A Novel Approach to the Characterization of Substrate Specificity in Short/ Branched Chain Acyl-CoA Dehydrogenase*

Rat and human short/branched chain acyl-CoA dehydrogenases exhibit key differences in substrate specificity despite an overall amino acid identity of 85% between them. Rat short/branched chain acyl-CoA dehydrogenases (SBCAD) are more active toward substrates with longer carbon side chains than human SBCAD, whereas the human enzyme utilizes substrates with longer primary carbon chains. The mechanism underlying this difference in substrate specificity was investigated with a novel surface plasmon resonance assay combined with absorbance and circular dichroism spectroscopy, and kinetics analysis of wild type SBCADs and mutants with altered amino acid residues in the substrate binding pocket. Results show that a relatively few amino acid residues are critical for determining the distance between the flavin ring of FAD and the catalytic base, and also that alteration of these residues influences different portions of the enzyme mechanism. Molecular modeling of the SBCAD structure suggests that position 104 at the bottom of the substrate binding pocket is important in determining the length of the primary carbon chain that can be accommodated. Conformational changes caused by alteration of residues at positions 105 and 177 directly affect the rate of electron transfer in the dehydrogenation reactions, and are likely transmitted from the bottom of the substrate binding pocket to β-sheet 3. Differences between the rat and human enzyme at positions 383, 222, and 220 alter substrate specificity without affecting substrate binding. Modeling predicts that these residues combine to determine the distance between the flavin ring of FAD and the catalytic base, without changing the opening of the substrate binding pocket.

The acyl-CoA dehydrogenases (ACDs) are a family of related enzymes that catalyze the α, β-dehydrogenation of acyl-CoA esters, transferring electrons to electron transferring flavoprotein (1–4). Deficiencies of these enzymes are important causes of human disease (3–7). Biochemical and immunological studies have identified at least 9 distinct members of this enzyme family, each having a characteristic substrate utilization pattern (8–13). Very long, long, medium, and short chain acyl-CoA dehydrogenases (VLCAD, LCAD, MCAD, and SCAD, respectively) catalyze the first step in the mitochondrial β-oxidation of fatty acids with substrate optima of 16, 16, 8, and 4 carbon chains, respectively (3, 14–15). A new ACD has been identified recently (ACAD9) that is also active against long chain acyl-CoA substrates (16). Isovaleryl-CoA dehydrogenase (IVD), short/branched chain acyl-CoA dehydrogenase (SBCAD, also known as 2-methyl branched chain acyl-CoA dehydrogenase), and isobutyryl-CoA dehydrogenase (IBD) catalyze the third step in leucine, isoleucine, and valine metabolism, respectively (2–4, 11, 12, 17, 18), whereas glutaryl-CoA dehydrogenase is active in lysine metabolism (19).

We have shown previously that SCAD, SBCAD, and IBD can all utilize butyryl-CoA as substrate (albeit with different efficiencies), whereas IVD, SBCAD, and IBD are most active with short branched chain acyl-CoAs as substrate. For the most part, the rat and human homologues of the ACDs have similar substrate specificities; however, rat and human SBCAD show key differences despite an amino acid homology of 85% (11, 12). Rat SBCAD is more active than human SBCAD with substrates with longer carbon side chains, whereas the human enzyme can utilize substrates with longer primary carbon chains. Previous studies have shown that the position of the active site catalytic glutamate, located at two different amino acid positions in the primary carbon backbone in the various ACDs, affects substrate specificity (20–22). These residues, however, are in the same position in rat and human SBCAD indicating that other amino acid residues contribute to the substrate specificity of SBCAD (22). Therefore, we have begun using SBCAD as a model to study the amino acid residues and motifs important in determining acyl-CoA substrate specificity.

The ACD enzyme mechanism is a complicated one consisting of a reductive half-reaction leading to the production of a stable substrate-enzyme intermediate (the charge-transfer complex) that resolves to reoxidized enzyme and product through interaction with electron transferring flavoprotein (ETF) during the oxidative half-reaction (Scheme 1) (23, 24). The manner in which different substrates interact with ACDs is not well understood. Most frequently, the interaction between an ACD and various substrates is studied by monitoring characteristic absorbance changes at 450 and 560 nm following the addition of substrate to enzyme under anaerobic conditions. A decrease in absorbance at 450 nm is characteristic of the reduction of the essential FAD coenzyme, whereas an increase in absorbance at 560 nm is because of a new resonance signal related to the charge-transfer complex in the enzyme reaction (1, 4, 25–27). In some situations, however, the observed changes at these wavelengths have been multiphasic, leading to difficulty in interpretation of the results. This has been postulated to be due to...
Characterization of ACD Substrate Specificity

**EXPERIMENTAL PROCEDURES**

Construction of SBCAD Prokaryotic Expression Vectors—The sequences for the mature form of wild type and human SBCAD cDNA (11, 12, 23) were inserted into the prokaryotic expression vector pET 21a (+) (Invitrogen) and altered at the 5′ end by PCR to reflect *Escherichia coli* codon usage bias as described previously (28–30). The sequences of 5′ FLT primers (reflecting the first 51 nucleotides of the mature coding region of each) are as follows: human, 5′-AAA TCT GCC TGG CTG CAC ACC ACC AAC GGC ACC CAC-3′, and rat, 5′-AAA GCC TGC ACC CCG GGA GCT CTG AGC GTA ACC AAC GAC GCT TTC TGC-3′. Altered nucleotides are shown in boldface and underlined. Amino acid numbering is relative to the amino terminus of the mature protein.

SBCADs with altered substrate specificity were constructed by PCR site-directed mutagenesis as described previously (21, 23). Mutagenic primers were synthesized by the Molecular Biology Core Facility of the Mayo Clinic. They are as follows (amino acid residues refer to the mature coding region of each): human, 5′-GGA ACC TTG ACA TTT TCG AAT GTT AAC GGG CAG GTG GAA-3′; F105L, 5′-TCT AAT CAG CAG TGT GAT AAT TAA TTT GCC GTG CTC AAG GAC GAC CAC AGA TGC-3′; S177N, 5′-CCC TTA TAT CCA ATG GTA GGG TCT ACA TTT GCC ATC ACC AGA AAG AGC CCT-3′; S177Y, 5′-TCT AAT CAG CAG TGT GAT AAT TAA TTT GCC GTG CTC AAG GAC GAC CAC AGA TGC-3′; S177L, 5′-TCT AAT CAG CAG TGT GAT AAT TAA TTT GCC GTG CTC AAG GAC GAC CAC AGA TGC-3′. Altered nucleotides are defined by PCR to reflect the overall substrate binding affinity only if the oxidative half-reaction was much faster than the reductive half-reaction (Scheme 1).

