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Hydration effects in protein unfolding ☆

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Abstract

The enthalpies and entropies of hydration of polar, aromatic and aliphatic groups upon unfolding of nine different globular proteins were calculated over a broad temperature range using information on the three-dimensional structures of the native states of these proteins and thermodynamic data on the transfer of various low molecular compounds modeling protein groups from the gaseous phase to water. Exclusion of these hydration effects from the calorimetrically determined enthalpy and entropy of unfolding of these proteins permitted us to estimate the energy of interactions between groups packed in the interior of the native protein, and also the entropy effects associated with the increase of configurational freedom of the backbone polypeptide chain and side chains. It is shown that the compact native state of a protein is stabilized by the enthalpic interactions between internal groups while the hydration effects of all the groups, except the aliphatic ones, which are exposed upon unfolding destabilize this state.

Key words: Hydration; Interactions; Protein; Unfolding; Thermodynamics

1. Introduction

It is clear that hydration effects should play an important role in the thermodynamics of unfolding/refolding of proteins in aqueous solution, i.e. in the energetics of the native protein structure. There have been many attempts to estimate hydration effects upon protein unfolding using information on transfer of various low molecular weight compounds to the aqueous environment [1–4]. Earlier we demonstrated for four globular proteins that the heat capacity, enthalpy and entropy of hydration upon unfolding can be estimated by simple summation of the hydration effects of individual groups which are exposed to water [6–10]. The thermodynamic parameters for hydration of individual groups were determined from transfer from the gaseous phase to water of various low molecular weight compounds modeling protein groups. In this paper, we present the results of an analysis of the contribution of hydra-

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tion effects to the energetics of nine globular proteins. The main criteria in selecting these proteins were the reversibility of the denaturation process modeling their unfolding, the completeness of unfolding, the reliability of calorimetric data specifying unfolding, and the precision of resolution of their three-dimensional structures. The knowledge of three-dimensional protein structures permits us to determine the water accessible surface areas (ASA) of polar and nonpolar groups in the native and unfolded states and, using thermodynamic data on transfer of various groups from the gaseous state to water, to estimate the hydration effects upon protein unfolding.

2. Structural and thermodynamic characteristics of the analyzed proteins

In Table 1 are given the structural characteristics of the globular proteins which were used in our analysis: the molecular weight, M_w, the number of amino acid residues, N_r; the number of S–S cross-links in the polypeptide chains, N_{ss}; water accessible surface area of polar, $\Delta^U_N ASA_{pol}$,

Table 1
Structural characteristics of the studied protein

	BPTI	Ubiquitin	RNase T1	Cytochrome c	Barnase	RNase A	Lysozyme	Interleukin 1β	Myoglobin
M.W.	6565	8433	11071	12830	12365	13600	14300	17381	17800
N_r	58	76	104	104	110	124	129	153	153
N_{SS}	3	0	2	0	0	4	4	0	0
ΔASA									
aliph	2350	3599	3375	3922	4317	4318	5180	6531	6842
arom	410	269	1482	1020	1296	955	1185	1374	1565
$\Sigma_{npl}\Delta ASA$	2760	3868	4857	4942	5613	5273	6365	7905	8407
Polar Parts									
Arg	76	124	103	214	350	234	362	101	268
Asn	3	25	197	190	220	201	291	29	30
Asp	62	80	80	5	231	143	175	104	153
Cys	0	0	175	109	0	442	437	113	0
Gln	87	254	108	177	93	326	80	364	207
Glu	195	71	219	105	86	178	88	320	447
His	0	7	84	80	48	125	38	29	309
Lys	125	117	45	193	181	87	114	247	255
Met	44	37	0	87	0	148	86	201	87
Ser	19	34	12	12	21	173	183	192	119
Thr	24	97	30	159	70	140	90	99	62
Trp	39	0	32	47	83	0	147	10	57
Tyr	39	36	291	198	213	179	83	101	83
-CONH-	737	1030	1415	1782	1517	1765	2057	2231	2716
$\Sigma_{pol}\Delta ASA$	1444	1912	2791	3358	3113	4141	4231	4141	4793

M.W.: molecular weight in daltons; N_r: number of amino acid residues; N_{SS}: number of disulfide bonds, ΔASA: water accessible surface area change. The surface area of a native protein was calculated using the CAVT66 program [24] using algorithm of Shrake and Rupley [25] and van der Waals radii of Chothia [26]. The surface area of the unfolded state was calculated as the surface area of a polypeptide of a given sequence in an extended conformation. The latter was generated using QUANTA (Molecular Simulations., Inc.). The detail on used structures from the Protein Data Bank [28] are reported elsewhere (BPTI [12]; ubiquitin [29]; RNase T1 [30]; barnase [31]; interleukin-1β [32]; RNase A, cytochrome c, lysozyme and myoglobin [7]). The listed buried ASA are lower than those reported earlier [7,12,29-32]. The reason for it is that the unfolded state in our previous work was computed as a sum of ASA obtained from the tripeptides Gly-X-Gly [27], which is not the best approxymation for the unfolded state.

and non-polar groups, $\Delta^U_N ASA_{npl}$. The analyzed proteins differ significantly in the molecular weight which varies from 6.5 kDa to 18 kDa, and in the relative extent of exposure of polar and non-polar surfaces.

In Table 2 are listed thermodynamic characteristics of unfolding for the considered proteins: the enthalpy, entropy and Gibbs energy of unfolding, determined in the temperature range 0–130°C. The values were obtained by extrapolation of the calorimetrically measured heat effects of protein denaturation. For the analysis we selected proteins for which denaturation is reversible, is approximated well by a two-state transition, and which show a good correspondence between the experimental heat capacity in the denatured state and that calculated for the unfolded polypeptide chain by summation of the heat capacities of the amino acid residues according to Makhatadze and Privalov [8]. The correspondence of the calculated and the measured heat capacity values was considered as a criterion of completeness of unfolding of polypeptide chain [11]. The enthalpies and entropies of unfolding were determined for the solvent conditions in which denaturation proceeds without noticeable residual structure and protein stability is maximal. Therefore they do not include effects of

Table 2
Thermodynamics characteristics of the studied proteins (molar enthalpy, $\Delta^U_N H^{exp}$, molar entropy, $\Delta^U_N S^{exp}$, and Gibbs energy of unfolding, $\Delta^U_N G^{exp}$)

		Temperature (°C)					
		5	25	50	75	100	125
BPTI	$\Delta^U_N H^{exp}$	72	130	200	259	303	323
	$\Delta^U_N S^{exp}$	87	288	514	690	809	864
	$\Delta^U_N G^{exp}$	47.8	44.2	34.0	18.9	1.2	−20.9
Ubiquitin	$\Delta^U_N H^{exp}$	88	27	1620	273	351	393
	$\Delta^U_N S^{exp}$	−444	−44	393	727	959	1068
	$\Delta^U_N G^{exp}$	35.4	40.1	35.1	20.0	−6.7	−32.1
RNase T1	$\Delta^U_N H^{exp}$	173	281	410	528	621	672
	$\Delta^U_N S^{exp}$	444	817	1233	1584	1845	1976
	$\Delta^U_N G^{exp}$	49.6	37.5	11.7	−23.2	−67.2	−114.4
Cytochrome c	$\Delta^U_N H^{exp}$	−53	89	268	421	532	593
	$\Delta^U_N S^{exp}$	−319	174	752	1210	1520	1681
	$\Delta^U_N G^{exp}$	35.7	37.1	25.1	−1.1	−35.0	−76.0
Barnase	$\Delta^U_N H^{exp}$	167	307	467	590	664	690
	$\Delta^U_N S^{exp}$	379	866	1384	1752	1959	2029
	$\Delta^U_N G^{exp}$	61.6	48.9	20.0	−19.7	−66.7	−117.5
RNase A	$\Delta^U_N H^{exp}$	220	294	405	512	603	664
	$\Delta^U_N S^{exp}$	641	896	1254	1574	1826	1989
	$\Delta^U_N G^{exp}$	41.8	27.0	−0.0	−35.8	−78.1	−127.6
Lysozyme	$\Delta^U_N H^{exp}$	111	242	408	562	683	753
	$\Delta^U_N S^{exp}$	164	618	1153	1615	1954	2138
	$\Delta^U_N G^{exp}$	65.4	57.8	35.6	−0.0	−45.8	−97.9
Interleukin-1β	$\Delta^U_N H^{exp}$	7	151	330	501	640	736
	$\Delta^U_N S^{exp}$	−99	401	1006	1516	1903	2155
	$\Delta^U_N G^{exp}$	34.5	31.5	5.1	−26.6	−69.8	−121.7
Myoglobin	$\Delta^U_N H^{exp}$	−231	6	291	555	774	920
	$\Delta^U_N S^{exp}$	−919	−116	805	1595	2207	2588
	$\Delta^U_N G^{exp}$	24.5	40.6	31.0	−0.1	−49.2	−110.0

$\Delta^U_N H^{exp}$ and $\Delta^U_N G^{exp}$ are in kJ mol^{-1} and $\Delta^U_N S^{exp}$ is in kJ K^{-1} mol^{-1}. Experimental data were taken from: BPTI [12]; ubiquitin [29]; RNase T1 [30]; barnase [31]; interleukin-1β [32]; RNase A, cytochrome c, lysozyme and myoglobin [7,10].

ionization of groups upon denaturation and their denaturation can be considered as an unfolding process.

