Structural and Thermodynamic Basis for Weak Interactions between Dihydrolipoamide Dehydrogenase and Subunit-binding Domain of the Branched-chain α-Ketoacid Dehydrogenase Complex

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The purified mammalian branched-chain α-ketoacid dehydrogenase complex (BCKDC), which catalyzes the oxidative decarboxylation of branched-chain α-keto acids, is essentially devoid of the constituent dihydrolipoamide dehydrogenase component (E3). The absence of E3 is associated with the low affinity of the subunit-binding domain of human BCKDC (hSBDb) for hE3. In this work, sequence alignments of hSBDb with the E3-binding domain (E3BD) of the mammalian pyruvate dehydrogenase complex show that hSBDb has an arginine at position 118, where E3BD features an asparagine. Substitution of Arg-118 with an asparagine increases the binding affinity of the R118N hSBDb variant (designated hSBDb*) for hE3 by nearly 2 orders of magnitude. The enthalpy of the binding reaction changes from endothermic with the wild-type hSBDb to exothermic with the hSBDb* variant. This higher affinity interaction allowed the determination of the crystal structure of the hE3/hSBDb* complex to 2.4Å resolution. The structure showed that the presence of Arg-118 poses a unique, possibly steric and/or electrostatic incompatibility that could impede E3 interactions with the wild-type hSBDb. Compared with the E3/E3BD structure, the hE3/hSBDb* structure has a smaller interfacial area. Solution NMR data corroborated the interactions of hE3 with Arg-118 and Asn-118 in wild-type hSBDb and mutant hSBDb*, respectively. The NMR results also showed that the interface between hSBDb and hE3 does not change significantly from hSBDb to hSBDb*. Taken together, our results represent a starting point for explaining the long standing enigma that the E2b core of the BCKDC binds E3 far more weakly relative to other α-ketoacid dehydrogenase complexes.

The mitochondrial α-ketoacid dehydrogenase complexes include the pyruvate dehydrogenase complex (PDC),3 the α-ketoglutarate dehydrogenase complex and the branched-chain α-ketoacid dehydrogenase complex (BCKDC) (1, 2). These macromolecular protein complexes catalyze the oxidative decarboxylation of α-keto acids according to Reaction 1.

\[
\text{RCOOOH + NAD}^+ + \text{CoASH} \rightarrow \text{RCOSCoA + CO}_2 + \uparrow + \text{NADH + H}^+ 
\] REACTION 1

Each α-ketoacid dehydrogenase complex consists of three catalytic components: a thiamine diphosphate-dependent decarboxylase/dehydrogenase (E1), a lipoyl transacylase (E2), and dihydrolipoamide dehydrogenase (E3). The overall reaction is the sum of individual reactions catalyzed by the three enzyme components, which are linked by substrate channeling. The α-ketoacid dehydrogenase complexes are organized around a symmetrical oligomeric E2 core, to which the E1 and E3 components are attached via noncovalent interactions. The E1 and E2 components are specific for each α-ketoacid dehydrogenase complex, whereas the E3 component is common among the three multienzyme complexes. The E1p, E1k, and E1b components (with the lowercase letter corresponding to each complex), similarly the E2p, E2k, and E2b components, belong to PDC, α-ketoglutarate dehydrogenase complex, and BCKDC, respectively. Both E1 and E3 components bind in a mutually exclusive manner to the subunit-binding domains (SBD) from the 24-meric E2 cores of α-ketoglutarate dehydrogenase complex and BCKDC and most bacterial PDCs. However, in yeast and mammalian PDCs, E3 is tethered to the E3-binding domain (E3BD) of the specialized E3-binding protein (E3BP), the latter being a constituent component of the 60-meric E2p core. The

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The atomic coordinates and structure factors (code 3RNM) 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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4 The abbreviations used are: PDC, pyruvate dehydrogenase complex; BCKDC, branched-chain α-ketoacid dehydrogenase complex; E1, α-ketoacid dehydrogenase/decarboxylase; E1p, E1 component of PDC; E2, dihydrolipoyl transacylase; E2b, E2 component of BCKDC; E3, dihydrolipoamide dehydrogenase; E3*, S456A mutant E3; E3BD, E3-binding domain of PDC; SBD, subunit-binding domain; hSBDb, subunit-binding domain of human BCKDC; hSBDb*, R118N variant of wild-type hSBDb; SBDp, SBD of PDC; hDDb, di-domain for human BCKDC; hDDb*, hDDb variant harboring the R118N mutation in hSBDb (hSBDb*); CHES, 2-(N-cyclohexylamino)ethane sulfonic acid; E3BP, E3-binding protein; r.m.s., root mean square; HSQC, heteronuclear single quantum coherence.
E1p component of yeast and mammalian PDC binds to the SBDb of the E2p core, thereby avoiding competition with E3 for binding to the same domain.

The E3 component is a flavoenzyme that re-oxidizes the reduced lipoyl moiety on E2 in the oxidative decarboxylation cycle of α-keto acids (Reaction 1) catalyzed by the α-ketoacid dehydrogenase complexes (1). E3 binds tightly to the E3BD of mammalian PDC (3) and SBDb of bacterial PDC (4). By contrast, E3 is only loosely associated with the SBDb of the mammalian BCKDC, as indicated by the absence of E3 in highly purified BCKDC preparations (5–7). The low-affinity binding of E3 to the E2b core explains the loss of most BCKDC activity during the purification of bovine BCKDC at high salt concentrations (8); BCKDC activity can be recovered by the addition of exogenous E3 to the assay mixture (5). The physiological significance of the unique weak binding of E3 to the E2b core of BCKDC is unclear. Because E1b binds the SBDb of BCKDC with high affinity, it has an advantage over E3 for binding to the E2b core. The increased number of dephosphorylated E1b molecules recruited to the E2b core may result in the positive regulation of BCKDC activity (9).

