Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265-275.
Pecora, R. (1972), *Annu. Rev. Biophys. Bioeng. 1*, 257-276.
Schaefer, D. W., and Berne, B. J. (1972), *Phys. Rev. Lett. 28*, 475-478.
Shelanski, M L., Gaskin, F., and Cantor, C. R. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70*, 765-768.
Tanford, C. (1961), Physical Chemistry of Macromolecules, New York, N.Y., Wiley.

Equilibrium Components of Tubulin Preparations†

Jon S. Gethner,‡ George W. Flynn, Bruce J. Berne, and Felicia Gaskin*

ABSTRACT: Laser light scattering techniques are used to examine the existence of a tubulin-ring or tubulin-aggregate equilibrium. Three principal components in addition to tubulin dimer have been observed. One large component having $D_{20,w} = 0.65 \pm 0.25 \times 10^{-7}$ cm^2/s is in equilibrium with tubulin (dimer) at 4 °C. A second large component having $D_{20,w} = 0.74 \pm 0.05 \times 10^{-7}$ cm^2/s is metastable at 4 °C and is formed when tubulin solutions are warmed to 36 °C and cooled back to 4 °C. The properties of the component reported in the previous paper (Gethner, J. S., Flynn, G. W., Berne, B. J., and Gaskin, F. (1977), *Biochemistry 16* (preceding paper in this issue)) to sediment at 10 S to 20 S are shown to be consistent with a small, metastable aggregate of tubulin.

The possibility that a tubulin-aggregate equilibrium may be present is suggested by the observation (reported in the preceding paper: Gethner et al., 1977a) of a large component having $D_{20,w} \sim 0.55 \times 10^{-7}$ cm^2/s in solutions which had been extensively centrifuged. In this paper we consider, in particular, the existence of a tubulin-ring or tubulin-aggregate equilibrium.[1] We have examined the equilibrium state of the system prior to temperature-induced assembly into microtubules and after cold-induced disassembly of the microtubules. Three components are described. Component A has $D_{20,w} \leq 3.2 \times 10^{-7}$ cm^2/s. Component B has $D_{20,w} = 0.65 \times 10^{-7}$ cm^2/s and is found before temperature-induced assembly. Component C has $D_{20,w} = 0.74 \times 10^{-7}$ cm^2/s and is found after temperature-induced assembly and disassembly. The possible identities of the various aggregates are discussed. Previously proposed mechanisms of assembly are evaluated in view of our results and limitations upon possible in vitro assembly mechanisms are discussed.

† From the Department of Chemistry, Columbia University, New York, New York 10027 (J.S.G., G.W.F., and B.J.B.), and the Departments of Pathology (Neuropathology) and Biophysics, Albert Einstein College of Medicine, Bronx, New York 10461 (F.G.). *Received February 2, 1977; revised manuscript received September 8, 1977.* Portions of this work have been presented at meetings of the American Physical Society (New York, February 1976) and the Biophysical Society (Seattle, February 1976). This work was taken, in part, from the Ph.D. Dissertation of Jon S. Gethner, Department of Chemistry, Columbia University, 1976. Support for the work was from grants from the National Science Foundation (NSF-CH-76-02414), the U.S. Public Health Service (NS-12418) and a Research Career Development Award to F.G.

‡ Present address: Laboratory of Chemical Physics, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014.

[1] In this context, an aggregate specifically refers to a polymeric structure formed from tubulin (dimer) subunits. An aggregate may be a ring structure; however, no additional biochemical data are available to identify an aggregate as being a ring. A ring specifically refers to a polymeric structure observed in the light scattering experiments under conditions where rings have been shown to be present in substantial concentrations.

Experimental Section

Tubulin preparations and laser light scattering techniques are described in the previous paper (Gethner et al., 1977a). Three additional tubulin samples were used in this work.

(i) *LSX (<60 S)* samples were prepared as described previously for HSX (<20 S) samples except that the sample was centrifuged for 20 min.