**Scheme 1**

| Reductive half-reaction | Oxidative half-reaction |
|-------------------------|-------------------------|
| Eox + S → EoxS           | Eox + 2ETFOx → Eox + 2ETF + P |
|                         | EoxP + S → EoxP + P       |

**Scheme 2**

| Eox + S + K1 → EoxK1 + I | EoxP + K2 → EoxP + I | K3 → FeredP | K4 → FeredP + P |

**Scheme 3**

| dNTP | dNTP | dNTP | dNTP |
|------|------|------|------|
| dATP| dCTP| dGTP| dTTP |

**Real Time Surface Plasmon Resonance—**Real time surface plasmon resonance experiments were performed on a BIACore T2000 instrument (BIAcore AB, Uppsala, Sweden). *Plasmidium falci-parum* acyl-CoA-binding protein (ACBP, generously provided by Dr. Terry Smith, University of Kentucky), 0.005% (v/v) surfactant P20 (Biacore AB), 5% glycerol, subse-

| NaCl | 0.005% (v/v) surfactant P20 (Biacore AB), 5% glycerol, subsequent experiments were performed at 4 °C with 50 mM sodium phosphate buffer, pH 7.4, 150 mM NaCl, 0.005% (v/v) surfactant P20 (Biacore AB), 5% glycerol, running buffer during the immobilization step was 50 mM sodium phosphate, pH 7.4, 150 mM NaCl, 0.0.05% (v/v) surfactant P20 (Biacore AB), 5% glycerol, running buffer during the immobilization step was 50 mM sodium phosphate, pH 7.4, 150 mM NaCl, 0.0.05% (v/v) surfactant P20 (Biacore AB), 5% glycerol, running buffer during the immobilization step was 50 mM sodium phosphate, pH 7.4, 150 mM NaCl, 0.0.05% (v/v) surfactant P20 (Biacore AB), 5% glycerol, running buffer during the immobilization step was 50 mM sodium phosphate, pH 7.4, 150 mM NaCl, 0.0.
RESULTS

In Vitro Mutagenesis—To explore the structural basis for the differential utilization of substrate by human and rat SBCAD, a structural model of SBCAD was generated based on its homology to the ACDs for which x-ray crystal data are available. Modeling based on the known structures of human IVD, porcine MCAD, and rat SCAD was used to generate predictions of the three-dimensional structure of human SBCAD. The positions of 10 amino acid residues predicted to be in or near the substrate binding pocket and that differ between rat and human SBCAD, along with the catalytic base, are shown. The images were generated with the Insight II 2000 software as described under “Experimental Procedures.” A model of a human SBCAD monomer is shown in A, and the surface of its substrate binding pocket is shown in B. FAD is represented in green, and the substrate (S)-2-methylbutyryl-CoA is shown in magenta. The 10 differing amino acid residues in the substrate pocket are highlighted as yellow balls, and the catalytic base is shown as a blue ball.

Characterization of ACD Substrate Specificity

To explore the structural basis for the differential utilization of substrate by human and rat SBCAD, a structural model of SBCAD was generated based on its homology to the ACDs for which x-ray crystal data are available. The modeling identified 10 amino acids in or near the substrate binding pocket that differ between the rat and human enzymes and thus are candidates to be important residues in modifying substrate specificity (Fig. 1).

To characterize the role of these residues in determining substrate specificity, in vitro mutagenesis was used to systematically change each residue in the human enzyme to its rat counterpart in various combinations. The mutant enzymes were then overexpressed in an E. coli system, and the activity in crude cellular extracts made from the induced cultures was measured with various short and branched chain substrates using the anaerobic fluorescent ETF reduction assay. The specific activities of crude cellular extracts from cells expressing the mutant enzymes as measured with (S)-2-methylbutyryl-CoA as substrate are shown in Table I along with the ratio of the specific activities measured with isobutyryl-CoA and hexanoyl-CoA relative to (S)-2-methylbutyryl-CoA. Extracts containing the rat and human enzymes had nearly equal specific activities when (S)-2-methylbutyryl-CoA was used as substrate. The human enzyme was more active than the rat enzyme toward hexanoyl-CoA, whereas this pattern was reversed for isobutyryl-CoA. Substitution of all 10 or 7 of 10 residues in the human enzyme with their rat counterparts (M10 and M7 mutants, respectively) led to a dramatic reduction in the ability of the enzyme to utilize hexanoyl-CoA as substrate, while slightly increasing the specific activity toward isobutyryl-CoA. Mutants V104L/F105L, S177N, and L220M/L222I/A383T (M2 + A in Table I) appeared to be of particular interest. The L220M/L222I (M2 in Table I) and A383T mutants showed a pattern of substrate specificity similar to the wild type human enzyme. The combination of either or both of the L220M and L222I mutations in combination with A383T, however, had enhanced activity toward isobutyryl-CoA compared with wild type human SBCAD. Introduction of V104L/F105L mutations into the human enzyme led to dramatically decreased activity toward hexanoyl-CoA compared with wild type human SBCAD, while leaving the specific activity toward isobutyryl-CoA essentially unchanged. Interestingly, the single F105L mutant had a substrate utilization pattern similar to the wild type human enzyme, implicating the V104L substitution in determining the rat SBCAD substrate utilization pattern. Unfortunately, the single V104L mutant was relatively unstable when expressed in E. coli (data not shown) and could not be studied further.
ever, the pattern of relative activity toward different substrates were higher than that measured in crude extract protein; however, the human SBCAD and the second to the identity of the rat residue. Because the M10 mutant containing the L220M, L222I, and A383T was stable, but still exhibited reduced activity toward all substrates compared with wild type rat, S177N, F105L enzymes, it was significantly reduced (about 1.5 times higher than the wild type human enzyme. Furthermore, the spectral absorbance profiles of all of these effects better, we purified the wild type rat and human SBCADs did not contain a bound Co-A persulfide molecule, and the enzymatic properties of the “degreened” recombinant ACDs expressed in E. coli and purified have been found previously to contain a bound Co-A persulfide molecule, resulting in a green color due to a characteristic spectral absorbance maximum in the 550–700 nm range (43–45). Removal of the Co-A persulfide by chemical reduction of the enzyme solution leads to a loss of the 550–700 nm absorption peak and a change of the enzyme preparation to a yellow color. The enzymatic properties of the “degreened” recombinant ACDs, however, have been reported to be unaltered from “green” ACDs (29–31). Wild type human and rat and the mutant SBCADs were purified to 95% pure as judged by SDS-PAGE (not shown). The purified SBCAD enzymes were all a pale yellow color, and dialysis against dithionite did not alter this. Furthermore, the spectral absorbance profiles of all of the enzymes were the same before and after dialysis against dithionite, lacking the characteristic absorption maximum of a bound Co-A persulfide. This indicates that the recombinant SBCADs did not contain a bound Co-A persulfide molecule and that chemical reduction of the enzyme preparation prior to subsequent use was not necessary.

Kinetic Properties of Purified Wild Type and Mutant SBCADs—ACDs expressed in E. coli and purified have been found previously to contain a bound Co-A persulfide molecule, resulting in a green color due to a characteristic spectral absorbance maximum in the 550–700 nm range (43–45). Removal of the Co-A persulfide by chemical reduction of the enzyme solution leads to a loss of the 550–700 nm absorption peak and a change of the enzyme preparation to a yellow color. The enzymatic properties of the “degreened” recombinant ACDs, however, have been reported to be unaltered from “green” ACDs (29–31). Wild type human and rat and the mutant SBCADs were purified to 95% pure as judged by SDS-PAGE (not shown). The purified SBCAD enzymes were all a pale yellow color, and dialysis against dithionite did not alter this. Furthermore, the spectral absorbance profiles of all of the enzymes were the same before and after dialysis against dithionite, lacking the characteristic absorption maximum of a bound Co-A persulfide. This indicates that the recombinant SBCADs did not contain a bound Co-A persulfide molecule and that chemical reduction of the enzyme preparation prior to subsequent use was not necessary.

