Analytical sedimentation studies of turkey gizzard myosin light chain kinase and telokin

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ABSTRACT Sedimentation equilibrium and velocity studies were performed with turkey gizzard myosin light chain kinase (MLCK) and telokin, a small protein apparently corresponding to the sequence of the COOH-terminal end of MLCK. The measurements carried out with MLCK give values for the monomer molecular weight (M_r), sedimentation coefficient ($s_{20°,w}$), and virial coefficient (A_2) of 108,000, 3.74 S, and -1.95×10^{-4} mol · ml · g^{-2}, respectively. In the case of telokin, M_r = 18,500; $s_{20°,w}$ = 1.63 S; and A_2 = 5.81×10^{-4} mol · ml · g^{-2}. Combination of the results of the two kinds of experiment shows that MLCK is a rod-shaped molecule (a/b = 18.9) with a Stoke's radius of 69 Å. Telokin is also elongated (a/b = 8.3) with a Stoke's radius of 29 Å. MLCK apparently exhibits self-association, with 15% of the protein sedimenting as a dimer in the experiments.

INTRODUCTION

Smooth muscle myosin light chain kinase (MLCK)[1] catalyzes the calmodulin-dependent phosphorylation of the M_r 20,000 myosin light chain (a rate-limiting reaction in smooth muscle contraction [cf. reviews by Small and Sobieszek, 1980; Hartshorne and Siemankowski, 1981; Perry et al., 1984; Hartshorne, 1987]). The full-length cDNA sequence shows that chicken gizzard MLCK contains 972 amino acid residues, corresponding to a calculated M_r of 107,534. The organization of the enzyme includes a catalytic domain (residues 526–762), a calmodulin-binding domain (residues 796–815) and an overlapping substrate-inhibitory domain (residues 787–807) (Olson et al., 1990). Curiously, an abundant protein of unknown function first isolated from smooth muscle by Dabrowska et al. (1977) proved to be an independently expressed gene product, apparently corresponding to amino acid residues 816–972 of smooth muscle MLCK. In view of its relationship to the COOH-terminal end of the kinase, Ito et al. (1989) have termed this protein "telokin."

This report contains the first detailed hydrodynamic study of smooth muscle MLCK. It provides quantitative information on the molecular weight distribution, partial specific volume, hydration, Stoke's radius, and radius of gyration for both the enzyme and telokin. It was instigated, in part, by discrepancies between the value of the molecular weight calculated from sequence and the values previously obtained by sedimentation equilibration (124,000) and sodium dodecyl sulfate ($NaDodSO_4$)–acrylamide gel electrophoresis (134,000) (Adelstein and Klee, 1981). Discrepancies between true molecular weights and the values deduced from electrophoresis experiments occur frequently, especially with highly charged basic (Panyim and Chalkley, 1971) or acidic proteins (Kleinschmidt et al., 1986; Takano et al., 1988).

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[1]*Abbreviations used in this paper:* MLCK, myosin light chain kinase; $NaDodSO_4$, sodium dodecylsulfate.

MATERIALS AND METHODS

Proteins

Turkey gizzard MLCK with a specific catalytic activity of 12 μmol ^{32}P/min per mg was prepared according to Sobieszek and Barylko (1985). Telokin was obtained from the same tissue source by following the methods of Ito et al. (1989) and Dabrowska et al. (1977). The homogeneity of the two proteins was demonstrated by $NaDodSO_4$ acrylamide gel electrophoresis (Fig. 1), performed on a 9–19% linear gradient minigel essentially according to Zhao et al. (1991). The amino acid compositions of the proteins (Table 1) were verified according to the method of Malencik et al. (1990).