The structure of the SBDb of E2p from the Geobacillus stearothermophilus (formerly Bacillus stearothermophilus) PDC bound to its cognate E3 shows that a single SBDb binds near to the 2-fold axis of the E3 homodimer (10). This mode of binding sterically excludes the binding of a second SBDb. In this Geobacillus SBDb/E3 complex, the interaction between the two proteins consists largely of electrostatic interactions between one α-helix of the SBDb and surface residues of E3. An SBDb/E1p complex structure of Geobacillus PDC demonstrates that the electrostatic nature of the SBDb/E3 interface is conserved, but much less surface area is buried at the SBDb/E1p interface than that of the SBDb/E3 (11). The structure of the E3/ SBDb complex of human PDC has also been determined (3, 12). The human E3/ SBDb structure shows that residues from E3 BD contact E3 across its 2-fold axis, resulting in overlapping SBDb binding sites on the homodimer. Consequently, the binding of E3BD to E3 sterically forbids the binding of a second ligand (3, 13, 14). Negligible conformational changes occur in E3BD upon its high-affinity binding to E3. Modifications of E3BD residues at the center of the E3BD/E3 interface impede E3 binding far more severely than those of residues on the periphery, validating the "hot spot" paradigm for protein interactions (3, 15); it is, however, interesting to note that the entire surface of E3 BD in contact with E3 serves as a hot spot, given the strict definition of that phenomenon (i.e. ΔG ≥ 2.0 kcal/mol) (16).

In the present study, we address the structural basis for weak binding of E3 to the E2b core of the mammalian BCKDC. The sequence of the hSBDb of the human BCKDC was aligned with cognate binding-domain sequences of the different α-ketoacid dehydrogenase complexes; the results show that the amino acid at position 118 of hSBDb is arginine, whereas the amino acid at the equivalent position in E3BD and other SBDbS with high binding affinities for E3 is occupied by an asparagine. Substitution of Arg-118 with an asparagine increased the binding affinity of the R118N hSBDb variant (designated hSBDb*) for E3 by about 70-fold. The high-affinity interactions between E3 and hSBDb* allowed the determination of the crystal structure of the E3/ hSBDb* complex to 2.4-Å resolution. The structure revealed that the presence of an arginine residue at position 118 poses a possible steric and/or electrostatic repulsion that hampers the interactions of E3 with the wild-type SBDb. Compared with the E3/E3BD (3) and E1/GsSBDb (10) structures, the E3/hSBDb* structure displays a smaller buried surface area and a specific set of interacting amino acid residues at the E3/hSBDb interface. Solution NMR data confirmed the hSBDb*/E3 interface that we observed in the crystal structure, and also indicated that this interface is not radically different in the hSBDb/hE3 complex. Structure-informed mutagenesis and calorimetry were used to dissect the nature of the interface. The hSBDb and hSBDb* constructs were found to have divergent mechanisms for hE3 association. The distinct mode of interactions of the hSBDb with E3 helps to account for the decades-old observation of markedly weaker E3 binding to the E2b core of BCKDC relative to other α-ketoacid dehydrogenase complexes.

EXPERIMENTAL PROCEDURES

Protein Expression, Purification, and Site-directed Mutagenesis—Recombinant hE3 protein was expressed and purified as previously described (17). Additionally a S456A point mutant, referred hereafter as hE3*, was created by site-directed mutagenesis as described below. To facilitate expression, the human subunit-binding domain (hSBDb) was fused with the lipoyl-bearing domain, with the linker region containing a tobacco etch virus protease-recognition site. This construct is referred to as the di-domain (hDDB) and contains the entire SBDb amino acid sequence from Gly-103 to Thr-152 of the hE2b protein. Two additional vector-derived amino acids (Leu-Glu) and a C-terminal His6 tag were included for purification purposes. The tobacco etch virus site (LENLYFQ ↓ G with the arrow showing the cleavage site) was introduced immediately N-terminal of the Gly-103 residue. The expression and purification of the hDDB construct was described previously (18). The hDDB fusion protein was digested with the tobacco etch virus protease followed by re-extraction with nickel-nitrilotriacetic acid resin. The resultant hSBDb protein was further purified on a HiLoad Sephadex 75 size-exclusion column that had been equilibrated with a buffer containing 50 mM potassium phosphate (pH 7.5), 100 mM KCl, and 5% (v/v) glycerol.

2H (and 15N) isotope enrichment of various samples for NMR study was achieved by growing the bacterial culture in minimal M9 media prepared with 99.9% 2H2O, and, if necessary, supplemented with 15N-NH4Cl. The purification of these samples was performed with standard protocols described above using buffers made with regular water leading to restoration of protons at labile amide positions.

Site-directed mutations were introduced into the hDDB and hE3 constructs using the QuikChange kit (Strategene, La Jolla, CA), according to the manufacturer’s instructions. The hDDB variant harboring the R118N substitution in SBDb is referred to as hDDB*. All mutations were confirmed by DNA sequencing. The above procedures for producing wild-type hSBDb were employed to obtain the hSBDb* variant.
**E3 Binding to BCKDC Subunit-binding Domain**

*Isothermal Titration Calorimetry*—The wild-type hDDb, hDDb*, and hE3 proteins were dialyzed exhaustively against a buffer containing 50 mM potassium phosphate (pH 7.5) and 100 mM KCl. 250 mM hDDb, hDDb*, hSBDb, or hSBDb* protein in a syringe was injected in 8-μl increments into the reaction cell holding 1.43 ml of 20 μM hE3 (based on the homodimer) at 20 °C in a VP-ITC microcalorimeter (MicroCal, Northampton, MA). Binding constants (K_d) and enthalpy (ΔHf) were derived by fitting a one-site binding model to the isothermal titration calorimetry binding isotherm using the ORIGIN version 7.0 software (MicroCal). The concentrations of hDDb and hSBDb were determined by absorbance at 280 nm using the calculated extinction coefficients (M⁻¹ cm⁻¹) were determined by absorbance at 280 nm using the calculated software (MicroCal). The concentrations of hDDb and hSBDb were determined by absorbance at 280 nm using the calculated extinction coefficients (M⁻¹ cm⁻¹) of 15,930 and 1,490, respectively. For E3, the absorbance of the flavin chromophore at 450 nm (ε_{450 nm} = 10,325 M⁻¹ cm⁻¹) was monitored. In addition, E3 was precipitated using trichloroacetic acid (TCA), washed with TCA, then dissolved in guanidinium chloride; the absorbance of E3 at 278 nm (ε_{278 nm} = 23,971 M⁻¹ cm⁻¹) was used to determine the concentration of E3.

