Size of Unfolded and Dissociated Subunits *versus* that of Native Multimeric Proteins

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Abstract. Two factors, unfolding and dissociation, act in opposition in determining the size of the unfolded state of multimeric proteins. An analysis has been presented to correlate relative expansion of the unfolded monomers in absence of disulfide bridges over the native state of different homo-multimeric proteins of varying molecular weights. The Stoke's radii of about 70 proteins of Mw between 6 kDa to 4000 kDa and ranging from monomers to dodecamers were calculated both under native and denatured condition induced by 8 M urea or 6 M guanidinium,HCl according to relations derived by Uversky [*Biochemistry* 32 (1993), 13288–13298]. Stoke's radii of monomeric proteins were found to increase by 1.6–2.2 times after denaturation as compared with the native state while that of the subunits of dimeric and tetrameric proteins were increased by factors 1.1–2.2 under the same conditions. For hexameric proteins this factor lies between 0.96–1.2. In each set the relative increment of the Stoke's radii followed a logarithmic relation with molecular weight and reached a minimum limiting value when Stoke's radii of native protein became almost equal to that of the unfolded monomer.

Key words: Denaturation, dissociation, molecular weight, multimeric proteins, Stoke's radius, subunits

1. Introduction

Unfolding of proteins are associated with a number of changes in their physical properties [1, 2]. One of them is the expansion of the molecule due to disruption of internal stabilizing forces. Though the exact shape of a protein in the unfolded state has not yet been established, it is certainly not a compact sphere like its native globular form [3]. An increase of Stoke's radius of the unfolded state is indicated by viscosity, sedimentation and diffusion measurements and also by size exclusion gel filtration chromatography [1, 4, 5]. This phenomenon is essentially true for proteins not linked by disulfide bridges. Excessive disulfide linkage resist denaturation as well as expansion e.g. soya bean trypsin inhibitor. This protein remains biologically

active even after heating at 100 °C or in 90% ethanol or at 9 M urea at pH 7.0, under which conditions most of the other proteins get denatured [6].

In case of noncovalently linked multimeric proteins without disulfide bridges the size of the unfolded state could not be predicted as such because the process also leads to dissociation of the subunits [7]. Thus two factors, expansion due to denaturation and decrease of size due to dissociation, act in opposition in determining the size of the unfolded state. This report presents a correlation between the relative size of the unfolded state of proteins -without intra or inter molecular disulfide links induced by denaturants like 8 M urea or 6 M guanidinium (Gdm) HCl, at neutral pH – to their native state and their multimericities.

2. Materials and Methods

2.1. CALCULATION OF STOKES' RADII OF NATIVE AND UNFOLDED PROTEINS

Stoke's radii of native (R_n) and unfolded (R_d) monomeric proteins (in Å) without inter and intra molecular disulfide links of known molecular weight in Da (M_w) were calculated according to the following equations after Uversky [5]:
for native proteins:

$$\log(R_n) = -(0.254 \pm 0.002) + (0.369 \pm 0.001) \log(M_w) \quad (i),$$

for proteins unfolded by 8 M urea:

$$\log(R_d) = -(0.657 \pm 0.040) + (0.524 \pm 0.001) \log(M_w) \quad \text{eq. (ii) and}$$

for proteins unfolded by 6 M Gdm.HCl:

$$\log(R_d) = -(0.543 \pm 0.004) + (0.502 \pm 0.001) \log(M_w) \quad \text{eq. (iii).}$$

These empirical relations are firmly based on experimental evidences and it is assumed that monomeric proteins with or without disulfide linkages have same Stoke's radii because the S-S linkages only stabilizes the molecules without inducing compactness.

2.2. GEL FILTRATION EXPERIMENTS

A sephacryl S-400 column (80 × 1.3 cm) equilibrated with 20 mM Na-phosphate, pH 7.0 containing 0.2 M NaCl was used to determine the Stoke's radii of alcohol dehydrogenase and creatin phosphokinase in the native state. The same column, equilibrated with phosphate buffer containing 8 M urea was used for determination of Stoke's radii of alcohol dehydrogenase, creatin phosphokinase, hemoglobin, UDP-galactose 4-epimerase (*E.coli*) and catalase. Since none of these proteins contains intra or inter molecular disulfide links, they were denatured by 8 M urea in absence of any reducing agent at equilibrium for 18 hr. Flow rate was 20 ml/hr and fraction size was 0.5 ml. The column was precalibrated with the following