Analytical ultracentrifuge analysis

Sedimentation velocity and sedimentation equilibrium runs were carried out on an analytical ultracentrifuge (model E; Beckman Instruments, Inc., Fullerton, CA) equipped with electronic speed and temperature control and photoelectric scanner. Sedimentation velocity analyses were performed in an An-F four-hole aluminum rotor using double-sector charcoal-filled Epon cells and sapphire windows. The boundaries were recorded routinely at 282 nm except when the absorbance would be higher than 0.8. In these cases, boundaries were recorded at suitable absorbancies in the wavelength range 282–290 nm. The analysis of the scans was carried out according to the method described by van Holde and Weischet (1978). The temperature of the rotor varied from one run to another within the range 20 ± 0.5°C. It was held constant during each run with the help of the RTIC temperature regulation unit of the ultracentrifuge. The buffer used in all the sedimentation experiments contained 0.2 M NaCl, 20 mM

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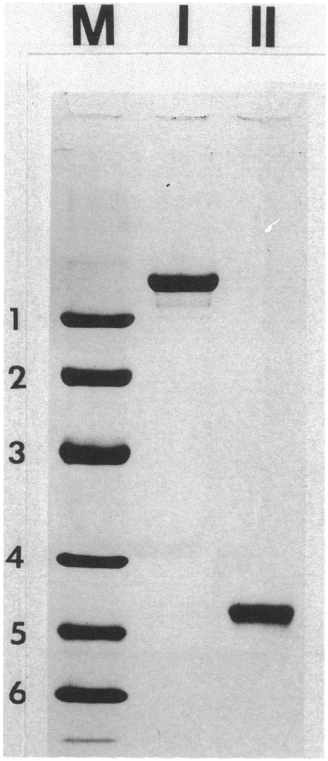

FIGURE 1 Acrylamide gradient (9–19%) $NaDodSO_4$ gel electrophoresis of (*I*) MLCK; (*II*) telokin; and (*M*) markers (*1*: phosphorylase b, M_r 96,000; *2*: BSA, M_r 67,000; *3*: ovalbumin, M_r 43,000; *4*: carbonic anhydrase, M_r 30,000; *5*: soybean trypsin inhibitor, M_r 20,000; *6*: α-lactalbumin, M_r 14,000). Markers from Pharmacia Fine Chemicals (Piscataway, NJ).

Tris-HCl, pH 7.5, 1 mM EDTA, and 1 mM β-mercaptoethanol. The density of this buffer was measured with a pycnometer at 20°C and was found to be 1.0088_4 g/cm^3. Apparent sedimentation coefficients (s) for a given buffer "b" and temperature "T" conditions were corrected to standard conditions (water and 20°C) according to

$$s_{20,w} = s \frac{\eta_{T,b}}{\eta_{20,\omega}} \cdot \frac{(1 - \phi_2' \rho_{20,w})}{(1 - \phi_2' \rho_{T,b})}, \quad (1)$$

where η is the viscosity of the solvent, ρ is the density of the solvent, and ϕ_2' is an apparent partial specific volume without a precise thermodynamic meaning. In a two-component system, $\phi_2' = \bar{v}_2$, which is the partial specific volume of component 2 (protein).

Sedimentation equilibrium runs were carried out either in an An-D two-hole aluminum rotor using six-channel charcoal-filled Epon cells or in an An-F four-hole aluminum rotor using double-sector charcoal-filled Epon cells with sapphire windows. 90–120-μl samples were loaded into the cells. The temperature of the rotor was adjusted to the desired value and maintained throughout the run using the Evapotrol refrigeration system of the ultracentrifuge. Interference (Rayleigh optics) and schlieren optics were used simultaneously (Chevrenka, 1969). The high speed meniscus depletion method was applied and the resulting interference patterns were analyzed as described by Yphantis (1964). The schlieren patterns were analyzed according to Lamm (1929) as described elsewhere (Malencik et al., 1989). The combination of the two optical systems provides information on the radial distribution of M_n^{app}, M_w^{app}, and M_z^{app} (Malencik et al., 1989). Refractometric units (in fringes) were converted to concentration units using the relation 4.1 fringes = 1 mg/ml (Babul and Stellwagen, 1969). The partial specific volumes of the proteins were estimated from the amino acid composition (Cohn and Edsall, 1943), using for these calculations the consensus values tabulated by Perkins (1986). Alternatively, these values were experimentally determined using density contrast variation analysis (see next section). The protein samples were thoroughly dialyzed against the buffer before each experiment.