**Crystallization of the hE3/hSBDb* Complex**—The hE3/hSBDb* complex was obtained by mixing the hE3 protein with the hSBDb* protein in a 1:2 molar ratio. The mixture was repeatedly diluted and concentrated in 20 mM HEPES (pH 7.5) and 20 mM β-mercaptoethanol in a 15-ml concentrator with a 30,000-Da cut off. The final concentration of the hE3/hSBDb* complex was 20 mg/ml. Crystals were obtained using the vapor-diffusion method at 20 °C by mixing 1.5 μl of the hE3/hSBDb* complex with 1.5 μl of well solution containing 18% (w/v) polyethylene glycol (PEG) 3350 and 0.1 mM CHES (pH 9.1). Crystals were cryoprotected in 20% (w/v) PEG 3350 and 0.1M CHES (pH 9.1), and 20% (v/v) ethylene glycol and flash-cooled by plunging into liquid nitrogen.

**X-ray Crystallography of the hSBDb*/E3 Complex**—Crystals of the hSBDb* complexed to hE3 exhibited the symmetry of space group P2₁, with approximate unit cell dimensions of a = 84.1 Å, b = 112.3 Å, c = 122.5 Å, and the unit cell angle β = 91.4°. X-ray diffraction data were acquired from a single hE3/hSBDb* crystal using beamline 19-ID at the Advanced Photon Source of Argonne National Laboratories, Argonne, IL. The data were indexed, integrated, and scaled with HKL3000 (19). Data processing statistics are found in Table 1. The data were processed to a d_min spacing of 2.4 Å. The phase problem was solved using molecular replacement. The search model was derived from the structure of hE3 that had NAD⁺ bound (13) (PDB accession code 1ZMC, which contains four hE3 homodimers). All substrates, cofactors, and water molecules were manually removed from this model, and only the AB homodimer of hE3 was used as the search model in Phaser (20, 21).

Two hE3 homodimers (i.e. four monomers) were located in the asymmetric unit of the hE3/hSBDb* crystals. The four monomers were designated A, B, C, and D. Therefore, the homodimers are called by the compound of their respective chain names: the “AB” dimer and the “CD” dimer. Electron density for the FAD cofactors was apparent. Moreover, associated with the AB homodimer was electron density consistent with hSBDb*; this protein chain was designated “E.” A de novo model of hSBDb* was built into the density using Coot (22), and the FAD cofactors were placed in their respective densities using the same program. Following an initial round of refinement in PHENIX (23), clear electron density for a molecule of hSBDb* bound to the CD homodimer was also observed. Protein chain E was used as a template to model this new density, and modifications were made as necessary. The new chain was designated “F.” No further copies of hE3 or hSBDb* were found. Thus, the total contents of the asymmetric unit are two homodimers of hE3 and two copies of hSBDb*, plus associated water molecules and buffer components. Each hE3 homodimer has one copy of hSBDb* associated with it. Iterative model building with Coot and refinement using the simulated-annealing, positional, translation libration screw, and isotropic individual B-factor refinement protocols available in PHENIX yielded the final model. There are significant portions of the hSBDb* not visible in the final difference electron density maps. Using the amino acid numbering of the mature E2b, chain E contains residues 110 through 152, two vector-derived residues, and the entire C-terminal His6 tag. The electron density for chain F in general is poorer than that for chain E, and only residues 111–152 plus one vector-derived residue are modeled; for many of these, the side chains are not visible in difference electron density maps. These maps reveal the presence of four molecules of CHES buffer associated with the proteins, and they are included in the final model (not shown). The final working and free R-values for the model are 0.175 and 0.232, respectively; further statistics regarding the quality of the

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**TABLE 1 Crystallographic and refinement data for the crystal structure presented in this work**

| Parameter          | Value               |
|--------------------|---------------------|
| Space group        | P2₁                 |
| Unit cell dimensions (Å) | 84.1, 112.3, 122.5, 91.4° |
| Resolution (Å)    | 44.9–2.4            |
| Completeness (%)   | 100 (99.9)          |
| Multiplicity       | 4.4 (2.4)           |
| Unique reflections | 88,688 (3,399)      |
| R_{cryst}          | 0.047 (0.649)       |
| I/σ(I)             | 21 (2.1)            |
| Wilson B (Å²)      | 39.5                |
| Average B-Factors  |                     |
| Non-solvent (Å²)   | 47.5                |
| Solvent            | 40.2                |
| R-values           |                     |
| R_{cryst}          | 0.181               |
| R_{free}           | 0.239               |
| Ramachandran statistics^a | 0    |
| Outliers (%)       | 96.4                |
| Most favored region (%) | 0.008             |
| R.m.s. deviations  |                     |
| Bonds (Å)          | 1.1                 |
| Angles (°)         |                     |

^a Values in parentheses are for the highest resolution shell.

^b R_{cryst} = Σ_{h,i} |F_{h,i}| - |F_{obs}| / Σ_{h,i} |F_{obs}|, where the outer sum (h) is over the unique reflections and the inner sum (i) is over the set of independent observations of each unique reflection.

^c From MolProbity (35).
model are found in Table 1. Molecular graphics figures were made using PyMol version 1.2 (DeLano Scientific LLC).

**Solution NMR Methods**—NMR data were acquired in Shigemi NMR tubes on a 500- or 800-MHz Bruker NMR spectrometer using the pulse programs with the TROSY enhancement scheme (24) provided with Topspin version 2.1 software (Bruker). The data were further analyzed using the Sparky version 3.112 software (25). Two-dimensional TROSY-HSQC spectra of 0.5 mM 2H,15N-hSBDb* and its complex with 1 mM 2H-hE3* were acquired at 12 °C in pH 7.5 buffer containing 50 mM potassium phosphate, 100 mM KCl, 5% (v/v) glycerol, and 7% (v/v) D2O. Spectra of hSBDb and its complexes with hE3* were obtained under identical conditions except in the same buffer containing an additional 800 mM NaCl. The TROSY cross-saturation transfer experiments (26) were performed on samples similar to mentioned above except with complexes prepared using unlabeled hE3*. The cross-saturation transfer was performed using 68.2 Hz band-selective inversion pulse centered at −0.4 ppm for a total saturation period of 1.5 s. The reference spectrum was acquired by moving the inversion pulse to −10 ppm. Although hSBDb* and hSBDb samples used in this study were highly perdeuterated, except at the amide positions, the dielectric of the protein was taken as 1, and that of solution was 80. The concentrations of monovalent ions were held at 0.15 M. APBS was used through the Plugin provided with PyMol version 1.2.

