Biophysical Chemistry 17 (1983) 29–34
Elsevier Biomedical Press

EFFECT OF MICROHETEROGENEITY ON THE SEDIMENTATION BEHAVIOR OF SELF-ASSOCIATING PROTEINS

John R. CANN and Nancy H. FINK

Department of Biochemistry/Biophysics/Genetics, University of Colorado Health Sciences Center, 4200 E. 9th Avenue, Denver, CO 80262, U.S.A.

Received 9th August 1982
Accepted 30th August 1982

Key words: Sedimentation theory; Microheterogeneity; Self-association

The effect of microheterogeneity on the sedimentation behavior of self-associating proteins has been examined theoretically with particular reference to rapidly equilibrating tetramerization reactions. Several models of microheterogeneity have been considered. Each one exhibits a sedimentation behavior departing substantially from that expected of homogeneous systems. It is anticipated that the results may be helpful for interpreting the sedimentation patterns of self-associating proteins when the patterns cannot be analyzed in terms of the Gilbert theory.

1. Introduction

Microheterogeneity refers to the property of certain highly purified proteins to show molecular heterogeneity when scrutinized by high-resolution immunochemical, biochemical or biophysical techniques [1–9]. Molecular heterogeneity can be due to genetic variations in primary structure as, for example, in the case of isozymes [2] and polyclonal antibodies [6,7]; intramolecular disulfide interchange in serum albumin [10]; hypothesized enzymatic nicking prior, perhaps, to isolation of concanavalin A [8,9]; or adventitious modification (e.g., deamidation of a few asparagine and glutamine residues and limited proteolytic attack on one or a few peptide linkages) during purification and/or subsequent manipulation. Strictly speaking, the term microheterogeneity should probably be restricted to polymorphism, but common usage includes all of the above. This aside, microheterogeneity can have pronounced effects on the electrophoretic and sedimentation behavior of interacting systems.

The classic research of Foster and co-workers [3–5,10] established that microheterogeneity accounts for the electrophoretic resolution of N and F forms of serum albumin undergoing rapid isomerization near pH 4. Recently [11], it has been shown that the heterogeneity of antibodies with respect to binding affinity provides an explanation for the trimodal shape of the analytical sedimentation patterns of univalent antigen-antibody and bivalent hapten-antibody reaction mixtures. Heterogeneity of pyruvate dehydrogenase multienzyme complex with respect to molecular weight causes excessive spreading of its sedimenting boundary, a conclusion reached by computer simulation [12]; and the nicked subunits present in some preparations of concanavalin A have a much decreased competency to self-associate as determined by sedimentation equilibrium [13]. Finally, heterogeneity is not restricted to proteins. Thus, the trimodal zonal sedimentation patters of *Escherichia coli* ribosomes have been explained [14] as being due to heterogeneity of formation constants of 70 S particles from 30 and 50 S subunits.

These examples along with poorly understood but suggestive observations in the literature prompted a theoretical investigation of the effect

0301-4622/83/0000-0000/$03.00 © 1983 Elsevier Biomedical Press

of microheterogeneity on the sedimentation behavior of self-associating proteins with particular reference to rapidly equilibrating, tetramerization reactions. The approach simulates experimental procedure in that analytical sedimentation patterns were computed for a range of protein concentrations by numerical solution of the appropriate set of transport equations and mass action expressions for several model systems.

2. Theory

The starting point of the calculations is the tetramerization of a homogeneous protein M schematized by the rapid association-dissociation equilibrium

$$4M \rightleftarrows M_4,\ K \qquad (1)$$

which assumes negligible concentrations of intermediate dimer and trimer. Gilbert [15,16] first described the salient features of the sedimentation behavior of such a system: At sufficiently low total protein concentration, the sedimentation pattern shows a single sedimenting peak of monomer which grows in area as the concentration is increased to a critical value determined by the value of the equilibrium constant K. Upon increasing the concentration still further, a second more rapidly sedimenting peak appears and grows in area, while the area of the slower peak now remains unchanged, the two peaks constituting a single reaction boundary. In general, this behavior is predicted for $nM \rightleftarrows M_n$ with $n \geq 3$ and critical concentration dependent upon n and K. It should be noted, however, that the Gilbert theory is an asymptotic theory which ignores the diffusional term in the transport equations, and Cox [17] has found that under certain conditions the predicted features of the sedimenting boundary shape may not be evident in the presence of diffusion. This is particularly so for weak association. Also, for sufficiently weak association, the interplay between the strength of association and the hydrodynamic concentration dependence of the sedimentation coefficients of the species in equilibrium may assume such proportion as to erase the expected bimodality of the reaction boundary [18,19]. Nevertheless, a number of self-association proteins show the features predicted by the Gilbert theory; e.g., the low-temperature tetramerization of β-lactoglobulin A at pH 4.65 [19–21] and the formation of skeletal muscle myosin filaments [22,23]. The question posed in the present study is: 'How might microheterogeneity with respect to association constant, other molecular parameters held constant, effect sedimentation behavior?'