TABLE 1 **Amino acid composition of MLCK and telokin***

	MLCK	Telokin
Asx	9.8	12.3
Glx	13.9	21.2
Ser	7.7	6.1
Thr	6.1	5.9
Gly	5.7	7.8
Ala	7.6	7.7
Arg	3.7	
Pro	5.2	5.5
Val	7.4	7.0
Met	2.0	1.4
Leu	5.9	4.3
Ile	4.6	3.6
Cys	2.5	3.3
Lys	10.3	7.2
His	1.2	1.3
Phe	2.9	0.7
Tyr	2.2	3.5
Trp	1.3	0.7

*Values are given in mol of residue/100 mol total residues. MLCK, myosin light chain kinase.

Measurement of the preferential hydration

The preferential hydration parameter of the proteins was determined using the density contrast variation analysis described by Eisenberg and Felsenfeld (1981) (see also Eisenberg, 1981). Sedimentation equilibrium experiments were performed in the presence of different sucrose concentrations and the preferential hydration parameter ξ_1, and the partial specific volumes $\bar{v}_2$ were evaluated from

$$(\partial \rho / \partial c_2)_\mu = (1 + \xi_1) - \rho(\bar{v}_2 + \xi_1 \bar{v}_1), \quad (2)$$

where $\bar{v}_2$ and $\bar{v}_1$ are the partial specific volumes of the particle and water, respectively, and ξ_1 is the preferential hydration parameter in g H_2O per g of protein. Taking $\bar{v}_1 = 1$, both $\bar{v}_2$ and ξ_1 can be evaluated from a plot of $M_2(\partial \rho / \partial c_2)_\mu$ against the medium density, ρ.

RESULTS

Sedimentation equilibrium in the analytical ultracentrifuge provides an absolute method for the measurement of molecular mass. Fig. 2 shows the distribution of

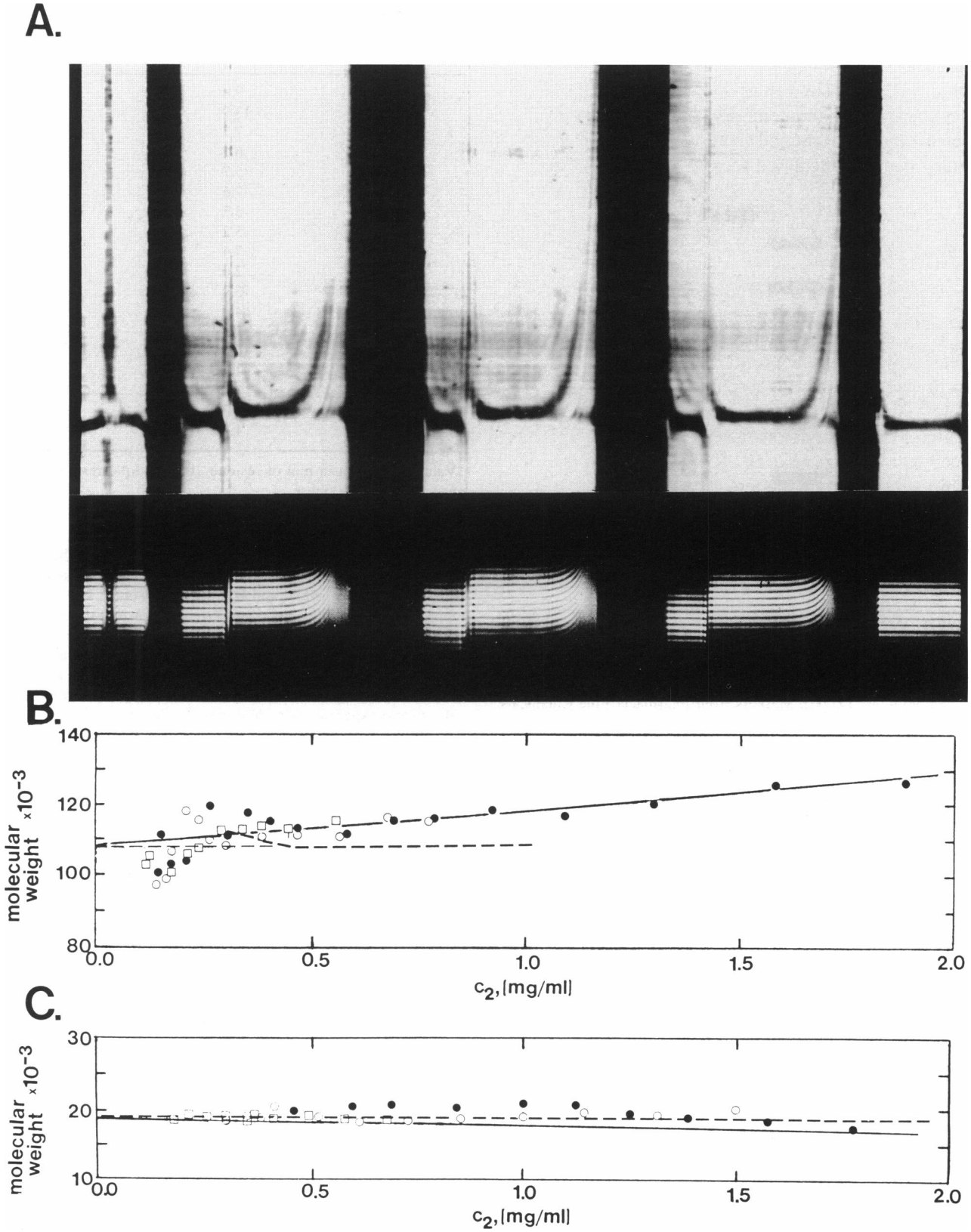
A.
B.
molecular weight x10-3
140
120
100
80
0.0
0.5
1.0
1.5
2.0
c2, [mg/ml]
C.
molecular weight x10-3
30
20
10
0.0
0.5
1.0
1.5
2.0
c2, [mg/ml]

