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Reinvestigation of the thermodynamics of spectrin self-association

Alissa Henniker, G.B. Ralston *

Department of Biochemistry, University of Sydney, Sydney, NSW 2006, Australia

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Abstract

The thermodynamics of the self-association reactions of human spectrin have been reinvestigated by means of sedimentation equilibrium over the temperature range 18–40°C. The experimental data were analysed in terms of a cooperative isodesmic model of association. The van't Hoff plot showed that the standard change in enthalpy for the heterodimer-tetramer step was temperature-dependent, leading to an estimate of -8.5 kJ mol^{-1} K^{-1} for the change in molar heat capacity, ΔC_p. Curvature in the van't Hoff plots, not detected in previous studies, was revealed through the increased precision of the data and the wider temperature range examined. On the assumption that ΔC_p reflects hydrophobic interactions in the tetramer that cannot be formed in the heterodimer, it can be estimated that approximately 50 CH_2 groups per heterodimer participate in hydrophobic interactions in the tetramer that cannot be formed in the heterodimer.

Keywords: Spectrin; Sedimentation equilibrium; Ultracentrifuge; Thermodynamics; Hydrophobic interactions; Van't Hoff

1. Introduction

Spectrin is the major structural protein of the erythrocyte membrane cytoskeleton, involved in maintaining and controlling membrane deformability and shape. The functional unit of erythrocyte spectrin is a heterodimer, composed of non-identical α- and β-chains with molecular masses of 280 kDa and 246 kDa, respectively, deduced from cDNA sequence analysis [1,2]. In previous studies, the molecular mass of spectrin heterodimer has usually been taken to be 480 kDa [3–5].

The α- and β-chains associate laterally and tightly in antiparallel fashion to form the heterodimer which is approximately 100 nm in length [6]. In electron micrographs of shadowed preparations the spectrin heterodimers appear as long flexible molecules exhibiting a wide variety of configurations, from linear rods to curved L-, C-, or S-shapes [6]. Two heterodimers are capable of associating head-to-head to form the predominant form of spectrin in vivo, the tetramer, which has a contour length twice that of the dimer [6]. This head-to-head assembly of tetramers involves reciprocal associations of the N-terminal region

* Corresponding author.

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of the α-subunit with the C-terminal region of the β-subunit [6–8]. In the heterodimer, the formation of these intramolecular interactions appears to be subject to some form of strain which destabilises the dimer with respect to the tetramer [4].

Spectrin is capable of association beyond the tetramer by the addition of further spectrin heterodimers [4,5,9–13]; i.e. the heterodimer is the protomer for association. This type of association reaction can be described as 'indefinite'; that is, the association reaction appears to proceed without limit. Oligomers larger than the tetramer have been visualised in electron microscopic studies [9] and in nondenaturing gel electrophoresis [8,10,11] and have been inferred from sedimentation equilibrium studies [12,13].

In a previous study of the self-association of human spectrin over the temperature range 21–35°C, Ralston [4] found that, over this temperature range and within the precision of the measurements, ΔH° was essentially constant ($\Delta H^\circ = -65$ kJ/mol), and that ΔC_p was therefore indistinguishable from zero. This conclusion implies that hydrophobic interactions are not of major importance in maintaining the tetramer. A constant value of ΔH° ($\Delta H^\circ = -130$ kJ mol^{-1}) was also obtained by Ungewickell and Gratzer [3] from sedimentation velocity studies over the temperature range 25–37°C. However, both of these earlier studies were based on a value of 480 kDa for the molecular mass of the spectrin heterodimer, a value now believed to be too low [1,2].

In attempting to assess the thermodynamic properties of a self-association reaction, it is of the utmost importance that (1) highly precise and accurate data be obtained at chemical equilibrium without perturbing the equilibrium and (2) an accurate and sensitive test be used: (a) to determine if chemical equilibrium had been achieved; and (b) to assess if there is a significant amount of protein incapable of taking part in the self-association (such as low molecular weight contaminants, association-incompetent protomers, or irreversible aggregates). The technique of sedimentation equilibrium meets both of these strict criteria [13,14].