Three different models of microheterogeneity have been considered. Each is for rapidly reversible tetramerization. The diffusional term is retained in the transport equations; but, in order to minimize the number of adjustable parameters, the concentration dependence of the sedimentation coefficients, which is of little consequence for strong associations [18], is ignored.

The first model assumes the protein to be a 1 : 1 mixture of either two polymorphic monomers or, possibly, native and modified forms of a monomorphic protein, designated as M(1) and M(2). The two have the same transport parameters, but they associate independently with different equilibrium constants according to the reactions.

$$4M(1) \rightleftarrows M(1)_4,\ K(1) \qquad (2a)$$

$$4M(2) \rightleftarrows M(2)_4,\ K(2) \qquad (2b)$$

A broad range of values for both $K(1)$ and $K(2)$ was explored; but the most systematic calculations were for $K(1) = 3.0720 \times 10^{15}\ M^{-3}$, with the ratio $K(1)/K(2)$ being treated as a parameter ranging in value from about 5×10^3 to 20. This range is not unrealistic considering that a preparation of concanavalin A containing about 50% nicked subunits had an apparent association constant for the dimer-tetramer equilibrium which was about 10-fold smaller than for a preparation free of nicked subunits [13]. In the second model the two monomers in 1 : 1 proportions interact to form hybrid tetramers as schematized by the reaction set

$$4M(1) \rightleftarrows M(1)_4,\quad K(1) \qquad (3a)$$

$$3M(1)+M(2) \rightleftarrows M(1)_3M(2),\quad K(1)^{3/4}K(2)^{1/4} \qquad (3b)$$

$$2M(1)+2M(2) \rightleftarrows M(1)_2M(2)_2,\quad K(1)^{1/2}K(2)^{1/2} \qquad (3c)$$

$$M(1)+3M(2) \rightleftarrows M(1)M(2)_3,\quad K(1)^{1/4}K(2)^{3/4} \qquad (3d)$$

$$4M(2) \rightleftarrows M(2)_4,\quad K(2) \qquad (3e)$$

with $K(1) = 3.0720 \times 10^{15}\ M^{-3}$ and $K(2) = 5.9259 \times 10^{11}\ M^{-3}$.

The third model is for rather extensive heterogeneity in which five classes of monomer associate independently,

$$4M(i) \rightleftarrows M(i)_4,\ K(i) \qquad (4)$$

where $i = 1,2 \ldots 5$. Two cases have been considered. The distribution of monomers in case I is 50% of class 1 with $K(1) = 4.736 \times 10^{16}\ M^{-3}$ and 12.5% each of the other four classes with $K(2) = 7.324 \times 10^{14}$, $K(3) = 7.407 \times 10^{13}$, $K(4) = 8.468 \times 10^{12}$ and $K(5) = 1.446 \times 10^{12}\ M^{-3}$. In case II the distribution is 20% of each class of monomer with the same association constants as in case I.

Sedimentation coefficients were assigned the values, $s_M = 4.3$ S and $s_{M_4} = 10.0$ S; diffusion coefficients, $D_M = 4.0 \times 10^{-7}$ cm^2/s and $D_{M_4} = 2.5 \times 10^{-7}$ cm^2/s; monomer molecular weight, 70000; rotor speed, 50750 rpm for sedimentation in a sector-shaped cell with the positions of the meniscus and the bottom of the cell at 5.9 and 7.4 cm from the center of rotation. The numerical procedures used to solve the set of simultaneous transport equations and mass action expressions were the same as described in detail on several previous occasions [11,24,25] and warrant no further comment except to say that computations were made on the University of Colorado's Cyber 172 electronic computer. Pilot calculations were for reaction 1 with $K = 7.407 \times 10^{10}\ M^{-3}$.

The computed sedimentation patterns are displayed as plots of concentration gradient, $\partial c/\partial r$ vs. the distance, r, from the center of rotation; c is the total concentration of protein in mg/ml. Vertical arrows at the top of the figures indicate where the peaks in the patterns would have been located had sedimentation been carried out on a mixture of noninteracting proteins having the same sedimentation coefficients as M and M_4. The area sustained by the monomer peak was taken to be twice the area from the meniscus to the apex of the peak, since this region of the cell was, for practical purposes, devoid of tetramer, and the peak had departed completely from the meniscus.