FIGURE 2

different apparent molecular weight averages, as a function of the radial concentration, for three different initial loading concentrations in a six-channel cell (Yphantis, 1964). The values of M_z^{app} and of the "ideal" molecular weight moment My_1, calculated from the experimentally determined values of M_n^{app} and M_w^{app}, are plotted. My_1 was calculated according to

$$(My_1)^{-1} = 2(M_n^{app})^{-1} - (M_w^{app})^{-1}. \quad (3)$$

We also calculated

$$My_2 = (M_w^{app})^2/(M_z^{app}). \quad (4)$$

For a homogeneous nonideal solute, $My_1 = M_2$, the molecular weight of the protein.

In the case of a nonideal self-associating system, My_1 and My_2 eliminate the "nonideality" effects (Roark and Yphantis, 1969; Yphantis and Roark, 1972). Within the concentration range analyzed, we found that $My_1 \equiv My_2$ (within the experimental error).[2] The partial specific volumes ($\bar{v}_2$) of MLCK and telokin were estimated from amino acid composition as described in the preceding section. The resulting values, $\bar{v}_2 = 0.731$ cm^3/g for MLCK and $\bar{v}_2 = 0.713$ cm^3/g for telokin, were employed in the calculation of M_2. Extrapolation of the data in Fig. 2 to zero protein concentration gives a molecular mass of 108,000 D in the case of MLCK. The slope is very close to zero, as would be expected for a monomer–dimer system (Roark and Yphantis, 1969). Similarly, the molecular mass of telokin was found to be 18,500 D.

Application of the equation

$$(M_z^{app})^{-1} = M_z^{-1} + 4A_2c_2 + \ldots, \quad (5)$$

with $M_z = M_2$ for a monodisperse homogeneous system, to the slopes of the plots shown in Fig. 2, *B* and *C*, allows calculation of the virial coefficients. We thus obtained $A_2 = -1.95 \times 10^{-4}$ mol · ml · g^{-2} for MLCK and $A_2 = 5.81 \times 10^{-4}$ mol · ml · g^{-2} for telokin.

Fig. 3 shows the results of sedimentation velocity experiments performed with the two proteins. Whereas telokin sediments as a single species (data not shown) with $s_{20,w} = 1.63$ S, MLCK exhibits a degree of self-association (Fig. 3*A*). In the latter case, ~15% of the sample sediments at an *s* value greater than that of the nonaggregated monomer, for which $s_{20,w} = 3.74$ S.