(ii) *HSX (<20 S)-Mg* samples were prepared by adding $MgCl_2$ to a concentration of 5 mM after the protein was dialyzed. Centrifugation was performed at 4 °C following the procedures previously described for the preparation of a HSX (<20 S) sample.

(iii) *HSX (<20 S)-colchicine* samples were prepared by adding colchicine to a concentration of 100 μM to the dialysis buffer before beginning the dialysis. Dialysis was carried out at 4 °C in the dark. Except for the addition of colchicine, no modifications were made in the HSX (<20 S) preparation procedure. Two samples were prepared at each concentration. One was kept at 4 °C the entire time. The other was incubated at 26 °C for 30 min, conditions usually sufficient to result in approximately 0.2 mol of colchicine bound per mol of tubulin dimer (F. Gaskin, unpublished data). The sample was then cooled to 4 °C for 30 min before centrifuging.

Sedimentation velocity experiments were performed on a HSX (<20 S) sample containing only 0.1 mM GTP at 4 °C in a Spinco Model E ultracentrifuge operated at 40 000 rpm. Velocity profiles were scanned by measuring A_{280} for the sample contained in a double-sector cell. (Laser light scattering measurements on samples containing 0.1 mM GTP gave the same results as samples containing 1 mM GTP.)

Results

Components in Solutions Centrifuged to Remove 20 S

(1) *Analysis at 4 °C, 9 °C, and in the Presence of Colchicine at 4 °C.* Data is shown in Table I for a variety of samples prepared at 4 °C under conditions such that structures having $S \geq 20$ should not be present. $\bar{D}_N$ in Table I is the average diffusion constant ($\bar{D}$) for a two component mixture at 4 °C which

TABLE I: Diffusion Constants of Tubulin Samples at 4 °C.

Sample no.	Concn (mg/mL)	$\bar{D}_N \times 10^7$ [a] (cm^2/s)	$\bar{D}_R \times 10^7$ [a] (cm^2/s)
D10-1	1.76	1.23	0.694
D10-2	1.60	1.36	0.788
D13-1	1.48	1.17	0.688
D13-2	0.89	2.33	0.746
D15-1[b]	1.72	1.76	1.83
D15-2[b]	1.02	1.97	2.38
D20-1	1.79	1.48	0.756
D20-2	0.94	2.20	0.819
D20-3	0.68	2.08	0.753
D20-4	0.57	2.20	0.666
D20-5[c]	3.6	1.46	1.03
D20-6[c]	1.7	1.50	1.50

[a] $\bar{D}_N$ is the average diffusion constant for solutions which have been maintained at 4 °C since completion of the last centrifugation step to remove 20 S. $\bar{D}_R$ is the average diffusion constant for the same solution which has been warmed to 36 °C for 30 min and then cooled back to 4 °C for at least 15 min prior to the measurement of $\bar{D}_R$. The values in the table are averages of two or three measurements performed on the same sample. Any single measurement deviates from the average by no more than 7% in all cases. [b] Dialysis buffer plus 100 μM colchicine. [c] Buffer is 10^{-2} M 2-(*N*-morpholino)ethanesulfonic acid, pH 6.55, containing 5×10^{-5} M $MgCl_2$ and 10^{-4} M EGTA.

has not been temperature cycled since centrifugation. Correction of $\bar{D}_N$ to obtain an estimate for the diffusion constant of the small component present gives $D^f = 2.86 \times 10^{-7}$ cm^2/s ($\sigma = 0.62 \times 10^{-7}$ cm^2/s). Tubulin (dimer) is expected to have $D_{20,w} = 4.7 \times 10^{-7}$ cm^2/s (Gethner et al., 1977a). The fact that $D^f < D$(tubulin) is a consequence of the presence of a component which we will call component A. The presence of component A in these solutions does not influence conclusions arrived at by changes in $\bar{D}_N$.