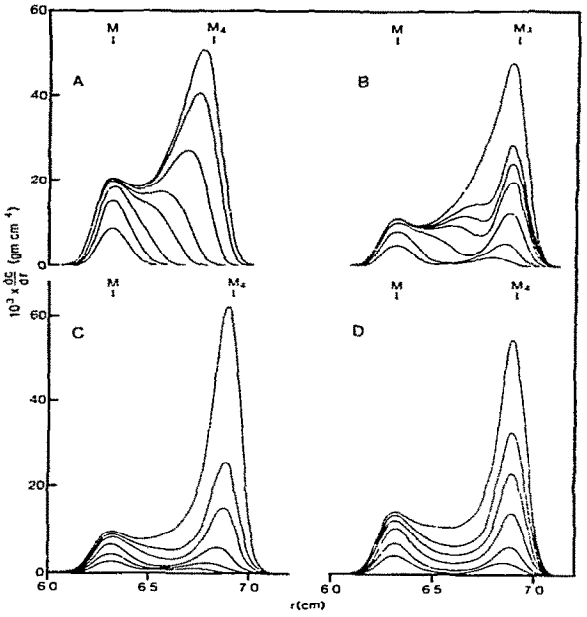

Fig. 1. Theoretical sedimentation patterns of rapidly equilibrating monomer-tetramer systems computed for a range of protein concentrations. (A) Homogeneous protein, reaction 1: Reading from bottom to top the initial constituent concentrations of protein are 1.75, 3.50, 5.25, 7.875, 10.5, 15.75, 21.0 and 24.5 mg/ml. (B) Microheterogeneous protein, reactions 2 with $K(1) = 3.0720 \times 10^{15}\ M^{-3}$ and $K(2) = 5.9259 \times 10^{11}\ M^{-3}$: 1.75, 3.50, 7.00, 10.5, 12.25, 14.0 and 21.0 mg/ml. (C) Microheterogeneous protein, reaction set 3: 0.875, 1.75, 3.50, 7.00, 10.5 and 21.0 mg/ml. (D) Microheterogeneous protein, reactions 4, case I: 1.75, 3.50, 7.00, 10.5, 14.0 and 21.0 mg/ml; essentially the same results were obtained for case II. Values of equilibrium constants for reaction 1, reaction set 3 and reactions 4 are given in section 2. Time of sedimentation, 5.60109×10^3 s.

3. Results

Theoretical sedimentation patterns and corresponding plots of the area of the monomer peak vs. constituent concentration of protein in the plateau region of the cell are displayed in figs. 1 and 2, respectively. The pilot calculations for tetramerization of a homogeneous protein (reaction 1) gave sedimentation patterns (fig. 1A) and a monomer peak area-concentration profile (fig. 2A) which agree qualitatively with the predictions of the Gilbert theory. Moreover, the evolution of the shape of the pattern with concentration is in accord with the findings of Cox [17].

This classical perception of the sedimentation behavior of self-associating proteins is signifi-

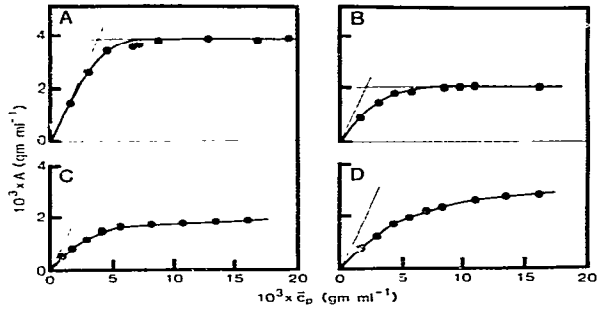

Fig. 2. Plots of area of monomer peak, A, against constituent concentration of protein in plateau region of centrifuge cell, $\bar{c}_p$. Panels A–D pair with correspondingly designated panels in fig. 1. The straight line intercepting at the origin has a slope of 1.