Kinetic parameters of the purified wild type and SBCAD mutants were measured with (S)-2-methylbutyryl-CoA, hexanoyl-CoA, and isobutyl-CoA as substrate using the ETF reduction assay (Table II and Scheme 1). All of the purified SBCADs were most active when (S)-2-methylbutyryl-CoA was used as substrate. Interestingly, both the V104L/F105L and F105L mutants had catalytic efficiencies that were higher than either wild type enzyme due to a greatly increased $k_{cat}$.

Human SBCAD utilized hexanoyl-CoA as substrate nearly 10 times more efficiently than isobutyl-CoA due to a lower

### Table I

**Comparison of specific activities of wild type and mutant SBCADs**

| Mutation position | 2meC4-CoA<sup>a</sup> activity | % 2meC4-CoA activity |
|-------------------|-------------------------------|---------------------|
|                    | Isobutyl-CoA                  | Hexanoyl-CoA        |
| Human             |                               |                     |
| M10               | 28 ± 2                        | 6                   |
| M7                | 9.4 ± 0.2                     | 17                  |
| A383T             | 51 ± 5                        | 12                  |
| A383T             | 56 ± 3                        | 38                  |
| M2                | 28 ± 3                        | 5                   |
| M2 + A            | 15 ± 0.8                      | 6                   |
| L220M/A383T       | 15 ± 0.4                      | 4                   |
| L222I/A383T       | 15 ± 0.2                      | 3                   |
| V104L/F105L       | 51 ± 4                        | 0.5                 |
| F105L             | 25 ± 20                       | 15                  |
| V104L             | 4.8 ± 0.3                     | ND                  |
| S177N             | 120 ± 20                      | 110                 |
| Rat               | 35 ± 3                        | 10                  |

*Substrate binding pocket mutation positions relative to the mature amino terminus. The first amino acid corresponds to the residue found in human SBCAD and the second to the identity of the rat residue.*

<sup>a</sup> (S)-2-Methylbutyryl-CoA.

<sup>b</sup> Milliunits/mg cellular protein assayed with 50 μM substrate.

<sup>c</sup> ND, not detectable.

### Table II

**Characterization of kinetic parameters of purified wild type and mutant SBCADs determined with the anaerobic ETF fluorescence reduction assay**

| Substrate | 2meC4-CoA | Hexanoyl-CoA | Isobutyl-CoA |
|-----------|-----------|--------------|--------------|
|           | $K_m$     | $k_{cat}$    | Catalytic efficiency | $K_m$     | $k_{cat}$    | Catalytic efficiency | $K_m$     | $k_{cat}$    | Catalytic efficiency |
|           | $\mu M$   | $s^{-1}$     | $\mu M^{-1} s^{-1}$ | $\mu M$   | $s^{-1}$     | $\mu M^{-1} s^{-1}$ | $\mu M$   | $s^{-1}$     | $\mu M^{-1} s^{-1}$ |
| hSBCAD    | 2.7       | 9.7 x 10<sup>5</sup> | 3.6 x 10<sup>5</sup> | 36 | 7.6 x 10<sup>3</sup> | 2.1 x 10<sup>2</sup> | 130 | 2.9 x 10<sup>3</sup> | 22 |
| rSBCAD    | 3.9       | 3.0 x 10<sup>4</sup> | 7.7 x 10<sup>4</sup> | 44 | 1.8 x 10<sup>5</sup> | 41 | 110 | 8.5 x 10<sup>3</sup> | 77 |
| M10       | 1.5       | 5.1 x 10<sup>4</sup> | 3.4 x 10<sup>4</sup> | 57 | 7.4 x 10<sup>4</sup> | 13 | 60 | 2.0 x 10<sup>3</sup> | 33 |
| L220M/A383T | 2.0         | 4.0 x 10<sup>4</sup> | 2.0 x 10<sup>4</sup> | 5.3 | 5.8 x 10<sup>3</sup> | 1.1 x 10<sup>3</sup> | 49 | 2.7 x 10<sup>3</sup> | 55 |
| L222I/A383T | 1.1         | 1.2 x 10<sup>5</sup> | 1.1 x 10<sup>5</sup> | 5.5 | 1.2 x 10<sup>5</sup> | 2.2 x 10<sup>5</sup> | 26 | 8.1 x 10<sup>3</sup> | 31 |
| F105L     | 2.6       | 2.6 x 10<sup>3</sup> | 1.0 x 10<sup>3</sup> | 35 | 2.5 x 10<sup>4</sup> | 7.0 x 10<sup>3</sup> | 95 | 2.9 x 10<sup>3</sup> | 30 |
| V104L/F105L | 10.7       | 2.1 x 10<sup>5</sup> | 2.0 x 10<sup>5</sup> | 150 | 3.2 x 10<sup>5</sup> | 2.1 | 85 | 3.5 x 10<sup>3</sup> | 41 |

<sup>a</sup> $K_m$ is the concentration of substrate needed for the overall reaction rate to reach half of its $V_{max}$ as shown in Scheme 1.

The M10 mutant contains all 10 residues that differ between rat and human SBCAD mutated from the human to the rat sequence. As seen for the wild type rat and V104L/F105L SBCADs, the activity of the M10 mutant toward hexanoyl-CoA was significantly reduced (—20-fold) compared with wild type human SBCAD, whereas its activity toward isobutryl-CoA was about 1.5 times higher than the wild type human enzyme. A mutant enzyme containing only the E107D/L112V/T115K/I117K alterations (M4) was not stable. The addition of S177N/F105L/V104L mutations (M7), however, restored the expressed enzyme, although it showed reduced activity to all substrates measured in crude cellular extracts compared with wild type enzyme. Because the M10 mutant containing the L220M, L222I, and A383T was stable, but still exhibited reduced activity compared with wild type rat, S177N, F105L enzymes, it appears that the E107D/L112V/T115K/I117K mutations might mediate the reduced activity seen in the M10 SBCAD.

