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Supplementary Material

Equilibrium unfolding thermodynamics of β_2-microglobulin analyzed through native-state H/D exchange

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Supplementary Material

n	type	301	303	303	305	307	309	310	311	312	313	315
6	K	4.4E-04 ± 4E-05	6.2E-04 ± 4E-05	4.6E-04 ± 8E-05	4.6E-04 ± 1.0E-04	1.0E-03 ± 3E-04	2.1E-03 ± 1.0E-03	1.3E-03 ± 5E-04	5E-03 ± 2E-03	1.7E-03 ± 1.1E-03	not detected	not detected
8	Q	overlapped	overlapped	overlapped	overlapped	overlapped	overlapped	overlapped	overlapped	overlapped	overlapped	overlapped
10	Y	5.7E-06 ± 1E-07	1.2E-05 ± 4E-07	1.1E-05 ± 6E-07	1.8E-05 ± 5E-07	3.9E-05 ± 9E-07	6.2E-05 ± 2E-06	9.5E-05 ± 2E-06	1.3E-04 ± 5E-06	1.7E-04 ± 7E-06	2.1E-04 ± 1E-05	4.9E-04 ± 3E-05
22	F	1.9E-04 ± 1E-05	2.9E-04 ± 1E-05	3.1E-04 ± 2E-05	3.7E-04 ± 5E-05	6.7E-04 ± 8E-05	5.4E-04 ± 1.1E-04	1.5E-03 ± 3E-04	1E-03 ± 3E-04	3.1E-03 ± 3E-04	not detected	not detected
23	L	overlapped	overlapped	overlapped	overlapped	overlapped	3.9E-05 ± 3E-06	5.6E-05 ± 1E-06	7.5E-05 ± 2E-06	1.1E-04 ± 4E-06	1.5E-04 ± 6E-06	2.6E-04 ± 9E-06
24	N	overlapped	overlapped	overlapped	1.9E-05 ± 7E-07	4.1E-05 ± 1E-06	6.7E-05 ± 1E-06	9.6E-05 ± 3E-06	1.4E-04 ± 6E-06	1.6E-04 ± 8E-06	2.2E-04 ± 1E-05	5.0E-04 ± 3E-05
25	C	6.6E-06 ± 1E-07	1.5E-05 ± 6E-07	1.2E-05 ± 6E-07	2.0E-05 ± 7E-07	4.7E-05 ± 2E-06	7.6E-05 ± 4E-06	1.1E-04 ± 3E-06	1.4E-04 ± 7E-06	1.9E-04 ± 1E-05	2.7E-04 ± 2E-05	4.7E-04 ± 3E-05
26	Y	3.2E-06 ± 9E-08	8.0E-06 ± 6E-07	5.3E-06 ± 2E-07	9.9E-06 ± 3E-07	2.4E-05 ± 7E-07	4.2E-05 ± 2E-06	6.9E-05 ± 2E-06	8.1E-05 ± 2E-06	1.1E-04 ± 4E-06	1.7E-04 ± 9E-06	3.0E-04 ± 2E-05
27	V	overlapped	overlapped	overlapped	overlapped	overlapped	overlapped	overlapped	5.9E-05 ± 2E-06	8.9E-05 ± 3E-06	1.3E-04 ± 6E-06	2.7E-04 ± 1E-05
28	S	1.6E-05 ± 5E-07	2.7E-05 ± 6E-07	2.9E-05 ± 1E-06	4.1E-05 ± 2E-06	6.7E-05 ± 2E-06	1.0E-04 ± 4E-06	1.3E-04 ± 4E-06	1.9E-04 ± 7E-06	2.0E-04 ± 9E-06	2.6E-04 ± 2E-05	5.1E-04 ± 3E-05
30	F	6.3E-04 ± 7E-05	8.5E-04 ± 8E-05	1.1E-03 ± 2E-04	1.5E-03 ± 5E-04	1.9E-03 ± 7E-04	1.2E-03 ± 7E-04	1.2E-03 ± 6E-04	not detected	not detected	not detected	not detected
35	I	7.6E-04 ± 2E-04	not detected	not detected	not detected	not detected	not detected	not detected	not detected	not detected	not detected	not detected
36	E	5.8E-06 ± 1E-07	9.5E-06 ± 1E-07	1.1E-05 ± 2E-07	1.4E-05 ± 3E-07	2.6E-05 ± 7E-07	4.4E-05 ± 2E-06	6.0E-05 ± 1E-06	7.1E-05 ± 2E-06	9.5E-05 ± 3E-06	1.4E-04 ± 6E-06	2.9E-04 ± 1E-05