marker proteins: carbonic anhydrase (Mw 29 kDa, Rn 23.6 Å), ovalbumin (43 kDa, 31.2 Å), BSA (bovine serum albumin) (66 kDa, 62.75 Å), thyroglobulin (669 kDa, 78.8 Å), *E.coli* epimerase (79 kDa, 39.6 Å) and BSA unfolded in 8 M urea (66 kDa, 76.8 Å) [5, 8]. In gel filtration chromatography elution of proteins are known to be dependent on Stoke's radii but independent of solvent compositions [5, 9]. Elution of proteins was monitored by A₂₈₀. Linear dependency of log Mw *versus* Ve (elution volume) and log Rn *versus* Ve were observed. Derived values have been mentioned in Table I within parenthesis.

2.3. OTHER METHODS

UDP-galactose 4-epimerase from *E.coli* and yeast *Kluyveromyces fragilis* were purified after [10] and [11] respectively. Hemoglobin (Sigma, USA) was further purified to homogeneity by absorbing in a DEAE-cellulose column equilibrated with 20 mM K-phosphate buffer, pH 8.5. After washing, it was eluted with a gradient of 0–0.5 M NaCl in the same buffer. Fractions were followed at 280 and 540 nm. Catalase and yeast alcohol dehydrogenase (Sigma) were further purified by passing through a Water's Protein Pak 300 SW size exclusion-HPLC column (0.78 × 30 cm) equilibrated with 20 mM K-phosphate buffer, pH 7.0 supplemented with 0.2 M NaCl. Elution of proteins was followed by A₂₈₀. Proteins which showed a single band in SDS-PAGE were used for further analysis.

3. Results and Discussion

The Stoke's radii of several proteins of molecular weight between 6 kDa to 4,000 kDa and multimericities between 2–12, all being homomultimers, have been listed in Table I. It also includes several monomeric proteins for comparison. The Stoke's radii of all these proteins, both for the native (Rn) and unfolded monomeric state (Rd) (induced either by 8 M urea or 6 M Gdm.HCl) were calculated using the eqns. (i–iii). The relations have been shown to hold good for a molecular weight range of 6 kDa to 6,000 kDa for the native state and also for unfolded states induced by 8 M urea [5]. Thus all proteins listed in Table I are within this specified limit. Moreover literature values of Stoke's radii (both native and unfolded states) as measured by different physical techniques and as determined in this study (described in methods) have been included in this table within parenthesis. Among 32 such proteins reported, theoretically derived values of Rn and Rd had maximum deviation of ± 11.16 and $\pm 17.16\%$ respectively from experimentally observed values. However, the result improved considerably when two proteins of wide deviation were excluded from each set; for example, the average deviations for Rn and Rd were reduced to ± 2.16 (n=31) and ± 10.76 (n = 21)% respectively. The wider variation of Rd in comparison to Rn is in agreement with the idea that the unfolded states of proteins are less well defined and they acquire different structures depending on the residual hydrophobic interactions still prevailing in the unfolded state particularly

Table I. Subunit compositions and Stoke's radii of different proteins under native and 8 M urea denatured state*