The temperature dependence of the enthalpy and entropy of unfolding was determined using the difference in the heat capacities of the native and denatured states

$$\Delta^{U}_{N}H(T) = \Delta^{U}_{N}H(T_t) + \int_{T_t}^{T} \Delta^{U}_{N}C_p(T)\, dT, \quad (1)$$

and

$$\Delta^{U}_{N}S(T) = \frac{\Delta^{U}_{N}H(T_t)}{T_t} + \int_{T_t}^{T} \Delta^{U}_{N}C_p(T)\, d \ln T, \quad (2)$$

where

$$\Delta^{U}_{N}C_p(T) = C^{U}_{p}(T) - C^{N}_{p}(T). \quad (3)$$

Here T_t is transition temperature, $\Delta^{U}_{N}H(T_t)$ is the calorimetrically measured enthalpy of protein unfolding at transition temperature, $C^{U}_{P}(T)$ is the heat capacity of the unfolded state which is determined by summation of the heat capacities of amino acid residues according to Ref. [8], and $C^{N}_{P}(T)$ is the heat capacity function of the native state which is determined by extrapolation of the calorimetrically measured partial heat capacities of the protein in the temperature range in which the protein exists in the native state. As has been shown, the heat capacity of the native state can be approximated by a linear function [8,9,12].

In Fig. 1a are presented temperature dependencies of the specific unfolding enthalpies calculated on a per mole of amino acid residue basis for the considered proteins. They all are monotonously increasing functions of temperature which asymptotically approach maximal values at 130°C, where the heat capacity difference between the native and denatured state become zero [9]. In contrast to what was observed on the smaller selection of proteins studied before [7,13] the nine proteins which we consider here do not show so clear convergence of the enthalpy functions at 130°C to the value of 5.5 J mol^{-1} of amino acid residues, which appeared as universal for globular proteins [14]: the specific enthalpies for barnase and RNase T1 are noticeably higher,

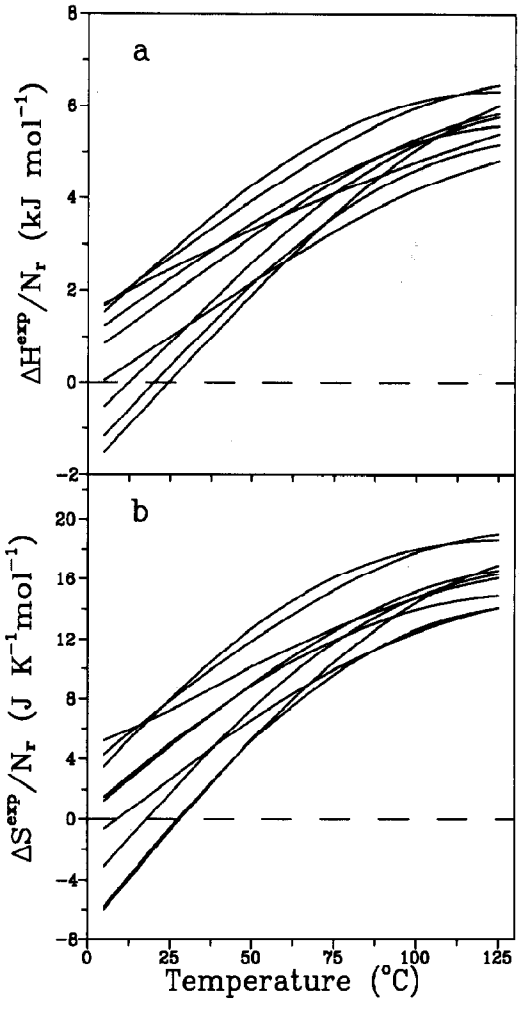

Fig. 1. Temperature dependencies of (a) the specific enthalpies of unfolding of some of the considered proteins, and (b) the specific entropies of unfolding of these proteins, calculated per mole of amino acid residues.

6.4 J mol^{-1} of amino acid residues, while for interleukin-1β it is lower, 4.8 J mol^{-1} of amino acid residues. Similar situation is observed with the entropy functions (Fig. 1b). The significant deviation of the enthalpy and entropy functions of barnase, RNase T1 and interleukin-β from that found for other, calorimetrically studied proteins was the main reason to include them in our analysis.

Table 3
Normalized values of the enthalpy entropy and Gibbs energy of hydration of various groups [a]

		Temperature (°C)					
		5	25	50	75	100	125
Aliphatic	$\Delta\hat{H}^{hyd}$	−166	−122	−70	−21	26	69
	$\Delta\hat{S}^{hyd}$	−730	−578	−409	−263	−134	−22
	$\Delta\hat{G}^{hyd}$	37	50	62	71	75	77
Aromatic	$\Delta\hat{H}^{hyd}$	−180	−148	−111	−77	−46	−18
	$\Delta\hat{S}^{hyd}$	−430	−319	−199	−98	−12	62
	$\Delta\hat{G}^{hyd}$	−61	−53	−47	−43	−42	−43
Arg	$\Delta\hat{H}^{hyd}$	−821	−827	−831	−833	−834	−833
	$\Delta\hat{S}^{hyd}$	−458	−478	−492	−497	−498	−495
	$\Delta\hat{G}^{hyd}$	−694	−685	−672	−660	−647	−635
Asn	$\Delta\hat{H}^{hyd}$	−871	−894	−915	−928	−936	−936
	$\Delta\hat{S}^{hyd}$	−575	−654	−723	−763	−783	−785
	$\Delta\hat{G}^{hyd}$	−711	−699	−681	−663	−643	−623
Asp	$\Delta\hat{H}^{hyd}$	−684	−715	−746	−768	−782	−788
	$\Delta\hat{S}^{hyd}$	−360	−469	−569	−636	−675	−691
	$\Delta\hat{G}^{hyd}$	−584	−575	−562	−547	−530	−513
Cys	$\Delta\hat{H}^{hyd}$	−309	−271	−218	−160	−98	−32
	$\Delta\hat{S}^{hyd}$	−535	−402	−232	−59	113	283
	$\Delta\hat{G}^{hyd}$	−160	−151	−143	−139	−140	−145
Gln	$\Delta\hat{H}^{hyd}$	−697	−703	−706	−706	−703	−697
	$\Delta\hat{S}^{hyd}$	−571	−591	−604	−603	−594	−579
	$\Delta\hat{G}^{hyd}$	−538	−527	−511	−497	−481	−467
Glu	$\Delta\hat{H}^{hyd}$	−549	−562	−573	−580	−583	−582
	$\Delta\hat{S}^{hyd}$	−392	−436	−473	−492	−500	−498
	$\Delta\hat{G}^{hyd}$	−440	−432	−420	−409	−396	−383
His	$\Delta\hat{H}^{hyd}$	−1084	−1128	−1188	−1247	−1301	−1349
	$\Delta\hat{S}^{hyd}$	−542	−693	−888	−1060	−1211	−1337
	$\Delta\hat{G}^{hyd}$	−933	−922	−901	−878	−848	−816
Lys	$\Delta\hat{H}^{hyd}$	−685	−714	−753	−789	−821	−847
	$\Delta\hat{S}^{hyd}$	−384	−482	−609	−716	−804	−870
	$\Delta\hat{G}^{hyd}$	−578	−570	−556	−540	−519	−498
Met	$\Delta\hat{H}^{hyd}$	−399	−473	−572	−672	−774	−869
	$\Delta\hat{S}^{hyd}$	−158	−412	−732	−1031	−1308	−1555
	$\Delta\hat{G}^{hyd}$	−356	−350	−335	−315	−283	−247
Ser	$\Delta\hat{H}^{hyd}$	−1015	−1045	−1078	−1104	−1126	−1140
	$\Delta\hat{S}^{hyd}$	−878	−983	−1089	−1168	−1227	−1265
	$\Delta\hat{G}^{hyd}$	−771	−752	−726	−698	−667	−636
Thr	$\Delta\hat{H}^{hyd}$	−1262	−1287	−1318	−1343	−1359	−1356
	$\Delta\hat{S}^{hyd}$	−971	−1053	−1156	−1232	−1274	−1265
	$\Delta\hat{G}^{hyd}$	−992	−972	−944	−916	−881	−850
Trp	$\Delta\hat{H}^{hyd}$	−1181	−1161	−1135	−1110	−1084	−1055
	$\Delta\hat{S}^{hyd}$	−766	−693	−615	−534	−460	−392
	$\Delta\hat{G}^{hyd}$	−968	−954	−936	−924	−912	−899