The present study employed sedimentation equilibrium to reinvestigate the self-association behaviour of spectrin over a wider temperature range than had been employed in the past, and using the revised molecular weight of spectrin dimer as 526 kDa, in order to resolve the apparent discrepancies in previously published work.

2. Experimental

2.1. Preparation of spectrin

Human spectrin was prepared from washed erythrocytes as previously described [5]. The purified spectrin was then dialysed against a buffer comprising 0.01 M sodium phosphate, pH 7.5, containing 0.1 M NaCl and 0.1 mM dithiothreitol.

In experiments employing Rayleigh optics in the Model E at temperatures above 30°C the addition of metrizamide was required to stabilise the protein concentration gradient [15]. For these experiments, spectrin was dialysed against the above buffer containing 1% (w/v) metrizamide. Metrizamide could not be used with the absorbance optics of the XL-A analytical ultracentrifuge, and attempts to use dextran as a density stabilising agent were unsuccessful. All buffers were purged with nitrogen to remove oxygen, in order to minimise oxidation of spectrin sulfhydryl groups.

2.2. Ultracentrifugation

Samples (120 μl) of the spectrin solution (usually ≈ 1.2, 0.6, 0.3 g/l) were placed in the sample channels of an ultracentrifuge cell fitted with a Yphantis 6-channel centrepiece. Aliquots of the dialysate (120 μl) were placed in the reference channels. The samples were centrifuged at 7000 rpm in a Beckman XL-A analytical ultracentrifuge, at temperatures between 18°C and 30°C, or at 7200 rpm in a Beckman Model E analytical ultracentrifuge, at temperatures between 18°C and 40°C. In each experiment, equilibrium was first attained at one temperature, after which the temperature was changed several degrees, and sedimentation was continued. At a given temperature, the estimated equilibrium constants were

independent of the direction in which the temperature was changed.

In experiments with the XL-A instrument, scans at 280 nm and 360 nm were taken at intervals after 24 h, the length of the interval depending on the temperature used, since the rate of equilibration was markedly lower at lower temperatures [3]. Sedimentation equilibrium was deemed to have been reached when the difference between consecutive scans was zero, within the precision of the measurements, indicating no significant change in concentration distribution. The 360 nm scans were subtracted from the 280 nm scans to correct for cell and window distortion and for any partial masking of the light pulses.

For experiments in the model E instrument, Rayleigh interference patterns were photographed at zero time and periodically after 24 h, the length of the interval depending on the temperature used. The pattern at zero time was used to correct for cell and window distortion [14]. Sedimentation equilibrium was deemed to have been attained when the Omega distributions overlapped within measurement error.

2.3. Data treatment

The equilibrium distribution was analysed by means of the omega function [16–18]. A reference concentration was chosen common to all three channels (usually 0.5 or 1.0 g/l). The omega data were fitted with the cooperative isodesmic (SEK III) reaction model [19] in order to estimate the equilibrium constant for the heterodimer–tetramer step, $K_{2,4,}$ and that for subsequent additions of heterodimer units, K_{iso}. The SEK III model has been judged the most appropriate and chemical equilibrium, and also as an indication of the absence of contaminants or association-incompetent spectrin. Data which did not display good overlap were rejected.

3. Results

3.1. The effect of Metrizamide on the association behaviour

At temperatures above 30°C, convection within the sample in the ultracentrifuge distorted the equilibrium concentration distribution, and led to a failure of Ω plots to overlap. The addition of 1% (w/v) Metrizamide [15] stabilised the concentration gradients at the higher temperatures studied (32°C, 35°C, 37°C and 40°C) in the Model E, provided that the minimum loading concentration of spectrin in any channel was 0.6 mg/ml. Comparison of the values of association constants obtained in the presence and absence of 1% (w/v) Metrizamide, between 18°C and 30°C, revealed that Metrizamide at this concentration did not perturb the equilibrium beyond the precision of the estimates.