**Electrostatics Calculations**—Calculations regarding the electrostatic surface potential of hE3 were carried out using APBS (27). The dielectric of the protein was taken as 1, and that of solution was 80. The concentrations of monovalent ions were held at 0.15 M. APBS was used through the Plugin provided with PyMol version 1.2.

**RESULTS AND DISCUSSION**

The Binding of hSBDb and hSBDb* to hE3—In a preliminary investigation, the binding of hSBDb to hE3 was studied using isothermal titration calorimetry (Fig. 1A). In all of the calorimetric experiments in this paper, we used a protein construct that contained both a lipoyl-bearing domain and the hSBDb; for simplicity, we simply refer to this construct as hSBDb.

In a preliminary investigation, the binding of hSBDb to hE3 was studied using isothermal titration calorimetry (Fig. 1A). In all of the calorimetric experiments in this paper, we used a protein construct that contained both a lipoyl-bearing domain and the hSBDb; for simplicity, we simply refer to this construct as hSBDb. However, some experiments were performed with the isolated domain, and it was found to have identical binding characteristics to the larger construct (not shown). We found that hSBDb bound to hE3 weakly and exhibited an equilibrium dissociation constant (Kd) of 4.5 μM (Table 2). Given the absence of E3 in the purified native mammalian BCKDC, the observed modest affinity was expected. For comparison, the hE3-binding domain (hE3BD) of the hE3-binding protein (hE3BP) binds to hE3 with a subnanomolar Kd (~80 pm) (3). The positive ΔH of the interaction (+6.2 kcal/mol) indicated an endothermic interaction;
E3 Binding to BCKDC Subunit-binding Domain

thus, this association is made favorable by a significant positive entropy (+44.2 cal/mol-K) of binding (Table 2). These experiments were carried out in a phosphate-containing buffer (see “Experimental Procedures”). Given that this buffer has a very low heat of ionization (−1.4 kcal/mol), it is not likely that the positive observed ΔH is due to buffer ionization effects, and thus it is probably close to the intrinsic heat of association. The data indicated that the molar ratio of the interaction between hSBDb and the hE3 homodimer is 1:1 (Fig. 1A). Because hE3 is known to exist as a homodimer in solution (3, 13), the true stoichiometry of the interaction is therefore 1:1. This stoichiometry has been observed before in other binding-domain interactions with hE3 and hE1 (3, 13).

As a first step in rationalizing the differences in the hE3-binding activities of hE3BD and hSBDb, we aligned the amino acid sequences of these binding domains (Fig. 2). In the hE3BD/hE3 complex, 12 residues are known to contact hE3 (3). The alignment reveals that the equivalent residues in hSBDb are not all identical. Indeed, at seven of the 12 equivalent positions in hSBDb, the amino acids are different (Fig. 2).

To ascertain whether any one of these amino acid differences between hE3BD and hSBDb could account for their divergent hE3-binding characteristics, we constructed seven mutants of hSBDb, each harboring a single amino acid change to the equivalent hE3BD residue. For example, we changed Leu-111 of hSBDb to arginine, which is the amino acid in the equivalent position in hE3BD. To distinguish residues of hSBDb from those of hE3 in this text, we append the abbreviation “bd” to the end of the amino acid designation. Thus, the leucine residue mentioned above shall be referred to as L111bd, and the mutant construct is L111Rbd. The seven mutant proteins are shown in Table 2. Most of the effects on Kd were small, in the 3–4-fold range, and K135Pbd showed 13-fold weakened binding.

One such mutant, however, had a dramatically positive effect on the Kd of hSBDb binding to hE3. The mutation of R118bd to asparagine resulted in a 70-fold decrease in Kd (to 68 nM) compared with the wild-type hSBDb. Furthermore, enthalpy of the R118Nbd-hE3 interaction is negative (ΔH = −5.2 kcal/mol), opposite that of the hSBDb-hE3 association. The entropic contribution of the interaction remains positive, but diminished (ΔS = +14.5 cal/mol-K). In the hE3BD/hE3 structure, the amide group of Asn-137 (the equivalent of position 118 in hSBDb) stacks on the guanidinium group of Arg-447 of hE3. This interaction is crucial to the association of hE3 and hE3BD; mutation of Asn-137 to alanine abrogates their association (3).

Although a similar contact is not made between R118Nbd and hE3 (see below), we concluded that R118bd is a unique, key determinant of the rather weak association between hSBDb and hE3. Hereafter, we refer to the R118Nbd construct as hSBDb*.

The Crystal Structure of Bound hSBDb*—We took advantage of the drastically tighter binding of hSBDb* to hE3 and grew crystals of the hE3/hSBDb* complex. The structure was determined using molecular replacement and refined at 2.4-Å resolution (see “Experimental Procedures” and Table 1). In this structure, a single hSBDb* binds to a homodimer of hE3. There are two hE3 homodimers (termed AB and CD here) present in the asymmetric unit of these crystals, each with a crystallographically independent hSBDb* bound. The overall folds of the two hSBDb* are nearly identical (Table 3). Because the electron density of the hSBDb* bound to the AB hE3 homodimer is significantly better, we confine our discussion of the structure to this protein chain, denoted as “hSBDb*AB” hereafter. Also, where it is necessary to distinguish them, we shall refer to the two different hE3 homodimers in the crystalline asymmetric unit as “hE3AB” and “hE3CD.”

Fifty-one amino acids were modeled for hSBDb*AB. As shown in Fig. 3A, near to its N terminus is a regular secondary structure that we named Loop 0 (L0; residues 110–113). Immediately following L0 is an α-helix termed H1

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### Table 3

| Protein Compared with | R.m.s. deviation | No. comparable Cn values |
|----------------------|-----------------|--------------------------|
| Complexed hSBDb*CD   | Complexed hSBDb*AB | 0.5          | 43   |
| Free hSBDb*          | Complexed hSBDb*AB | 1.1          | 36   |
| Free hE3BD           | Complexed hSBDb*AB | 1.5          | 45   |
| Complexed hE3BD      | Complexed hSBDb*AB | 1.3          | 42   |
| Complexed hSBDbp     | Complexed hSBDb*AB | 1.1          | 42   |
| Complexed pSBDbp     | Complexed hSBDb*AB | 1.1          | 40   |
| Complexed EcSBDbk    | Complexed hSBDb*AB | 1.2          | 33   |
| Free hE3             | hE3 in the hE3/hSBDb*AB complex | 0.4 | 943 |
| hE3 in the hE3/hE3BD complex | hE3 in the hE3/hSBDb*AB complex | 0.4 | 943 |
(residues 114–122). After H1 is L1, a loop that comprises residues 123 to 140. Finally, there is α-helix H2, which extends from residue 141 to 152. Helices H1 and H2 pack together in a parallel arrangement. Residue 152 marks the end of the wild-type sequence of hSBDb* that was used. H2 actually extends further, including four residues (LEHH) that are part of the affinity tag used to purify the domain. Indeed, there is a loop region after H2 called Lt (Loop terminus) that includes four more histidine residues.