Table 3 (continued)

		Temperature (°C)					
		5	25	50	75	100	125
Tyr	$\Delta\hat{H}^{hyd}$	−824	−854	−889	−921	−946	−963
	$\Delta\hat{S}^{hyd}$	−314	−415	−531	−625	−695	−742
	$\Delta\hat{G}^{hyd}$	−735	−730	−717	−703	−686	−667
CONH	$\Delta\hat{H}^{hyd}$	−1662	−1702	−1745	−1785	−1823	−1862
	$\Delta\hat{S}^{hyd}$	−890	−1026	−1162	−1278	−1383	−1481
	$\Delta\hat{G}^{hyd}$	−1415	−1396	−1370	−1340	−1307	−1272

[a] Data from Refs. [7,10]; $\Delta\hat{H}^{hyd}$ and $\Delta\hat{G}^{hyd}$ are in J mol^{-1} Å^{-2} and $\Delta\hat{S}^{hyd}$ is in J K^{-1} mol^{-1} Å^{-2}. The reduced thermodynamic parameters listed in this table are obtained using the surface areas calculated as described in the legend to Table 1. The surface area strongly depends on the choice of the van der Waals radii. Consequently, one should recalculate the hydration parameters for the new value of ASA obtained using different set of van der Waals radii [10].

3. Hydration effects of protein unfolding

As was shown earlier the hydration effects, $\Delta^U_N L^{hyd}_k(T)$, of protein groups exposed to water upon unfolding can be assumed to be proportional to the water accessible surface area of the group [7,10],

$$\Delta L^{hyd}_k(T) = \sum_i \Delta^U_N ASA_{k,i} \times \Delta\hat{L}^{hyd}_{k,i}(T), \qquad (4)$$

Table 4
Enthalpies of hydration of aliphatic, $\Delta^U_N H^{hyd}_{alp}$, aromatic, $\Delta^U_N H^{hyd}_{arm}$, and polar groups, $\Delta^U_N H^{hyd}_{pol}$, upon unfolding of studied proteins

		Temperature (°C)					
		5	25	50	75	100	125
BPTI	$\Delta^U_N H^{hyd}_{alp}$	−274	−202	−116	−35	43	114
	$\Delta^U_N H^{hyd}_{arm}$	−136	−112	−84	−58	−35	−14
	$\Delta^U_N H^{hyd}_{pol}$	−1879	−1912	−1942	−1967	−1986	−2002
Ubiquitin	$\Delta^U_N H^{hyd}_{alp}$	−597	−439	−252	−76	94	248
	$\Delta^U_N H^{hyd}_{arm}$	−48	−40	−30	−21	−12	−5
	$\Delta^U_N H^{hyd}_{pol}$	−2395	−2454	−2517	−2574	−2625	−2671
RNase T1	$\Delta^U_N H^{hyd}_{alp}$	−560	−412	−236	−71	88	233
	$\Delta^U_N H^{hyd}_{arm}$	−267	−219	−165	−114	−68	−27
	$\Delta^U_N H^{hyd}_{pol}$	−3362	−3437	−3516	−3584	−3644	−3696
Cytochrome c	$\Delta^U_N H^{hyd}_{alp}$	−720	−529	−304	−91	113	299
	$\Delta^U_N H^{hyd}_{arm}$	−229	−189	−141	−98	−59	−23
	$\Delta^U_N H^{hyd}_{pol}$	−4365	−4466	−4578	−4677	−4764	−4842
Barnase	$\Delta^U_N H^{hyd}_{alp}$	−717	−527	−302	−91	112	298
	$\Delta^U_N H^{hyd}_{arm}$	−233	−192	−144	−100	−60	−23
	$\Delta^U_N H^{hyd}_{pol}$	−3829	−3921	−4018	−4105	−4181	−4249
RNase A	$\Delta^U_N H^{hyd}_{alp}$	−717	−527	−302	−91	112	298
	$\Delta^U_N H^{hyd}_{arm}$	−172	−141	−106	−74	−44	−17
	$\Delta^U_N H^{hyd}_{pol}$	−4614	−4716	−4823	−4914	−4992	−5058
Lysozyme	$\Delta^U_N H^{hyd}_{alp}$	−860	−632	−363	−109	135	357
	$\Delta^U_N H^{hyd}_{arm}$	−213	−175	−132	−91	−55	−21
	$\Delta^U_N H^{hyd}_{pol}$	−5023	−5124	−5226	−5313	−5387	−5451
Interleukin 1β	$\Delta^U_N H^{hyd}_{alp}$	−1084	−797	−457	−137	170	451
	$\Delta^U_N H^{hyd}_{arm}$	−247	−203	−153	−106	−63	−25
	$\Delta^U_N H^{hyd}_{pol}$	−5048	−5178	−5320	−5449	−5567	−5675
Myoglobin	$\Delta^U_N H^{hyd}_{alp}$	−1205	−885	−508	−152	189	501
	$\Delta^U_N H^{hyd}_{arm}$	−327	−269	−202	−140	−84	−33
	$\Delta^U_N H^{hyd}_{pol}$	−6292	−6450	−6625	−6784	−6928	−7062

All values in kJ mol^{-1}.

where $\Delta^{U}_{N}ASA_{k,i}$ is the change of water accessible surface areas of type k of a group i in protein upon unfolding and $\Delta\hat{L}^{hyd}_{k,i}(T)$ is the normalized per square angstrom the hydration enthalpy, entropy or heat capacity change of this type of a group in protein. These normalized hydration effects were determined by transfer of model compounds from the gaseous phase to water and their values are listed in Table 3. The transfer characteristics were corrected for the volume effect to exclude the effect of thermal liberation as suggested by Ben-Naim [15,16]. The procedure of determination of the normalized per square angstrom of surface area enthalpies, entropies and heat capacities of hydration of polar and non-polar groups in protein were described [6,7,9,10]. The values of normalized hydration effects are given in Table 3.

As follows from Table 3, at room temperature the hydration enthalpy and entropy of both polar and non-polar groups are negative, but they are much larger for the polar groups than for the non-polar groups. The other difference between hydration effects of these groups is that with increasing temperature the hydration effects of non-polar groups are increasing, becoming even positive, while the hydration effects of polar groups are decreasing to more negative values. This is because the heat capacity change upon hydration of polar and non-polar groups precedes with opposite sign: it is negative for the polar groups and positive for the non-polar groups [5,6,17]. It is notable that the normalized hydration effects of the aliphatic groups and aromatic groups differ considerably in magnitude and in their dependencies on temperature. As a result,

Table 5
Entropies of hydration of aliphatic, $\Delta^{U}_{N}S^{hyd}_{alp}$, aromatic, $\Delta^{U}_{N}S^{hyd}_{arm}$, and polar groups, $\Delta^{U}_{N}S^{hyd}_{pol}$, upon unfolding of studied proteins