With the data obtained from the sedimentation equilibrium and the sedimentation velocity measurements, it is possible to further analyze the conformational parameters of the two proteins. The frictional properties of the sample can be obtained from the relationships

$$f = \frac{M_2}{s_{20,w}\mathcal{N}}\left(\frac{\partial\rho}{\partial c_2}\right)_\mu \quad (6)$$

and

$$\left(\frac{\partial\rho}{\partial c_2}\right)\mu \equiv (1 - \phi_2'\rho), \quad (7)$$

where f is the frictional coefficient of the protein, M_2 is the molecular weight of the monomer, $\mathcal{N}$ is Avogadro's number, ρ is the density of the solution, c_2 is protein concentration, and ϕ_2' is the operationally defined apparent partial specific volume (Eisenberg, 1981). The values of the relative frictional ratio f/f_0 can be calculated and used to evaluate the shapes of the molecules. f_0 is the frictional coefficient of a hypothetical equivalent sphere of radius R_0.

$$f_0 = 6\pi\eta R_0 \quad (8)$$

with

$$R_0 = \left[\frac{3M_2}{4\pi\mathcal{N}}(\bar{v}_2 + \xi_1\bar{v}_1)\right]^{1/3}. \quad (9)$$

The frictional ratio can be assigned to a prolate ellipsoid with a semiaxial ratio a/b (Tanford, 1961).

$$f/f_0 = \frac{(1 - b^2/a^2)^{1/2}}{(b/a)^{2/3}\ln\dfrac{(1 + b^2/a^2)^{1/2}}{b/a}} \quad (10)$$

[2]This fact can be used in conjunction with Eqs. 3 and 4 to reconstruct the values of M_n^{app} and M_w^{app} from Fig. 2*B*. That is, in this particular case M_w essentially equals $\sqrt{My_1M_z^{app}}$. $M_n^{app} = 2[(My_1)^{-1} + (M_w^{app})^{-1}]^{-1}$.

FIGURE 2 (*A*) Schlieren and Rayleigh interference patterns obtained with a six-channel centerpiece in a high speed sedimentation equilibrium experiment on MLCK at 22,000 rpm and 18.2°C. (*B*) Concentration dependence of the observed apparent Z-average molecular weight (M_z^{app}) (*solid line*) of MLCK as a function of the radial protein concentration within the cell for the run shown in *A*. The initial concentrations of the samples loaded in each of the channels, starting with the most centripetal, were 0.1% (●), 0.05% (○), and 0.025% (□). The heavy dashed line represents the least-squares curve fitting for the concentration dependence of My_1 as calculated from $(My_1)^{-1} = 2(M_n^{app})^{-1} - (M_w^{app})^{-1}$ (see the text for more details). (*C*) Concentration dependence of My_1 (*dashed line*) (as above) for telokin. The initial concentrations of the samples loaded in the cell were 0.12% (●), 0.06% (○), and 0.03% (□). In this case, three-double sector cells were simultaneously run in a four-hole An-F rotor. The solid line in this case represents the least-square fitting for the M_z^{app} distribution as averaged over the three channels. The speed of this run was 44,000 rpm and the temperature 20.3°C.