38	D	2.9E-06 ± 7E-08	9.3E-06 ± 4E-07	7.8E-06 ± 6E-07	1.2E-05 ± 8E-07	2.5E-05 ± 7E-07	3.9E-05 ± 2E-06	6.1E-05 ± 2E-06	8.7E-05 ± 3E-06	1.2E-04 ± 4E-06	1.7E-04 ± 9E-06	3.4E-04 ± 2E-05
39	L	2.5E-04 ± 2E-05	2.9E-04 ± 1E-05	3.4E-04 ± 2E-05	3.9E-04 ± 4E-05	5.4E-04 ± 3E-05	6.3E-04 ± 9E-05	8.1E-04 ± 5E-05	8.5E-04 ± 1E-04	9.7E-04 ± 1.0E-04	1.0E-03 ± 2E-04	1.3E-03 ± 2E-04
40	L	1.0E-06 ± 6E-08	1.8E-06 ± 4E-08	2.0E-06 ± 9E-08	2.6E-06 ± 1E-07	7.4E-06 ± 1E-07	1.6E-05 ± 1E-06	2.2E-05 ± 4E-07	2.9E-05 ± 7E-07	5.0E-05 ± 2E-06	7.8E-05 ± 3E-06	1.7E-04 ± 5E-06
41	K	overlapped	overlapped	overlapped	overlapped	overlapped	overlapped	overlapped	overlapped	overlapped	overlapped	overlapped
42	N	7.3E-04 ± 9E-05	7.9E-04 ± 7E-05	1.2E-03 ± 3E-04	1.4E-03 ± 4E-04	1.6E-03 ± 4E-04	9.4E-03 ± 2E-04	3E-03 ± 3E-03	1.4E-03 ± 6E-04	not detected	not detected	not detected
44	E	1.3E-05 ± 2E-07	2.4E-05 ± 3E-07	3.0E-05 ± 5E-07	3.6E-05 ± 9E-07	6.2E-05 ± 1E-06	9.3E-05 ± 3E-06	1.3E-04 ± 3E-06	1.6E-04 ± 5E-06	2.2E-04 ± 7E-06	2.8E-04 ± 1E-05	5.5E-04 ± 3E-05
46	I	overlapped	overlapped	overlapped	overlapped	overlapped	overlapped	overlapped	9.4E-05 ± 3E-06	1.3E-04 ± 4E-06	1.7E-04 ± 7E-06	3.6E-04 ± 1E-05
63	Y	2.4E-04 ± 2E-05	3.7E-04 ± 3E-05	4.9E-04 ± 6E-05	5.8E-04 ± 1.3E-04	6.0E-04 ± 6E-05	1.2E-03 ± 3E-04	9.6E-04 ± 1.8E-04	1.3E-03 ± 3E-04	1.0E-03 ± 3E-04	not detected	not detected
64	L	overlapped	overlapped	overlapped	overlapped	overlapped	overlapped	overlapped	overlapped	overlapped	1.1E-04 ± 3E-06	2.1E-04 ± 6E-06
65	L	overlapped	overlapped	overlapped	1.5E-04 ± 8E-06	1.4E-04 ± 5E-06	2.9E-04 ± 2E-05	3.0E-04 ± 1E-05	3.0E-04 ± 2E-05	3.8E-04 ± 2E-05	4.1E-04 ± 3E-05	6.6E-04 ± 5E-05
66	Y	5.6E-07 ± 6E-08	1.6E-06 ± 6E-08	1.5E-06 ± 7E-08	2.4E-06 ± 9E-08	4.3E-06 ± 9E-08	1.0E-05 ± 8E-07	1.3E-05 ± 3E-07	1.8E-05 ± 4E-07	3.0E-05 ± 1E-06	5.4E-05 ± 3E-06	9.5E-05 ± 2E-06
67	Y	1.3E-05 ± 2E-07	2.0E-05 ± 4E-07	3.0E-05 ± 8E-07	3.5E-05 ± 1E-06	3.8E-05 ± 8E-07	7.5E-05 ± 3E-06	9.4E-05 ± 3E-06	9.7E-05 ± 3E-06	1.3E-04 ± 4E-06	1.7E-04 ± 6E-06	3.8E-04 ± 1E-05
68	T	3.1E-05 ± 1E-06	5.1E-05 ± 1E-06	5.2E-05 ± 2E-06	6.6E-05 ± 3E-06	1.2E-04 ± 4E-06	1.6E-04 ± 8E-06	2.4E-04 ± 1E-05	2.8E-04 ± 2E-05	3.4E-04 ± 2E-05	4.6E-04 ± 3E-05	7.7E-04 ± 6E-05
70	F	1.0E-05 ± 9E-07	1.7E-05 ± 5E-07	1.8E-05 ± 1E-06	2.7E-05 ± 1E-06	4.5E-05 ± 2E-06	7.8E-05 ± 6E-06	1.0E-04 ± 1E-05	1.2E-04 ± 2E-05	1.7E-04 ± 2E-05	2.3E-04 ± 4E-05	5.9E-04 ± 3E-05
79	A	overlapped	overlapped	overlapped	overlapped	overlapped	overlapped	overlapped	overlapped	overlapped	overlapped	overlapped