The correlation functions obtained for the samples before recycling were resolved into slow and fast exponential decays. $D_{20,w} = 0.65 \pm 0.25 \times 10^{-7}$ cm^2/s has been obtained[2] for the large component, which we call component B. This value is different from the value ($D_{20,w} \sim 0.55 \times 10^{-7}$ cm^2/s) reported in the preceding paper for similar samples probably due to the limited number of samples run to obtain the present value. Analytical ultracentrifugation was attempted on HSX (<20 S) samples at 4 °C and suggested the presence of a small amount of protein sedimenting rapidly relative to tubulin. Instrumental limitations prevented an accurate determination of a value of its sedimentation constant. However, the data obtained were consistent with a structure sedimenting between 20 S and 40 S.

Examination of $\bar{D}_N$ as a function of total protein concentration reveals a decrease in $\bar{D}_N$ at higher protein concentrations. This is consistent with a shift in an equilibrium for a reaction of the form

$$n(\text{dimer}) \overset{4\ ^\circ C}{\rightleftharpoons} (\text{dimer})_n$$

$\bar{D}_N$ approaches 2.2×10^{-7} cm^2/s at concentrations less than 1.1 mg/mL. This limit which is less than the diffusion constant for tubulin dimer is presumably just due to the presence of component A which has $D_{20,w} \leq 3.2 \times 10^{-7}$ cm^2/s.

The effect of temperature was investigated in a temperature-jump experiment from 4.1 °C to 9.1 °C. Correlation functions were measured at the two temperatures. To within experimental error, $\bar{D}$ was independent of temperature. This is contrary to what would be expected if an equilibrium is present. However, examination of semilogarithmic plots of the correlation functions reveals qualitative changes which could be consistent with an equilibrium model even though the changes are not observed in the value of $\bar{D}$.[3]

One further test for an equilibrium is to examine the effect of a perturbation by binding colchicine to the tubulin (dimer). At a protein concentration of 1.72 mg/mL and in the presence of colchicine, $D_{20,w} = 1.78 \times 10^{-7}$ cm^2/s. This value is slightly larger than for a HSX (<20 S) sample containing component B at such a protein concentration. Multiple exponential behavior is resolvable, however, indicating that a large component (probably component B) is present at a lower relative concentration. The larger value of $\bar{D}$ observed with colchicine is thus consistent with the binding of colchicine to tubulin resulting in a shift in the tubulin–component B equilibrium away from the formation of the aggregate. A sample containing 100 μM colchicine at a lower protein concentration (1.02 mg/mL) has a slightly larger diffusion constant ($\bar{D}_{20,w} = 1.97 \times 10^{-7}$ cm^2/s). This is precisely what is expected if a tubulin–colchicine equilibrium exists in competition with the tubulin–aggregate equilibrium. These results do not exclude the possibility of the formation of a colchicine-induced structure. In fact, the presence of a colchicine-induced structure in addition to an equilibrium aggregate may explain why $\bar{D}$ for the 1.0 mg/mL sample is slightly smaller than $\bar{D}$ for sample D20-2 (Table I).

(2) *Analysis after Raising the Sample to 36 °C and Returning to 4 °C.* The samples shown in Table I were warmed to 36 °C for 30 min and then cooled back to 4 °C. Using electron microscopy, assembly of the tubulin into microtubules at 36 °C was verified in all cases. Cooling a solution containing microtubules back to 4 °C resulted in diassembly of the microtubules. The diffusion constants obtained from the samples after cooling back to 4 °C are shown in Table I as $\bar{D}_R$. A significant increase in the turbidity of the sample accompanied the decrease in $\bar{D}_N$ to $\bar{D}_R$ indicating an increase in the concentration of high molecular weight components. Correlation functions obtained from these samples were dominated by the presence of the slow exponential decay. Multiple exponential behavior is evident; however, the small contribution of the fast exponential to the correlation function makes the fast exponential impossible to resolve. From the values of $\bar{D}_R$, we find $D_{20,w} = 0.738 \times 10^{-7}$ cm^2/s ($\sigma = 0.052 \times 10^{-7}$ cm^2/s) for a component C. To within experimental error this is the same as obtained for the large component B present in the non-temperature-cycled samples, though it appears to be experimentally slightly larger. After recycling, $\bar{D}_R$ and the turbidity were constant for at least 6 h. These results indicate that at 4 °C component C is metastable and is effectively not in equilibrium with tubulin (dimer). Analysis of electron micrographs suggests slightly more aggregation in the temperature-cycled solution than in the non-temperature-cycled solution. However, neither contained double rings.