Together these findings implicated amino acid residues at positions 220 and 222 acting in concert with that at position 383 as critical in determining activity toward isobutryl-CoA, whereas residues positions 104 and 105 play a key role in determining activity toward hexanoyl-CoA. To characterize these effects better, we purified the wild type rat and human enzymes along with the M10, L220M/A383T, L222I/A383T, F105L, and V104/F105L mutants. The specific activities of pure enzymes at the same substrate concentration (50 μM) were higher than that measured in crude extract protein; however, the pattern of relative activity toward different substrates remained the same (data not shown).
$K_m$ and higher $k_{cat}$ value with hexanoyl-CoA. The human enzyme was also nearly 5-fold more efficient than the rat enzyme at utilizing hexanoyl-CoA as substrate. In contrast, rat SBCAD had a nearly 2-fold higher catalytic efficiency with isobutyryl-CoA than with hexanoyl-CoA and utilized isobutyryl-CoA as substrate 3.5 times more efficiently than did the human enzyme. The mechanism for the differential utilization of the SBCADs in using these non-optimal substrates is difficult to discern because the relative catalytic efficiencies with the sub-

Fig. 2. Spectrophotometric analysis and reductive titration of purified human and rat wild type, F105L, and V104L/F105L SBCADs with (S)-2-methylbutyryl-CoA or hexanoyl-CoA. Purified SBCADs were diluted in assay buffer as described under “Experimental Procedures” and scanned in a Beckman DU 7500 spectrophotometer, and then substrate was added under anaerobic conditions at room temperature in increasing concentrations. Scans were taken after each substrate addition. Only selected spectra are shown for clarity. The substrate concentration for each line is indicated in the legend for each panel. A, C, E, and G, analysis of human, rat, F105L, and V104L/F105L SBCAD (starting enzyme concentrations 8.0, 8.2, 8.5, and 8.7 nM, respectively) with (S)-2-methylbutyryl-CoA. B, D, F, and H, titrations of human, rat, F105L, and V104L/F105L SBCAD (starting enzyme concentrations 8.0, 8.2, 8.5, and 8.7 μM, respectively) with hexanoyl-CoA. The absorbance of indicator dye, glucose oxidase, and turbidity was measured separately and subtracted from all spectra.

The substitution of L220M/A383T or L222I/A383T in human SBCAD resulted in a decreased catalytic efficiency toward (S)-

The limitation may be due to substrate binding or any subsequent step. Thus, the relative $K_m$ values as measured with the ETF reduction assays do not necessarily reflect substrate binding differences.
efficiency than both wild type enzymes when (compared with 6% for the wild type human SBCAD). Thus, the A383T mutant also more efficiently used hexanoyl-CoA as substrate (2.6- and 1.4-fold increase in catalytic efficiency). The L220M/A383T mutant showed a decrease in these absorbance maxima (55 and 20% as compared with 6% for the wild type human SBCAD). Thus, utilization of all nonoptimal substrates was more efficient by this mutant enzyme compared with the wild type.

The V104L/F105L mutant enzyme had a higher catalytic efficiency than both wild type enzymes when (S)-2-methylbutyryl-CoA was used as substrate. Interestingly, however, the effect of these mutations was much more specific than was seen for the 220/222/383 position mutants as the V104L/F105L double mutant showed a 100-fold reduction in catalytic efficiency toward hexanoyl-CoA. There was an almost 5-fold increase in the Km of this enzyme to hexanoyl-CoA as compared with the wild type human SBCAD. A less dramatic reduction was seen in the ability of the double mutant to utilize isobutyryl-CoA. This suggests that the main effect of the V104L/F105L mutation was on substrate binding. Amino acid residue 104 appears to play a key role in mediating this effect as the F105L single mutant enzyme actually utilized hexanoyl-CoA nearly as efficiently as wild type human SBCAD, whereas its catalytic efficiency with isobutyryl-CoA as substrate was unchanged.

Overall, these results substantiate significant effects of alterations in key amino acid residues located in the substrate binding pocket on the substrate specificity differences seen between rat and human SBCAD. Amino acids at positions 220, 222, and 383 influence activity toward isobutyryl-CoA and, to a lesser extent, hexanoyl-CoA, whereas residues at position 104 and 105 affect more greatly the activity toward a longer chain substrate (i.e. hexanoyl-CoA). To investigate the mechanism of these effects further, binding of substrate to the wild type and mutant SBCADs was investigated with spectrophotometric assays not dependent on the completion of the second half of the ACD enzyme mechanism (transfer of electrons from the intermediate charge transfer complex to the secondary electron acceptor).

Absorbance Spectroscopy of Wild Type and Mutant SBCADs—All ACDs including SBCAD show a characteristic spectral absorption pattern with maxima around wavelengths 365 and 450 nm related to the non-covalently bound FAD coenzyme (2, 45–47). When incubated with optimal substrate under anaerobic conditions, reduction of FAD occurs, and decreases in these absorbance maxima occur. At the same time, a new broad, higher wavelength maximum appears at ~560 nm, reflecting establishment of the productive charge-transfer enzyme-substrate intermediate between the highest occupied molecular orbital of the flavin and the lowest unoccupied molecular orbital of the polarized substrate/product. The ACD absorbance spectra usually exhibits two isosbestic points, one at about 500 nm and the other one at about 340 nm (25, 35, 48–50).

| Substrate          | S,2meC4-CoA | Hexanoyl-CoA |
|--------------------|-------------|--------------|
|                    | % reduction | % reduction  |
|                    | with excess | with 1:1      |
|                    | substrate:enzyme | substrate:enzyme |
| hSBCAD             | 43          | 30           |
| rSBCAD             | 42          | 23           |
| L220M/A383T        | 33          | 26           |
| F105L              | 51          | 25           |
| V104L/F105L        | 64          | 20           |
|                    | 59          | 44           |
|                    | 62          | 5.0          |
|                    | 56          | 26           |
|                    | 71          | 40           |
|                    | 19          | 2.0          |

The V104L/F105L mutant enzyme had a higher catalytic efficiency than both wild type enzymes when (S)-2-methylbutyryl-CoA was used as substrate. Interestingly, however, the effect of these mutations was much more specific than was seen for the 220/222/383 position mutants as the V104L/F105L double mutant showed a 100-fold reduction in catalytic efficiency toward hexanoyl-CoA. There was an almost 5-fold increase in the Km of this enzyme to hexanoyl-CoA as compared with the wild type human SBCAD. A less dramatic reduction was seen in the ability of the double mutant to utilize isobutyryl-CoA. This suggests that the main effect of the V104L/F105L mutation was on substrate binding. Amino acid residue 104 appears to play a key role in mediating this effect as the F105L single mutant enzyme actually utilized hexanoyl-CoA nearly as efficiently as wild type human SBCAD, whereas its catalytic efficiency with isobutyryl-CoA as substrate was unchanged.

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![Absorbance Spectroscopy of Wild Type and Mutant SBCADs](image)

**Fig. 2** shows the effect of the addition of increasing concentrations of (S)-2-methylbutyryl-CoA (A, C, E, and G) or hexanoyl-CoA (B, D, F, and H) to wild type and mutant SBCADs. An isosbestic point at about 503 nm was present in all of the purified wild type and mutant SBCADs titrated with (S)-2-methylbutyryl-CoA, with development of the absorbance maximum characteristic of the charge-transfer complex. The human and most of the mutant SBCADs demonstrated a second isosbestic point at about 336 nm, whereas this occurred at 342 nm in rat SBCAD (Fig. 2, A, C, E, and G). For other ACDs, the isosbestic points in the spectra shift slightly when substrate is added in greater than 4-fold molar excess compared with protein. This has been interpreted to implicate a multistep reaction (26) and is also seen in the current experiments.