Protein	Subunits (kDa)	No. of subunit	Native Mw (KDa)	Rn ^a (Å)	Rd ^b (Å)	f = Rd/Rn ^c
MONOMER						
Ubiquitin	8.5	1	8.5	15.7±0.26 (13.2) ⁿ	25.24±2.55 (26.3) ⁿ	1.61±0.14 (1.9)
Cytochrome C	11.7	1	11.7	17.7±0.25 (17) ^e	29.84±3.03 (26.2) ^f	1.69±0.14 (1.54)
Ribonuclease	13.7	1	13.7	18.7±0.26 (19.3) ^e	32.41±3.29 (32.4) ^e	1.73±0.15 (1.68)
α-lactalbumin	14.2	1	14.2	18.9±0.27 (20.1) ^g	33.0±3.51 (34.0) ^h	1.74±0.15 (1.69)
Lysozyme	14.3	1	14.3	19±0.27 (20.5) ⁿ	33.14±3.37 (34.6) ^d	1.74±0.15 (1.69)
Myoglobin	16.9	1	16.9	20.2±0.29 (20.2) ^e	36.18±3.69 (35.8) ⁿ	1.79±0.15 (1.77)
Soyabean trypsin inhibitor	20.0	1	20	21.5±0.31 (22.6) ^g	39.5±4.2	1.84±0.16
α-chymotrypsin	21.6	1	21.6	22.1±0.32 (20.9) ^g	41.1±4.4 (40.1) ^h	1.86±0.16 (1.92)
Trypsin	23.8	1	23.8	22.9±0.33 (19.4) ^g	43.3±4.6	1.89±0.16
Chymotrypsinogen	25.7	1	25.7	23.6±0.35 (21.7) ^e	45.06±4.61 (45.0) ^e	1.91±0.16 (2.07)
Carbonic anhydrase	28.8	1	28.8	24.6±0.36 (23.6) ^e	47.83±4.91 (50.1) ^e	1.94±0.17 (2.12)
<i>S.aureus</i> β-lactamase	30.0	1	30	25±0.37	48.87±4.8	21.95±0.17
Pepsinogen	40.0	1	40	27.8±0.42	56.82±5.84	2.04±0.18
Ovalbumin	43.5	1	43.5	28.7±0.44 (30.2) ^e	59.37±6.11 (59.4) ^e	2.07±0.18 (1.97)
BSA	66.3	1	66.3	33.5±0.52 (33.9) ^e	74.04±7.65 (76.8) ^e	2.21±0.19 (2.26)
DIMER						
Insulin	2.97	2	5.78	13.62±0.18	14.55±1.46	1.07±0.95
β-lactoglobulin	17.5	2	35	26.47±0.41 (27.1) ^e	36.84±3.76 (37.8) ^e	1.39±0.12 (1.39)
Rhodanase	18.5	2	37	27.02±0.41 (28.5) ⁱ	37.93±3.87	1.4±0.12
Bovine growth hormone	25	2	48	29.74±0.45	44.41±4.54	1.49±0.13

Table I. Continued

Protein	Subunits (kDa)	No. of subunit	Native Mw (KDa)	Rn ^a (Å)	Rd ^b (Å)	f = Rd/Rn ^c
O-Acetyl serine sulfydrylase A	34	2	68	33.82±0.53	52.18±5.36	1.54±0.13
Glycerol 1- phosphate dehydrogenase	40	2	78	35.58±0.56	56.82±5.84	1.6±0.14
UDP-galactose 4-epimerase (<i>E. coli</i>)	39	2	79	35.75±0.57 (39.6) ^j	56.07±5.76 (55.0) ^j	1.57±0.13 (1.40)
Creatin phosphokinase	40	2	80	35.91±0.57 (37.5) ^k	56.82±5.84 (56.0) ^k	1.58±0.14 (1.50)
Alkaline phosphatase	43	2	86	36.0±0.58 (34.6) ^g	59.0±6.3 (61.3) ^f	1.64±0.14 (1.77)
Enolase	41	2	82	36.24±0.57	57.56±5.9	21.59±0.13
α-amylase	48.2	2	97.6	38.65±0.62	62.65±6.4	61.62±0.14
Haptoglobin	50	2	100	38.99±0.63 (45.3) ^g	63.86±6.58	1.64±0.145
Mammmary glucose 6- phosphate dehydrogenase	63	2	130	42.96±0.70	72.09±7.45	1.68±0.14
L-aminoacid oxidase	70	2	135	43.56±0.71	76.18±7.88	1.75±0.15
UDP-gal 4-epimerase (<i>K. fragilis</i>)	75	2	150	45.29±0.74 (45.3) ^l	78.98±8.17	1.74±0.15
Carboxylesterase	85.5	2	167	47.12±0.78	84.6±8.77	1.8±0.15
Tryptophanase	110	2	220	52.16±0.88	96.54±10.03	1.85±0.16
Phycocyanin	134	2	266	55.95±0.95 (52.6) ^g	107.05±11.2	1.91±0.17
Lipovitellin	200	2	400	65.04±1.14	132.05±13.8	2.03±0.17
Fatty acid sythetase	230	2	450	67.93±1.19	142.08±14.9	2.09±0.19
Thyroglobulin	335	2	669	78.63±1.42 (85.0) ^m	173.03±18.2	2.2±0.2
TETRAMER						
Neusporamalate dehydrogenase	13.5	4	54	31.06±0.48	32.16±3.27	1.04±0.09
Haemoglobin	16	4	64.5	33.17±0.52 (33.2) ^e /(35.1) ^k	35.15±3.58 (37.5) ^k	1.06±0.09 (1.12)