[2] The error limit includes the maximum and minimum values of $D_{20,w}$, obtained from the entire set of data. This value is the same as $D_{20,w}$ reported for the large component found in HSX (<20 S) solutions in the preceding paper.

[3] Computer generated data are discussed elsewhere in which it is apparent that graphical presentation of data may reveal changes which are not apparent when the data are reduced to a single parameter in a computer fit (Gethner, 1976). This is especially true when the data are being fit to a power series expansion which effectively calculates the value of the first derivative at $t = 0$.

The actual structure formed after temperature recyclization may be sensitive to the buffer conditions. Table I also contains data obtained from tubulin which had been dialyzed against a low ionic strength buffer. The correlation functions obtained for these samples, D20-5 and D20-6, after temperature cycling are dominated by the presence of the slow exponential. However, $\overline{D}_R = 1.03 \times 10^{-7}$ cm^2/s indicates that a more compact hydrodynamic structure has formed in the modified buffer. Samples prepared with colchicine present at a concentration of 100 μM do not exhibit any significant changes upon temperature cycling. It should be noted that tubulin is not capable of assembly in the modified buffer or in 100 μM colchicine.

Ring Containing Solutions

Several groups have reported that ring-like structures are present in tubulin preparations. Though the solution conditions reported are different from our HSX solutions, the diffusion constants we have obtained for components B and C are not too different from what would be expected for a ring structure (Gethner, 1976). We therefore examined solutions under conditions generally believed to contain a significant concentration of ring components.

Low speed extracts were prepared under conditions to pellet 60 S. The light scattering resulted in correlation functions characteristic of a heterogeneous solution. In most cases clear evidence of double exponential behavior was not obtained. At 4 °C, $D_{20,w} = 0.61 \times 10^{-7}$ cm^2/s was found. The reproducibility of the experiments on different preparations is extremely good, especially when compared with HSX preparations. Weak angular dependence of $\overline{D}$ was found indicating the presence of at least some fairly large anisotropic particles (Gethner, 1976). In 2 out of 5 LSX (<60 S) preparations at 4 °C, double rings similar to those described by Kirschner et al. (1974) were found using electron microscopy. All samples assembled into microtubules at 37 °C. After recycling back to 4 °C all samples contained double rings and $D_{20,w} = 0.52 \times 10^{-7}$ cm^2/s.

High concentrations of Mg^{2+} and Ca^{2+} induce the formation of rings (Weisenberg and Timasheff, 1970; Weingarten et al., 1974; Kirschner et al., 1974; Frigon and Timasheff, 1975a). Our samples routinely contain only 0.5 mM $MgCl_2$. In the presence of 5 mM $MgCl_2$ the amount of protein in the pellet was somewhat greater when centrifuged to remove 20 S. This is consistent with Mg^{2+} inducing the formation of large aggregates. Scattering from the supernatants containing 5 mM $MgCl_2$ was observed after at least 2 h had elapsed since centrifugation. The results of two determinations for samples prepared in an identical manner on different days did not agree. Solution 1 shows $\overline{D} = 0.7 \times 10^{-7}$ cm^2/s. Solution 2, however, shows $\overline{D} = 2.1 \times 10^{-7}$ cm^2/s with no tendency to decrease with time. Warming the solution 2 to 36 °C for 1 h (assembly does not take place due to the high Mg^{2+} concentration) and then observing the scattering at 4 °C after cooling back down reveals an irreversible change to $\overline{D} = 0.6 \times 10^{-7}$ cm^2/s. Count rate data are consistent with solutions 1 and 2 (recycled) containing the same components. There is some indication that the large component formed in solution 2 (recycled) may be sensitive to the laser illumination.