When the SBCADs were titrated with hexanoyl-CoA (Fig. 2, B, D, F, and H), a decrease of absorbance of the ~365- and ~450-nm maxima also occurred; however, absorbance either did not increase at long wavelengths or changed only minimally even when the substrate was present in 4–80-fold excess. This suggests that the stable charge-transfer intermediate seen with optimal substrate did not form when SBCAD was incubated with hexanoyl-CoA. Moreover, isosbestic points in the hexanoyl-CoA titration curves differed when excessive substrate was present in the reaction mixture, suggesting that the substrate might be reentering the reaction (Scheme 2) (26). Given this, it is possible that the minimal long wavelength changes that appear in some of the curves might actually represent the complex between reduced enzyme and substrate.

Table III summarizes the percent of maximal reduction of the ~450-nm maximum for each enzyme induced by the addition of a 1:1 ratio of substrate:enzyme or a saturating amount of substrate. Reduction of the ~450-nm maximum with (S)-2-methylbutyryl-CoA was similar for both wild type enzymes at a 1:1 substrate ratio, and neither showed complete reduction even with saturating concentrations. This is in agreement with our findings reported previously (22). Addition of hexanoyl-CoA to human SBCAD at a 1:1 molar ratio, in contrast, led to significantly greater reduction of this peak than was seen with the rat enzyme (5 versus 44%, respectively), although saturating substrate concentrations caused equal reduction of both enzymes. Increases in absorption at the ~560 nm maximum corresponding to the establishment of the charge transfer complex were not observed or were minimal in this case. This
suggests that the lower catalytic efficiency seen for rat SBCAD with hexanoyl-CoA as compared with the human enzyme is due to a differential ability of the two enzymes to bind substrate. Because the L220M/A383T and L222I/A383T enzymes showed a reduced $K_m$ and $k_{cat}$ value toward hexanoyl-CoA as compared with human SBCAD in the ETF reduction assay, the absorbance reduction spectra of L220M/A383T titrated with hexanoyl-CoA were also characterized (Table III). Addition of hexanoyl-CoA to the L220M/A383T SBCAD at 1:1 molar ratio led to a 26% reduction of the 450-nm absorbance maximum and a minimal increase in absorbance at 560 nm, similar to the findings with wild type human SBCAD. This suggests the increased catalytic activity of this mutant with hexanoyl-CoA in the ETF assay is not simply related to differential substrate binding as compared with the wild type human enzyme.

Because of the relatively clear effect of the V104L/F105L mutation on the ability of the human enzyme to utilize hexanoyl-CoA as substrate, this enzyme was further examined. As shown in Table III, the double mutant enzyme showed a similar reduction of the ~450 nm absorption maximum as compared with the wild type enzymes upon addition of (S)-2-methylbutyryl-CoA. In contrast, addition of a 1:1 molar ratio of hexanoyl-CoA to the double mutant enzyme resulted in reduction of this peak by just 2%, similar to that seen for rat (5%) rather than human (44%) SBCAD. When only the F105L mutation was present, the human pattern was preserved. This correlates with the kinetic studies with these enzymes. Thus, the presence of V104L mutation is necessary to confer the difference in hexanoyl-CoA utilization seen between the rat and human enzymes. Interestingly, the increase in absorbance in the 515–700-nm wavelength range characteristic of formation of the charge-transfer complex was absent upon addition of even saturating concentrations of hexanoyl-CoA for the human

**Fig. 3. Absorbance spectra for SBCADs titrated by isobutyryl-CoA.** Absorbance spectrometric assays were carried out as described in Fig. 2. Human (A), L220M/A383T mutant (B), and rat SBCAD (C) were titrated with a serial of isobutyryl-CoA concentrations as indicated in the legend for each panel. The starting concentration of each enzyme was 8.0, 8.5, and 8.2 µM, respectively.

**Fig. 4. CD spectra of wild type and mutant SBCADs.** The CD spectra of purified human and rat wild type, F105L, and V104L/F105L mutant SBCADs are shown in A. The concentration of each enzyme was 5 µM. The CD signal of a blank cuvette containing buffer was subtracted from each spectra. The subtracted CD spectra of mutant and rat SBCADs minus the wild type human SBCAD signal are shown in B. The CD spectra of oxidized form in human SBCAD and different substrate-saturated human SBCAD in the 280–500-nm wavelength range are shown in C. Free substrate does not exhibit a CD signal in the visible range. The signal of a blank cuvette containing free substrate and buffer was subtracted from the spectra of substrate/enzyme mixture.
SBCAD and F105L mutant, even though the amount of reduction of the ~450-nm maximum was similar to that obtained with (S)-2-methylbutyryl-CoA. This suggests that even though binding of the non-optimal substrate led to perturbation of the electronic environment of the flavin ring, the resultant enzyme-substrate (or product) intermediate differed from the bona fide charge-transfer complex involving the optimal substrate. Of note, when hexanoyl-CoA concentration was not excessive, the long wavelength intermediate was not observed in all the SBCADs. This implies that the enzyme-substrate complex present under these conditions was nonproductive and that substrate in the complex was in its neutral rather than anionic form. Thus the enzyme-substrate complex under these conditions appeared to be present mainly as a Michaelis-Menten rather than charge-transfer complex (Scheme 3) (51).

Fig. 3 shows the incubation of SBCADs with increasing concentrations of isobutyryl-CoA under anaerobic conditions. When isobutyryl-CoA was incubated with the various SBCADs, the change in absorbance at the ~450 nm absorbance maximum at an equimolar substrate:enzyme ratio was less than 5% of the absorbance of the free oxidized form enzyme at 450 nm (Fig. 3). Although there was significant reduction of absorbance observed at ~450 nm when isobutyryl-CoA was present in 10–50-fold excess, the development of a long wavelength maximum was either absent or minimal. This indicates that the binding of isobutyryl-CoA to the SBCADs was poor there. Interestingly, for each enzyme, it was gradual reduction of the absorption maxima upon addition of substrate at lower enzyme:substrate ratios than a sudden decrease in these maxima at a particular ratio. The discontinuity occurred at different enzyme:substrate ratios and at varying absorbance levels among different SBCADs (Fig. 3). The 450-nm maximum after the gap also shifted 10–20 nm to the blue. Such a phenomenon has not been reported previously in studies of other ACDs and suggests that the charge-transfer complex of isobutyryl-CoA with SBCADs differs from SBCADs with optimal substrate.

**CD Analysis**—Circular dichroism spectroscopy is a sensitive technique for detecting changes in protein tertiary structure. The CD signal detects the movement of amino acid residues with chiral centers including tryptophan, which dominates the 275–305-nm region of the CD spectrum (37, 53). There are only two tryptophans in SBCAD, Trp-174 and Trp-355. Both are preserved in human and rat SBCAD and are predicted to be at the surface of the protein structure. Trp-355 is located at an alpha-helix homologous to helix 1 G in other ACDs and distant from the substrate binding pocket. Mutations in the substrate binding pocket are not likely to significantly affect its orientation in the SBCAD structure and thus its CD signal. In contrast, Trp-174 is on beta-sheet 3 and makes close contact with the isoalloxazine ring of FAD. Trp-174 is also important in stabilizing the reduced flavin ring, and thus alterations in the conformation of this residue are likely to affect the catalytic rate of the enzyme. The CD maximum at 450 nm in ACDs is believed to be dominated by the signal from the flavin ring of FAD because amino acid residues lack absorbance in the visible wavelength (54, 55).