Since Metrizamide absorbs strongly at 280 nm, it could not be used with the absorbance optics of the XL-A instrument, and dextran was incapable of sufficient stabilisation of the protein concentration gradient. This restricted the accessible temperature range with the use of the XL-A instrument to between 18 and 30°C.

3.2. The temperature dependence of $K_{2,4}$

The temperature dependence of the spectrin dimer–tetramer association is marked. The equi-

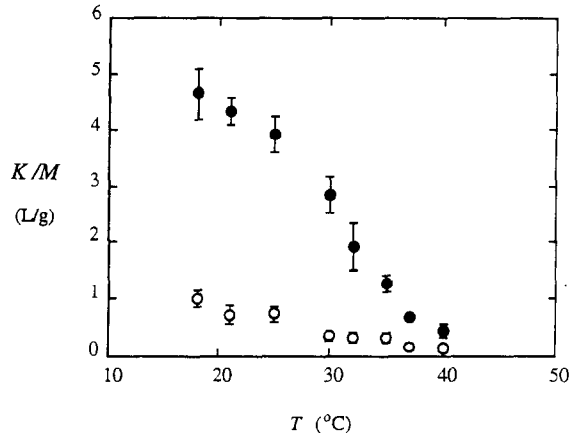

Fig. 1. Temperature dependence of $K_{2,4}$ (•) and K_{iso} (○) for the self-association of human spectrin. Data are shown as K/M, where K is the relevant equilibrium constant in the molar scale, and M is the molar mass of the heterodimer (526 kg/mol). The error bars indicate the standard error of the mean from replicate experiments ($n = 3$ at 40°C, 2 at 37°C, 5 at 35 and 32°C, 7 at 30°C, 8 at 25°C, 5 at 21°C, and 4 at 18°C).

plot was fitted well with a quadratic function, from which the slope could be calculated numerically at each temperature. The standard enthalpy change for the dimer tetramer reaction at each temperature was calculated from the local slope

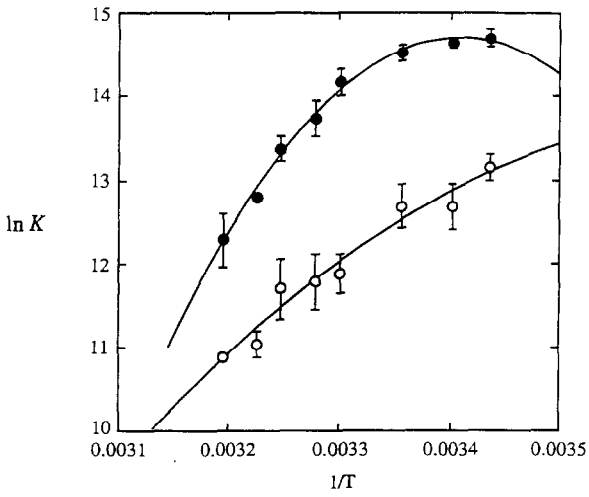

Fig. 2. van't Hoff plots for $K_{2,4}$ (•) and K_{iso} (○) (in the molar scale) for the self-association of human spectrin. The error bars indicate the SEM from replicate experiments. Quadratic functions have been fitted to the data by means of unweighted linear regression.