Discussion

The use of laser light scattering techniques to characterize the in vitro tubulin self-assembly system has resulted in the identification of several components other than 6S tubulin dimer. These components are indicated in Figure 1.

Component A. If component A were an oligomer of tubulin dimer, we estimate it would contain 3–5 tubulin dimers. [Application of the Svedberg equation and using $D_{20,w} = 3.2 \times 10^{-7}$ cm^2/s, 10 S $\leq s \leq$ 20 S, $\bar{v} = 0.736$ (Lee and Timasheff, 1974), we find 290 000 $\leq M \leq$ 580 000.]

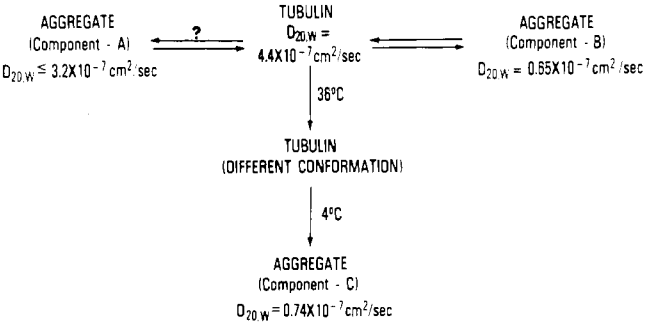

FIGURE 1: Summary of the components in tubulin preparations. The equilibria shown are at 4 °C. Although nontubulin high molecular weight proteins are present, they are not explicitly included in the present scheme since we do not know if they exist freely or are complexed with other components or tubulin (see Discussion section).

Component A might also be explained by an average over several small oligomers of tubulin. In fact, Frigon and Timasheff (1975a,b) have recently reported evidence for a 26-step association reaction of tubulin into a closed "ring" and at low tubulin concentration only the first few steps may contribute. Because of instrumental limitations, it is not possible to distinguish between the case of a multi-step equilibrium and a tubulin-oligomer mixture. No concentration dependence of the diffusion constant for component A was found (see Gethner et al., 1977a). If component A is an oligomer of tubulin and a multi-step equilibrium is present in addition, the contribution of the oligomer to the correlation function must be large enough to obscure the shift of the equilibrium as the concentration is decreased. We have attempted to shift the equilibrium, if present, by observing these samples in the presence of 100 μM colchicine and under conditions of high salt (Gethner, 1976; Gethner et al., 1977b). The small component formed was found to be stable with respect to dissociation by these methods.

A third possibility is that component A is a nontubulin component such as the high molecular weight (HMW) protein(s) of about 350 000 that copurify through several cycles of assembly and disassembly (Gaskin et al., 1974). The HMW proteins form the filamentous coating seen on tubules (Dentler et al., 1975; Murphy and Borisy, 1975) and may also promote the formation of ring structures (Vallee and Borisy, 1976). HMW proteins are present to approximately the same extent in HSX (<10 S) and HSX (<20 S) samples. Thus it is unlikely that the component preferentially sedimented is HMW protein.

The fact that component A is present after purification involving an assembly/disassembly procedure suggests that if component A is not a metastable aggregate of tubulin, it is either incorporated into the microtubule or associated to it. By considering the amplitude of component A relative to tubulin dimer, we estimate that component A may account for as much as 10% of the protein present (Gethner, 1976).

Components B and C. While components B and C exhibit experimentally similar values for their diffusion constants, equilibrium considerations suggest that they are different. At 4 °C, both before and after temperature cycling, both the average diffusion constant and the intensity of scattered light are constant for at least 6 h. However, the substantially greater intensity of scattering after recycling indicates an increase in

the concentration of a large component. These results suggest that components B and C are distinct and that the component formed upon temperature cycling is metastable at 4 °C.