80	C	overlapped	overlapped	overlapped	overlapped	overlapped	overlapped	overlapped	overlapped	overlapped	1.7E-04 ± 5E-06	3.5E-04 ± 1E-05
81	R	4.8E-06 ± 9E-08	1.1E-05 ± 4E-07	1.0E-05 ± 6E-07	1.6E-05 ± 4E-07	4.0E-05 ± 1E-06	5.8E-05 ± 2E-06	8.9E-05 ± 2E-06	1.2E-04 ± 5E-06	1.6E-04 ± 6E-06	2.2E-04 ± 1E-05	4.6E-04 ± 3E-05
82	V	2.6E-06 ± 7E-08	7.1E-06 ± 4E-07	5.2E-06 ± 1E-07	1.1E-05 ± 8E-07	2.2E-05 ± 5E-07	3.5E-05 ± 9E-07	5.5E-05 ± 1E-06	7.6E-05 ± 2E-06	overlapped	overlapped	overlapped
83	N	9.0E-06 ± 2E-07	1.9E-05 ± 4E-07	1.5E-05 ± 3E-07	2.6E-05 ± 8E-07	5.7E-05 ± 1E-06	8.9E-05 ± 4E-06	1.3E-04 ± 3E-06	1.8E-04 ± 7E-06	2.0E-04 ± 8E-06	3.0E-04 ± 2E-05	5.4E-04 ± 3E-05
84	H	2.0E-03 ± 7E-04	2.3E-03 ± 9E-04	not detected	not detected	not detected	not detected	not detected	not detected	not detected	not detected	not detected
86	T	3.9E-04 ± 3E-05	6.1E-04 ± 4E-05	5.6E-04 ± 6E-05	6.4E-04 ± 1.0E-04	1.2E-03 ± 1E-04	1.2E-03 ± 3E-04	1.2E-03 ± 2E-04	1.5E-03 ± 4E-04	1.5E-03 ± 6E-04	not detected	1.7E-03 ± 9E-04
87	L	1.9E-04 ± 6E-06	3.0E-04 ± 8E-06	3.4E-04 ± 2E-05	4.2E-04 ± 4E-05	5.3E-04 ± 3E-05	7.7E-04 ± 9E-05	9.0E-04 ± 8E-05	9.1E-04 ± 1.3E-04	1.3E-03 ± 3E-04	1.3E-03 ± 3E-04	1.8E-03 ± 6E-04
91	K	overlapped	overlapped	overlapped	overlapped	overlapped	overlapped	overlapped	overlapped	overlapped	overlapped	1.0E-03 ± 7E-05
93	V	4.4E-06 ± 4E-07	8.6E-06 ± 3E-07	8.4E-06 ± 6E-07	1.0E-05 ± 2E-07	2.5E-05 ± 6E-07	4.1E-05 ± 1E-06	6.5E-05 ± 2E-06	7.7E-05 ± 2E-06	1.2E-04 ± 3E-06	1.5E-04 ± 5E-06	3.2E-04 ± 1E-05
95	W	1.5E-05 ± 4E-07	2.6E-05 ± 6E-07	2.7E-05 ± 5E-07	4.3E-05 ± 2E-06	6.7E-05 ± 1E-06	1.1E-04 ± 5E-06	1.5E-04 ± 5E-06	1.9E-04 ± 7E-06	2.7E-04 ± 2E-05	3.1E-04 ± 2E-05	6.0E-04 ± 3E-05