To examine the effects of the amino acid differences between the two wild type enzymes and the various SBCAD mutations on tertiary structure, we determined the CD spectra of purified wild type rat and human SBCADs, and the F105L and V104L/F105L mutant enzymes with and without bound substrate (Fig. 4). As a control, a series of SBCAD concentrations from 1.25 to 15 μM were characterized. CD signals for each enzyme were observed to be proportional to the enzyme concentration (data not shown). Comparison of the CD spectra of the SBCADs at visible wavelengths revealed that the rat and mutant V104L/F105L SBCADs had maximal signals at 450 nm. The CD signal in this wavelength range was lower for the F105L mutant and lower still in human SBCAD.

Both the FAD and protein moieties of the ACD contribute to the CD signal in the near-UV range (250–320 nm). Because the oxidized form of FAD has a predominant signal at 267 nm and no absorbance around 305 nm, the negative peak at ~270 nm in the SBCAD spectrum is likely related to FAD, whereas the area around 280–305 nm is dominated by tryptophan. It is notable that the human wild type SBCAD CD spectrum differs from the wild type rat enzyme in the near-UV range and that the spectrum of the V104L/F105L mutant is similar to that seen for the rat enzyme (Fig. 4A). The negative peak at ~270 nm and the positive peak at ~450 nm are similar for both rat SBCAD and V104L/F105L, but both spectra differ from human SBCAD, consistent with a differing FAD conformation. Although the difference in the CD spectra of rat SBCAD, V104L/F105L, and human SBCAD were dominated by the FAD signal, additional differences at ~290 nm were present. There is a shoulder at ~294 nm in the spectra of rat SBCAD, F105L, and V104L/F105L that is absent in the spectrum of human SBCAD.

Subtracting the CD spectrum of human SBCAD from the spectra of rat SBCAD, F105L, and V104L/F105L (Fig. 4B) emphasizes that the primary difference between the CD spectrum of F105L and human SBCAD is a peak at 284 nm, representing the signal from tryptophan. This supports our previous conclusion that the F105L mutation changes the conformation of Trp-174 in human SBCAD indirectly by altering beta-sheet 3 where this residue is located (31, 56). Interestingly, the wide negative peak centered at ~270 nm is similar in both rat SBCAD and double mutant V104L/F105L.

The CD spectra of SBCADs reduced by substrate were also examined. The signals in the near-UV wavelength were overwhelmed by an absorption maximum centered at 300 nm (Fig. 4C), likely caused by anionization of substrate (56). Reduced FAD may have a CD signal at 300 nm as well, but it is relatively weaker (54). These factors limited our ability to identify other changes related to conformation shifts around this wavelength. It is notable, however, that the large positive maximum at 300 nm seen when SBCAD was reduced with (S)-2-methylbutyryl-CoA was much smaller when hexanoyl-CoA was used, suggesting that substrate anionization is minimum in the SBCAD-hexanoyl-CoA complex.

Titration of wild type human SBCAD with additional (S)-2-methylbutyryl-CoA or hexanoyl-CoA beyond an excess substrate:enzyme ratio did not lead to additional changes in either the absorbance (Fig. 2) or the CD signals in the visible range (Fig. 4C and not shown). Comparing absorbance and CD spectra of different substrate-complexed SBCAD at this point, there were no significant difference between the absorbance spectra, and in contrast, the CD spectra were different. The (S)-2-methylbutyryl-CoA-reduced human SBCAD has a negative peak centered at 416 nm in visible wavelength range; however,
this peak is absent in the CD signal of hexanoyl-CoA-reduced SBCAD (Fig. 4C). Such a difference between the absorbance and CD spectra suggests a difference in the conformational shift of the reduced form of enzyme when it complexed with different substrates (25, 27, 36, 52). This could be due to alterations in the conformation of the flavin isoalloxazine ring itself or in its position relative to an amino acid residue that can alter the flavin signal. The same discrepancy in absorbance and CD spectra changes was also seen with the F105L mutant when reduced by excess hexanoyl-CoA; however, the CD signal changes for wild type rat and V104L/F105L mutant SBCADs induced by hexanoyl-CoA were minimal (data not shown). In conjunction with earlier results, these findings suggest that even though human SBCAD can be reduced by excess hexanoyl-CoA, the charge-transfer complex formed is in a conformation less able than that formed with (S)-2-methyl-butyryl-CoA to subsequently interact with or transfer electrons to ETF.

Surface Plasmon Resonance Analysis of the Interactions of SBCADs with (S)-2-Methylbutyryl-CoA and Hexanoyl-CoA—To examine substrate binding to SBCADs, we have developed a solution competition assay based on SPR measurement with a BIAcore™ 3000 instrument as described under “Experimental Procedures.” It was difficult to directly detect binding of substrate to SBCAD tetramer by SPR measurement because the molecule mass of SBCAD is about 210 times higher than its substrate, and the relatively small change in molecular mass related to substrate binding was too small to be detected (40, 58). It was also not possible to immobilize substrate to the sensor chip in a way that allowed continued recognition of native enzyme. This is likely due to the fact that substrate is almost completely buried when it binds to ACDs (59). We discovered, however, that ACBP competed with SBCAD for substrates in ETF assays (data not shown). ACBP is a relatively stable monomer of molecular mass (9.5 kDa) only 11 times higher than acyl-CoA substrate, and this subsequently allowed us to use it to determine quantitatively substrate concentration using a competitive solution SPR assay. The KD value between (S)-2-methylbutyryl-CoA and ACBP was determined to be 1.6 μM, whereas the KD value between hexanoyl-CoA and ACBP was 1.3 μM. A substrate concentration near the KD was chosen and kept constant in all subsequent experiments. The solution affinity of a SBCAD for substrate was
determined by pre-incubating increasing enzyme concentrations with the substrate at 4 °C and measuring the SPR signal (Table IV). A typical solution competition assay is shown in Fig. 5. The sensorgrams of a series concentration of hexanoyl-CoA injected over the ACBP surface are shown in Fig. 5A, and the calibration curve for calculating free hexanoyl-CoA in the enzyme/substrate mixture is shown in Fig. 5B. The plot of sensorgrams following incubation of increasing concentrations of rat SBCAD with hexanoyl-CoA is shown in Fig. 5C. The free hexanoyl-CoA concentration in the reaction mixture calculated from the calibration curve was then plotted against the concentration of rat SBCAD, and the solution affinity \( K_D \) value was calculated as described under “Experimental Procedures” (Fig. 5D). The absorbance spectra of the SBCADs with and without added substrate remained stable after 2 h or overnight incubation at 4 °C, under incubation conditions identical to those used for the BIAcore experiments, indicating that reoxidation of enzyme in the enzyme/substrate mixture was negligible (data not shown).

