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Stopped-flow kinetics and cysteine residue protonation of human isobutyryl-CoA dehydrogenase

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ABSTRACT

Human Isobutyryl-CoA Dehydrogenase, IBD, is involved in the valine amino acid catabolism. It catalyzes with a high specificity the reduction of the isobutyryl-CoA to methylacrylyl-CoA in contrary to the rat Short-Branched Chain Acyl-CoA Dehydrogenase, SBCAD, which is able to utilize both 2-methylbutyryl-CoA and isobutyryl-CoA that emerged from Ile and Val degradation, respectively. Here, the IBD stopped-flow kinetic was studied and it was found that IBD reduction reaction has two phases, the faster one was exponential at pH 9.0 while it was much slower and multi exponential in pH 6.5. Also, it was found that isobutyryl-CoA binds IBD in two steps, where the first step was the faster one. The protonation of the high IBD Cys content was computed by the *H*++ server and the results are discussed. In conclusion, one or more of Cys residues interact and involved in the p*K*a value of the active site Glu-376. It could thus be concluded that Cys residue(s) has crucial role(s) in the conformation stability and biocatalytic activity of the IBD.

Human Isobutyryl-CoA Dehydrogenase (IBD, EC 1.3.99.12) or ACAD8 is one of the Acyl-CoA dehydrogenases (ACADs) that participates in the catabolism of the amino acid valine. IBD is responsible for the third step in the breakdown of valine at which it converts isobutyryl-CoA into methylacrylyl-CoA [1,2]. ACADs are a family of nuclear encoded, mitochondrial flavoenzymes that catalyze the α, β-desaturation of acyl-CoA to corresponding trans-2-enoyl-CoA in the catabolism of fatty acids and branched chain amino acids [3-7]. ACADs are classified according to the acyl moieties in their substrates into two subclasses. First subclass comprises five members: Very long 1and 2 (VLCAD1, VLCAD2), Long (LCAD), Medium (MCAD) and Short chain acyl-CoA dehydrogenase (SCAD) utilizing "straight chain" acyl-CoA substrates, that in turn, enable the sequential degradation of the substrates in ß-oxidation cycle [8]. The second subclass includes the "branched chain" acyl-CoA degrading enzymes. This includes Isovaleryl-CoA dehydrogenase (IVD), Short-Branched Chain-CoA Dehydrogenase (SBCAD), Glutaryl-CoA Dehydrogenase (GCAD) and Isobutyryl-CoA dehydrogenase (IBD). ACADs are homotetrameric enzymes, with exception of VLCAD1 which is homodimer, with one Flavin Adenine Dinucleotide (FAD) non-covalently bound to each monomer [7]. Inherited deficiencies of these enzymes are important causes of human disease such as Sudden Infant Death Syndrome (SIDS) and other mitochondrial fatty acid oxidation disorders [9-11]. It is worth to mention the link between the Reye Syndrome, which is known to be triggered by aspirin ingestion in children and teenagers with fatty acid oxidation disorders, where Aspirin was found to exert its effect on long-chain fatty acid transport into mitochondria [12].

Three monomethyl branched-chain thioesters, 2-methylbutyryl-CoA, isovaleryl-CoA, and isobutyryl-CoA, are derived from the catabolism of the branched chain amino acids isoleucine, leucine, and valine, respectively. These thioesters are catalyzed by SBCAD, IVD and IBD, respectively. The three enzymes share similar sequences, catalytic mechanisms, and structural properties; however, the positions of their catalytic residues are not conserved in their primary sequences [13-15]. IBD deficiency is a very rare autosomal recessive metabolic disorder. So far, 22 *ACAD8* mutations have been identified in patients with IBD deficiency [1,16-18].

IBD crystallographic structure data indicated that Glu-376 is the catalytic base and the substrate binding cavity is shorter and wider relative to SCAD, and hence it allows the optimal binding of the isobutyryl-CoA substrate. Another difference, ACADs have conserved

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Tyr or Phe that defines a side of the binding cavity is replaced by Leu residue (Leu-375) in IBD. In comparison with IVD, the lateral expansion of the binding cavity in IVD is not observed in IBD [19].