The degree of structural conservation among the folds of the E3-binding domains is striking. Pairwise superimpositions of the structures of these domains are shown in Fig. 3A, and statistics on the comparisons are in Table 3. The comparable Cα atoms of the structures have r.m.s. deviations of ±1.5 Å (Table 3). The structures of helices H1 and H2 are well preserved in all of the structures. The folds mainly differ in the loop regions and in the number of residues that can be modeled at the C terminus. Therefore, our structure of hSBDb*AB comports with earlier assertions that the binding domains associated with the α-ketoacid dehydrogenases are similar, modular polypeptides that utilize a common fold to facilitate the association of their respective binding partners to these large multienzyme complexes (3, 11, 13).

Overall Features of the E3-hSBDb*AB Complex—A representation of the crystal structure of an hE3 homodimer bound to hSBDb* is shown in Fig. 4A. Like hE3BD (3), only a single molecule of hSBDb* binds to a homodimer of hE3. As noted above, only hSBDb*AB will be discussed, but all of the conclusions drawn for this copy of hSBDb* hold for the second one as well. In addition, we denote the origin of hE3 residues with a superscript; hence, Y438B represents tyrosine 438 from monomer B.

In comparing the present structure of hE3 to those established previously (3), we find that there are no significant structural perturbations of this enzyme caused by the complex formation with hSBDb*. As shown in Table 3, the r.m.s. deviations obtained when comparing the current hE3 structures to those heretofore described are about 0.4 Å. Thus, the previous notion...
of hE3 as a rigid enzyme that does not change conformation upon association with its binding partners (3) is here upheld.

hSBDb*AB binds to hE3 across the dyad axis that relates the A and B monomers of hE3 (Fig. 4, B and C). Thus, residues from both of the hE3 monomers are involved in binding hSBDb*.

Because hE3 is a homodimer, the enzyme has two putative binding sites for hSBDb. However, the fact that the binding site extends across the dyad axis forces the two binding sites to partially overlap. This binding mode sterically precludes a second molecule of hSBDb* from interacting with hE3 once the first one has bound. Notably, the footprint of hSBDb*AB binding on hE3 is discontinuous (Fig. 4B). Apart from the main body of the interaction footprint (Fig. 4B), the Oγ of the side chain of N118bd has a van der Waals interaction with the side chain carbon atom of A346B (Fig. 5A). As noted above, N118bd is the residue whose mutation from arginine of wild-type hSBDb created hSBDb*, which binds tightly to hE3. More details concerning the disposition of this residue are discussed later in this report. The total surface area buried upon the association of hSBDb* and hE3 is \( \sim 1,190 \text{ Å}^2 \).

TheLt portion of hSBDb*AB is stabilized through the interaction with a second hE3 in the NAD+/NADH binding site (not shown). This interaction, however, is not observed in hSBDb*CD. Thus, the binding of Lt at this site is likely an artifactual consequence of its non-native sequence and the non-physiological crystallographic conditions. It is important to note that the orientation of hSBDb*AB with respect to hE3AB is not affected by this adventitious interaction. This assertion may be made because the relative orientations of hSBDb*AB to hE3AB and hSBDb*CD to hE3CD are essentially identical (not shown).

**A Detailed Analysis of the hE3/hE3BD*AB Interactions**—A number of polar and non-polar interactions define the interface between hE3 and hSBDb*AB (Table 4). The side chains of only four residues from hSBDb*AB are involved in apparent hydrogen bonds or salt bridges with the enzyme: T113bd, K135bd, K141bd, and E142bd (Fig. 5B). None of these residues reside in...
### TABLE 4

| Protein | $K_d$ (µM) | $\Delta H$ (kcal/mol) | $\Delta G$ (cal/mol/K) |
|---------|------------|------------------------|------------------------|
| Wild type | 4.5 ± 0.3 | +6.2 ± 0.4 | −7.2 ± 0.5 | +44.2 ± 2.8 |
| T113Abd | 5.2 ± 0.4 | +4.2 ± 0.3 | −7.05 ± 0.5 | +69.2 ± 2.6 |
| P114Abd | 5.2 ± 0.8 | +0.94 ± 0.08 | −7.1 ± 0.6 | +26.4 ± 2.2 |
| A115Mbd | NM | NM | NM |
| R118Abd | NM | NM | NM |
| K135Abd | NM | NM | NM |
| D136Abd | 2.6 ± 0.2 | +4.7 ± 0.3 | −7.5 ± 0.5 | +40.2 ± 2.6 |
| K141Abd | NM | NM | NM |
| E142Abd | 11.6 ± 0.7 | +7.1 ± 0.5 | −6.6 ± 0.5 | +45.2 ± 3 |

R118Nbd background

| Protein | $K_d$ (µM) | $\Delta H$ (kcal/mol) | $\Delta G$ (cal/mol/K) |
|---------|------------|------------------------|------------------------|
| T113Abd | 0.98 ± 0.08 | −3.5 ± 0.3 | −8.1 ± 0.7 | +15.1 ± 1 |
| P114Abd | NM | NM | NM |
| E136Abd | NM | NM | NM |
| R138Abd | NM | NM | NM |
| K141Abd | NM | NM | NM |
| E142Abd | NM | NM | NM |

*NM, not measurable.

*All the following mutations were actually double mutations: the noted mutation in addition to R118Nbd. These are cited here and in the text as (amino acid change) Abd. For example, the T113Abd mutant in the R118Nbd background is shown here and in text as T113Abd.

