

Structural and biochemical characterization of 20 β -hydroxysteroid dehydrogenase from *Bifidobacterium adolescentis* strain L2-32

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Figure S1. Active site mutant thin layer chromatography (TLC) isothermal titration calorimetry (ITC) and circular dichroism (CD).

Figure S2. Structural analysis of 20 β -HSDH.

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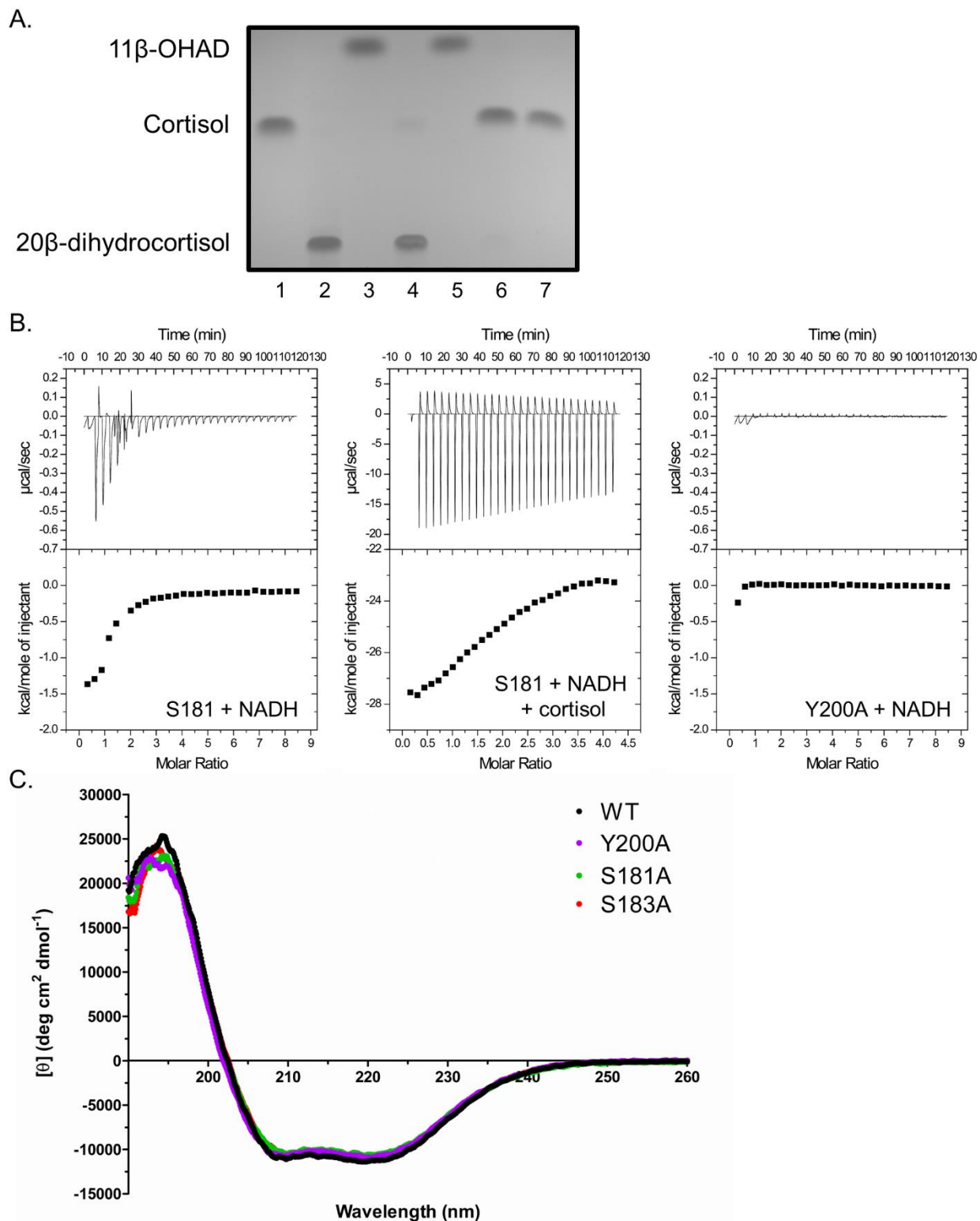