		Temperature (°C)					
		5	25	50	75	100	125
BPTI	$\Delta^{U}_{N}S^{hyd}_{alp}$	−1206	−955	−676	−434	−221	−36
	$\Delta^{U}_{N}S^{hyd}_{arm}$	−325	−241	−150	−74	−9	47
	$\Delta^{U}_{N}S^{hyd}_{pol}$	−1151	−1262	−1356	−1428	−1483	−1523
Ubiquitin	$\Delta^{U}_{N}S^{hyd}_{alp}$	−2627	−2080	−1472	−947	−482	−79
	$\Delta^{U}_{N}S^{hyd}_{arm}$	−116	−86	−54	−26	−3	17
	$\Delta^{U}_{N}S^{hyd}_{pol}$	−1378	−1581	−1783	−1945	−2088	−2206
RNase T1	$\Delta^{U}_{N}S^{hyd}_{alp}$	−2464	−1951	−1380	−888	−452	−74
	$\Delta^{U}_{N}S^{hyd}_{arm}$	−637	−473	−295	−145	−18	92
	$\Delta^{U}_{N}S^{hyd}_{pol}$	−1910	−2164	−2415	−2615	−2783	−2915
Cytochrome c	$\Delta^{U}_{N}S^{hyd}_{alp}$	−3167	−2507	−1774	−1141	−581	−95
	$\Delta^{U}_{N}S^{hyd}_{arm}$	−548	−406	−254	−125	−15	79
	$\Delta^{U}_{N}S^{hyd}_{pol}$	−2489	−2836	−3192	−3483	−3727	−3925
Barnase	$\Delta^{U}_{N}S^{hyd}_{alp}$	−3151	−2495	−1766	−1135	−578	−95
	$\Delta^{U}_{N}S^{hyd}_{arm}$	−557	−413	−258	−127	−16	80
	$\Delta^{U}_{N}S^{hyd}_{pol}$	−2115	−2433	−2740	−2994	−3209	−3382
RNase A	$\Delta^{U}_{N}S^{hyd}_{alp}$	−3152	−2496	−1766	−1136	−579	−95
	$\Delta^{U}_{N}S^{hyd}_{arm}$	−411	−305	−190	−94	−11	59
	$\Delta^{U}_{N}S^{hyd}_{pol}$	−2802	−3154	−3495	−3759	−3981	−4148
Lysozyme	$\Delta^{U}_{N}S^{hyd}_{alp}$	−3781	−2994	−2119	−1362	−694	−114
	$\Delta^{U}_{N}S^{hyd}_{arm}$	−510	−378	−236	−116	−14	73
	$\Delta^{U}_{N}S^{hyd}_{pol}$	−3004	−3349	−3675	−3925	−4134	−4296
Interleukin 1β	$\Delta^{U}_{N}S^{hyd}_{alp}$	−4768	−3775	−2671	−1718	−875	−144
	$\Delta^{U}_{N}S^{hyd}_{arm}$	−591	−438	−273	−135	−16	85
	$\Delta^{U}_{N}S^{hyd}_{pol}$	−2924	−3372	−3827	−4201	−4534	−4809
Myoglobin	$\Delta^{U}_{N}S^{hyd}_{alp}$	−5298	−4195	−2969	−1909	−973	−160
	$\Delta^{U}_{N}S^{hyd}_{arm}$	−782	−580	−362	−178	−22	113
	$\Delta^{U}_{N}S^{hyd}_{pol}$	−3514	−4060	−4616	−5080	−5483	−5824

All values in $J\ K^{-1}\ mol^{-1}$.

they become zero at different temperatures. It is remarkable that these temperatures are different for the enthalpy and entropy of hydration [7,10].

In Tables 4, 5 and 6 are listed the hydration enthalpy, entropy and Gibbs energy upon unfolding of the considered proteins. It is remarkable that the Gibbs energies of hydration of polar groups are large and negative and their absolute values increase in magnitude with decreasing temperature. The Gibbs energies of hydration of aliphatic groups are positive and their absolute values decrease with decreasing temperature. As for the Gibbs energy of hydration of aromatic groups, which were always regarded as typical non-polar groups, their Gibbs energies of hydration are negative, but much smaller in magnitude than the Gibbs energies of hydration of polar groups [10,18].

In Fig. 2 are presented the hydration effects of non-polar groups for two proteins, myoglobin and RNase A, which are two extreme cases from the point of view of the relative number of non-polar contacts. The interior of myoglobin is much more saturated by non-polar contacts than that of RNase A (see Table 1). The hydration effects of polar groups of these proteins are shown in Fig. 3. These hydration effects are calculated per mole of amino acid residues (specific values) to facilitate comparison of these two proteins which differ in molecular weight. It appears (see also Tables 4–6) that *it is only hydration effects of aliphatic groups which stabilize the compact native*

Table 6
Gibbs energies of hydration of aliphatic, $\Delta^U_N G^{hyd}_{alp}$, aromatic, $\Delta^U_N G^{hyd}_{arm}$, and polar groups, $\Delta^U_N G^{hyd}_{pol}$, upon unfolding of studied proteins

		Temperature (°C)					
		5	25	50	75	100	125
BPTI	$\Delta^U_N G^{hyd}_{alp}$	61	83	102	117	124	127
	$\Delta^U_N G^{hyd}_{arm}$	−46	−40	−36	−33	−32	−33
	$\Delta^U_N G^{hyd}_{pol}$	−1559	−1536	−1504	−1470	−1433	−1396
Ubiquitin	$\Delta^U_N G^{hyd}_{alp}$	133	180	223	256	270	277
	$\Delta^U_N G^{hyd}_{arm}$	−16	−14	−13	−12	−11	−12
	$\Delta^U_N G^{hyd}_{pol}$	−2012	−1983	−1941	−1897	−1846	−1793
RNase T1	$\Delta^U_N G^{hyd}_{alp}$	125	169	209	240	253	260
	$\Delta^U_N G^{hyd}_{arm}$	−90	−79	−70	−64	−62	−64
	$\Delta^U_N G^{hyd}_{pol}$	−2831	−2792	−2736	−2674	−2606	−2536
Cytochrome c	$\Delta^U_N G^{hyd}_{alp}$	161	217	269	308	325	334
	$\Delta^U_N G^{hyd}_{arm}$	−78	−68	−60	−55	−54	−55
	$\Delta^U_N G^{hyd}_{pol}$	−3673	−3621	−3547	−3465	−3374	−3280
Barnase	$\Delta^U_N G^{hyd}_{alp}$	160	216	268	307	324	332
	$\Delta^U_N G^{hyd}_{arm}$	−79	−69	−61	−56	−54	−56
	$\Delta^U_N G^{hyd}_{pol}$	−3241	−3196	−3133	−3063	−2984	−2903
RNase A	$\Delta^U_N G^{hyd}_{alp}$	160	216	268	307	324	332
	$\Delta^U_N G^{hyd}_{arm}$	−58	−51	−45	−41	−40	−41
	$\Delta^U_N G^{hyd}_{pol}$	−3835	−3776	−3694	−3606	−3507	−3407
Lysozyme	$\Delta^U_N G^{hyd}_{alp}$	192	259	321	368	389	399
	$\Delta^U_N G^{hyd}_{arm}$	−72	−3	−56	−51	−50	−51
	$\Delta^U_N G^{hyd}_{pol}$	−4188	−4126	−4039	−3947	−3845	−3741
Interleukin 1β	$\Delta^U_N G^{hyd}_{alp}$	242	327	405	464	490	503
	$\Delta^U_N G^{hyd}_{arm}$	−84	−73	−65	−59	−58	−59
	$\Delta^U_N G^{hyd}_{pol}$	−4235	−4173	−4084	−3987	−3876	−3761
Myoglobin	$\Delta^U_N G^{hyd}_{alp}$	269	363	450	515	544	559
	$\Delta^U_N G^{hyd}_{arm}$	−111	−96	−85	−78	−76	−78
	$\Delta^U_N G^{hyd}_{arm}$	−5315	−5240	−5134	−5016	−4883	−4744

All values in $kJ \cdot mol^{-1}$.

state of protein molecule. Hydration effects of aromatic, and especially of polar groups destabilize compact native state of protein molecule.

It should also be noted that the calculated values of the enthalpy and entropy of hydration of polar groups in proteins represent perhaps the maximal values of the expected hydration effects. As we discussed earlier [7], if there is a distant influence of the polar group on the state of water, i.e. not only the groups which are on the surface of protein are affecting water but also the groups which are not in direct contact with water, one can expect that the hydration effects of exposure of polar groups to water would be smaller than what follows from the additive calculation. However, since at the present time the range of the influence of the polar group on water is unclear, in this paper we ignore this possibility.

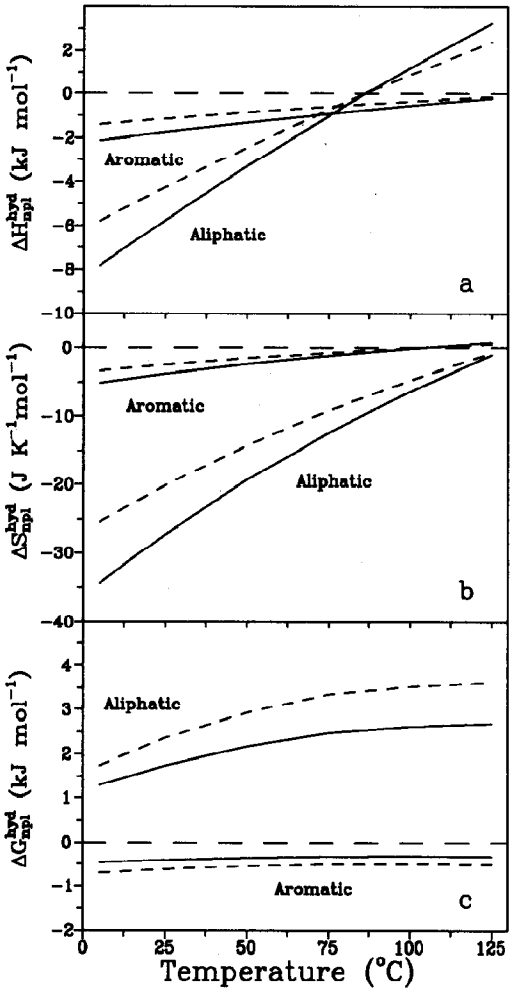

Fig. 2. The specific enthalpy (a), entropy (b) and Gibbs energy (c) of hydration of aromatic and aliphatic groups in RNase A (solid line) and myoglobin (dashed line), calculated per mole of amino acid residues.