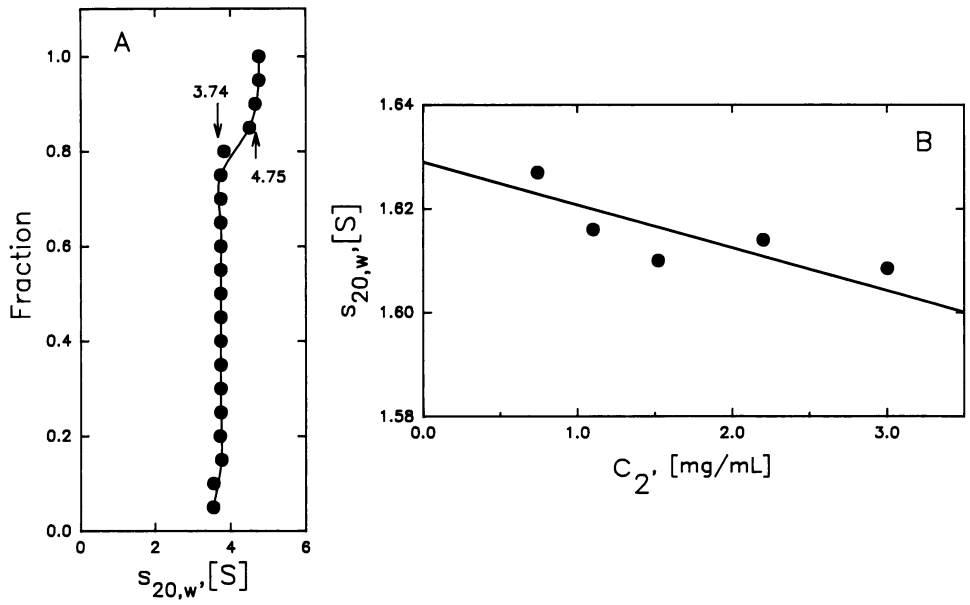

FIGURE 3 (*A*) Sedimentation velocity analysis of MLCK according to the method of van Holde and Weischet (1978). The ordinate measures the fraction of material with $s_{20,w}$ less than or equal to the value given on the abscissa. The run was performed at 48,000 rpm and 20.5°C. (*B*) Concentration dependence of the sedimentation coefficient for telokin, which exhibited a single component when analyzed by the method of van Holde and Weischet. The rotor speed was 48,000 rpm. The buffer in *A* and *B* was the same: 0.2 M NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 1 mM β-mercaptoethanol.

the volume

$$V_h = \frac{M_2}{\mathcal{N}}(\bar{v}_2 + \xi_1\bar{v}_1) = 4/3\pi ab^2 \quad (11)$$

and the Stokes radius

$$R_s = f/f_0 \cdot R_0. \quad (12)$$

V_h also can be related to a rigid rod of equivalent length ($L = 2a$) and volume V_h

$$V_h = \pi L d^2/4, \quad (13)$$

with a radius of gyration

$$R_G = L/(12)^{1/2}. \quad (14)$$

An important quantity in the evaluation of shape is ξ_1, the preferential hydration parameter. We measured this value using the density contrast variation approach described by Eisenberg (1981), with the results shown in Fig. 4. From the slopes and the intercepts of the lines in Fig. 4, *B* and *C*, it is possible to calculate ξ_1 as well as $\bar{v}_2$. From these graphs we have estimated that $\xi_1 = 0.275$ g H_2O/g protein and $\bar{v}_2 = 0.717$ cm^3/g for MLCK and that $\xi_1 = 0.342$ g H_2O/g protein and $\bar{v}_2 = 0.714$ cm^3/g for the telokin. Table 2 summarizes the conformational parameters for both MLCK and telokin.

DISCUSSION

Application of sedimentation equilibrium to turkey gizzard MLCK has provided a molecular weight (108,000) that is in good agreement with values calculated from amino acid sequence −107–534 (Olsen et al., 1990) and from stoichiometric fluorescence titrations with calmodulin, in which the concentration of the enzyme was calculated on the basis of M_r = 124,000 and $E^{1\%}_{280}$ = 10. The latter demonstrated the binding of 1.2 mol calmodulin/mol enzyme, a value consistent with M_r = 103,000 (Malencik et al., 1982). The difference between our values and that (124,000) reported by Adelstein and Klee (1981) may be due to a number of factors. We note that their sample contained only 0.04 mg/ml enzyme, corresponding to an absorbance (1.2 cm path) of 0.05 at 278 nm, the wavelength at which the measurements were performed. The accuracy of the M_r values obtained with the high speed meniscus depletion method of Yphantis (1964) is strongly dependent on the identification of the baseline defining zero concentration. Because of the intrinsic noise obtained with the scanner unit of the model E analytical ultracentrifuge, this can be particularly difficult when absorption methods are used. Refractometric methods and, in particular, inter-