Figure S1. Active site mutant thin layer chromatography (TLC) isothermal titration calorimetry (ITC) and circular dichroism (CD). (A) Wild-type and active site mutant 20 β -HSDH overnight reaction products separated by TLC. (1) Cortisol standard, (2) 20 β -dihydrocortisol standard, (3) 11 β -hydroxyandrostenedione standard, (4) WT + NADH + cortisol, (5) WT + NADH + 11 β -OHAD, (6) S181A + NADH + cortisol, (7) Y200A + NADH + cortisol. (B) Ligand binding order of S181A and Y200A studied

by ITC. Left panel is 2 mM NADH binding to S181A, middle panel is 1 mM cortisol binding to S181A with 2 mM NADH, right panel is 2 mM NADH binding to Y200A. (C) CD spectra of purified recombinant 20 β -HSDH and its active site mutants.

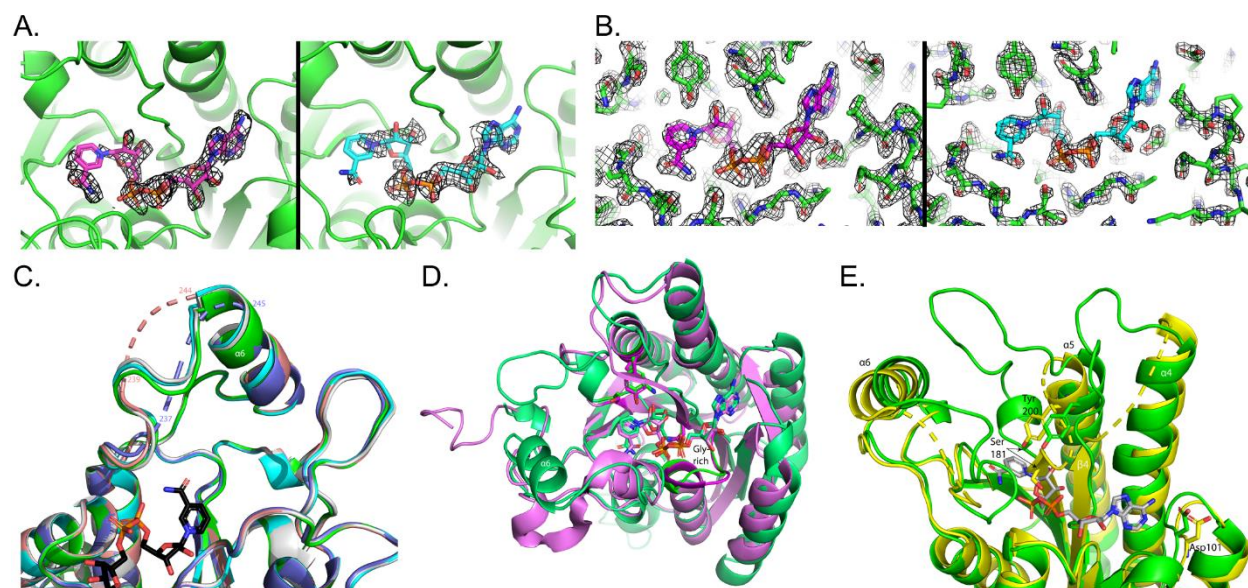


Figure S2. Structural analysis of 20 β -HSDH. (A) Fo-Fc omit map of NADH from Chain A and E shown at 2 sigma. (B) 2Fo-Fc map of NADH and surrounding backbone chains from Chain A and E shown at 2 sigma. (C) Alignment of 5 of the monomers from the NADH-bound structure showing the flexibility of loop 235-245 near where cortisol is predicted to bind. The 3 monomers not shown look similar to those shown. (D) Comparison of 20 β -HSDH (green) to 3 α , 20 β -HSDH from *Streptomyces hydrogenans* (pink). The Gly-rich region is highlighted in brighter colors near the adenine of NADH and the side chains of the residues making up the catalytic tetrad are shown. Ser181 of the catalytic tetrad has been mutated to an alanine in 20 β -HSDH. (E) Superposition of apo (yellow) and S181A mutant holo structure with NADH bound (green).