Table 1: Observed exchange rates (k_{obs}) for all detected amide hydrogens in the 11 experiments performed.

ensemble	distribution	slope (K)	intercept (kcal/mol)	R^2
10,36,38,40,44,66,68,70,82,93,95	experimental	317 ± 4	5.8 ± 0.4	1.00
	theoretical random	307	6.79	n.a.
10,36,38,40,44,66,68,70,82,95	experimental	318 ± 3	5.7 ± 0.3	1.00
	theoretical random	306	6.86	n.a.
10,36,38,40,44,68,70,82,93,95	experimental	314 ± 3	6.1 ± 0.3	1.00
	theoretical random	306	6.73	n.a.
10,36,38,40,44,68,70,82,95	experimental	315 ± 1	5.97 ± 0.13	1.00
	theoretical random	306	6.79	n.a.

Table 2: Regression parameters of enthalpy-entropy linear fittings, using different data ensembles. n.a. not available. The statistical assessment of the compensation robustness is required to exclude the effect of possible artifacts. In our case, artifacts may arise because ΔG_{obs} and T values are restricted in the range 4-9.1 kcal/mol and 301-315 K, respectively; so the linear trends of the thermal variation of ΔG_{obs}, in the considered range of temperatures, may appear concurrent because forced within limited intervals of the $\Delta G(T)$ space. The restricted free energy and temperature windows may explain why also low-precision points (such as His-84, Leu-64 and Cys-80) have a very good correlation in the enthalpy-entropy plot (not shown). On the basis of these caveats, we considered only the most precise data subset showing EX2-like behavior (reported in this table), to assess the significance of the observed enthalpy-entropy correlation (Fig. 3 in the main text).

ensemble	DOF	slope *t*-value	*p*-value	intercept *t*-value	*p*-value
10,36,38,40,44,66,68,70,82,93,95	9	2.7	2.3E-2	2.5	3.6E-2
10,36,38,40,44,66,68,70,82,95	8	3.9	4.8E-3	3.5	8.4E-3
10,36,38,40,44,68,70,82,93,95	8	2.8	2.3E-2	2.5	3.7E-2
10,36,38,40,44,68,70,82,95	7	6.9	2.3E-4	6.2	4.4E-4

Table 3: Student's *t*-tests about the null hypothesis that the experimental enthalpy-entropy correlation line is compatible with the random theoretical correlation lines. In fact, if the observed correlation were fully artifactual, the most probable random values of slope and intercept would respectively be the harmonic weighted mean of the experimental temperatures and the weighted mean of the experimental free energy differences (1), reported as theoretical random values in table 2. In the table 3, we checked whether our data could randomly derive from the Gaussian distribution around these mean values (1). The statistical significance is improved if data of Tyr-66 and Val-93 are discarded, on the basis that their residuals to Eq. 19 (see text) are outliers according to the Peirce criterion with t-values of 2.3 and 2.0, respectively. According to these tests, the null hypothesis

could be rejected, at a significance level varying among considered ensembles. DOF degrees of freedom.