cantly modified by microheterogeneity. Thus, the patterns for a 1 : 1 mixture of two monomeric forms having the same sedimentation and diffusion coefficients but tetramerizing independently with different association constants (reactions 2) can show three peaks, depending upon initial constituent concentration (fig. 1B). This is a direct result of superimposing the individual patterns of the two admixed systems. Thus, the slowest sedimenting peak is a mixture of the two monomers. To a first approximation, the fastest peak corresponds to the faster of the two peaks which the more strongly associating system would show, if it were to be isolated and examined alone; while the intermediate peak belongs to the weaker system. The monomer peak at first grows with concentration even though the fastest peak has already emerged, but, as one would expect, its area remains constant once the intermediate peak appears (fig. 2B). These results are for $K(1) = 3 \times 10^{15}$ M^{-3} and $K(1)/K(2) = 5 \times 10^3$, but trimodal patterns have been obtained for $K(1)/K(2)$ as small as 20 depending upon concentration: e.g., $K(1)/K(2) = 200$ at a concentration of 3.5 mg/ml; 50 at 2.205 mg/ml; and 20 at 1.624 mg/ml. Nor need $K(1)$ be so large, $K(1) = 7 \times 10^{12}$ M^{-3} with $K(2) = 1.4 \times 10^{11}$ M^{-3} giving a trimodal pattern at 16.8 mg/ml.

The picture is quite different when the two monomers interact to form hybrid tetramers (reaction set 3). In that case, the patterns show only two peaks at all concentrations (fig. 1C), and the area of the monomer peak growns continuously with increasing concentration over the broad range examined (fig. 2C). Furthermore, the area apparently does not approach a limiting value, a behavior probably related to the change in distribution of material among the various tetramers with increasing constituent concentration. *

In practice, it would be difficult to distinguish between hybrid formation and rather extensive microheterogeneity with the various components self-associating independently of one another (reactions 4). Comparison of fig. 1C and D points out the similarity between the sedimentation patterns predicted for the two models. On the other hand, in the case of rather extensive microheterogeneity the area of the monomer peak does approach a limiting value (fig. 2D), where the gradient of each of the five components of the mixture is bimodally distributed along the centrifuge cell. Only the weakest associating one is not as yet bimodal at the highest concentration examined.

4. Discussion

Although the foregoing results are for tetramerization reactions, the qualitative conclusions also apply to higher-order associations even when the equilibrium constants are quite large and, thus, may bear on the in vitro formation of smooth

* The mass action characteristics of reaction set 3 are such that the equilibrium distribution of tetramers for a constituent concentration of protein equal to 3.5 mg/ml is 26.8% of the constituent protein bound into $M(1)_4$ on a weight basis, 11.6% bound into $M(1)_3M(2)$, 50% $M(1)_2$ $M(2)_2$, 2.2% $M(1)$ $M(2)_3$ and 0.9% $M(2)_4$. Increasing the concentration to 21 mg/ml decreases the percentage of $M(1)_4$ while increasing all others, the distribution changing to 23.0, 18.4, 14.8, 11.9 and 9.5%, respectively. At extremely high concentrations the distribution converges on 20% of each tetramer. That the percentage-constituent concentration curve for $M(1)_4$, but not for the other tetramers, shows a maximum (at 3.5 mg/ml) is a consequence of the relatively large value of $K(1)$ with respect to the graded values of the other K parameters.

muscle myosin filaments [23]. * Unlike rabbit skeletal muscle myosin, the sedimentation behavior of calf aorta smooth muscle myosin under self-associating conditions cannot be analyzed in terms of the Gilbert theory. The sedimentation patterns show two peaks between pH 6 and 8 over a wide range of protein concentrations. But, there is no critical concentration beyond which the area of the monomer peak remains constant, the areas of the monomer and n-mer peaks increasing together over nearly the entire concentration range. Lack of reversiblity or equilibration or presence of 'incompetent' monomer (i.e., incapable of associating) seem to be improbable causes of this behavior. The investigators allude to possible isozymes, and the SDS-gel electrophoretic pattern of their material distinctly shows heterogeneity in the region of the heavy chains. Indeed, the shape of the sedimentation patterns and the monomer area-concentration profile are strikingly similar to those displayed in figs. 1D and 2D for rather extensive microheterogeneity. It may be pertinent that plasminogen activator is found in highest concentration in vascular structures [27], specifically in vascular endothelium, and that rabbit muscle is reported to contain none [28]. It is conceivable that the aorta myosin preparations may have contained nicked molecules due to extra- or intracellular proteolytic attack.

In conclusion, the results of the present investigation broaden the theoretical base for biophysical studies on the architectural and regulatory roles played by self-associating proteins in vivo. A rather provocative finding is that microheterogeneity with respect to association constant can give sedimentation patterns exhibiting three peaks. Mediation by two different ligands acting in a stepwise fashion is another mechanism which can give trimodal patterns for rapidly equilibrating self-association [25]. It is essential, therefore, that several biophysical methods, including fractionation, be brought to bear in order to characterize as precisely as possible the nature of associating systems.