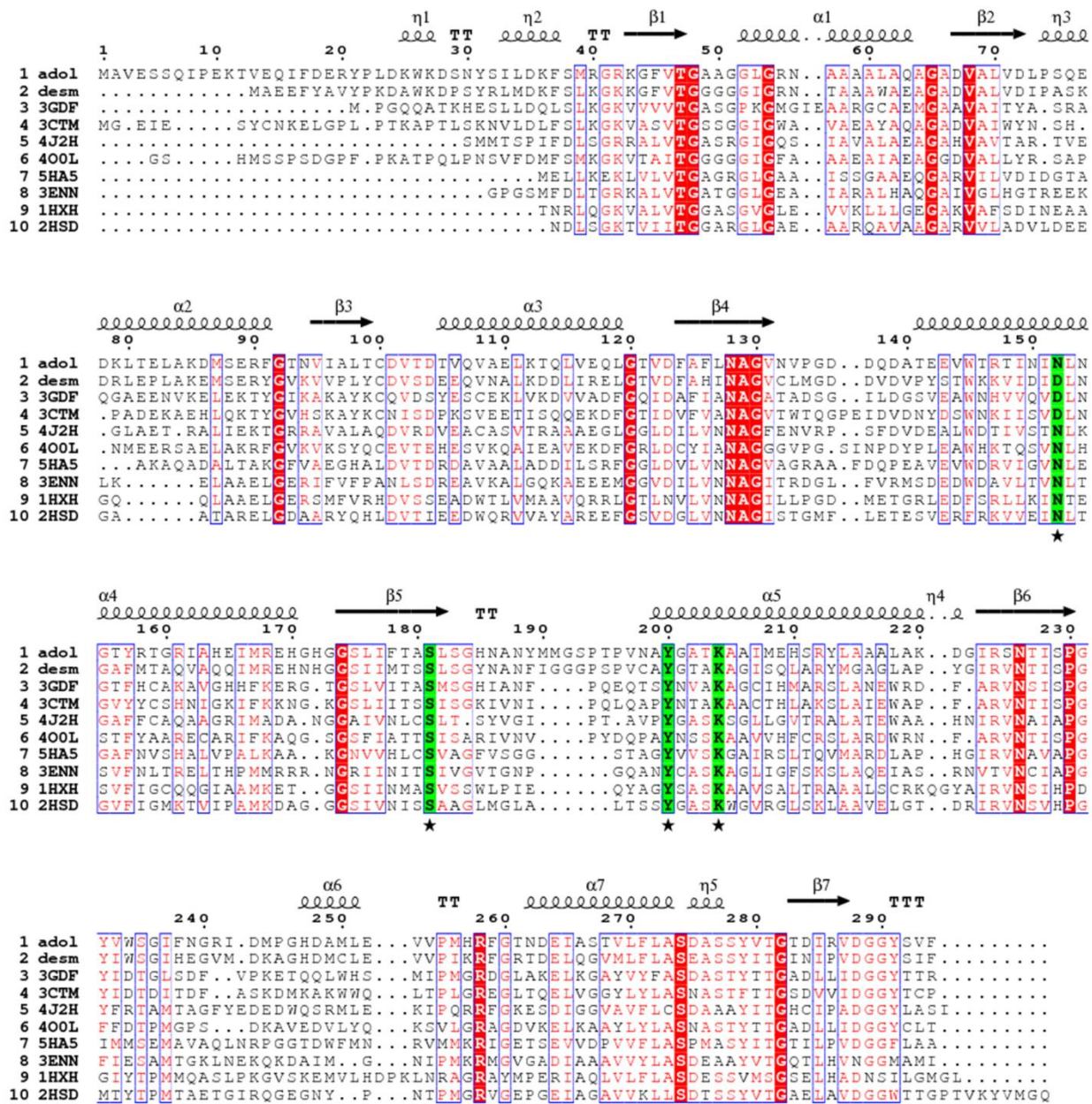


Figure S3. Sequence alignment of 20β-HSDH found in *B. adolescentis* and other structurally similar SDR members. Red highlights indicate identical residues, green highlights and stars indicate active site residues, blue boxes indicate conserved residues. Secondary structural elements of apo-20β-HSDH are displayed on the top of the alignment. The abbreviations of protein names are as follows: adol, 20β-HSDH from *Bifidobacterium adolescentis* strain L2-32; desm, 20β-HSDH from *Butyricoccus desmolans* ATCC 43058; remaining labels correspond to PDB IDs. The sequences were aligned with Clustal Omega (1.2.4) and secondary structure rendered by ESPrnt 3.0 web server.