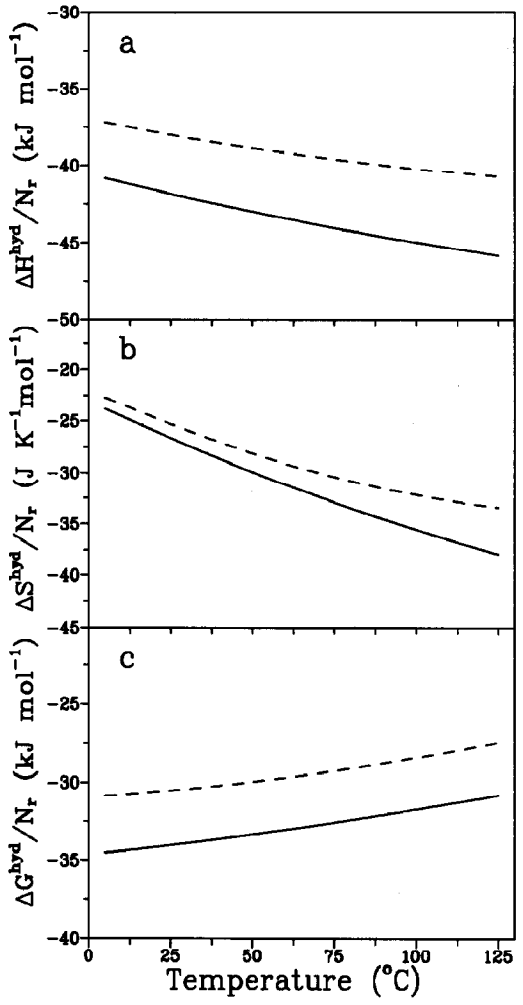

Fig. 3. The specific enthalpy (a), entropy (b) and Gibbs energy (c) of hydration of polar groups in RNase A (solid line) and myoglobin (dashed line), calculated per mole of amino acid residues.

4. Enthalpy of intramolecular interactions in proteins

Since we know the enthalpy of protein unfolding and we know the enthalpy of hydration of groups which are exposed to water upon unfolding, we can determine the enthalpy of disruption of internal interactions in a protein molecule upon unfolding by excluding the hydration enthalpy from the calorimetrically measured enthalpy of protein unfolding. These enthalpies of internal interactions in proteins, which are actually the energies of proteins unfolding in vacuum, are listed in Table 7. The specific values of these enthalpies, calculated per mole of amino acid residues, are rather similar for all the considered proteins. They do not depend significantly on temperature, only slightly decreasing with increasing temperature (Fig. 4), as one could expect because of thermal expansion of protein structure.

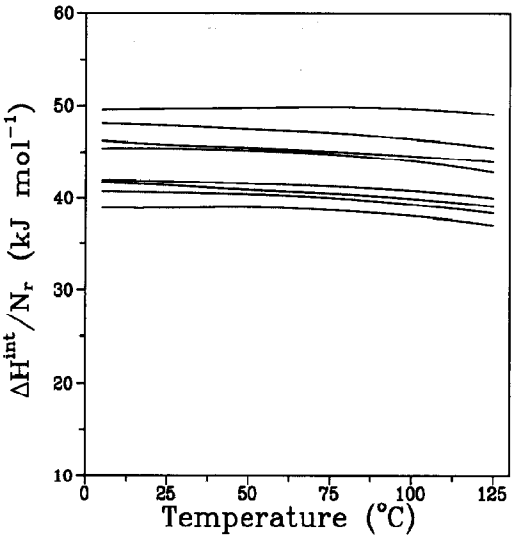

Fig. 4. Temperature dependencies of the enthalpies of internal interactions in the considered proteins, calculated per mole of amino acid residues.

The specific values of the enthalpies of internal interactions in proteins do not show a clear correlation with the molecular weight of protein (Fig. 5a), but they definitely correlate with proportion of polar surface which is exposed upon protein unfolding $[(\Delta^U_N ASA_{pol})/N_r]$ (Fig. 5b). It is most interesting that the enthalpy of internal interactions depends much more strongly on the relative extent of the polar contacts in protein than on the non-polar ones. The energy value of a polar surface in protein therefore appears to be much higher than that of a non-polar surface.

The other important conclusion which follows from the found dependencies is that the specific enthalpy of internal interactions in proteins extrapolated to zero content of polar surface amounts to (140 ± 70) J mol^{-1} A^{-2}. This is the value of enthalpy of internal interactions in proteins in the absence of polar contacts, i.e. without hydrogen bonds. It appears that *the energy contribution of van der Waals interaction to the stabilization of the compact native structure of a protein is*

Table 7
Enthalpies of internal interactions, $\Delta^U_N H^{int}$, upon unfolding of studied proteins

	Temperature (°C)					
	5	25	50	75	100	125
BPTI	2361	2355	2342	2319	2281	2225
Ubiquitin	2953	2960	2961	2943	2895	2821
RNase T1	4362	4349	4327	4297	4245	4162
Cytochrome c	5261	5273	5291	5287	5242	5159
Barnase	4946	4946	4931	4885	4792	4664
RNase A	5723	5678	5636	5590	5527	5441
Lysozyme	6207	6173	6128	6075	5990	5868
Interleukin 1β	6386	6329	6260	6193	6100	5985
Myoglobin	7593	7611	7626	7631	7597	7514

All values in kJ mol^{-1}.

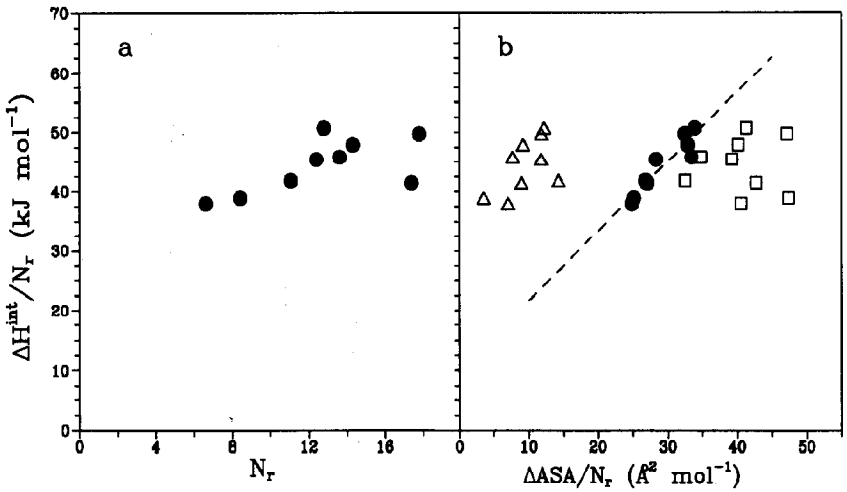

Fig. 5. Plot of the specific enthalpy of internal interactions in proteins at 25°C versus (a) the molecular weight of considered proteins ($R = 0.64$), (b) the relative extent of the buried polar surface, $(\Delta^U_N ASA_{pol})/N_r$ (●), aromatic surface, $(\Delta^U_N ASA_{arm})/Nr$, (△) and aliphatic surface, $(\Delta^U_N ASA_{alp})/N_r$ (□). Correlation coefficients 0.93, 0.04 and 0.54, respectively.

not zero as it was supposed before (see e.g. Refs. [17,19]).

5. Configurational entropy of protein unfolding

Excluding the entropy of hydration of groups in a protein from the calorimetrically determined entropy of protein unfolding, we get the entropy associated with the increase of configurational freedom of polypeptide chain and side chains upon protein unfolding, $\Delta^U_N S^{cnf}$. These values are smaller than that reported before [10] because in determining the surfaces exposed upon unfolding of protein we used here the extended conformation of polypeptide chain which gives smaller values of ASA than a simple summation of the ASA of amino acid residues determined in Gly-X-Gly tripeptides (see footnote to Table 1). It should be noted that the entropy of hydration of groups, and particularly of polar groups, reflects not only the decrease of configurational freedom of water molecules in the presence of these groups but also the restrictions in configurational free-

Table 8
Configurational entropies, $\Delta^U_N S^{cnf}$, upon unfolding of studied proteins

	Temperature (°C)					
	5	25	50	75	100	125
BPTI	2767	2744	2697	2630	2517	2374
Ubiquitin	3678	3701	3700	3649	3525	3332
RNase T1	5451	5401	5323	5236	5086	4865
Cytochrome c	5882	5919	5969	5963	5831	5613
Barnase	6203	6202	6146	6013	5747	5416
RNase A	7008	6846	6703	6568	6386	6162
Lysozyme	7455	7333	7178	7026	6782	6465
Interleukin 1β	8182	7982	7774	7579	7308	7009
Myoglobin	8675	8713	8749	8770	8663	8445

All values in J K^{-1} mol^{-1}.

