the difference in trajectory of the G- and E-helices that line the binding cavity. These two factors together allow for the expansion of the binding cavity away from the FAD, which allows for the binding of longer substrates.

The binding of long acyl-CoAs to SCAD, bSCAD, and IVD is significantly affected because the side chains of Ile-251 (SCAD), Leu-258 (IVD) or Ile-88 (bSCAD) are blocking the bottom of the binding cavity. The insertion of an extra residue in helix E results in the bending of the helix toward the active site in SCAD/bSCAD/IVD. In MCAD, on the other hand, a proline substitution in the G-helix makes the helix bend away from the active site. For SCAD and IVD the blocking residue (Ile-251/Leu-258) is on one side of the binding cavity, whereas for bSCAD it (Ile-88) is on the other side. The second structural difference appears to be the position of the peptide backbone in the region of the binding cavity. All of the enzymes have subtle differences in the position of the main chain atoms. In the vicinity of the binding cavity, these differences result in the movement of amino acid side chains further into the binding cavity, as is the case with SCAD/bSCAD/IVD, or further away from the binding cavity, as is the case in MCAD and, presumably, LCAD/VLCAD. The combination of the differences in the residues, combined with the trajectory of the helices they are
1. Molscript (44) and Raster3D (45) were used to render the image.

2. In contrast, the oxygen reactivity of butyryl-CoA reduced complexed with certain substrate analogs. Rat SCAD also reduced FAD of pig MCAD was increased up to 3,600-fold when Wang and Thorpe (38) showed that the half-life of the photoreduction by molecular oxygen of the flavins is dramatically slowed.

The molecular basis of oxygen reactivity of ACDs has been a subject of some debate (36–38). When reduced flavins of mammalian ACDs are bound to certain acyl-CoA ligands, reoxidation by molecular oxygen of the flavins is dramatically slowed. Wang and Thorpe (38) showed that the half-life of the photo-reduced FAD of pig MCAD was increased up to 3,600-fold when complexed with certain substrate analogs. Rat SCAD also shows a similar result when bound to substrate/product analogs. In contrast, the oxygen reactivity of butyryl-CoA reduced bSCAD is ~5,000-fold higher than for substrate-reduced MCAD (37). As described above, the opening of the binding cavity and shape of the pantothene binding regions in the cavity are very similar in the four ACD structures. Because the binding cavity of bSCAD is not significantly wider than any other binding cavity, it would be unlikely that the access of molecular oxygen to the isoalloxazine ring occurs through the active site opening. Djordjevic et al. (15) showed that the flavin in the structure of bSCAD complexed with acetoacetyl-CoA is more exposed to the solvent than the flavin in MCAD, mainly because of the substitution of Phe-160 in bSCAD for Trp-166 in MCAD. They also concluded that these findings are in agreement with the mechanism proposed by Wang and Thorpe (38) in which desolvation of the active site due to ligand binding is responsible for protection from molecular oxygen. Our current structure of rat SCAD shows that the isoalloxazine ring of FAD is also shielded from the solvent in the MCAD structure. To investigate the solvent accessibility of the isoalloxazine ring in the SCAD structure, we have compared cavities in the vicinity of the si-face of the isoalloxazine ring in the structures of all four ACDs. In SCAD and bSCAD, there are cavities at the dimethylbenzene side of the flavin ring (Fig. 5). This cavity in SCAD (diameter of ~3Å) is smaller and more deeply buried than that in bSCAD (diameter of 5.3 Å in the longest dimension), which is very close to the surface. As proteins are dynamic in solution, it is reasonable to propose that upon modest motion of the molecules these cavities, illustrated in Fig. 5, could become contiguous with the surface, confirming the results of Djordjevic et al (15). The previous observation that bulky substrates enhance the oxygen sensitivity of MCAD may also be due to an enlargement of this cavity, allowing greater access of molecular oxygen to the reduced FAD, or to the creation of a temporary cavity analogous to what is seen in bSCAD (36–38).