Table I. Continued

Protein	Subunits (kDa)	No. of subunit	Native Mw (KDa)	Rn ^a (Å)	Rd ^b (Å)	f = Rd/Rn ^c
Avidin	18	4	68.3	33.88±0.53	37.39±3.81	1.1±0.09
Concanavalin A	17.5	4	71	34.36±0.54 (38.2) ⁱ	36.84±3.76	1.07±0.09
Liver alcohol dehydrogenase	20	4	80	35.91±0.57 (35.9) ⁱ	39.51±4.04	1.1±0.1
Tryptophanyl transfer ribonucleic acid synthetase	27	4	110	40.39±0.65	46.24±4.74	1.14±0.1
Spinach leaf aldolase	30	4	120	41.71±0.68 (46.0) ⁿ	48.87±5.01	1.17±0.1
Tryptophan oxygenase	31	4	122	41.96±0.68	49.71±5.1	1.18±0.1
Tyrosinase	32	4	128	42.71±0.7	50.55±5.19	1.18±0.1
Yeast alcohol dehydrogenase	37.5	4	150	45.29±0.74 (48.0) ^k	54.93±5.64 (55.0) ^k	1.21±0.105 (1.15)
Muscle aldolase	40	4	160	46.38±0.76 (46.6) ^d	56.82±5.84 (54.0) ⁿ	1.23±0.11 (1.16)
Phosphoenol pyruvate carboxylase	49.2	4	198	50.17±0.84	63.33±6.53	1.26±0.11
Glutamine phosphoribosyl pyrophosphate amido transferase	50	4	200	50.36±0.84	63.86±6.58	1.27±0.11
Tryptophanase	55	4	220	52.16±0.88	67.14±6.93	1.29±0.11
Formyl terahydrofolate synthetase	58	4	230	53.02±0.9	69.03±7.12	1.30±0.11
Catalase	58	4	232	53.19±0.9 (52.0) ^e	69.03±7.12 (65.0) ^k	1.3±0.11 (1.25)
Ovine glutamine synthetase	65	4	263	55.71±0.95	73.28±7.57	1.32±0.11
Phosphorylase A	92	4	370	63.19±1.1 (64.7) ⁱ	88.16±9.14 (90) ⁿ	1.4±0.12 (1.39)
β-galactosidase	130	4	520	71.65±1.27 (68.5) ⁱ	105.37±10.97 (118.0) ⁿ	1.47±0.12 (1.7)

Table I. Continued

Protein	Subunits (kDa)	No. of subunit	Native Mw (KDa)	Rn ^a (Å)	Rd ^b (Å)	f = Rd/Rn ^c
Pyruvate carboxylase	165	4	660	78.24±1.41	119.39±12.46	1.53±0.13
Propionyl carboxylase	175	4	700	79.95±1.44	123.13±12.85	1.54±0.13
HEXAMER						
C-reactive protein	21.5	6	129	42.84±0.71	41.04±4.21	0.96±0.08
Asparaginase	22	6	139	44.03±0.72	41.54±4.25	0.94±0.08
Phosphoribosyl ATP synthetase	36	6	215	51.72±0.87	53.77±5.51	1.04±0.09
Anthranilate synthetase complex	40	6	240	53.86±0.92	56.82±5.84	1.05±0.09
Glucose 6-phosphate dehydrogenase	43	6	240	53.86±0.92	59.01±6.07	1.1±0.09
Phytochrome	42	6	252	54.84±0.93	58.29±6.1	1.06±0.09
Acetoacetate decarboxylase	62	6	340	61.25±1.06	71.48±7.39	1.17±0.11
Urease	83	6	483	69.72±1.23 (61.8) ⁱ	83.29±8.81	1.19±0.1
OCTAMER						
Hemeerythrin	13.5	8	108	40.12±0.65	32.16±3.28	0.80±0.06
Ceruloplasmin	18	8	151	45.40±0.75	37.39±3.85	0.82±0.07
Glutamine dehydrogenase	250	8	2000	117.78±2.26	148.43±15.5	1.26±0.10
DECAMER						
Histidine decarboxylase	19	10	190	49.42±0.83	38.47±3.925	0.78±0.07
Mitochondrial ATP synthetase	26	10	284	57.32±0.98	45.34±4.64	0.79±0.06
Liver acetyl coA carboxylase	409	10	4100	153.5±3.05	192.11±20.2	1.25±0.11

Table I. Continued

Protein	Subunits (kDa)	No. of subunit	Native Mw (KDa)	Rn ^a (Å)	Rd ^b (Å)	f = Rd/Rn ^c
DODECAMER						
Glutamine synthetase	48.5	12	592	75.16±1.35	62.85±6.47	0.84±0.07
Chlorocruorin	250	12	2750	132.47±2.58	148.43±15.5	1.12±0.09
Erythrocrunin	250	12	3000	136.79±2.67	148.43±15.5	1.09±0.09

*All proteins listed have noncovalently linked subunits [17].