IBD has many unique features including its high substrate specificity to isobutyryl-CoA and high Cysteines content (13 residues) which were shown to be important for its activity [20]. Here we study the IBD substrate, isobutyryl-CoA, binding and reduction by using the Stopped-flow kinetic analysis. In addition, we study the protonation states of the Cys residues at different pH using *H*++ molecular modeling and simulations program.

Materials and Methods

Instruments: UV/Vis spectra were carried out using Kontron Uvikon 930 instrument. Rapid reaction studies were conducted with a stopped-flow spectrophotometer with a 1.0 cm path length cell and a diode array detector (J&M Aalen), using BioKine [21]. The software used for the evaluation of kinetic traces was "Program A". Graphical work and fitting routines were done with the program KaleidaGraph. Analytical and preparative high-performance liquid chromatography (HPLC) was carried out with a Kontron System equipped with a diode array UV/Vis detector and a Kontron SFM 25 fluorescence detector. HPLC column was C18 column from Merck (250 × 4 mm, Lichrosorb RP-18) and guarded by preparative guard cartridge from Waters Corporation, USA.

Methods. The enzyme was purified to homogeneity using the method described before [20]. Stopped-flow experiments were carried out using Stopped-flow spectrophotometer where the fastest acquisition time of 1 spectrum/0.7 ms in the range of 300- 650 nm. In general, blocks of 120 spectra each were recorded for the first 100 milliseconds (ms) and 1- 2 s intervals and, for a third, variable length of time up to 5 min according to the reaction time to end. Stopped-flow experiments were conducted at 25°C. The concentrations mentioned are those of the reagents after mixing, i.e., 1/1 dilution, from the initially prepared solutions [21]. Isobutyryl-CoA was prepared from CoA-SH according to previously published method [22]. The concentrations of saturated acyl-CoAs and FAD were calculated according to their extinction coefficient (ε_{260}) values at 260 nm, 15.4 mM^{-1} cm^{-1} [23] and 10.8 mM^{-1} cm^{-1} [24], respectively. All anaerobic reactions and pH dependent activities were carried out as described in [20]. Protonation of the Cys residues at different pHs were simulated using *H*++ atomistic molecular modeling and simulations web server (http:// biophysics.cs.vt.edu/) using the PDB files 1rx0.pdb and 2JIF.pdb for human isobutyryl-CoA and short branched-chain acyl-CoA dehydrogenases, respectively, downloaded from Research Collaboratory for Structural Bioinformatics-Protein Data Bank database (RCSB PDB) (http://www.rcsb.org) [25,26].

Results and Discussion

The interaction of medium chain acyl-CoA dehydrogenase (MCAD), as a well-studied ACAD, with its acyl-CoA substrates can be affected by the pH, buffer and composition and ionic strength as documented in various studies [27-29]. Here we study the effect of the pH of the reaction buffer and isobutyryl-CoA conc. on both isobutyryl-CoA binding and reaction rate.

Upon addition of the substrate, isobutyryl-CoA, to the enzyme spectral changes indicating the isobutyryl-CoA binding and the FAD reduction are started simultaneously as shown in **Fig. 1**. These changes are pH dependent as it differs in their behavior at pH 9.0 or 6.5. The absorption traces upon addition of the substrate are recorded at 445 and 575 nm at pH 9 and 449 and 575 nm at pH 6.5. The changes at these specific wavelengths are initiated by a rapid absorbance increase up to approximately 0.1 s.

This is followed by a much slower process, the beginning of the reduction phase. The rate of this spectral perturbation depends on substrate concentration. This representation reflects a two-step process [30] as represented in **Scheme 1**. The first step (k_{b1}) reflects very rapid formation of a first equilibrium E_{ox}…S complex (K_{b1}). In the second equilibrium (K_{b2}), the E_{ox}…S complex is converted to the E_{ox}~S complex. At pH 9, the data points are fitted to a single exponential equation using KaleidaGraph software and showed k= 20 s^{-1}. While at pH 6.5, data points are fitted using 3 exponents and showed k_1= 214, k_2= 19 and k_3= 1.7 s^{-1}. The reduction reaction was faster at pH 9.0 comparing to that at pH 6.5 and hence, the X-Axis (time) is linear with pH 9 and logarithmic with pH 6.5.