Mutation Sites—The amino acid sequence identity between rat and human SCAD is 92%, and therefore it is reasonable to assume that the structures are identical. Ten missense mutations leading to SCAD deficiency in humans have been reported in the literature (R22W, G66S, G68C, R83C, W153R, A168V, S329L, R356W, R359C) along with two polymorphisms (R147W and G185S) (27–30). The location of these mutations are indicated by the spheres in Fig. 6. We have modeled these mutations into the SCAD structure to attempt to determine why these amino acid substitutions are detrimental to the function of the enzyme. The mutations R83C and R359C appear to interfere with local bonding interactions between adjacent helices or strands. Disruption of local bonding and steric hindrance of the introduced amino acid appear to be responsible in the R22W, R301W, and R356W mutations. Arg-22 from helix A lies on the outside of the molecule and forms a salt bridge to Glu-80 of helix D. A comparison of the sequences in the dehydrogenase family shows that both Arg-22 and Glu-80 are conserved for the mammalian straight chain acyl-CoA dehydrogenases, and therefore this salt bridge may be important in the folding of all the straight chain dehydrogenases. The guanidinium of Arg-83 of helix D forms salt bridges with Glu-26 and Asp-264. Replacing Arg-83 with tryptophan would disrupt the bonding between helices A, D, and G. The MCAD residue homologous to SCAD Arg-22 (Arg-28) is also mutated in human disease (R28C). The SCAD mutation is not conducive to proper folding, as shown by a lack of protein in eukaryotic expression systems, in contrast to the significant expression seen with the MCAD R28C mutation (39, 40). This lack of expression in the SCAD mutation is likely due to steric effects on folding caused by the bulky indole ring of tryptophan compared with the relatively non-bulky cysteine residue in the MCAD mutant. Arg-83 is found only among the short chain dehydrogenases where it is salt-bridged with Glu-26/Asp-264. In MCAD, Arg-83 is replaced with tyrosine, which still forms a hydrogen bond with Asp-264. G66S, W153R, A168V, and S329L are all pathogenic by virtue of introducing a longer or bulkier side chain into a local environment that cannot accommodate it. The final mutation, G68C, is interesting because it exhibits activity upon in vitro expression (27). Gly-68 is located in the same loop as Gly-66. There is limited space between the Cα of the glycine and the backbone of Pro-61/Glu-62, and the introduction of any side chain at position 68 would alter the trajectory of this loop. However, the G68C mutant does have activity (40% of wild-type) when expressed at 26 °C, indicating that there must be enough flexibility in this loop to allow for correct folding when allowed to fold slowly at lower temperatures (27).

None of the currently identified human SCAD mutations appears to be involved with the tetramer interface, substrate binding, flavin binding, or catalysis. It has been documented previously that some patients have an absence of immunoreactive protein in their cells and that there are mutations that appear to affect folding in a temperature-sensitive manner (41–43). The mutations either disrupt hydrogen bonding or salt bridge networks and/or disrupt local topology by the introduction of a bulky amino acid side chain, thus affecting monomer folding and oligomer formation. This should not be surprising, as the majority of residues in most enzymes are not involved in the actual catalytic function of the enzyme per se but are required to hold the functional residues in such a position that the reaction the enzyme catalyzes can go forward.

The polymorphisms lie in positions in which there are few, if any, structural elements impinging on the position of the substituted side chain. Arg-147 is exposed to solvent on β-strand 2

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*M. Stankovich, personal communication.*
and does not appear to participate significantly in other interactions. The solvent exposure may allow the tryptophan to orient itself in a favorable position, which would allow for the observed activity (45% of wild type at 37 °C, 85% at 26 °C, and 13% at 41 °C) (27). Gly-185 lies at the beginning of β-strand 5 after a convoluted loop region connecting strands 4 and 5. This convoluted loop region lies adjacent to the beginning of the binding cavity where substrate binds. Modeling the G185S polymorphism can place the serine side chain less than 2 Å from Asp-178 and Arg-181. The introduction of this mutation likely remodels the topology in the loop between sheets 4 and 5, as well as the beginning of strand 5, thereby interfering with substrate binding. The distances between the serine side chain in the G185S mutant and the neighboring atoms can vary significantly depending on the orientation of the side chain, and therefore the adoption of a favorable conformation of the serine side chain to allow productive folding may explain the activity (136% of wild-type at 37 °C, 183% at 26 °C, and 58% at 41 °C) and stability of this mutation (27).

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