Ensemble	(Number of positive)/(number of negative) values of residuals to random distributions	*p*-value
10,36,38,40,44,66,68,70,82,93,95	5/6	1
10,36,38,40,44,66,68,70,82,95	4/6	0.75
10,36,38,40,44,68,70,82,93,95	4/6	0.75
10,36,38,40,44,68,70,82,95	4/5	1

Table 4: Sign tests for the null hypothesis that the relationship between enthalpy and entropy is dictated by the limited interval of the $\Delta G(T)$ space (see text). Presented *p*-value is the two-tailed binomial probability. According to these data, the null hypothesis can not be rejected; this result could derive from the fact that both observed and theoretical distributions are made using the same experimental data.

ensemble	Number of runs	*p*-value
10,36,38,40,44,66,68,70,82,93,95	4	0.11
10,36,38,40,44,66,68,70,82,95	2	9.5E-3
10,36,38,40,44,68,70,82,93,95	4	0.19
10,36,38,40,44,68,70,82,95	2	1.6E-2

Table 5: Wald-Wolfowitz runs tests for the null hypothesis that the relationship between enthalpy and entropy is dictated by the limited interval of the $\Delta G(T)$ space (see text). Observed enthalpy values exceeding random function values are marked with “+” and other data with “–”. Then data were ordered according to the magnitude of enthalpy value: a run is then defined as a sequence of adjacent equal “+” or “–” elements. The probability values presented is one-tailed, since we are only interested in finding less runs than the expectation. According to these values, the null hypothesis could be rejected for the second and fourth data sets.

ensemble	Composition of first row	Contingency matrix	*p*-value
10,36,38,40,44,66,68,70,82,93,95	36,44,68,70,95	$\begin{vmatrix} 0 & 5 \\ 4 & 1 \end{vmatrix}$	4.8E-2
10,36,38,40,44,66,68,70,82,95	36,44,68,70,95	$\begin{vmatrix} 0 & 5 \\ 4 & 1 \end{vmatrix}$	4.8E-2
10,36,38,40,44,68,70,82,93,95	36,44,68,70,95	$\begin{vmatrix} 0 & 5 \\ 4 & 1 \end{vmatrix}$	4.8E-2
10,36,38,40,44,68,70,82,95	36,44,68,95	$\begin{vmatrix} 0 & 4 \\ 4 & 0 \end{vmatrix}$	2.9E-2

Table 6: Fisher exact tests for the null hypothesis that the relationship between enthalpy and entropy is dictated by the limited interval of the $\Delta G(T)$ space (see text). The number of data is distributed into a 2×2 matrix; columns sharing was made according to the positive (left) or negative (right) sign of the residuals to random distribution, while rows were made according to the magnitude of enthalpy values in comparison to the median value (lower values above, higher values below; values equal to median were discarded). According to two-tailed *p*-values, the null hypothesis could apply to no ensembles. Note that, while sign-tests (table 4) and Wilcoxon signed-ranks tests (not shown) indicate that residuals to random distributions have an average close to zero, Wald-Wolfowitz and Fisher exact tests (tables 5 and 6) suggest that the residuals distribution depends on enthalpy values.

References of Supplementary Material

1. Krug, R., W. Hunter, and R. Grieger. 1976. Enthalpy-entropy compensation. 1. Some fundamental statistical problems associated with the analysis of van't Hoff and Arrhenius data. J. Phys. Chem. 80:2335-2341.