Although their measured diffusion constants are consistent with those expected for rings or spirals, components B and C may well be other aggregates of tubulin. Tubulin dimer has been shown to undergo a conformational change at temperatures greater than 20 °C (Ventilla et al., 1972). Thus it is possible that the high temperature conformation allows a structure to form which does not readily disassemble when the temperature is lowered. It is also possible that the metastable aggregates formed at high temperature or after disassembly result from the formation of intermolecular disulfide bonds (Mellon and Rebhun, 1976). Preliminary experiments using mercaptoethanol and glutathione to block the formation of intermolecular bonds suggest that such a mechanism is not responsible for the aggregate formation which we observe (F. Gaskin, unpublished data). Since trace impurities present in glycerol have been reported to be responsible for intermolecular cross-linking of proteins (Bello and Bello, 1976), the possibility must be considered that either or both of components B and C form as a result of the fact that glycerol has been used to prepare our solutions and trace amounts of glycerol remain after dialysis. Such a nonspecific cross-linking reaction may account for the formation of component C at high temperature. The existence of an equilibrium between tubulin and component B argues that a nonspecific cross-linking is not responsible for the formation of component B.

Role of the Components. Before discussing the nature and possible role of these components in the assembly of tubulin, we want to point out that there is little evidence to support the hypothesis by Kirschner et al. (1974) and Erickson (1974) that rings and spirals are the key intermediates in microtubule assembly. In fact recent data from several laboratories suggest that assembly can occur quite well in the absence of rings and spirals and that oligomeric intermediates may be important (Weisenberg, 1974; Frigon and Timasheff, 1975a,b; Doenges et al., 1977). Studies on vinblastine-induced tubulin assembly show an accumulation of a component in the 9-15S range before assembly into rings, spirals, and helices (Weisenberg and Timasheff, 1974; Ventilla et al., 1975). Our component A has a $10\ S \leq s \leq 20\ S$ and may be a critical nucleation intermediate similar to that found for vinblastine-induced tubulin assembly. Using electron microscopy we find no double rings or spirals in our high speed extracts at 4 °C before assembly and after disassembly. This suggests that they are not required for assembly and are not a breakdown product of disassembled microtubules. However, we must keep in mind the possibility that electron microscopy is not sensitive enough to detect a trace amount of rings. Since the low speed extracts frequently contain double rings and spirals, it would seem plausible that a component bound to the rings or spiral is necessary for its formation and removal of the double ring by centrifugation would also remove a component necessary for ring formation. Microtubules formed from highly centrifuged solutions would not be expected to contain double rings upon tubule disassembly. Two possibilities for a component necessary for ring formation are HMW proteins or the τ protein (Weingarten et al., 1975). HMW proteins are present to approximately 6% in all our <20S and <10S supernatants (based on polyacrylamide gel analysis). This implies that the HMW protein(s) is not the required component for the double ring to form or is not in high enough concentration in our high speed extracts to promote double ring formation. The amount of component B formed may thus be limited by the HMW protein present. Our gel electrophoresis system is not good enough to quantitate τ protein. However, since τ has been reported to be necessary for tubule elongation (Witman et al., 1976) as well as initiation (Weingarten et al., 1975) in similar tubulin preparations, we expect that τ is present in all preparations capable of assembly.

Summary

We have found that tubulin solutions prepared by the method of Shelanski et al. (1973) are highly heterogeneous and contain at least three components in addition to tubulin (dimer). One of the components is apparently in equilibrium with tubulin (dimer) and could, therefore, be important in the details of tubulin assembly. Two of the components are apparently metastable and their presence may influence the amount of tubulin which is competent to assemble in a preparation.

Though we cannot rule out the presence of a trace concentration of rings or spirals, our results suggest that assembly may be capable of proceeding by a mechanism which does not require double rings or spirals as intermediates.

Acknowledgment

We would like to thank Professor C. R. Cantor for allowing us to use his facilities for some of the biochemical work.