UniProt ID	Extended N-terminus	
Ado1 WP_003810233.1	AVE---SSQI--PEKTVEQI-FDERYPLDKWKDSNYSILDKFS	MRGR-KGFVTGAAGGLG 54
A0A1A9H573_HELPX	N-----IRGIIKIIRGLAL----DNGR---WREKESQK-VAVITGASSGI	39
U4WVT8_HELPX	-----MGVGEKEEKKESQK-VAVITGASSGI	26
A0A083YF40_HELPX	-----MG-----VGEK---EKKESQK-VAVITGASSGI	26
T2SNM1_HELPX	-----MG-----VGEK---EKKESQK-VAVITGASSGI	26
A0A0B2EPQ3_HELPX	-----MG-----VGEK---EKKESQK-VAVITGASSGI	26
A0A0U1A5J2_9MYCO	GHWLPFSHPQVLAATTELIDAVSGNQPGRGLRRAEMGKSRRF	FEDQ-LVVITGGGSGI 235
A0A0N8HB56_9ACTN	-----MAEHAEHAEHAEQAEYPTGRAR-SVVITGASRGL	34
A0A101BC04_9MYCO	-----MAEPRSGDERSCGKR-TVVITGASRGL	27
A0A2D6MTV7_9DELT	SGM-----TRRSRSP-----ARISEEDKMKGALGYEGK-TVVITGAASGM	51
A0A2E5YH49_9DELT	PGM-----KRKRSS-----TRISEGYKMKNALGYEGK-TVVITGAASGM	51
A0A1X1T1G8_9MYCO	-----MTGIDGLWRHLGYHCR-RVVVTGCASGI	28
A0A379BZG6_9NOCA	-----MRI-LGHGYPGIDLKGA-RVLITGARGI	28
A0A3P8L1B1_TSUPA	-----MNL-FSSRDHLARLDGA-LVVVTGARGI	28
A0A2Z5YF10_MYCMR	-----M-ADSTTIGVVRDK-VIVITGARGI	26
A4ETZ5_9RHOB	A-R---GWTV--P----PK-----QSTTFNSGDLEIKMMDIKGK-TVVITGASRGI	55
A3JLM7_9RHOB	-----MNFEGMKMSMQGK-VVFITGASRGI	26
A3STW2_9ULSN	-----P-----RAGLIDDEGNYTMDMTGK-TVMITGASRGI	40
A0A0A3XQW2_BRAJP	-----MTRNAHHLRQAMTR-TILITGSSSGI	26
A0A103KGN7_9BURK	-----MRLRLSNLACWKTLDMSK-TILITGASSGI	30
A0A174GG33_9BACT	GHRER-GDKA--VTAPCEPAEVQGGTGE--RHGRRLERGA	VAPGSA-WALVTGAGSGI 113
U4E9H0_9VIBR	-----MKTSTDKTEVNIMK-TAFITGATSGI	26
U4KB55_9VIBR	-----MKTSTDKTEVNIMK-TAFITGATSGI	26
A0A1E3LA45_9BACL	-----MQYVYCDNMVKLANK-VVLITGASSGI	27
A0A3S4VN67_MYCAU	-----MTKWTAAADVDPQSGR-VAVITGANTGI	27
A0A2X1S640_MYCXE	-----MRWTAADLPSFAGR-TVVVTGANSGL	26
A0A378YLY9_9NOCA	-----MAWKPSEIPDQSGR-TVVITGANGGI	26
A0A174QRR8_BACVU	GIISKIKSKL--SYKEVTPY-DMDDL--RDAYQTSVVGGS	LKGR-IALVTGATSGI 57
D6D469_9BACE	SIKKYLKRAF--V-----F-LLHGIPERHVIANITKLAPNEM	LKGR-TALITGATSGI 51
O53547_MYCTU	-----MK---LTESNRSRPR-TTNTTDL	LSGK-VAVVTGAAAAGL 33
A0A1R3Y4F5_MYCBO	-----MK---LTESNRSRPR-TTNTTDL	LSGK-VAVVTGAAAAGL 33
Q0S7K5_RHOJR	-----MNAV-ADRDNVVDGK-VAVVTGAGSGL	26
J1RDG0_9NOCA	-----MFMNAV-ADRDNVVDGK-VAVVTGAGSGL	28
A0A376F856_9MICO	-----MKLS---RRTAPSHAGR-CVLVTGASGL	26
A0A0M3C339_9SPHI	-----MFAF---IIDYNRTGMDISKLF	LSGK-TAIITGGAAGI 36
3BHDP_RUMGV	-----MNFGGFIMGRFDEK-IMLVITGATSGI	26
A2WJD3_9BURK	-----MHVNGTHDPAQLPLAGR-TALVTGGGRGL	29
A0A378YJ86_9NOCA	-----MRLNP--FGGSRRTRYA-DAVVTGAGSGI	27
A0A3B8M323_9ACTN	-----MV---LVAFWINT--GDMSNRLNGL-TAIISGARGI	32