H1; instead, they are in L0 (T113bd), L1 (K135bd), and H2 (K141bd and E142bd) (Fig. 1). Helix H1, which harbors N118bd, does contact hE3, but it uses only van der Waals interactions, not polar ones. Three of the four residues noted above are charged at physiological pH (and the pH of the crystal; see “Experimental Procedures”). The charges of these residues are complemented by the charges of their interaction partners on hE3. This charge complementarity is likely to be a prominent component of the association of hSBDb* and hE3.

An unusual interaction between hSBDb*Abd and hE3 is observed at the dyad axis of the latter (Fig. 5C). Here, the benzyl side chains of Y438Abd and Y438B of hE3 form an offset stacking interaction, as seen in other hE3 structures (17). Engaging in an edge-to-face interaction with the side chain of Y438B is the guanidinium group of R138bd (Fig. 5C). The other face of the guanidinium group is contacted by L111bd. We observe no compensatory negatively charged atoms interacting with the guanidinium group of R138bd in this structure. We note, however, that electrostatic calculations demonstrate that the hSBDb-binding site on hE3 is predominantly negatively charged (Fig. 4C). It is therefore possible that the guanidinium group of R138bd is interacting with this charge nonspecifically, rather than forming a specific salt bridge with a negatively charged amino acid side chain. Both Y438Abd and Y438B serve as the C-terminal residues of the respective α-helices in which they reside. Thus, R138bd is close to the partial negative charges generated by the dipole moments of these helices, which probably contribute to the overall negative charge near to the binding site. Additionally, there is significant evidence that R138bd adopts a poorly occupied alternative conformation in which the face of its guanidinium group stacks on the edge of the benzyl group of Y438Abd (not shown). This conformation was included in the model and refined to an occupancy of 0.4. The positive charge of this arginine conformer comes closer (but not within hydrogen-bonding distance) to D444B and D136bd (not shown).

Given that this conformation is in the minority, it does not appear that the nearby putatively or negatively charged residues are significantly stabilizing for this position of the guanidinium group.

Finally, the disposition of N118bd is of particular interest. In wild-type E2b, this position harbors arginine, not asparagine. The mutation that exists in hSBDb* causes that protein to associate about 70-fold more strongly than the wild-type hSBDb (see above). Thus, it might be expected that N118bd would be involved in a particularly favorable interaction with hE3. The crystal structure defies this expectation. The side chain of N118bd has only the long-range (3.9 Å) interaction with A346B noted above (Fig. 5A). We therefore posit that the R118Nbd mutation rids hSBDb* of a disadvantageous interaction, rather than introducing an advantageous one. It is noteworthy that R447Abd is in the region of N118bd (Fig. 5A). Juxtaposition of the two positively charged residues R447Abd and R118bd, as might occur if the amino acid at position 118 was arginine, could be deleterious to hSBDb binding to hE3. Furthermore, 87% of the preferred rotamers of arginine, if placed in the hSBDb*/hE3 structure, would sterically clash with hE3.

Comparisons to Other hE3/Binding Domain Structures—In the reported hE3BD/hE3 structures (3, 12), the hE3BD domain occupied a site on hE3 that is very similar to the hSBDb*-binding site we observe in the current structure (Fig. 4B). However, the association of hE3 and hE3BD buried 1,490 Å² of surface area, 300 Å² more than the hE3/hSBDb* interface. Consequently, the footprint of hE3BD on hE3 is larger than that of hSBDb (Fig. 4B). The smaller footprint of the hE3/hSBDb* interaction appears to be a result of the binding domain taking up a different conformation relative to hE3 (Fig. 6A). In comparing the positions of the two binding domains, it is clear that helices H1 and H2 of hSBDb*Abd are rotated away from hE3 by about 30° relative to the same helices in hE3BD. The orientations of H1 and H2 relative to one another in the two structures are conserved, which appear to be a rigid unit. Unlike the helices, loops L0 and L1 adopt similar orientations relative to the hE3 in their respective structures. As described above, residues in these regions are responsible for many of the contacts between the binding domains and hE3.

In the GsSBDp/GsE3 structure of the PDC from G. stearothermophilus (11), the GsSBDp binding site on GsE3 differs from the structures discussed above in that it does not cross the dyad axis of GsE3. Nevertheless, it comes very close to the axis, and Mande et al. (10) speculated that this proximity would sterically prohibit the binding of a second GsSBDp to GsE3, as is the case for the hE3/hE3BD and hE3/hSBDb* interactions. The orientation of GsSBDp relative to GsE3 is significantly different from the orientation of hSBDb*Abd relative to hE3 (Fig. 6B). Although the differences between the hE3/hE3BD and hE3/ hSBDb*Abd structures could be characterized as a simple tilting of the hSBDb*Abd helices away from hE3, no similarly facile relationship between the GsE3/GsSBDp and hE3/hSBDb*Abd structures is present. To adopt the conformation that is observed in the hE3/hSBDb*Abd structure, the GsSBDp would have to tilt away from GsE3 by about 27° and rotate around its H1 axis away from the GsE3 equivalent of the B monomer by about 70° (not shown).
E3 Binding to BCKDC Subunit-binding Domain

Solution NMR Studies of the hSBDb-hE3 Interface—Likely because of the micromolar dissociation constant of wild-type hSBDb to hE3, we were unable to obtain diffraction quality crystals of these two proteins. Therefore, to examine the question of whether the mode of wild-type hSBDb and hSBDb* binding to hE3 was significantly different, we turned to solution NMR studies. Intriguingly, we observed deterioration of the NMR spectrum with time, indicating damage to hSBDb presumably by the cryptic proteolytic activity of hE3 (29). These observations likely are the compounded effects of the high protein concentration and the long acquisition times typically used in the NMR studies and can be alleviated by using an hE3 S456A mutant, referred to here as hE3*. The hE3* protein exhibited wild-type enzyme activity with a binding affinity for hSBDb also similar to that of wild-type hE3 (not shown).