* It has been estimated [23] that here $N \leq 30$ and $K' \approx 10^{13}$ (concentrations in units of g/100 ml). As an adjunct to our numerical computations, asymptotic sedimentation patterns for such extensive self-association were calculated as described [26]. For a model of microheterogeneity analogous to reactions 2 the patterns show three peaks for the following values of the parameters: $K'(1) = 10^{13}$, $K'(2) = 10^{11}$, constituent concentration 0.8 g/100 ml; $K'(1) = 10^{15}$, $K'(2) = 10^{13}$, 0.68 g/100 ml; and $K'(1) = 10^{15}$, $K'(2) = 10^{11}$, 0.82–0.94 g/100 ml. By inductive reasoning, a model analogous to reactions 4 would in practice show only two peaks, small satellite peaks near the centripetal foot of the dominant polymer peak being washed out by diffusion and lost in an elevated baseline. These insights provided by the Gilbert theory lend confidence in the application to strong higher-order associations of the qualitative conclusions drawn from the results of our computations for tetramerization reactions.

Acknowledgement

This study was supported in part by Research Grant RO1 GM 28793-31 from the National Institute of General Medical Sciences, National Institutes of Health, U.S. Public Health Service.

References

1 H.E. Schultze and J.F. Heremans, Molecular biology of human proteins, vol. 1, ch. 1 in section I and ch. 1 in section III (Elsevier, New York, 1966)
2 C.L. Markert, Isozymes I Molecular structure (Academic Press, New York, 1975).
3 M. Sogami and J.F. Foster, J. Biol. Chem. 238 (1963) 2245.
4 H.A. Petersen and J.F. Foster, J. Biol. Chem. 240 (1965) 2503.
5 H.A. Petersen and J.F. Foster, J. Biol. Chem. 240 (1965) 3958.
6 E.A. Kabat, Adv. Protein Chem. 32 (1978) 1.
7 H.N. Eisen, Immunology. An introduction to molecular and cellular principles of the immune responses, 2nd edn., ch. 16 and 19 (Harper and Row, Hagerstown, MD, 1980).
8 J.L. Wang, B.A. Cunningham and G.M. Edelman, Proc. Natl. Acad. Sci. U.S.A. 68 (1971) 1130.
9 J.L. Wang, B.A. Cunningham, M.J. Waxdal and G.M. Edelman, J. Biol. Chem. 250 (1975) 1490.
10 M. Sogami, H.A. Petersen and J.F. Foster, Biochemistry 8 (1969) 49.
11 J.R. Cann, Mol. Immunol. 19 (1982) 505.
12 G.A. Gilbert and L.M. Gilbert, J. Mol. Biol. 144 (1980) 405.
13 D.F. Senear and D.C. Teller, Biochemistry 20 (1981) 3076.
14 J.B. Chaires and G. Kegeles, Biophys. Chem. 7 (1977) 173.
15 G.A. Gilbert, Disc. Faraday Soc. 20 (1955) 68.
16 G.A. Gilbert, Proc. R. Soc. A250 (1959) 377.
17 D.J. Cox, Arch. Biochem. Biophys. 129 (1969) 106.
18 G.A. Gilbert, Proc. R. Soc. A276 (1963) 354.

19 T.F. Kumosinski and S.N. Timasheff, J. Am. Chem. Soc. 88 (1966) 5635.
20 R. Townend, R.J. Winterbottom and S.N. Timasheff, J. Am. Chem. Soc. 82 (1960) 3161.
21 R. Townend and S.N. Timasheff, J. Am. Chem. Soc. 82 (1960) 3168.
22 R. Josephs and W.F. Harrington, Biochemistry 5 (1966) 3474.
23 J. Megerman and S. Lowey, Biochemistry 20 (1981) 2099.
24 W.B. Goad, in: Interacting macromolecules. The theory and practice of their electrophoresis, ultracentrifugation, and chromatography, ed. J.R. Cann (Academic Press, New York, 1970) ch. 5.
25 J.R. Cann, Biophys. Chem. 16 (1982) 41.
26 J.R. Cann, Interacting macromolecules. The theory and practice of their electrophoresis, ultracentrifugation, and chromatography (Academic Press, New York, 1970) ch. 3 and 4.
27 E.W. Davie and O.D. Ratnoff, in: The proteins composition, structure, and function, 2nd edn., vol. 3, ed. H. Neurath (Academic Press, New York, 1965) ch. 16.
28 O.K. Albrechtsen, Acta Physiol. Scand. 47, Suppl. 165 (1959) 1.