To study the effect of isobutyryl-CoA conc. on the reaction rates, K_{obs} were measured at pH range 6.5-9.0. The reduction reaction takes place in two phases, and the observed k_{obs} rates exhibit saturation behavior at pH 7.5-9.0 but not with pH 6.5-7.0 as shown in **Fig. 2**. The faster reduction rates were plotted versus pH as shown in **Fig. 3**.

$$E_{OX} + iC_4\text{-CoA} \underset{}{\overset{K_{b1}}{\rightleftharpoons}} E_{OX} \ldots iC_4\text{-CoA} \overset{K_{b2}}{\rightleftharpoons} E_{OX} \sim iC_4\text{-CoA} \overset{K_{r1}}{\rightleftharpoons} E_{Red} \sim Int \overset{K_{r2}}{\rightleftharpoons} E_{Red} + \text{Methyl-Acrylyl-CoA}$$

Scheme 1: Kinetic scheme describing steps and species involved in the reaction of human IBD with the substrate isobutyryl-CoA (iC4CoA).

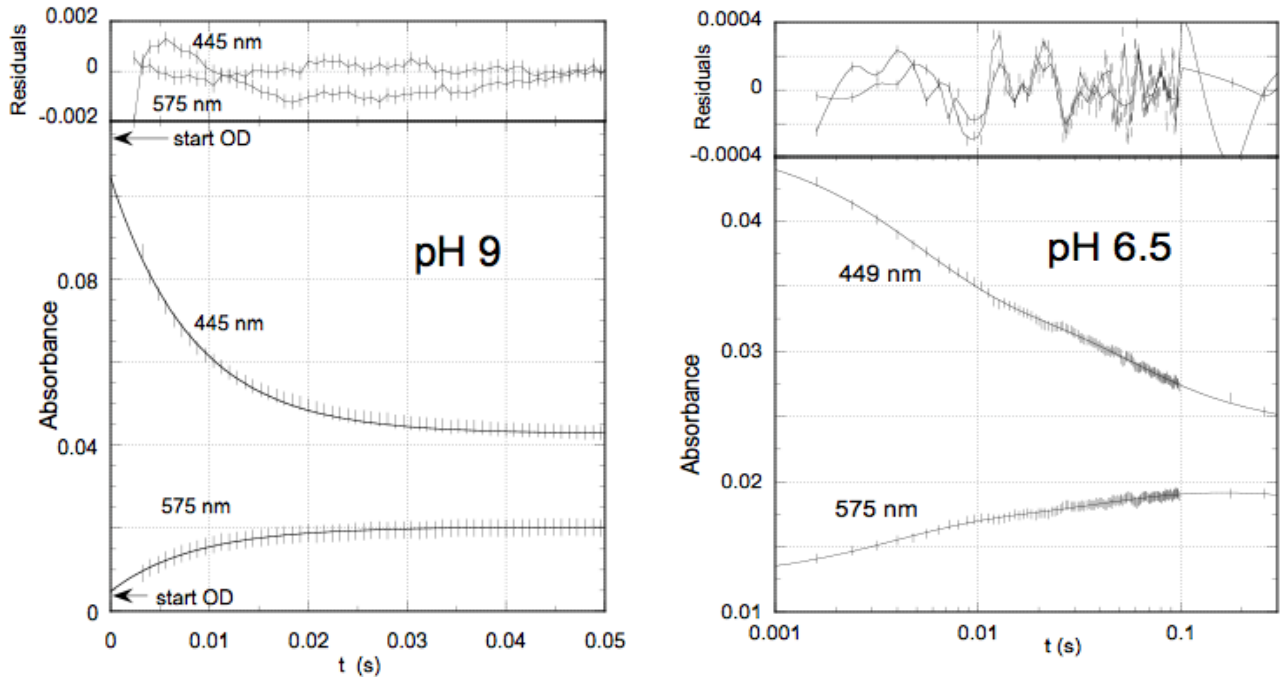

Fig (1): Time course of the spectral changes accompanying the reaction of IBD with isobutyryl-CoA at pH 9.0 (left) and 6.5 (right). Isobutyryl-CoA conc. is 25 µM. The data points at pH 9 are fitted to a single exponential