We conducted two types of experiments. In one, 2H,15N-labeled hSBDb or hSBDb* was subjected to two-dimensional 15N-TROSY-HSQC experiments (Fig. 7) (24). hSBDb resonance assignments were previously performed at 22 °C (30). Peaks for many important interface residues cannot be observed at this temperature likely due to fast exchange with water, but almost every practically observable residue can be seen by lowering the temperature to 12 °C. These new peaks were then assigned using standard triple-resonance experiments. Similarly, the hSBDb/hE3* complex gives rise to resonances that at 22 °C appear in intermediate to the slow exchange regime of NMR time scale. Because of the endothermic nature of this reaction, the equilibrium can be modulated by the ionic strength by the addition of 800 mM NaCl. Such a fast exchange regime of NMR time scale regime by simultaneously dropping the sample temperature to 12 °C and increasing the ionic strength by the addition of 800 mM NaCl. Such a fast exchange regime leads to population-weighted averaging of the free- and bound-hSBDb chemical shifts leading to movement of peaks, particularly those originating from the interface residues. Lower temperatures are generally not favored in NMR studies due to increased solvent viscosity, which leads to unfavorable broad line shapes particularly for a sample as big as ~110 kDa. We persisted in performing our studies at 12 °C, because it facilitated unambiguous assignment of the bound-hSBDb resonances and provided immensely useful chemical shift perturbation data. The hSBDb or hSBDb* residues that experienced statistically significant chemical shifts perturbation upon the addition of hE3* were considered as contacting hE3. But such perturbation can also be caused by a conformational change as a result of binding at the distal location, and hence we further carried out a second set of experiments known as cross-saturation transfer experiments (26) on hSBDb*/hE3* and hSBDb/hE3* mixtures. Generally, large intensity changes in these experiments were taken as evidence of those residues being in immediate contact with hE3; although sometimes such changes can also be associated with spin diffusion. Taken together, these NMR results confirmed the presence of interactions between Arg-118 and Asn-118 of hSBDb and hSBDb*, respectively, and hE3 (Fig. 7 and Table 5). We mapped (Fig. 8) these interactions on the crystal structure of hSBDb* (which is very similar to the solution structure of hSBDb; Table 3). We find that the interface between hE3 and hSBDb or hSBDb* is also similar, regardless of the proteins or experimental conditions. Because the structures of hE3 or hSBDb do not significantly change upon binding (see above), most of the entropic gain from the interaction likely originates from desolvation of the two proteins. Protein interactions can sometimes have a negative heat capacity change (ΔCp); in fact, the binding of GsSBDb to GsE3 is one such interaction (4). The effect of a negative ΔCp is that the heat of the interaction will become lower at higher temperatures. It is even possible that

FIGURE 6. Comparisons of the orientations of binding domains bound to E3s. A, comparing the binding modes of hE3BD (cyan) and hSBDb*AB. The structures were aligned using the respective hE3 coordinates from the hE3BD/hE3 and the hE3/hSBDb*AB structures. B, comparing the binding modes of GsSBDb (black) and hSBDb*AB. The structures were aligned using only the respective E3 coordinates from the GsSBDb/GsE3 and hE3/hSBDb*AB structures.
the $\Delta H$ of hSBDb binding to hE3 would be negative at physiological temperature. Although this seems favorable, this phenomenon is often almost perfectly compensated by a commensurate lowering of the entropy of the interaction (31). In that way, the overall $\Delta G$ (and thus the $K_d$) is maintained roughly constant at achievable temperatures. The fact that mutating residues A115bd, K135bd, K141bd, or E142bd results in diminished binding comports well with the crystal structure, as all of these residues are in contact with hE3 (Table 5). These findings emphasize the importance of the electrostatic interactions made between hE3 and hSBDb. They also underscore the necessity of the region of hSBDb around Ala-115 to approach hE3 closely; replacement of Ala-115 with methionine introduces a bulky side chain that likely disallows such an approach.

Mutations T113Abd, P114Abd, and D136Abd have similar $K_d$ values to the wild-type protein, despite the fact that these residues have close contacts to hE3 in the crystal structure, and T113bd appears to bind to hE3 in the NMR data (Fig. 7, Table 5). However, it is notable that all of these mutant proteins have significantly different heats of interaction ($\Delta H$) compared with the wild-type protein (Table 4). Thus, the different heats must be nearly compensated by commensurately different entropies of interaction ($\Delta S$). This phenomenon has been observed before in protein-protein interactions that have a modest association constant (32). We conclude that the mutation of these residues affects the overall thermodynamic properties of the interaction, but these residues are not vital for the association of the two proteins. Obviously, residue R118bd is centrally impor-
E3 Binding to BCKDC Subunit-binding Domain

TABLE 5
Residues of hSBDb* in contact with hE3 or hE3*

| Residue | Crystal structure | R118N NMR data | Wild-type NMR data |
|---------|------------------|----------------|------------------|
| T110bd  | X                | Δδ             | X                |
| L111bd  | A                | X              | Δδ, l            |
| T113bd  | P                | ST, Δδ, l      | I                |
| P114bd  | A                | N/O            | N/O              |
| A115bd  | A                | X              | X                |
| V116bd  | X                | o              | ST, l            |
| R117bd  | A                | l              | X                |
| R118bd  | N/A              | N/A            | I                |
| N118bd  | A                | ST             | N/A              |
| L119bd  | N/O              | Δδ             | l                |
| A120bd  | X                | Δδ             | X                |
| M121bd  | A                | o              | Δδ               |
| E122bd  | X                | ST             | l                |
| N123bd  | X                | ST             | I                |
| N124bd  | X                | X              | I                |
| K135bd  | P                | Δδ             | Δδ               |
| D136bd  | A                | X              | X                |
| R138bd  | A                | X              | X                |
| L140bd  | A                | X              | X                |
| K141bd  | P                | X              | ST               |
| E142bd  | P                | Δδ, l          | Δδ               |
| D143bd  | X                | ST, l          | X                |
| I144bd  | X                | ST             | ST               |
| L145bd  | A                | o              | X                |

* Interactions were divided into two categories: “P” represents polar (hydrogen bonds, salt bridges) and “A” represents apolar (van der Waals) interactions. For the latter, a maximum atom-atom distance of 4 Å was enforced. An X denotes that the interaction was not observed.

* Three different types of interactions were considered: ST, meaning that the ratio $I_{-10ppm}/I_{0ppm}$ was less than 0.9; Δδ, meaning that Δδ < Δδ + σ and I, meaning that the intensity of the peak of the residue in the TROSY-HSQC spectrum was significantly lowered or broadened. Furthermore, an X denotes that the interaction was not observed, and an “o” means that the interaction may be obscured by peak overlap.