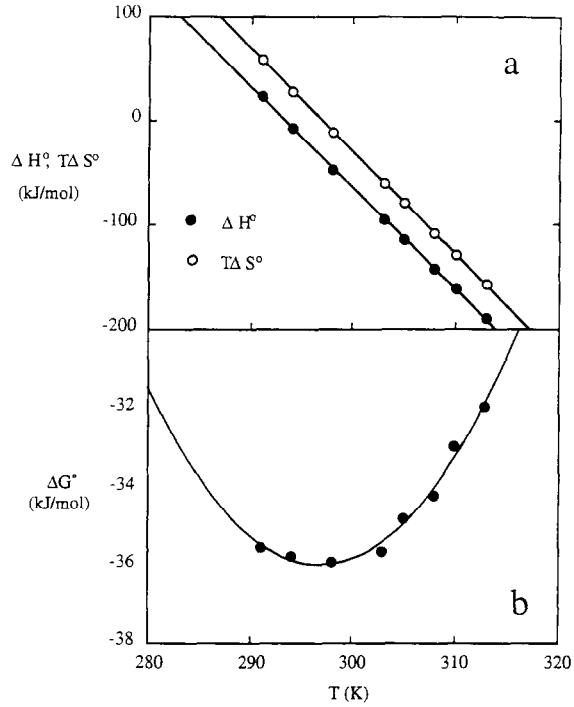

Fig. 3. Thermodynamic functions for the dimer-tetramer equilibrium of human spectrin: (a) $\Delta H°$ (•) and $T\Delta S°$ (○); (b) $\Delta G°$. The uncertainty in the values of $\Delta G°$ are estimated to be 1 to 2%, that in $\Delta H°$ between 3 and 5%, and the estimated uncertainty in $\Delta S°$ may be from 10 to 20%.

($\Delta H° = -R$ d ln K/d$(1/T)$), and the standard change in molar entropy was calculated from the relationship $T\Delta S° = \Delta H° - \Delta G°$. These data are shown in Fig. 3.

Both $\Delta H°$ and $T\Delta S°$ for the heterodimer–tetramer step were found to decrease markedly with increasing temperature (Fig. 3a). $\Delta G°$ showed pronounced temperature dependence, with a minimum at 23°C (Fig. 3b); at this temperature $\Delta S°$ was zero. The change in heat capacity, ΔC_p, for the formation of tetramer from dimer, was obtained from the relationship $\Delta C_p = \mathrm{d}\Delta H° / \mathrm{d}T$ and was calculated to be -8.5 ± 1.4 kJ mol^{-1} K^{-1}.

Since the rates of the association/dissociation reactions for spectrin decrease markedly at lower temperatures [3], the possibility exists that the levelling off of the van't Hoff plot at temperatures below 25°C might be due to kinetic limitations rather than a reflection of the thermody-

namics. To further assess the association behaviour at low temperatures (18°C and 21°C), sedimentation equilibrium experiments were conducted for an extended period of time in both the XL-A and model E ultracentrifuges.

Samples of spectrin were centrifuged in three separate cells of the XL-A. One sample had been incubated in 0.1 M NaCl at 30°C for 4 h at a concentration of 5 g/l to promote the extensive formation of tetramer. After incubation, the sample was chilled on ice and diluted to 2.5, 1.2 and 0.6 g/l. A second sample comprised freshly prepared spectrin dimer, diluted with ice-cold buffer and held at 2–4°C until the experiment. The third sample comprised freshly prepared spectrin dimer, diluted to 2.5, 1.2 and 0.6 g/l; the loaded cell was held at 30°C for 4 h to promote partial association to tetramer. All three cells were then centrifuged at 7000 rpm and 21°C for a prolonged period, and scans were taken at appropriate intervals. A sample which had been incubated in 0.1 M NaCl at 30°C for 4 h at a concentration of 5 g/l, was then chilled on ice and diluted to 2.5, 1.2 and 0.6 g/l. This sample was simultaneously centrifuged in the model E instrument at 7200 rpm and 21°C.

After 67 h at 21°C, all three sets of solutions in the XL-A and the single set in the model E were deemed to have reached combined chemical and sedimentation equilibrium; there was no detectable change in the concentration distributions between 67 and 139 h of centrifugation, and omega data from all samples showed good overlap.

At this point, the temperature was lowered to 18°C, and centrifugation was continued. After an additional 40 h, a new equilibrium was deemed to have been reached, and the concentration distribution did not change further over an additional 90 h of centrifugation. Even after this time, satisfactory overlap was found with all samples. At both 18°C and 21°C, the data from both ultracentrifuges were in good agreement with each other and with that shown in Figs. 1 and 2; at a given temperature the estimates of $K_{2,4}$ from the three cells in the XL-A were comparable, and were independent of the past history of the samples.