equation (k = 20 s^{-1}). Those at pH 6.5 are fitted using 3 exponents (449 nm trace: k_1= 214, k_2= 19, k_3= 1.7 s^{-1}, and Δ1= 0.011, Δ2 = 0.0082, Δ3= 0.004 AU; 575 nm trace: k_1= 279, k_2= 23, k_3= 1.5 s^{-1}, and Δ1= 0.004, Δ2= 0.003, Δ3= 0.0014 AU). Note that at pH 9 the time axis is linear, that at pH 6.5 is logarithmic.

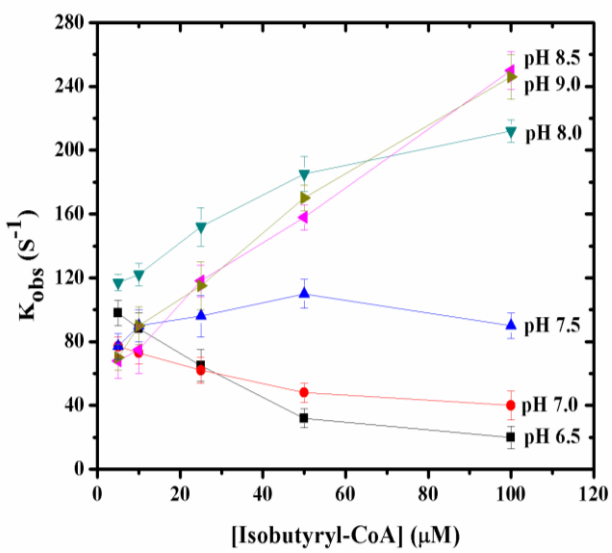

Fig. 2. Dependence of observed rates of IBD reduction on the isobutyryl-CoA concentration at the indicated pH values. (this is the plot of the "faster" rate when more than one is observed).

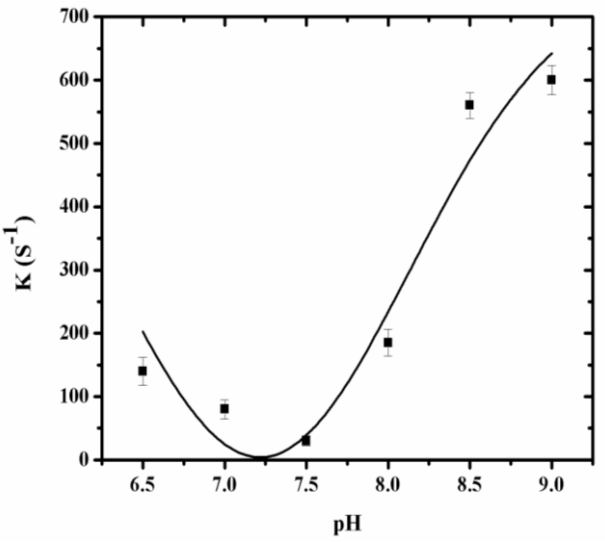

Fig. 3. Dependence of the rate of the fast(er) rate of enzyme reduction and of its reverse on the reaction pH.

In an attempt to understand the effect of the pH on the IBD reaction with the isobutyryl-CoA, the binding constants at different pHs were plotted as shown in **Fig. 4**. The isobutyryl-CoA binding constant values are varying strongly with the pH, as their logarithmic values decrease with increasing the pH with better binding at pH 7.5-9.