Figure S4. SDR family subset with extended N-terminus multiple sequence alignment. A Clustal Omega (1.2.4) alignment was performed with 555 sequences labeled as SDR family, hydroxysteroid dehydrogenase, and of bacterial origin from the UniProt database. This excerpt from the total sequence alignment includes 39 sequences with a ≥ 10 amino acid residue extended N-terminus. Conserved residues are highlighted, bolded residues indicate the *B. adolescentis* 20 β -HSDH extended N-terminus, and the line indicates the beginning of the *B. adolescentis* 38-residue extended N-terminus.

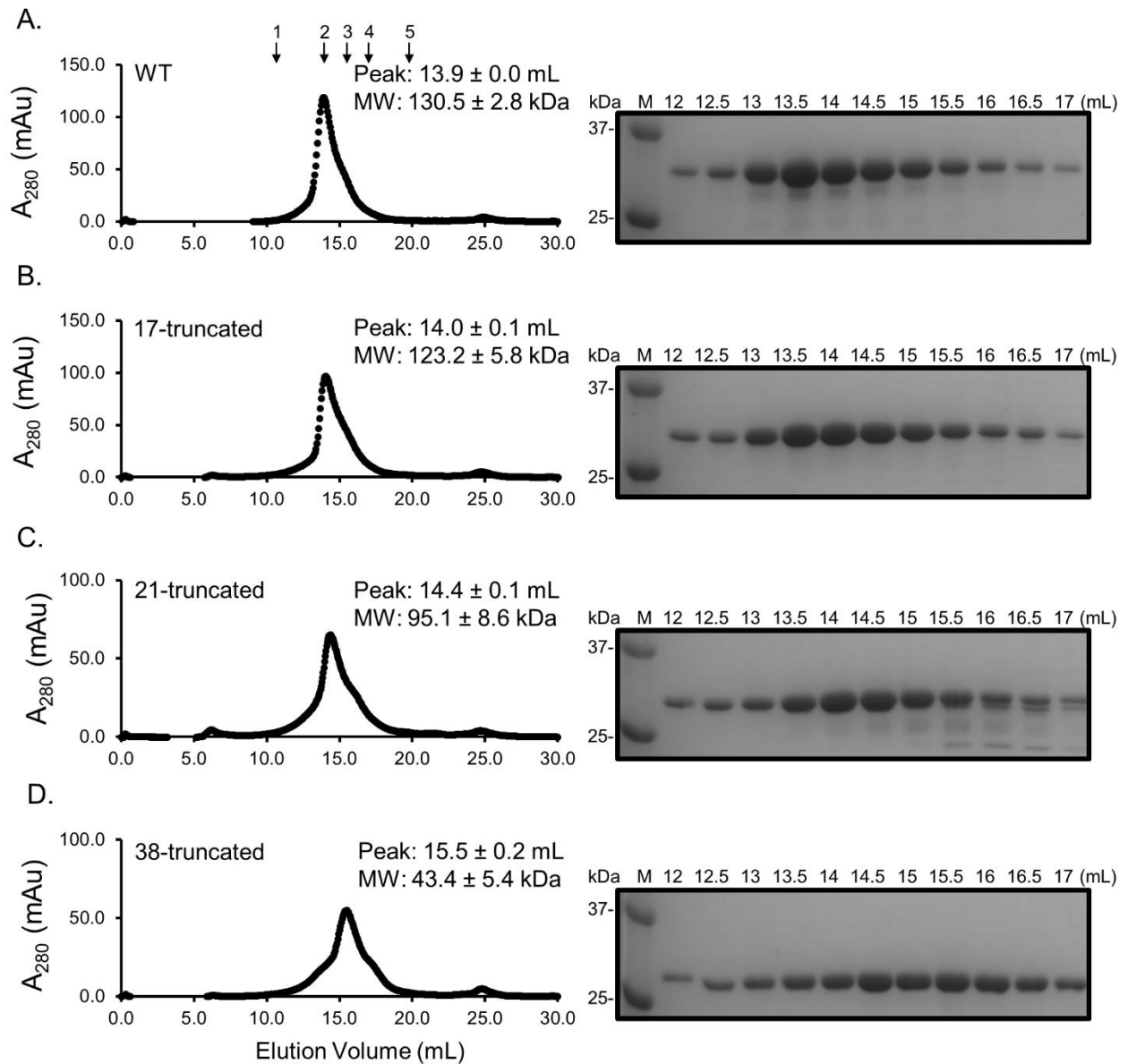


Figure S5. Gel filtration chromatography of 20 β -HSDH truncation mutants. Native molecular weight estimates were based on elution time of 10 mg/ml WT and truncated 20 β -HSDH from a Superose 6 10/300 GL analytical column. Fractions