These results indicate that the levelling off of the van't Hoff curve at low temperatures seen in Fig. 2 actually reflects the thermodynamics of the equilibrium reaction, and is not due to insufficient time of ultracentrifugation. In these protracted time periods no proteolysis was evident and no irreversible aggregation was detectable by means of gel electrophoresis.

In the present study, the data of Ralston [4] was reanalysed using the revised molecular mass of spectrin heterodimer of 526 kDa. The van't Hoff plot obtained could be fitted with a straight line, giving a constant standard change in enthalpy of -66 kJ/mol, a value very close to that of -65 kJ mol^{-1} obtained in Ralston's study. This indicates that the small difference in the value used for the molecular mass of spectrin does not significantly affect the derived standard change in enthalpy. Because of the scatter in the experimental data, any curvature in this plot is obscured.

3.3. Temperature dependence of K_{iso}

The value of K_{iso} also decreased by almost an order of magnitude between 18°C and 40°C (Fig. 1), but curvature in the van't Hoff plot was less marked (Fig. 2). For this reaction, a value of -2.4 ± 1.8 kJ mol^{-1} K^{-1} was estimated for ΔC_p, the molar change in heat capacity, although this value is subject to a large uncertainty.

4. Discussion

Curvature of the van't Hoff plot implies variation of $\Delta H°$ with temperature, a reflection of the difference in heat capacity between two moles of the heterodimer and one of the tetramer. This temperature dependence is almost certainly due to the participation of hydrophobic interactions in the tetramer that cannot be formed in the dimer.

The value of ΔC_p (-8.5 ± 1.4 kJ mol^{-1} K^{-1}) is referred to two moles of the dimer; thus the value of ΔC_p for one dimer is -4.2 ± 0.7 kJ mol^{-1} K^{-1}. This value is relatively large, and is comparable with that associated with the folding of a small protein [20]; changes in heat capacity

of this magnitude almost certainly arise from hydrophobic interactions. If the partial molar heat capacity per CH_2 group exposed to water is taken to be 0.09 kJ mol^{-1} K^{-1} [21] then the observed change in heat capacity could be rationalised by the exposure to water of approximately 50 CH_2 groups in the spectrin dimer that are removed from water during the formation of the tetramer.

Since the association of spectrin heterodimers is proposed to result from an exchange of *intra*molecular for *inter*molecular interactions, the magnitude of ΔC_p cannot be accounted for adequately in terms of the direct interactions at the α–β interface alone. The change in heat capacity presumably reflects changes elsewhere in the spectrin molecule that are required to permit the formation of the tetramer.

It has been proposed that a 'hairpin' bend occurs in the α-chain [7] (which is longer than the β-chain), to permit the intramolecular interactions between the α- and β- termini. Such a hairpin bend in the α-chain may require partial unfolding of the α-chain which exposes nonpolar groups to the surrounding water. In forming the tetramer, the 'hairpin' bend is lost and folding is restored, removing solvent-exposed nonpolar groups from the surrounding water (Fig. 4).

This explanation is consistent with recent crystallographic data of a repetitive segment from *Drosophila* α-spectrin [22]. This study revealed that a number of hydrophobic interactions is involved in interactions between the ends of successive triple-α-helical repeated segments. The postulated involvement of 3 leucines, 1 isoleucine and 1 valine at this interface would contribute 19 methylene or methyl groups to the interactions. Disruption of these interactions between 2 or 3 successive domains to allow intramolecular α–β interactions at the tetramer interface could account for the magnitude of the observed change in heat capacity.

4.1. Other interactions influencing the thermodynamic parameters

The magnitude and sign of ΔC_p are consistent with the involvement of hydrophobic interactions in the formation of the tetramer of spectrin.