Table IV shows the overall binding constants of human, rat wild type SBCAD, mutant F105L, and mutant V104L/F105L with (S)-2-methylbutyryl-CoA and hexanoyl-CoA. The binding affinity of rat SBCAD and the double mutant V104L/F105L for hexanoyl-CoA was much lower than for human SBCAD (overall \( K_D \) value was 20 and 30 times higher, respectively, than for human SBCAD), whereas the binding affinity between the single F105L mutant enzyme for hexanoyl-CoA was very close to that of the human wild type enzyme. As discussed above, when substrate is in limiting or in moderate excess, the interaction between SBCADs and hexanoyl-CoA appeared to be mainly driven by Michaelis-Menten complex formation. Therefore, the binding constant between SBCADs and hexanoyl-CoA as measured with the BIAcore solution affinity assay directly reflects the binding affinity of the enzyme for substrate. Our results thus indicate that hexanoyl-CoA binds to human SBCAD better than to rat SBCAD, and in combination with our earlier findings, we conclude that this effect is mediated by amino acid residue 104. In contrast, even though the L220M/A383T mutant enzyme showed greater enzymatic activity and had a lower \( K_m \) value than wild type human SBCAD as measured with the ETF reduction assay, the overall \( K_D \) value of this enzyme for hexanoyl-CoA directly measured with the BIAcore solution affinity technique was similar to that obtained with wild type human SBCAD (data not shown). Thus, the L220M/A383T mutations do not appear to affect the binding of hexanoyl-CoA. Rather, the alteration in function of this mutant.

![Molecular models of the residues studied for substrate specificity in human SBCAD models.](image)
must be related to another part of the enzyme mechanism.

When the optimum substrate (S)-2-methylbutyryl-CoA reacts with SBCAD without a secondary electron acceptor present, both Michaelis-Menten ($E_{\text{cat}}$) and charge-transfer complexes (I, productive intermediate) may coexist in the reaction mixture (Scheme 3). Therefore, the overall binding constant as measured with the BIAcore solution assay would be affected not only by the substrate binding constant but also the on and off rates of the $E_{\text{cat}}$ turning to I as described in Scheme 3 (57).

It is notable that the overall $K_D$ values of V104L/F105L and F105L toward (S)-2-methylbutyryl-CoA as measured with the BIAcore assay were higher than for wild type SBCADs, whereas the $K_D$ values of rat and human SBCADs toward (S)-2-methylbutyryl-CoA were very close to each other (Table IV). Previous studies have shown that the reductive half-reaction is the rate-limiting step in the ACD dehydrogenation reaction (Scheme 1) when an acyl-CoA dehydrogenase reacts with its optimum substrate (61, 62). Because both the V104L/F105L and F105L mutants exhibit stabilization of the reduced flavin ring of FAD and increased catalytic rates for the overall reaction, the turnover rate for these two mutants of $E_{\text{cat}}$ to I should remain at least as fast as in the wild type enzyme (Table II). Thus, substrate binding is likely the main factor contributing to the differences seen in the reactions between the V104L/F105L, F105L, and wild type enzymes with (S)-2-methylbutyryl-CoA. The elevated overall $K_D$ value observed therefore must reflect the binding of substrate rather than the rate of $E_{\text{cat}}$ turning into $I$.

The overall $K_D$ value of F105L and V104L/F105L with (S)-2-methylbutyryl-CoA as determined with the BIAcore assay and the $K_v$ value for the full reaction as measured with the ETF assay are similar. This supports the hypothesis that the rate-limiting step in catalysis of (S)-2-methylbutyryl-CoA for these mutant enzymes is the binding of substrate by enzyme. The overall $K_D$ values of wild type human and rat SBCAD toward 2-methylbutyryl-CoA were lower than the $K_v$ value measured with the ETF assay. This is likely due to the low sensitivity of the ETF assay when substrate is in very low concentration (nanomolar). The dissociation constant determined by the BIAcore solution affinity assay of human wild type SBCAD with its product, tiglyl-CoA, was 0.47 ± 0.01 μM, very close to its affinity for substrate (S)-2-methylbutyryl-CoA. This is also seen with other ACDs with their product (62).

It should be noted that our BIAcore assay failed to detect consistent binding of isobutyryl-CoA to wild type human and rat SBCAD or the L220M/A383T mutant enzyme. This suggests that the binding between SBCADs and isobutyryl-CoA is too weak to compete with the acyl-CoA-binding protein in a measurable concentration range. We are currently developing new capture ligands to better demonstrate the interaction of suboptimal substrates with SBCAD.

**Molecular Modeling**—To examine further the structural basis for our findings, the mutant residues were introduced into the SBCAD sequence, and their models were made using the homology module of the Insight II software as described under “Experimental Procedures” (Fig. 6). Replacement of Val-104 and Phe-105 in human SBCAD with Leu-104 and Leu-105 as found in the rat enzyme significantly reduced the binding affinity of the enzyme toward hexanoyl-CoA. It appears that position 104 is more important in mediating this difference because the F105L mutation did not affect the binding of hexanoyl-CoA. Molecular modeling predicts that amino acids 104 and 105 are at the bottom of the substrate binding pocket (Fig. 6A and C). Substitution of the longer leucine side chain for a valine in position 104 is predicted to make the bottom of the binding pocket more shallow (2.7 versus 5.3 Å), thus interfering with the binding of substrates with longer primary carbon chain such as hexanoyl-CoA. Because substitution of V104L and F105L also affected the binding of (S)-2-methylbutyryl-CoA to enzyme, it appears that the binding pocket may not only become shallower but also narrower. In this regard, our SBCAD model predicts that a longer leucine side chain at position 104 would be closer than a valine to Tyr-380 (3.2 versus 4.5 Å). The latter is 3.9 Å away from C-2 of the substrate and movement to accommodate Leu-104 would reduce the distance between Tyr-380 and C-2 of the substrate to 3.6 Å. This would narrow the substrate binding pocket (Fig. 6, A-C). This agrees with the slightly elevated $K_D$ value seen for binding of (S)-2-methylbutyryl-CoA to the V104L/F105L mutant enzyme. In wild type rat SBCAD, the combined effect of V104L/F105L leading to a narrowing of the substrate binding pocket (Fig. 6, A-C) is offset by the widening effect of the L220M/L222I/A383T residues, and thus its binding affinity toward (S)-2-methylbutyryl-CoA is comparable with the human enzyme.

The change of V104L/F105L also potentially allows two extra H-bonds to form between Asp-151/Ser-150 and the adenine group of the FAD (not shown). This agrees with the observation that the FAD signals in CD spectra of the oxidized form of rat...
wild type and mutant V104L/F105L SBCADs are similar, although they are slightly different from those of human wild type and F105L mutant, especially at wavelength ~ 270 nm. Interestingly, the catalytic efficiency of V104L/F105L toward its optimal substrate was elevated compared with the mutant F105L and wild type human SBCAD. We propose that this further reflects the indirect effect of V104L/F105L on FAD conformation, as the additional hydrogen bond with FAD could also stabilize the coordination between the isoalloxazine ring of FAD and Tyr-380 and Trp-174, the latter stabilizing the reduced form of the flavin ring. This in turn would lead to more rapid extraction of electrons from substrate (35).