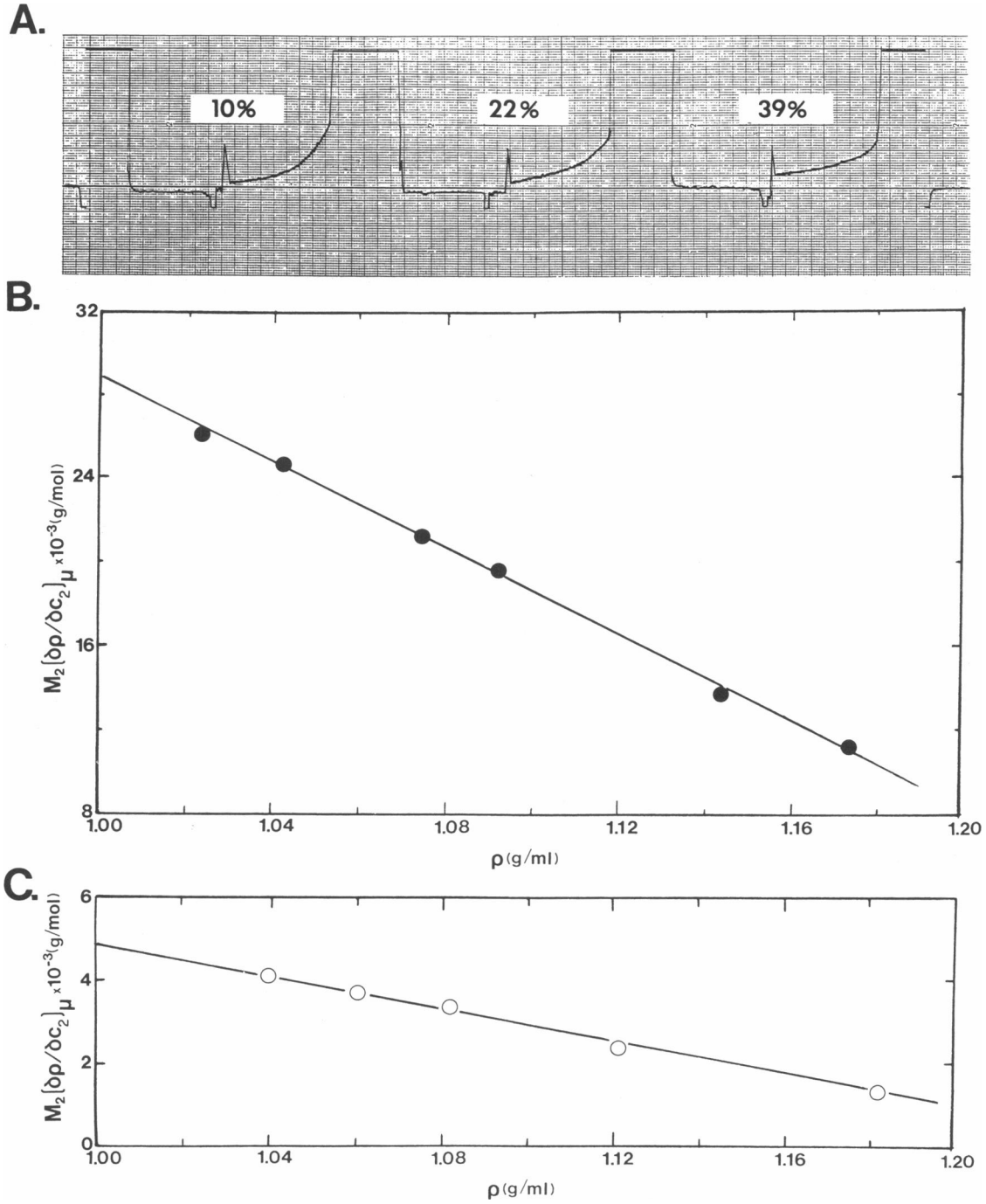

FIGURE 4 (*A*) Ultraviolet scanner trace at 282 nm obtained at equilibrium for a MLCK sample in 10, 22, and 39% sucrose in 0.2 M NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 1 mM β-mercaptoethanol. The scans were collected after 96 h at 15,000 rpm and at 20.1°C. *B* and *C* contain the slopes $M_2(\partial\rho/\partial c_2)\mu$ from sedimentation equilibrium vs. medium density ρ, carried out in sucrose. (*B*) MLCK and (*C*) telokin.

ference optics, which we have used in the present study, are preferred (Yphantis, 1964). Finally, Adelstein and Klee (1981) used a least-squares curve-fitting method in order to analyze the gradient at equilibrium over the whole column length. In the case of a degree of protein association, this may lead to overestimation of M_r.