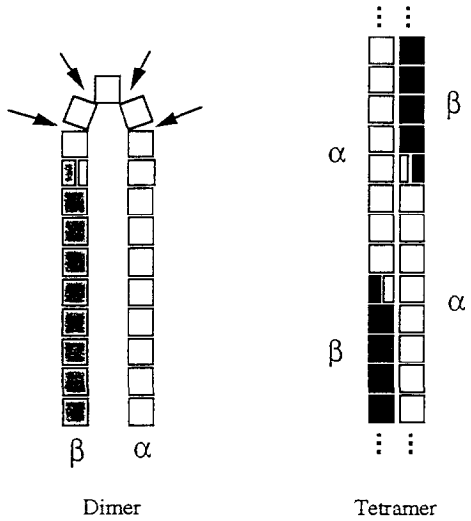

Fig. 4. Diagrammatic representation of the postulated exposure of nonpolar groups in the dimer to the surrounding water (indicated by the arrows) accompanying the reorganisation of the molecule to accommodate intramolecular interactions. Hydrophobic interactions between nonpolar groups in adjacent folded domains in the tetramer reduce the area exposed and lower the heat capacity. The dotted lines indicate that the tetramer is much longer than is shown.

Below 25°C, the reaction is entropically driven; the positive sign of both $\Delta H°$ and $\Delta S°$ at low temperatures is characteristic of the hydrophobic interaction. However, between 25°C and 40°C both $\Delta H°$ and $\Delta S°$ are negative; since $\Delta H°$ and $\Delta S°$ are both positive for hydrophobic interactions, other factors must contribute to the negative quantities determined experimentally.

Potential sources of a negative $\Delta S°$ include loss of rotational entropy or changes in vibrational modes on formation of the tetramer [4]. The unfavourable changes in entropy noted between 25°C and 40°C mean that the reaction must be enthalpy-driven in this temperature range. Sources of negative $\Delta H°$ could include hydrogen bonding or electrostatic interactions occurring in the tetramer which do not occur in the dimer, or steric strain in the dimer which is relieved in the tetramer. An electrostatic contribution has been indicated from pH dependence [4] and ionic strength dependence [23] of the reaction. These contributions may also arise from the partial unfolding of the α-chain upon 'hairpin' bend forma-

tion [7], which may decrease both hydrogen bonding and electrostatic interactions between the α- and β-chains.

4.2. Comparison of the present study with past studies

Extending the temperature range downwards to 18°C and upwards to 40°C and improving the precision of the data, has revealed curvature in the van't Hoff plot that was not detected in two earlier studies [3,4].

The study by Ungewickell and Gratzer [3] of the thermodynamics and kinetics of the self-association of human spectrin encompassed the temperature range 25–37°C. A single value of $\Delta H°$, of -130 kJ mol^{-1} sufficed in this range, corresponding to the more steeply-sloping portion of the van't Hoff plot in Fig. 2. The $K_{2,4}$ values of the Ungewickell and Gratzer study and those of the present study are quite similar, although those of Ungewickell and Gratzer are slightly lower. When the van't Hoff plot in the present study is analysed from 25 to 37°C only, the curvature is not so evident, and fitting to a linear relationship yields a $\Delta H°$ value of -116 ± 10 kJ mol^{-1}, in good agreement with that of Ungewickell and Gratzer [3].

The study by Ralston [4] covered the somewhat lower temperature range of 21–35°C, corresponding to a shallower portion of the van't Hoff plot in Fig. 2. In the 1991 study, curvature in the van't Hoff plot was obscured by the relatively large experimental errors, and a constant enthalpy of -65 kJ mol^{-1} was obtained from a linear fit. Fitting the data of the present study over the restricted temperature range 21–35°C with a linear fit yielded a change in standard enthalpy of -62 ± 10 kJ mol^{-1}, in excellent agreement with the results of the previous study [4]. The equilibrium constants from the earlier study [4] were also noticeably lower than those in the present study. There are several possible explanations for this. Firstly, small amounts of association-incompetent dimer, insufficient to display non-coincidence of the omega plots, would be expected to lower the measured equilibrium constants. Secondly, the maximum observable concentration at the cell bottom in the 1991 study was only 2 g/l, while in the present study, fringes could be resolved routinely to a concentration of 3–4 g/l. A wider concentration range allows more precise resolution of the separate equilibrium constants, $K_{2,4}$ and K_{iso}. Additionally, the obscuring of the fringes at the cell bottom in the earlier work may have arisen in part from rotor precession, which in turn may lead to some distortion of the fringe pattern.