On the other hand, the Cys residues have role(s) in the reactivity of the IBD as mentioned in our previous work [20]. To further study the role of these Cys residues we study the protonation states of the Cys residues which they depend on complex electrostatic interactions between Cys residues themselves and with the surrounding environment including neighbor residues and the pH of the buffer. Here we used *H++* as a web

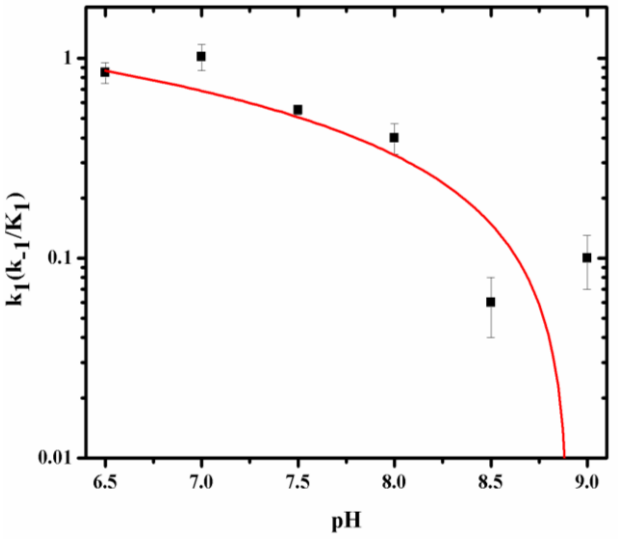

Fig. 4. pH dependence of the isobutyryl-CoA "binding steps".

server that automates the prediction of the pK values for titratable amino acids, including Cys residues, based on the standard continuum solvent methodology within the framework of the Poisson Boltzmann (PB) model [26,31,32]. IBD has 13 Cys residues at positions 12, 94, 107, 124, 128, 137, 183, 195, 229, 261, 329, 340 and 344. *H*++ predicted that 7 Cys residues at positions 107, 124, 128, 229, 261, 340 and 344 are fully protonated, which mean their they are fully protonated in all pHs. However, the 6-remaining Cys residues have different protonation states depending on the surrounding pH. These protonation states of the 6-remaining titratable Cys residues are shown in **Fig. 5**. In spite of the IBD is homotetrameric protein (A, B, C, D subunits), these subunits seem to be not identical in IBD complex to their surrounding environment. If all IBD subunits are identical, it will be expected that all corresponding Cys residues are identical in their protonation states. However, *H*++ was predicted that the Cys-12 is fully protonated at subunit A while it is titratable on the other three subunits. Similarly, Cys-94, Cys-137, Cys-183, Cys-195 and Cys-329 as shown in Figure 5. Interestingly enough, the protonation prediction shows four Cys residues that are protonated with wide range of protonation states depending on the pH and it is the same number of Cys residues that can be modified with DNTB and dramatically affect the IBD activity.

SBCAD which has the closest ACAD structure to the IBD has only 5 Cys residues and only two of them, 175 and 261, are titratable residues as shown in **Fig. 6**.