We have also measured the virial coefficients of MLCK and telokin. Under the buffer conditions used

TABLE 2 **Physical parameters of MLCK and telokin**

	MLCK	Telokin
M_2 sedimentation equilibrium, D*	108,000	18,500
$\bar{v}_2$, cm^3/g*	0.731	0.713
$s_{20,w}$, S	3.74	1.63
ξ_1, g H_2O/g protein	0.275	0.342
f/f_0	1.95_2	1.45
R_0, Å	35.1	19.8
R_s, Å	68.5	28.7
a/b‡	18.9	8.3
a, Å‡	249.5	81.3
b, Å‡	13.2	9.8
d, Å	21.5	15.9
R_G, Å	144	46.9

*Partial specific volume ($\bar{v}_2$) was determined from amino acid composition as described in text. ‡A prolate ellipsoid shape is assumed. $s_{20,w}$, sedimentation coefficient; ξ_1, preferential hydration parameter; f and f_0, frictional coefficients; R_0, radius; R_s, Stoke's radius; d, cross-sectional diameter of a rigid rod of length $L = 2a$; R_G, radius of gyration for a rigid rod.

(see Materials and Methods), MLCK exhibits a negative virial coefficient, a phenomenon that usually arises from self-association. That this is indeed the case is supported by the results of sedimentation velocity studies (Fig. 3 *A*). MLCK consists mainly of two sedimenting components, with 85% of the protein sedimenting at 3.74 S. A second minor component sediments faster, with $s_{20,w}$ = 4.75 S. Although we have not determined whether these two species are in slow equilibrium with each other, they appear to be related since $NaDodSO_4$ gel electrophoresis reveals no contaminating bands above that corresponding to MLCK (see Fig. 1). Assuming that the 4.755 component is indeed an enzyme dimer, the association of two MLCK molecules would lead to a highly asymmetric molecule. If we further assume that neither the partial specific volume nor the ξ_1 change upon association,[3] it is possible to combine Eqs. 6 and 8 in order to estimate $f/f_0 = 2.44$ for the dimer. This frictional ratio corresponds to that of a prolate ellipsoid with an axial ratio $a/b = 32$, which is almost twice the axial ratio found for the monomer (Table 2). Perhaps association of MLCK takes place in either a head-to-head or head-to-tail fashion.

In the case of telokin, we found a positive virial coefficient, with the sample sedimenting as a single species. A positive virial coefficient in this case is indicative of nonideal behavior under the buffer conditions used. Second virial coefficients for noninteracting molecules usually result from excluded volume effects. In the absence of charge effects, it is possible to evaluate the magnitude of the excluded volume contribution to A_2. Thus, for a rod of length L and cross-sectional diameter d,

$$A_2 = \frac{L}{d}\frac{\bar{v}_2}{M_2} \tag{15}$$

(van Holde, 1985).

From the values in Table 2, we calculate that $A_2 = 3.94 \times 10^{-4}$ mol · ml · g^{-2}, which is quite close to the experimental value. From the hydrodynamic analysis performed, we conclude that both MLCK and telokin are quite asymmetric. The axial ratio observed for MLCK is consistent with a rod-shaped molecule ($a/b = 18.9$). Telokin is somewhat more compact, with an axial ratio $a/b = 8.3$. It is possible that the value of A_2 calculated for telokin represents an overestimate and that repulsive charge effects need to be additionally invoked in order to account for the experimental value of A_2. Indeed, this would not be surprising considering the amino acid composition of this protein (Table 1).

Quantitative sedimentation studies of rabbit skeletal muscle MLCK revealed an asymmetric structure corresponding to $a/b = 12$, $R_s = 52$ Å, and $M_r = 70{,}300$ (Mayr and Heilmeyer, 1983). Preliminary studies by Adelstein and Klee (1981) indicated that turkey gizzard MLCK also is elongated. However, the sedimentation coefficient that they reported (4.45S) probably is an unresolved average value for the monomer and dimer. As a result of the differences in M_r and in $s_{20,w}$, the Stoke's radius reported by these authors (75 Å) is higher than our value (69 Å).

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[3]We calculated f/f_0 for two extreme situations: $\xi_1 = 0$ (no hydration) and $\xi = 0.5$. In the first case, $f/f_0 = 2.71$ and $a/b = 41.5$, whereas, in the second case, $f/f_0 = 2.28$ and $a/b = 27.7$. Thus, even with an unlikely large increase in hydration, the presumed dimer would still be highly asymmetric.

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