Substitution of F105L did not affect hexanoyl-CoA binding. Our studies indicate, however, that it is important in adjusting the conformation of \( \beta \)-sheet 3. Amino acid residues on \( \beta \)-sheet 3, especially Trp-174, are not only important in coordinating the isoalloxazine ring of FAD but are also involved in transfer of electrons to ETF and in protecting the reduced FAD from molecular oxygen in other ACDs (35, 43, 59). In our SBCAD model, Phe-105 is in close proximity to Ser-177, the residue at the end of \( \beta \)-sheet 3. Alteration of F105L reduces its distance to Ser-177 from 3.7 to 3.6 Å (Fig. 6C). More significantly, the distance between Trp-174 and FAD correspondingly changed from 3.0 Å in human wild type to 3.4 Å in F105L and 3.5 Å in V104L/F105L mutant SBCAD (Fig. 6, A–C). This demonstrates that substitution of F105L shifts the position of Ser-177 and changes the conformation of \( \beta \)-sheet 3 including residue Trp-174 and Ser-176. The CD signal change in the Leu-105 mutants supports this hypothesis. Because both Ser-176 and Trp-174 are predicted to be involved in a series of hydrogen bonds with the isoalloxazine ring of FAD, changes in these residues should also alter the CD signal related to FAD. This was in fact seen. Overall, our data and modeling indicate that amino acid residue 104, located at the bottom of the substrate binding pocket, directly influences the length of primary chain of substrate that can be utilized by SBCAD. They also highlight that the conformation of \( \beta \)-sheet 3 is important in optimizing the electron extracting and transferring functions of the enzyme, therefore affecting the overall turnover rate.

Replacement of L220M or L222I, together with A383T enhanced the activity of human SBCAD toward isobutyryl-CoA, mimicking its rat counterpart. Our studies suggest, however, that the binding affinity between isobutyryl-CoA and SBCADs is very low. The difference in the catalytic efficiency between human and rat SBCAD utilizing isobutyryl-CoA was only detected when substrate was present in great excess. This suggests that binding of isobutyryl-CoA is not likely the main contributor to the difference in catalytic efficiency of the overall reaction with this substrate. In the SBCAD model, Ala-383 is predicted to be near the hydroxyl group of the ribose ring of adenine group in FAD, whereas Leu-222 is in approximation to the end of the isoalloxazine ring (Fig. 6, D and E), with two apparent hydrogen bonds connecting substrate and FAD in between. The A383T replacement would allow a hydrogen bond to form with the hydroxyl group on the ribose ring of FAD, moving the adenine group of FAD slightly closer to the catalytic base. Because the two hydrogen bonds between FAD and substrate remain unchanged in the mutant (Fig. 6E), the other end of the FAD molecule (the isoalloxazine ring) might consequently be positioned further from the catalytic base. Alteration of L222I increases its distance from the bottom of the flavin ring from 3.4 to 4.2 Å. Leu-220 is on the same protein backbone turn as Leu-222, and the L220M alteration is predicted to indirectly increase the distance between Leu-222 and the isoalloxazine ring by altering the conformation of this turn. The combination of A383T and L222I or L220M would increase the space between the isoalloxazine ring and the catalytic base, potentially altering the capacity to utilize suboptimal substrates, especially isobutyryl-CoA. The A383T alteration alone, however, would not widen the opening of the substrate binding pocket and thus it would not be predicted to increase the accessibility of the substrate binding pocket for isobutyryl-CoA. This agrees with the observation that mutants A383T/L222I and A383T/L220M utilized suboptimal substrates better than the wild type enzyme without affecting their binding affinity.

The amino acid residues hypothesized to be important for substrate specificity in all of the known ACDs are compared in Table V. Tyr-380 is conserved in all the ACDs that efficiently use straight chain acyl-CoA substrates, including human SCAD, MCAD, LCAD, and SBCAD but not in the ACDs with predominant branched chain substrate specificity (IVD and IBD).

Comparison of the known structures of SCAD, MCAD, IVD, and our SBCAD model (Fig. 7) reveals that the conformation of Tyr-367 in SCAD and Tyr-375 in MCAD are very similar. In both proteins, the aromatic rings of the homologous tyrosine residues are vertical to the isoalloxazine ring of FAD, a position relative to the catalytic base that makes binding of branch chain substrate unfavorable. The aromatic ring of Tyr-380 in SBCAD, however, is predicted to be more parallel to the flavin ring of FAD, oriented ~ 45° away from the position in MCAD and SCAD. This would allow more space near the catalytic base Glu-381 for accommodation of substrates with a methyl group at C-2. Notably, our model does not allow a branched chain at C-3 to fit well. In IVD and IBD, the bulky side chain of tyrosine is replaced by a glycine or leucine with a smaller or more flexible side chain, respectively, again leading to enough space adjacent to the FAD to accommodate branched chain substrates.

The residues homologous to SBCAD Trp-174 are similar in all the ACDs. This residue is a tryptophan in human SBCAD, SCAD, IVD, MCAD, whereas it is a phenylalanine in human LCAD, IBD, VLCAD, and ACAD-9. Our data indicates that Trp-174 plays a role in determining the overall catalytic rate of SBCAD. It has been suggested previously that Trp-174 is not only involved in stabilizing reduced FAD but may also play an important role in transferring electrons to ETF. The conformation of Trp-174 is similar in the three published ACD structures and is predicted to be similar in our SBCAD model. Interestingly, Asn-177 is conserved in all ACDs (including rat SBCAD) except human IBD and SBCAD, which have a glycine and serine, respectively, at this position. We hypothesize that the position of Trp-174 in the human SBCAD structure is secondarily altered by the serine at position 177, thus leading to altered substrate utilization. We predict that Gly-172 in human IBD may similarly affect the position of Phe-169.

Because the conformation of the bottom of the substrate binding pocket is slightly different in the known ACD structures, it is not surprising, therefore, that Val-104 and Phe-105, which are predicted to be in this position in SBCAD, are not well conserved among the other ACDs (Table V). The position corresponding to SBCAD 383 is a threomine in most ACDs but not human SBCAD and IBD, whereas Leu-220 and Leu-222 are not conserved among the ACDs. These positions are on opposite ends of the FAD isoalloxazine ring, and because Thr-383 is probably relatively flexible, alteration of the residues at Leu-222 or Leu-220 could lead to alteration in the distance between the flavin ring and catalytic base, and thus change substrate specificity.

DISCUSSION

Through a combination of techniques and systematic mutagenesis studies of amino acid residues in the SBCAD substrate binding pocket, we have demonstrated that relatively
few amino acid residues are critical for determining the difference in substrate specificity seen between the human and rat enzymes, and that alteration of these residues affects different portions of the enzyme mechanism.

We have developed a new technique based on SPR for measuring binding of acyl-CoA substrates to acyl-CoA dehydrogenases. Importantly, the solution affinity BIAcore assay has allowed us to study the reaction between enzyme and substrate under conditions of relatively limiting or moderately excessive substrate concentrations rather than vast substrate excess as is typical of other methods. This allows a closer approximation to physiological conditions, because reaction between ACD and its substrate is multiphasic when substrate concentration is excessive (Scheme 2) (26). Previous attempts to accomplish this by equilibrium dialysis were unsuccessful as the method proved not to be sensitive enough to generate reliable results. In contrast the high sensitivity of the BIAcore™ 3000 system makes it possible for us to measure an overall binding constant.


to the gene family and the structural determinants that influence should provide valuable insight into the catalytic mechanism in

plexes between ACDs and non-optimal substrate often lack

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