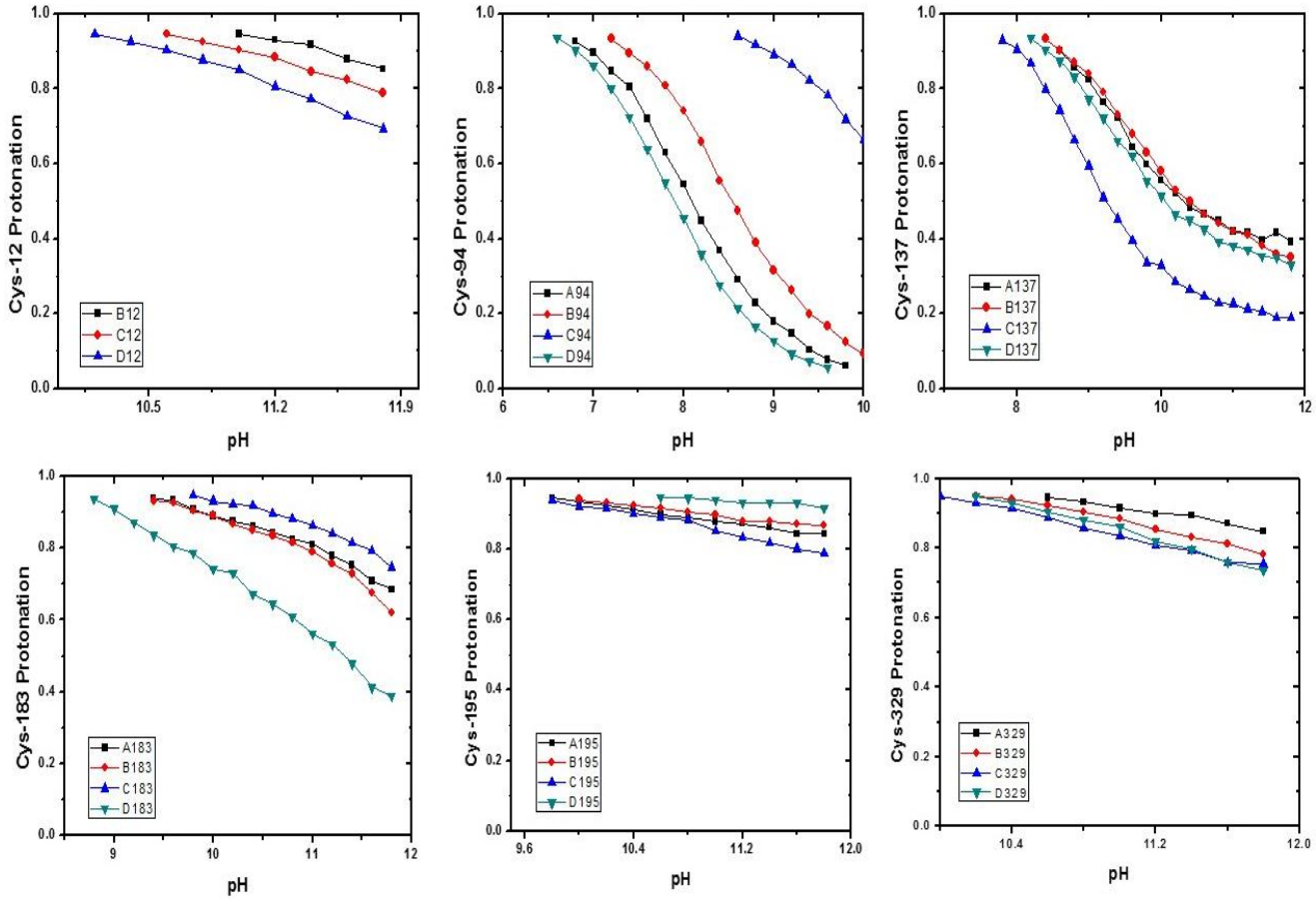

Fig (5): Prediction of protonation states for titratable IBD Cys residues number 12, 94, 137, 183, 195 and 229 at indicated pH ranges using the *H*++ web server.