^a Rn stands for Stoke's radius of native multimeric proteins (including all subunits) and ^b Rd for Stokes radii of proteins denatured and dissociated by 8 M urea. Both Rn and Rd were calculated after [5].

^c Average of Rd and Rn were used to calculate the mean f value. The deviations were drawn from the maximum and minimum values of Rn and Rd respectively.

^d [24], ^e [5], ^f [19], ^g [20], ^h [21], ⁱ (values computed from diffusion coefficient values obtained from [17], ^j [8], ^k (this report), ^l [18], ^m [22] and ⁿ [23].

Values of Stoke's radii in parenthesis are from various experimental measurements.

at the core region [2, 3]. The proteins analyzed, though not very large in numbers, corroborated with Uversky's relation within limits of error.

It may be mentioned that no matter whether the monomeric or multimeric proteins are linked by inter and/or intra molecular disulfide linkages, in all cases the unfolded state of the proteins are defined as completely reduced of disulfide bridges, dissociated and denatured. Therefore no attempt has been made to determine the Stoke's radii of proteins under denatured state with intact or partially reduced disulphide bonds.

Dependency of the factor f (= Rd/Rn) on native molecular weight of the listed proteins of varying multimericity is presented in Figure 1 (A, monomer; B dimer, tetramer and hexamer). Since the abundance of proteins of higher multimericities lies within a certain molecular weight range, all the curves of Figure 1 could not be presented in a single profile. The best fitting of the coordinates of each set has been presented in the respective panel; all pairs were found to satisfy logarithmic relations as follows:

$$f = 0.291 \ln(\text{Mw}) + 0.972, R^2 = 0.997 \text{ for monomers;}$$

$$f = 0.246 \ln(\text{Mw}) + 0.529, R^2 = 0.978 \text{ for dimers;}$$

$$f = 0.197 \ln(\text{Mw}) + 0.236, R^2 = 0.992 \text{ for tetramers and}$$

$$f = 0.199 \ln(\text{Mw}) - 0.023, R^2 = 0.934 \text{ for hexamers;}$$

where Mw stands for molecular weight in kDa and R stands for regression coefficient. Proteins of multimericity ≥ 8 are rare and consequently their f values have not been graphically represented. Figure 1A shows that for monomeric proteins the

Figure 1. Dependence of the factor f [Stoke's radii of proteins denatured by 8 M urea (R_d) divided by native state (R_n)] on molecular weights of proteins of different multimericities. A: monomeric and B: dimeric (a) tetrameric (b) and hexameric (c).

Stoke's radii become 1.61–2.21 times larger after denaturation with urea. This corresponds to an increase in volume of the molecules by a factor 4.2–10.8 assuming a sphere-like shape in both cases. For dimeric, tetrameric and hexameric proteins increase of Stoke's radii ratio varies between 1.07–2.2, 1.04–1.54 and 0.96–1.2 respectively. This indicates that as the multimericity of proteins increases, relative expansion of the unfolded state over native state becomes less pronounced.

Since $f = 1$, indicates no change of Stoke's radius upon unfolding, proteins of such f values represent a limiting case for which unfolding and dissociation could hardly cause any expansion of the molecules. This can be precisely determined from Uversky's relation for proteins of different multiplicities as follows:

$$f = [-0.657 + 0.524 \log (Mw/n)] / [0.254 + 0.369 \log (Mw)] \quad \text{eq. (iv)}$$

Figure 2. Dependence of f (R_d/R_n) on $\log$ multimericity of proteins of defined molecular weight. The coordinates were derived from the plots of Figure 1 A and B. Two points on y coordinate (corresponding to multimericity one) were obtained after extrapolation of the curve in Figure 1A. The profiles (□-□) and (■-■) represent proteins of Mw 100 and 600 kDa respectively. The dotted line ($f = 1$) indicates no change of Stoke's radii of a multimeric protein after dissociation and denaturation by 8 M urea.

where n = number of subunits.