FIGURE 8. A map of the interaction surface on the surface of the crystal structure of hSBDb*. For uniformity of comparison, all interaction surfaces are mapped on the crystal structure of hSBDb* A, a key for the parts that follow. The surface is divided according to the hSBDb* residue that contributes the corresponding surface and the corresponding residue number is noted. Note that residues A120bd and D143bd (Table 5) are not visible upon the NMR data. All residues are colored blue except those that are noted as P (red) or A (gold) in Table 5, indicating polar or apolar interactions with hE3, respectively. C, the interaction surface between hSBDb* and hE3 based upon the NMR data. All residues are gray except as noted. The hE3-contacting residues were colored as follows in the following order of precedence: green residues had $I_{-0ppm}/I_{0ppm}$ ≤ 0.9 in the cross-saturation transfer experiment; blue residues had Δδ < Δδ + σ in the two-dimensional 15N-TROSY-HSQC spectra; and pink residues had a significant broadening or lowering of intensity in the two-dimensional 15N-TROSY-HSQC spectra. D, the interaction surface between hSBDb and hE3* based upon the NMR data. The coloration is the same as in B.
characterized by scanning alanine mutagenesis. In short, the theory states that only a few amino acid residues at a protein-protein interface are responsible for the majority of the binding energy. Other residues observed making interprotein contacts are not as important because (i) the interactions lost by their mutation to alanine can be replaced by water molecules, buffer components, or other side chains; and/or (ii) the main responsibility of the energetically neutral residues is water occlusion from the hot spot, a function that is not diminished by their mutation to alanine. Thus, many protein interfaces have a dual character, featuring some contacts that are energetically vital, and others that appear to be energetically neutral. In many interaction surfaces, a cluster of hot spot residues in the center of the surface is surrounded by an energetically unimportant “O-ring” (16). This observation is explained above (point i). Because this clustering phenomenon is not present in the hE3/hSBDb interaction, we deem this mechanism mostly irrelevant for the following discussion.

Within this theoretical context, we may hypothesize that hSBDb and hSBDb* utilize two distinct binding modes to interact with hE3. We term these modes “R” and “N” after the identity of the residue at position 118 of hSBDb and hSBDb*, respectively. It is important to note that, given our solution NMR data, these modes do not differ significantly in the interaction surface that hSBDb (or hSBDb*) uses to associate with hE3. In mode R, charged residues R118bd, K135bd, and K141bd are crucial for the formation of the complex (i.e. they qualify as hot spot residues; see Table 4). Mutating other binding domain residues to alanine has little to no effect on the interaction. Because no O-ring is evident in this interaction, mode R exhibits the dual character noted above: water molecules or other protein side chains may be thermodynamically compensating for the interactions lost due to the mutagenesis. All of these results are in keeping with hot spot theory. On the other hand, mode N is very different from mode R. Mutation to alanine of binding domain residues known to be in contact with hE3 abrogates the interaction (Tables 4 and 5, and Fig. 8), except in the case of T113bd. Thus, the dual character of mode R is mostly absent in mode N. Mode N is similar to the binding mode observed in the hE3/hE3BD interaction (3), in that nearly every (tested) residue at the interface satisfies the definition of a hot spot (ΔΔGbinding ≥ 2.0 kcal/mol) (16). Although it is clear that water or other side chains are unable to substitute for the interactions lost upon mutation, the origin of this inability is obscure.

Because the only difference between hSBDb and hSBDb* is the identity of the residue at position 118, this residue must be a key to the interaction mode. We therefore hypothesize that the suboptimal environment that R118bd finds itself in upon hE3 binding endows the interface with the dual character consistent with hot spot theory. On the other hand, changing that residue to asparagine relieves the negative interactions of R118bd with hE3, but strips the interface of the ability to adapt to mutation. The possibility exists that mutations to hSBDb* (or hE3BD) cause a significant change in the free energy of the unbound binding domain, a fact that could partially account for our calorimetric observations. However, all of the hSBDb and hSBDb* mutants that we examined in this study exhibited iden-

tical behavior on a size exclusion column, ruling out gross conformational changes (i.e. folding defects) in these constructs relative to the wild-type protein. Other free energy changes, like those resulting from differential solvation, cannot be ruled out. Obviously, further experimentation is required to explore these hypotheses.

Conclusions—We have observed that a single mutation to hSBDb, R118Nbd (hSBDb*), significantly enhances the affinity of this domain for hE3. This fact was used to facilitate the structural determination of the complex of hE3 and hSBDb*. The interaction between them is dominated by the interaction of complementarily charged residues on the respective surfaces of the proteins. The co-crystallization of wild-type hSBDb with hE3 was not successful due to the low binding affinity, with Kd in the micromolar range (Table 2). However, our solution NMR data show that the interfacial surface of hSBDb does not change significantly from hSBDb to hSBDb*. Thus, the uniquely tight interaction of N118bd with hE3 in the mutant hSBDb*/hE3 structure strongly suggests that the presence of R118bd in wild-type hSBDb poses a possible steric and electrostatic incompatibility that could impede hE3 interactions with wild-type hSBDb. These potentially deleterious interactions apparently cause a change in the mode of the interaction from one that is tolerant of some mutations (mode R) to one that is more intolerant (mode N). The present study provides a starting point for explaining at the structural level the significantly weaker binding of hE3 to the E2b core of the BCKDC, compared with other α-ketoacid dehydrogenase complexes. Clearly, further characterization of this system using kinetic, thermodynamic, and computational means would shed more light on the properties of this interaction.

The question of why evolution has favored an hSBDb with a low affinity for hE3 remains open. It is clear that, all other things being equal, hE1b will easily dominate the competition for binding sites on the hSBDb of BCKDC. This situation arises from the much higher affinity of hE1b for hSBDb (Kd = 205 nm) compared with that of hE3. However, BCKDC requires bound hE3 for the efficient activity of the enzyme (5). Among the many possibilities that could account for this paradox are: (i) hE1b molecules bind to the complex, but cannot reach full occupancy due to steric constraints; hE3 molecules could bind to the vacant binding sites, (ii) conditions in vivo are significantly different from those in vitro, allowing hE3 to effectively compete with hE1b for binding to hSBDb, or (iii) the concentration of hE1b is modulated in the mitochondrion in response to protein contents in the diet, which controls the number of hE1b bound to the core as suggested previously (36).

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