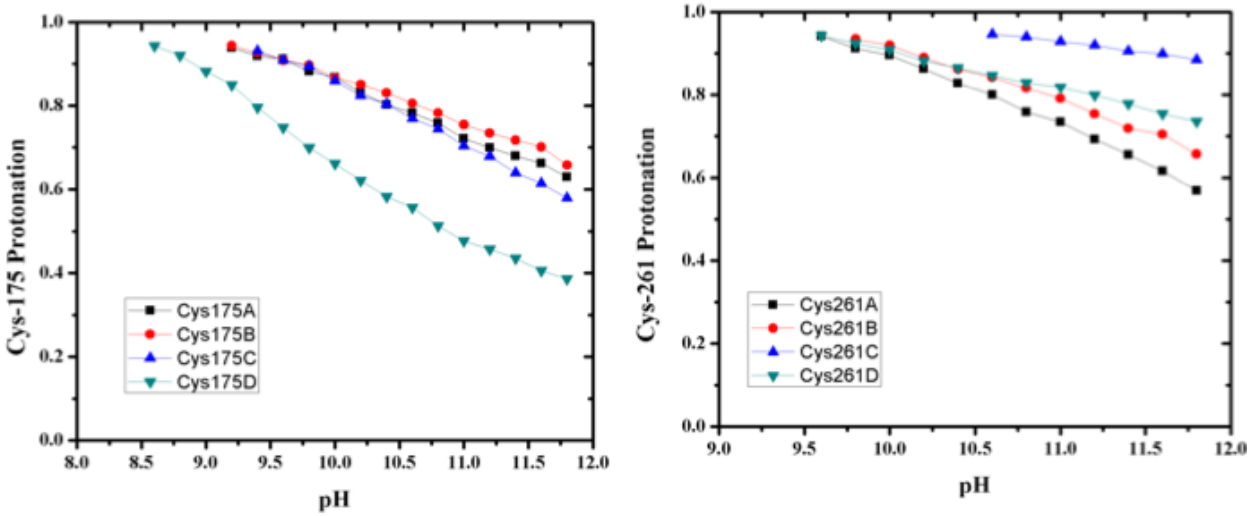

Fig (6): Prediction of protonation states for titratable SBCAD Cys residues number 175 and 261 at indicated pH ranges using the *H*++ web server.

Table (1): Significant contributions to pK of active site Glu-376 from proximate Cys-residues.

Cys-residue #	Non-titratable groups	Titratable groups
Cys-94	0.07	0.35
Cys-107	-0.049	1.02
Cys-124	0.04	0.21
Cys-128	0.02	0.19
Cys-137	-0.05	0.71
Cys-183	0.007	0.26
Cys-195	-0.03	0.46
Cys-261	-0.2	1.16
Cys-329	0.1	0.69
Cys-340	-0.07	0.42
Cys-344	-0.02	0.29
Cys-12 (NS)*	-0.001	0.15

* NS: Neighboring subunit.

From the table above we notice the contribution of the titratable groups (-SH) of 11 Cys residues out of 13 on the p*Ka* value of Glu-376 active site and Cys-12 from the neighboring subunit indicating both the importance of the Cys residues for the right pKa value of Glu-376 and in the same time the cooperative interactions between the four subunits in the whole IBD enzyme.

This IBD subunits interaction was also proposed from the IBD crystallography that showed binding of only two isobutytryl-CoA molecules to the homotetrameric whole enzyme instead of four [19]. It is worth to mention the titratable groups contribution of the Tyr residues 68, 100 and 136 on p*Ka* value of Glu-376 as shown in **Table 2**.

The large number of the Cys residues in IBD ensures that it has different role in the IBD conformations and hence activity. As we showed in previous work, the modification of the Cys residues with DTNB rend the activity dramatically [20] and here we showed the contribution of most of the Cys residues in p*Ka* value of active site Glu-376.

Table (2): Significant contributions to pK of active site Glu-376 from proximate Tyr-residues.

Tyr-residue #	Non-titratable groups	Titratable groups
Tyr-68	-0.07	1.17
Tyr-100	0.1	0.66
Tyr-136	-0.08	1.49

It can be concluded that the proper redox state of the Cys residue(s) is very important for IBD fidelity in general and might be the Cys residues influences the FAD binding similar to Cys-108 in D-Amino Acid Oxidase (AAO) from *Trigonopsis variabilis*. In AAO, Cys108 is presumably found in the vicinity of FAD binding domain, but remote from the active center. Oxidation of Cys-108 causes a global conformational response that affects the protein environment of the FAD cofactor and hence the biocatalyst function and stability of the AAO [33].

In conclusion, IBD reduces the isobutyryl-CoA with two phases process. The rate of each phase is dramatically pH dependent; it is faster at pH 9.0 than that at pH 6.5 being in the same time dependent on substrate concentration. Similarly, IBD binds isobutyryl-CoA in two steps, faster one followed by slower step and again the binding rates are pH-dependent. On the other hand, one or more of Cys residues interact and is involved in the p*K*a value of the active site residue, Glu-376. It seems that Cys residue(s) has crucial role(s) in the conformation stability and biocatalytic activity of the IBD, and their roles need further investigations.

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