were collected and analyzed on SDS-PAGE for visualization. (A) WT, (B) 17-truncated, (C) 21-truncated, (D) 38-truncated. Numbers indicate elution time of standard proteins (1) thyroglobulin, (2) γ -globulin, (3) ovalbumin, (4) myoglobin, (5) vitamin B₁₂.

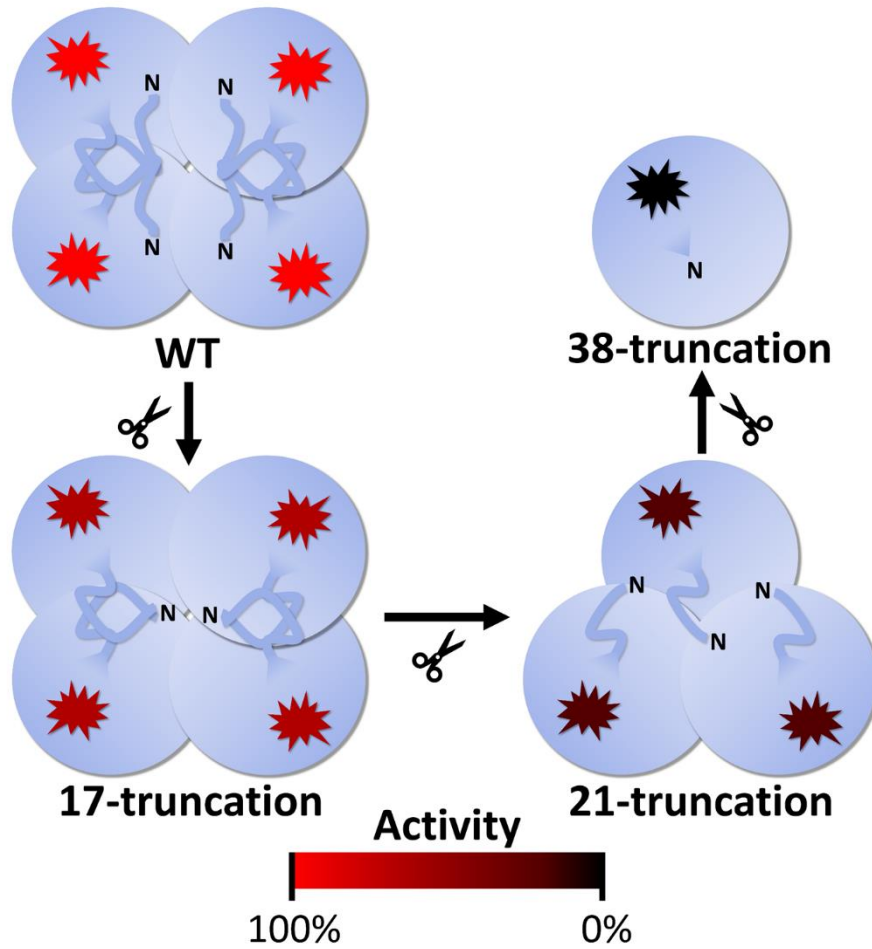


Figure S6. Model of 20β-HSDH N-terminal truncation effects. Oligomerization is shown by the number of blue circles, or subunits, and enzymatic activity is depicted by the color of the explosion shape. WT 20β-HSDH has 100% relative activity and is likely tetrameric. When the first 17 residues are truncated, the protein exhibits 62% activity, but remains a tetramer. When 21 residues are truncated, the protein loses quaternary structure and has only 2% activity. The full 38-residue truncation results in no activity and a mixed dimeric and monomeric form.