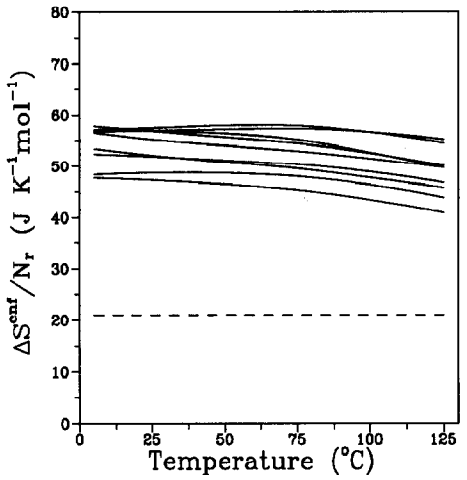

Fig. 6. Temperature dependencies of the specific configurational entropy of unfolding of the considered proteins, calculated per mole of amino acid residues. Dashed line represents the configurational entropy of the polypeptide backbone [21].

dom of the groups due to their interactions with water (namely hydrogen bonding with water in the case of polar groups). Using the molar concentration scale in considering transfer of groups to water, we exclude automatically the contribution of translational motion of solute from the hydration effect [15,16] but we do not exclude contribution of a change of rotational freedom of a group caused by its interactions with water [20]. However, there is no way quantitatively to evaluate this effect separately. We therefore assign all the entropy effects caused by the presence of water to the hydration entropy. Thus, the entropy which we get after exclusion of hydration entropy from the overall entropy of protein unfolding does not include the effects associated with the presence of water. It is actually an entropy of unfolding of protein in vacuum. The values of this entropy calculated per mole of protein and the values calculated per mole of amino acid residues are listed in Table 8.

In Fig. 6 is presented the temperature dependencies of the specific configurational entropies of proteins calculated per mole of amino acid residues. These entropies are not identical, as one could expect for polypeptide chains of different length, sequence and number of disulfide cross-links. But the spread of their values is rather small, less than 8% at room temperature. Their values decrease with increasing temperature, which is understandable because increasing temperature leads to an increase of the entropy of the polypeptide chain in the native conformation. At room temperature these values are significantly larger than those expected from the consideration of the configuration of the polypeptide backbone [21] which is shown by the dotted line

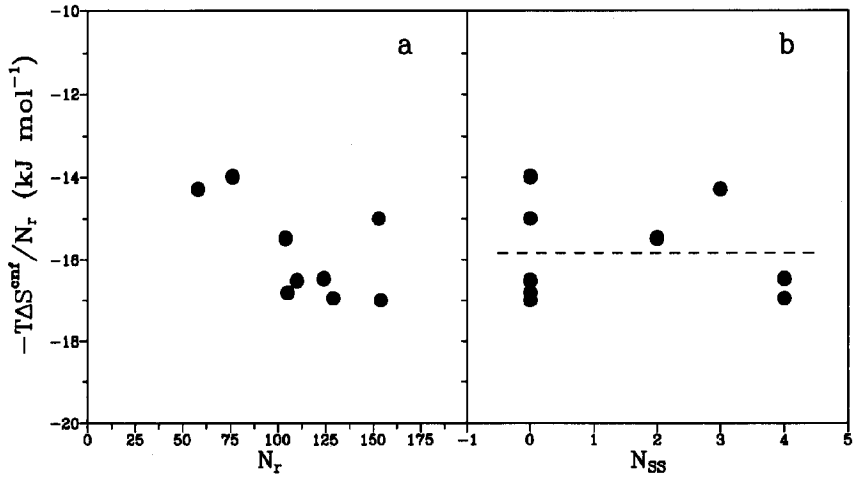

Fig. 7 Plot of the specific configurational entropies versus (a) the number of amino acid residues, N_r, in the proteins; (b) the number of S–S cross-links in the polypeptide chains, N_{ss}.

in the figure (21 J K^{-1} mol^{-1} of amino acid residues). The difference between these values might be associated with the entropy contribution of unpacking the side chains and also with delocalization of protons and electrons which are involved in hydrogen and van der Waals bonding in the native protein. These effects have not before been taken into account.

In Fig. 7a is presented the plot of the specific configurational entropies versus the number of amino acid residues in the proteins, N_r. It shows that the specific configurational entropy calculated per mole of amino acid residue decreases with a decrease of the number of amino acid residues in the polypeptide chain, i.e. with a smaller molecular weight of the protein. One can explain this by increase of the proportion of surface residues, which may be somewhat looser than internal residues.

In Fig. 7b is presented the plot of the specific configurational entropies versus the number of S–S cross-links in the polypeptide chains, N_{ss}. It does not show a simple correlation, which was expected after Flory [22], partly because this correlation is screened by the strong dependence of entropy on the molecular weight, and partly because of the error in determination of the configurational entropy. This error is of the order of the expected effect of cross-linking (< 10%). It is not excluded, however, that the configurational entropy of the polypeptide chain is not a simple function of its composition and depends on the rigidity of the initial (native) and final (denatured) states (see also Ref. [23]).

6. Factors stabilizing and destabilizing compact protein structure

As follows from the above, hydration of polar and aromatic groups destabilize the compact native conformation of protein because the Gibbs energy of hydration of these groups is negative (Table 3). Hydration of aliphatic groups stabilizes the compact protein structure, but its positive contribution is much less than the negative contribution of the first two hydration effects. Thus in total, hydration effects destabilize the native

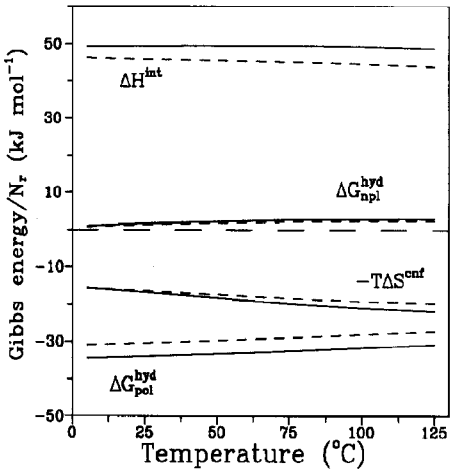

Fig. 8. Temperature dependencies of various factors stabilizing and destabilizing the compact native state in protein for the examples of RNase (solid line) and myoglobin (dashed line). ΔH^{int} the enthalpy of interactions between the protein groups; $T\Delta S^{cnf}$ the contribution of increase of configurational freedom of polypeptide chain upon unfolding; ΔG^{hyd} the Gibbs energies of hydration of various groups which are exposed to water upon unfolding.

state of the protein molecule and this destabilizing action increases with decreasing temperature which leads to cold denaturation of proteins at low enough temperatures. A thermal dissipative force which is proportional to a gain of configurational entropy upon protein unfolding and increases with increase of the absolute temperature, $-T\Delta^{U}_{N}S^{cnf}$, also destabilize protein.

The negative factors destabilizing the compact state of protein are counterbalanced by the enthalpic interactions between the groups tightly packed in the protein interior. These are interactions between polar groups (hydrogen bonding) and non-polar groups (van der Waals interactions) having positive contribution to the overall internal enthalpy. It also includes negative contribution of deformation of bonds and angles in the native state. The stabilizing effect of entropy of hydration of aliphatic groups is much lower than that of the enthalpic interactions, i.e. van der Waals interactions and hydrogen bonding (Fig. 8).

However, if we split the Gibbs energies of hydration into the enthalpic and entropic compo-

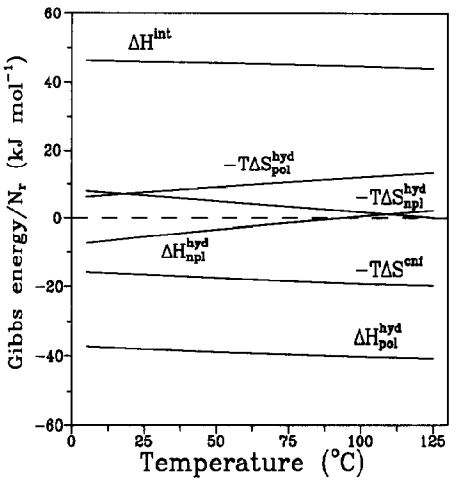

Fig. 9. The balance of the enthalpic and entropic factors in stabilization of the RNase native structure.

nents, it becomes clear that a significant role in stabilization of the compact native state is played by the entropy of hydration of polar groups (Fig. 9). This contribution notably exceeds the contribution of the entropy of hydration of non-polar groups, which was commonly regarded as a dominant factor in the stabilization of the compact protein state.