References

Bello, J., and Bello, H. R. (1976), *Arch. Biochem. Biophys. 172,* 608-610.

Dentler, W. L., Granett, S., and Rosenbaum, J. L. (1975), *J. Cell Biol. 65,* 237-241.

Doenges, K. H., Nagle, B. W., Uhlmann, A., and Bryan, J. (1977), *Biochemistry 16,* 3455-3459.

Erickson, H. P. (1974), *J. Supramol. Struct. 2,* 393-411.

Frigon, R. P., and Timasheff, S. N. (1975a), *Biochemistry 14,* 4559-4566.

Frigon, R. P., and Timasheff, S. N. (1975b), *Biochemistry 14,* 4567-4573.

Gaskin, F., Kramer, S. B., Cantor, C. R., Adelstein, R., and Shelanski, M. L. (1974), *FEBS Lett. 40,* 281-286.

Gethner, J. S. (1976), Ph.D. Dissertation, Department of Chemistry, Columbia University.

Gethner, J. S., Flynn, G. W., Berne, B. J., and Gaskin, F. (1977a), *Biochemistry 16* (preceding paper in this issue).

Gethner, J. S., Flynn, G. W., Berne, B. J., and Gaskin, F. (1977b), The Observation of Salt Induced Aggregation in Tubulin Solutions (in preparation).

Kirschner, M. W., Williams, R. C., Weingarten, M., and Gerbart, J. C. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 1159-1163.

Lee, J. C., and Timasheff, S. N. (1974), *Biochemistry 13,* 257-265.

Mellon, M. G., and Rebhun, L. I. (1976), *J. Cell Biol. 70,* 226-238.

Murphy, D. B., and Borisy, G. G. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 2696-2700.

Shelanski, M. L., Gaskin, F., and Cantor, C. R. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 765-768.

Vallee, R. B., and Borisy, G. G. (1976), *Biophys. J. 16,* 177a.

Ventilla, M., Cantor, C. R., and Shelanski, M. (1972), *Biochemistry 11,* 1554-1561.

Ventilla, M., Cantor, C. R., and Shelanski, M. L. (1975), *Arch. Biochem. Biophys. 171,* 154-162.

Weingarten, M. D., Lockwood, A. H., Hwo, S.-Y., and Kirschner, M. W. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 1858-1862.

Weingarten, M. D., Suter, M. M., Littman, D. K., and Kirschner, M. W. (1974), *Biochemistry 13*, 5529-5537.
Weisenberg, R. C. (1974), *J. Supranol. Struct. 2*, 451-465.
Weisenberg, R. C., and Timasheff, S. N. (1970), *Biochemistry 9*, 4110-4116.
Witman, G. B., Cleveland, D. W., Weingarten, M. D., and Kirschner, M. W. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73*, 4070-4074.

The Shape of Myosin Subfragment-1. An Equivalent Oblate Ellipsoid Model Based on Hydrodynamic Properties†

Jen Tsi Yang* and Chuen-Shang C. Wu

ABSTRACT: The molecular weights of the two heads of myosin subfragment-1, S-1(A1) and S-1(A2), based on sedimentation equilibrium are 120 000 and 110 000. Hydrodynamically, the two heads are indistinguishable, with intrinsic viscosity, $[\eta]$, of 0.064-0.065 dL/g and sedimentation coefficient, $s^0_{20,w}$, of 5.8 S. Together with the rotational correlation time taken from the literature (235 ns), all three hydrodynamic properties can be better fitted with an equivalent oblate ellipsoid of revolution than a prolate model. The width of the equatorial axis of the ellipsoid is about 135 Å (the axial ratio is about 6). Probably, the S-1(A1) and S-1(A2) molecules have a half-doughnutlike or a flattened pearlike shape rather than an elongated one.