This equation directly leads for $f = 1$, to:

$$\log(n) = -0.769 + 0.295 \log(\text{Mw}) \quad \text{eq. (v)}$$

Therefore, for dimeric ($n = 2$), tetrameric ($n = 4$) and hexameric ($n = 6$) proteins the threshold values for $f = 1$, are 4.2, 50 and 176 kDa respectively (total molecular weight). In nature, dimeric and tetrameric proteins of such low molecular weight hardly exist. The tetrameric Neusporamalate dehydrogenase and haemoglobin of Mw 54 and 64.5 kDa are nearest to the theoretically computed value of 50 kDa which give ' f ' of 1.04 and 1.06 respectively. With haemoglobin this has been experimentally verified from gel filtration of its native (Mw 64 kDa; R_n 33.2–35.1 Å) and denatured state (unfolded by 8 M urea, Mw 16 kDa, R_d 37.5 Å). The elution volumes of hemoglobin in native and denatured state from a Sephacryl S-400 column equilibrated with respective solvent systems were almost identical –61.9 and 61.5 ml respectively as expected. In this context it may be mentioned that though the shape of the protein in unfolded state could not be determined, it is the Stoke's radius which guides its movement in solvents or in gel filtration matrix.

The derived eq. (v) further predicts what multimericity should be for a protein of assigned molecular weight to have $f = 1$. Two proteins of molecular weight 100 and 600 kDa were considered for such calculations irrespective of their existence.

It shows that hexameric ($n > 5.1$) and nonameric ($n > 8.7$) compositions are required for proteins of 100 and 600 kDa respectively. A relation between f and multimericity of proteins having the same assigned native molecular weight could also be derived from Figure 1. A logarithmic relation gives a good fit for both cases (Figure 2). The relations presented are:

for 100 kDa, $f = -1.81 (\log \text{ multimericity}) + 2.27$, $R^2 = 0.997$ and

for 600 kDa, $f = -2.08 (\log \text{ multimericity}) + 2.82$, $R^2 = 0.995$.

It is interestingly that the points for monomeric proteins were constructed by extrapolation of the curve in Figure 1A and were found to fit well in the derived plot. It shows that for proteins of 100 and 600 kDa, $f = 1$ may be reached by hexameric and nonameric proteins respectively – the same conclusions predicted earlier

Gdm.HCl, an ionic denaturant, is also frequently used like the nonionic denaturant urea in protein folding studies. Usually it is considered that 8 M urea is equivalent to 6 M Gdm.HCl at neutral pH in terms of denaturing potentials of proteins [12]. Therefore similar analysis were done for Gdm.HCl as described in Table I. It was observed that for monomeric, dimeric and tetrameric proteins, f *versus* Mw profiles become indistinguishable for the two denaturants (results not shown).

Compared to the high resolution structures of proteins derived under native conformation, corresponding unfolded states are less characterized primarily due to the presence of high concentration of denaturants. Direct determination of radius of gyration from light scattering and low angle x-ray scattering, intrinsic viscosity, sedimentation coefficient, spectral properties like circular dichroism etc certainly indicate that the unfolded state is expanded [1]. Acid-base titration, NMR, circular dichroism spectrum, accessibility to reagents for chemical modification, hydrodynamic properties etc., on the other hand also indicate that the unfolded state is neither a completely linear polypeptide chain nor a totally random coil structure [1, 13]. Evidences favor that one or more rigid hydrophobic core structures are still retained in the unfolded state which act as nucleus during refolding [4]. Analysis presented on Stoke's radius thus accommodates the notion that the unfolded state has defined hydrodynamic dimensions.

The f *versus* molecular weight profiles presented may be used to ascertain the Stoke's radii of unfolded and dissociated states of any multimeric proteins, provided the molecular weight of the native state is known. This study would thereby help in excluding phenomena like aggregation, incomplete unfolding, association of intermediates etc., which often accompany denaturation. Nevertheless, there are certain limitations to the present analysis, for example, only homomultimeric proteins were considered; only native and fully unfolded states of proteins have been addressed – it often happens that dissociation precedes complete unfolding [14, 15] and in that case partially unfolded states may have similar or even smaller size than the native state. This is relevant in case of trans membrane movement and chaperone binding of proteins [16]. Proteins of multimericity higher

than six are uncommon and their behavior has not been studied properly. Moreover proteins of higher multimericities have not been considered since determination of native molecular weight and estimation of multimericities are often ambiguous in such cases. Finally, proteins have been considered to be composed of polypeptides only – contribution of carbohydrates, lipids, cofactors etc. were ignored. Nonetheless a monotonic behavior of multimeric proteins has been observed in this analysis. It is true that denaturation by 8 M urea or 6 M guanidinium, HCl is not an *in vivo* phenomenon, but it does provide a limiting case of unfolding allowing an insight into the thermodynamic and kinetic forces responsible for denaturation.

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