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Discussion to the paper by G.I. Makhatadze and P.L. Privalov

Short note from the Editor: The discussion below pertains to the previous version of the paper where ASA in the unfolded chain has been calculated using Gly-R-Gly tripeptides. Partially as the result of the discussion, the authors decided to calculate ASA in the unfolded state from the actual sequence in the extended conformation. This resulted in values that less drastically disagreed with the expected values of thermodynamic parameters of folding. Texts, however, differ very little, and most points of the discussion below remain valid. They also suggest that the

change in the way of calculating of ASA adopted by the authors does not represent neither unique nor the best justified way to achieve more reasonable results. The use of a different set of atomic radii with traditional way of calculations of ASA (tripeptide) may suffice.

Comments

By K.P. Murphy

There seems to be inconsistency in your tabulation of configurational entropies. In Fig. 7 you show ΔS^{conf} values of about 700 J K^{-1} g^{-1} while in Fig. 8 you give $T\Delta S^{conf}$ of about 250 J K^{-1} g^{-1}. Which is correct?

In your discussion of the enthalpy of internal interactions in proteins you show that there is a strong correlation with buried polar surface, but virtually no correlation with buried apolar surface. This would seem to imply that the apolar surface makes very little contribution to ΔH^{int} and is consistent with the small correlation between the sublimation ΔH and apolar surface for the N-acetyl amino acid amides given by Barone et al. (this Issue). While you do observe a finite intercept in plotting ΔH^{int} versus the polar ΔASA, you observe nearly the same intercept if you plot ΔH^{int} versus the total ΔASA. These results do not seem to support a sizable contribution of the apolar surface to ΔH^{int}. Might the intercept be due to other contributions, perhaps electrostatic?

By A. Rashin

It looks like many if not most of your intriguing conclusions come from significantly larger magnitude of hydration ΔH, ΔG and $T\Delta S$ for NH, OH and the peptide group compared to everything else (about two fold difference). The fact that ΔH of hydration is so large for the peptide unit dictates a necessity for compensating hydrogen bonds that seem to be about twice stronger than usually expected [1,2]. The fact that $T\Delta S$ of hydration is so large for the peptide unit dictates a necessity for unexpectedly large compensating loss of conformational entropy upon folding, etc.

The question therefore arises whether thermodynamic characteristics of hydration derived for these groups in Refs. [1,2] are reliable. Not that I expect or intend to prove them to be wrong, but I do not feel comfortably assured that they are correct for the following reasons (I concentrate on only a few that seem to have a more assured basis).(1) When you fold a protein you not only, as you mention, do not loose all of its hydration enthalpy because polar groups still feel distant solvent, but because you basically substitute the water solvent of the polar groups by 'protein atoms' solvent for the same groups. According to the Born formula [4] for solutes forming cavities of the same size in water (dielectric constant = 78) and, say, hydrocarbon (dielectric constant = 2) the difference in solvation energies is about two fold. This means that upon transfer of polar groups between two such solvents polar groups loose only about a half of the enthalpy compared with water to vacuum transfer. In fact, cavity sizes may not be the same, and thus it may be $\frac{2}{3}$. Nevertheless, it would significantly reduce the enthalpy of hydrogen bonds (or non-polar interactions) required to compensate for the loss of hydration enthalpy in water to vacuum transfer. This is a reasonably well established physics [4] and it is not explicitly, considered in your derivations.

(2) In Ref. [3] we use experimental entropies of hydration from your compilation [2] to obtain parameters for $T\Delta S$/ASA for different types of groups. For $T\Delta S$ our agreement with experiment [3] is about as good as yours. But with our parameters we get for the peptide unit $T\Delta S = 1.28$ kcal/mol at room temperature, which is about a half of your value. With this value we would not have an excessive loss of configurational entropy to compensate for the gain of the entropy of hydration. We would get close to the expected values. Note that in our scheme average $T\Delta S$/ASA for the peptide unit is about the same as for alkanes. We found previously that if ΔG/ASA is equal for all buried atoms in proteins the loss of conformational entropy needed to counterbalance it comes to about expected values [5].

(3) Note that your estimate of $T\Delta S$/ASA for CO group is very low (0.5 kcal/mol) and most of $T\Delta S$ for CONH comes from the NH group. How-

ever, in your database you have data mostly for terminal NH_2 groups which are much more exposed and, thus, more hydrated. NH group in peptides have a low accessible area (11 Å^2 in our calculations) and even your two fold exaggerated $T\Delta S/ASA$ for NH_2 groups would yield only 0.6–0.7 kcal/mol (I took ASA for this group from ASA of polar part of Lys in your Table 3 [1]). Thus total for CONH would be 1.2 kcal/mol. You get much larger values apparently because you take average $T\Delta S/ASA$ (that includes total NH_2 group) and multiply it by the total area of CONH that comes mainly from CO group (50 Å^2 in our calculations). Thus, you apparently assign $T\Delta S/ASA$ for NH_2 group to CO group. This may lead to a significant exaggeration.

(4) In any case the fact that our parametrization of $T\Delta S$ for the same set of small molecules yields agreement with expenment similar to that obtained with your parametrization suggest that the parametrization is not unique. The fact that our parametrization does not produce an unexpectedly large hydration entropy for the peptide unit suggests that your conclusions stemming from this high entropy value can be artifactual due to the non-uniquiness of the parametrization.

My analysis is rather superficial and does not pretend on finality. I think arguments on possibility of overestimation of $T\Delta S$ for the peptide unit are more serious than the rest. It is for you to reanalyze your data and either agree, disagree or find something completely different. But I hope that at least some of these comments are worth analyzing, and at least mentioning in your future publications. I, of course, would appreciate your response in this Discussion.

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By B.K. Lee

(1) The plots of ΔH and ΔS with respect to temperature (Figs. 1a and 1b) no longer show the convergence phenomenon. Does this mean that all the discussions on the convergence temperatures that have been going on for the last few years were a meaningless exercise?

(2) The slope of the correlation between ΔH^{int} and the polar surface area (Fig. 5b) is an important information since it can be related to the energy of an intra-molecular hydrogen bond in a protein. Please give this number.

(3) In the last part of Section 4, the zero polar surface area intercept of ΔH^{int} is interpreted as "the value of enthalpy of internal interactions in proteins in the absence of polar contacts". However, (1) there is no protein which does not have internal polar contact and (2) it is possible that there is a contribution to ΔH^{int}, which is polar but which is constant per gram and not proportional to the surface area. These caveats must be stated. With proper caveats, a more appropriate label for this term may be the "nonpolar contribution to ΔH^{int}".

(4) Contrary to the statement at the end of the first paragraph of Section 5, Table 8 lists the ΔS^{conf} values per mole of protein only. It *would* be nice to have the same data per amino acid residue also.

(5) In the second paragraph of Section 5 and in connection with Fig. 6, it is noted that the calculated ΔS^{conf} values are much larger than a previous estimate. As possible causes of this discrepancy, side-chain unpacking and delocalization of protons and electrons are mentioned. However, there is another possible cause. The ΔS^{conf} values are obtained as the remainder part of the observed total entropy change after the hydration part has been subtracted. Since the observed total entropy change is nearly zero at room temperature, high ΔS^{conf} values will result if the magnitude of the hydration part was overestimated. The hydration part is indeed likely to be overestimated since it is proportional to the ΔASA and that the latter was assumed to be the difference between the fully stretched conformation and the native, folded state. Lee [1] estimated that the actual ΔASA upon protein unfolding is only about half of this ΔASA. The fact that ΔC_p correlates well with ΔASA from the native to the fully stretched conformation is not a

proof that the protein is fully stretched in the unfolded state.

(6) In the legend to Fig. 6, please specify whether the plot is for RNase T1 or for RNase A.

(7) At the very end of the paper, the entropy change upon hydration of the polar groups, $\Delta S^{hyd,pol}$, is emphasized over that of the non-polar groups, $\Delta S^{hyd,np}$. While I agree that this need to be emphasized, it may be argued that the total free energy change is the sum of many terms and which term is considered "dominant" depends on how these different terms are combined. Anticipating such objection, I would like to put this analysis in perspective.

The total free energy change upon unfolding, ΔG, is broken into six terms in this article as follows:

$$\Delta G = \Delta H^{hyd,pol} - T\Delta S^{hyd,pol} + \Delta H^{hyd,np} - T\Delta S^{hyd,np} + \Delta H^{int} - T\Delta S^{cnf}.$$

In terms of values, this equation is

$$27 = -7759 + 1496 - 916 + 1127 + 8970 - 2891$$

for RNase A at 25°C, where the numbers are the values, in kJ/mol, of the terms in the previous expression in the same order. The traditional way of combining these terms is

$$\Delta G = \left[(\Delta H^{hyd,pol} - T\Delta S^{hyd,pol}) + \Delta H^{int,pol}\right] + \left[\Delta H^{hyd,np} + \Delta H^{int,np}\right] - T\Delta S^{hyd,np} - T\Delta S^{cnf},$$

where ΔH^{int} is further broken into the polar, $\Delta H^{int,pol}$, and nonpolar, $\Delta H^{int,np}$, components. The combination of terms in the first square bracket is mainly the hydrogen bonding contribution, that in the second square bracket is the van der Waals contribution, and $T\Delta S^{hyd,np}$ is approximately the hydrophobic contribution. The reason that the $T\Delta S^{hyd,np}$ term was considered dominant was because the square bracketed terms were considered small because of cancellations; intra-protein versus protein–water intermolecular hydrogen bonds in the case of the first square bracketed term and intra-protein versus protein–water intermolecular van der Waals interactions in the case of the second bracketed term. Actual numbers, according to this paper, are

$$27 = [(-7759 + 1496) + 8970f] + [-916 + 8970(1-f)] + 1127 - 2891 = [-6263 + 8970f] + [-916 + 8970(1-f)] + 1127 - 2891,$$

where f is the polar fraction of ΔH^{int}. Unfortunately, this paper does not give the value of f explicitly. If one assumes that the change in the van der Waals interaction is zero, as was recently suggested (Lee [2]), the terms in the second square brackets add up to zero and f becomes 89.8%. The terms in the first square brackets then add up to 1791 kJ/mol. This is indeed a large stabilization compared to the hydrophobic term of 1127 kJ/mol and emphasizes the importance of the polar, hydrogen bonding interaction, as suggested by Lee [1]. The $T\Delta S^{hyd,pol}$ term is one reason for such a large hydrogen bonding contribution. This is presumably the entropy gain upon folding due to the release of water molecules that were hydrogen bonded to the polar groups when the latter were exposed to water in the unfolded state.

[1] B. Lee, Proc. Natl. Acad. Sci. USA 88 (1991) 5154.
[2] B. Lee, Protein Sci. 2 (1993) 733.

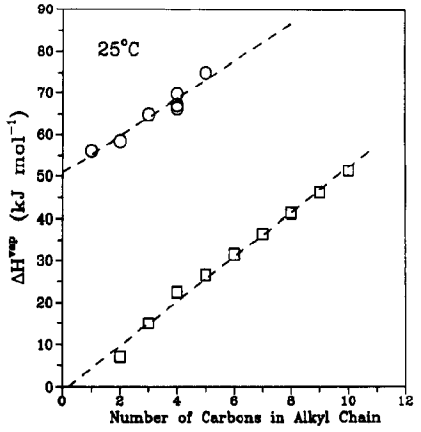

Fig. 1. Vaporization enthalpies, ΔH^{vap}, versus the number of carbon atoms in the alkyl chain of the *N*-*n*-alkylamides (○ and *n*-alkanes (□). Data for *N*-*n*-alkylamides were taken from Barone et al. (Fluid Phase Equilibria 21 (1985) 157) and Starzewski et al. (J. Chem. Thermodynamics 16 (1984) 331).

Responses by G. Makhatadze and P. Privalov to Comments

To K. Murphy

The data of Barone et al. in this Issue on the N-acetyl amino acids is not extended enough (only four points) to make any rationale conclusion out of them. However, the work of Barone et al. (1985) and Starzewski et al. (1984) on the enthalpies of vaporization of *N-n*-alkylamides is much more extended and is more suitable for the analysis. Fig. 1 shows the dependence of the enthalpies of vaporization for *n*-alkanes and *N-n*-alkylamides on the number of carbon atoms in the alkyl chain. The dependencies are both linear with the similar slopes 5.1 for alkanes and 4.5 for amides. The slope represents the contribution of interactions between non-polar groups (van der Waals interactions) giving about 145 ± 10 J mol^{-1} per square angstrom of aliphatic surface. This number is in a good agreement with the results of our previous analysis 126 J mol^{-1} Å^{-2} (Makhatadze and Privalov [1]). There is a difference in the intercept with *y* axis which is essentially zero in case of alkanes and 52 kJ mol^{-1} for *N-n*-alkylamides. Obviously this intersept for the *N-n*-alkylamides represents the enthalpy of hydrogen bonding in these molecules. Again this value is in amazingly good agreement with the values we obtained before for the enthalpy of hydrogen bonding in the absence of hydration (mean value 52 kJ mol^{-1} [1]). Recalculating per square angstrom, the enthalpy of disruption of interactions between polar groups will be about 1.4 kJ mol^{-1} Å^{-2} of polar surface area. This means that one square angstrom of polar surface area contributes approximately ten times more to the enthalpy of internal interactions in proteins than the non-polar surface. On the other hand, there is approximately 1.7 times less buried polar surface area than non-polar surface area and this ratio does not depend significantly on the molecular weight of the protein (Fig. 2). Consequently, total buried polar surface area contributes six times more to the enthalpy of internal interactions than the non-polar. That is why the enthalpy of internal interactions correlates so well with the buried polar surface area and not with

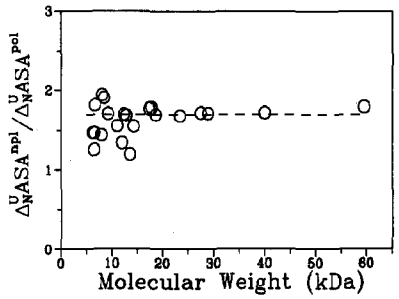

Fig. 2. Dependence of the ratio of non-polar to polar buried water accessible surface areas, $\Delta^U_N ASA^{npl} / \Delta^U_N ASA^{pol}$, on the molecular weight of proteins.

buried non-polar surface area. All this is a strong argument that the enthalpy of internal interactions depends mostly on the buried polar ΔASA, although, non-polar ΔASA also contributes to it much but to a lesser extent. However, if one will take into account the hydration, then the situation changes dramatically. The the enthalpy gain from the disruption of interactions between polar groups is overcompensated by the enthalpy loss due to their hydration, resulting in the enthalpically unfavorable burial of polar groups. The enthalpy of interactions between non-polar groups is much larger than the enthalpy of their hydration. The product of disruption of internal interactions between non-polar groups and their hydration is ethalpically favorable and stabilizes native protein structure. One can argue how well *N-n*-alkylamides model the interior of proteins, but they are definitely not worse that N-acetyl amino acid amides or diketopiperazines.

[1] G.I. Makhatadze and P.L. Privalov, J. Mol. Biol. 232 (1993) 639–659.

To A. Rashin and B.K. Lee

In this paper we analyzed the existed experimental data on protein denaturation and transfer of low molecular weight compounds into water, based on the assumption that groups of protein contribute additively to the overall hydration effects of unfolding. The results of this analysis deviate in many points from what was expected in theory. This might mean that either the existing experimental data are not correct (but at the

present time we do not have anything better), or additivity principle has some limitations (but we do not know much about them), or that the theoretical understanding of the process of protein unfolding and hydration of groups is far from perfect. For example, in considering the configurational entropy of protein unfolding the contributions of vibrational modes are usually neglected. But this modes cannot be the same in the folded and unfolded states. Nobody has considered yet the entropy effect of delocalization of protons upon disruption of hydrogen bonds, but could we believe that it is zero? These theoretical aspects of protein unfolding certainly require more attention. In considering overall effects of protein unfolding the hydration contribution of groups are usually just summed up as if they are independent. But even in the unfolded proteins these groups are not absolutely independent. The surfaces calculated assuming that the polypeptide chain is in an extended conformation or β-conformation are different. Assuming independence of groups in the unfolded state, perhaps we overestimated the surfaces which are exposed in the random coiled conformation of protein and correspondingly overestimated the hydration effects and hence the enthalpy of hydrogen bonding of protein and the configurational entropy of unfolding. But nobody has yet studied in detail the ASA of groups in the unfolded proteins.

This certainly requires special attention and we are sure these surfaces could be estimated using computer modeling. As for the experimental data they certainly need further refinement. This requires the increase in precision of calorimetric measurements and expansion of collection of studied examples. Expansion of denaturation data showed us already that convergence of the enthalpy and entropy of protein unfolding is a rule not without exceptions. It is just those exceptions which might be most interesting for the understanding of protein energetics.