The present study has covered a broader temperature range, encompassing both temperature ranges used in the earlier studies. Over the wider range, and with vastly more data and more precise data, the present study revealed curvature in the van't Hoff plot which was not evident in the earlier studies, and which explains the apparent discrepancies in the earlier published data. Because of this curvature, both sets of earlier data [3,4] are consistent with those of the present study. The substantial negative change in heat capacity on forming the tetramer indicates a role for hydrophobic interactions in stabilising the tetramer.

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References

[1] K.E. Sahr, P. Laurila, L. Kotula, A.L. Scarpa, E. Coupal, T.L. Leto, A.J. Linnenbach, J.C. Winkelmann, D.W. Speicher, V.T. Marchesi, P.J. Curtis and B.G. Forget, J. Biol. Chem. 265 (1990) 4434.

[2] J.C. Winkelmann, J. Chang, W.T. Tse, A.L. Scarpa, V.T. Marchesi and B.G. Forget, J. Biol. Chem. 265 (1990) 11827.

[3] E. Ungewickell and W.B. Gratzer, Eur. J. Biochem. 88 (1978) 379.

[4] G.B. Ralston, Biochemistry 30 (1991) 3076.

[5] G.B. Ralston, Biophys. Chem. 44 (1992) 175.

[6] D.M. Shotton, B.E. Burke and D. Branton, J. Mol. Biol. 131 (1979) 303.

[7] D.W. Speicher, T.M. deSilva, K.D. Speicher, J.A. Ursitti, P. Hembach and L. Weglarz, J. Biol. Chem. 268 (1993) 4227.

[8] F. Shahbakhti and W.B. Gratzer, Biochemistry 25 (1986) 5969.
[9] J.M. Tyler, W.R. Hargreaves and D. Branton, Proc. Natl. Acad. Sci. USA 76 (1979) 76.
[10] J.S. Morrow and V.T. Marchesi, J. Cell Biol. 88 (1981) 463.
[11] J.S. Morrow, W.B. Haigh and V.T. Marchesi, J. Supramol. Struct. 17 (1981) 275.
[12] M.B. Morris and G.B. Ralston, Biochem. Biophys. Acta 788 (1984) 132.
[13] M. Morris and G.B. Ralston, Biochemistry 28 (1989) 8561.
[14] D.C. Teller, Methods Enzymol. 27 (1973) 346.
[15] G.B. Ralston, D.C. Teller and T. Bukowski, Anal. Biochem. 178 (1989) 198.
[16] B.K. Milthorpe, P.D. Jeffrey and L.W. Nichol, Biophys. Chem. 3 (1975) 169.
[17] M. Morris and G.B. Ralston, Biophys. Chem. 23 (1985) 49.
[18] G.B. Ralston and M.B. Morris, in: Analytical ultracentrifugation in biochemistry and polymer science eds. S.E. Harding, A.J. Rowe and J.C. Horton, (Royal Society of Chemistry, Cambridge, 1992) p. 253.
[19] E.T. Adams, L.-H. Tang, J.L. Sarquis, G.H. Barlow and W.M. Norman, in: Physical aspects of protein interactions, ed. N. Catsimpoolas (Elsevier, Amsterdam, 1978) p. 1.
[20] C. Tanford, Advan. Protein Chem. 24 (1970) 1.
[21] G.I. Makhatadze and P.L. Privalov, J. Mol. Biol. 213 (1990) 375.
[22] Y. Yan, E. Winograd, A. Viel, T. Cronin, S.C. Harrison and D. Branton, Science 262 (1993) 2027.
[23] N. Cole and G.B. Ralston, Biochim. Biophys. Acta 1121 (1992) 23.