The cross-section of striated muscle fibers shows that the thick filaments of myosin are arranged in a hexagonal lattice and each filament is surrounded by six thin filaments of actin (Huxley, 1969). The two heads of myosin known as subfragment-1 constitute the cross-bridges linking the heavy and thin filaments; the heads are believed to be functionally different and are termed as S-1(A1) and S-1(A2) (Weeds and Taylor, 1975), but the shape of the molecules remains to be solved.

Recently, the methods of preparation for muscle proteins and their enzymatically digested fragments have been much modified. In this work we determine the molecular weights and hydrodynamic properties of myosin S-1(A1) and S-1(A2) rather than those of their mixture. Traditionally, globular proteins are approximated by the prolate ellipsoids of revolution, although sperm whale myoglobin, the first protein whose structure was solved by x-ray diffraction methods, has dimensions of 44 × 44 × 24 Å^3 (quoted by Dickerson and Geis, 1969), which is equivalent to an oblate model having an axial ratio of about two. We will show that the myosin subfragment-1 molecule hydrodynamically fits an oblate model better than a prolate one.

Experimental Section

Preparation of Proteins. Myosin from the back muscle of rabbit was prepared as mentioned previously (Wu and Yang, 1976), but without the purification step on a DEAE-Sephadex column.[1] The protein in 50% glycerol, 20 mM Tris-HCl, 0.5 M KCl, and 0.5 mM dithioerythritol (pH 6.8) was stored at −40 °C. The stock solution was diluted with 10 vol of cold water before use and centrifuged; the precipitate was redissolved in an appropriate buffer.

Myosin subfragment-1 (S-1) was obtained by digesting myosin filaments with α-chymotrypsin (Weeds and Taylor, 1975), and the two myosin heads, S-1(A1) and S-1(A2), were separated on two columns: a DEAE-cellulose (Whatman DE 52) column for S-1(A1) and impure S-1(A2), followed by rechromatographing the latter fraction on a CM-cellulose (Bio-Rad Cellex CM) column to obtain S-1(A2). Both purified fractions were concentrated on an Amicon filter XM 50 and dialyzed against 0.1 M NaCl, 20 mM sodium phosphate buffer, 1 mM EDTA, and 0.5 mM dithioerythritol (pH 7.0). Sodium azide (1 mM) was added to prevent bacterial growth.

Disc gel electrophoresis in a nondissociating buffer was done on 5% polyacrylamide gels without sample gel and stacking gel (Davis, 1964). Sodium dodecyl sulfate gel electrophoresis was carried out on 10% gel with 50 mM sodium phosphate buffer (pH 7.0) (Weber and Osborn, 1969).

Methods. Viscosities were measured in a suspension-type Ubbelohde capillary viscometer (specially made by our glassblower). The solvent flow time were about 2100 s at 5 °C and 1200 s at 25 °C. The data were treated according to the equation:

$$(t - t_0)/t_0C = [\eta]_{uncorr} + k'[\eta]_{uncorr}^2C$$

where t and t_0 are the flow times of the solution and solvent, C is the protein concentration, and k' is a constant. Because myosin S-1 has low intrinsic viscosities, we used long solvent flow time to improve the precision of the data. Even for the most dilute solutions used, $(t - t_0)$ was at least more than 35 s. The protein concentrations based on the micro-Kjeldahl method, assuming a 16% nitrogen content, ranged from 0.5 to 1.3%. The intrinsic viscosity is further corrected for the density difference between solution and solvent by (Tanford, 1955):

$$[\eta] = [\eta]_{uncorr} + (1 - \bar{v}\rho)/100$$

where $\bar{v}$ is the partial specific volume of the protein and ρ the solvent density. The density correction amounts to about +0.003 dL/g.

Sedimentation velocity at 5 and 25 °C and sedimentation equilibrium at 5 °C were studied with a Spinco Model E analytical ultracentrifuge. The schlieren patterns showed a single,

† From Cardiovascular Research Institute and Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143. *Received May 11, 1977.* This work was aided by U.S. Public Health Service Grants GM-10880 and HL-06285 (Program Project).

[1] Abbreviations used are: DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance.