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THE MOLECULAR WEIGHT OF α-CHYMOTRYPSINOGEN

PHILIP E. WILCOX, J. KRAUT*, R. D. WADE AND HANS NEURATH

Department of Biochemistry, University of Washington, Seattle, Washington (U.S.A.)

Recent estimates of the molecular weight of crystalline α-chymotrypsinogen have varied within the range of 22,000–25,000[1], which is considerably lower than the original value of approximately 36,000 reported by KUNITZ AND NORTHROP[2]. In view of the widespread interest in this protein as a precursor of a family of proteolytic enzymes[1,3,4] and as a highly purified and relatively homogeneous product[5,6,7], it appeared of importance to define the molecular weight of α-chymotrypsinogen within narrow limits of experimental error. To this end, a combination of chemical and physical methods of measurement has been employed, including amino acid analysis, light scattering, sedimentation rate, and diffusion. Molecular weight calculations deduced from X-ray diffraction data of salt-free crystals, already reported by BLUHM AND KENDREW[8], have formed part of the present, cooperative project. All of these data converge toward 25,000 as the most probable value for the molecular weight of α-chymotrypsinogen.

EXPERIMENTAL

Material. α-Chymotrypsinogen was obtained from Worthington Biochemical Laboratory, Freehold, New Jersey, as a once-crystallized filter cake and was recrystallized seven times with am-

* Fellow of the Howard Hughes Medical Institute.

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monium sulfate[5] and twice with alcohol[9]. As previously reported[10], this sample was electrophoretically nearly homogeneous at pH 4.97; less than 5% of the protein appeared outside the main peak after seven hours of electrolysis. When tested against acetyl-L-tyrosine ethyl ester as substrate, the zymogen was found to contain 0.02% active chymotrypsin.

AMINO ACID ANALYSIS*

A solution of the protein was dialyzed against dilute HCl and then was passed over a column of Amberlite IR-120 on the H^+-cycle in order to remove ammonium and metallic ions or peptide impurities which might be present. Samples of protein were hydrolyzed with 5 *N* HCl at 110° ± 1° for 12, 24, 36, 48 and 72 hours. The amino acids in each hydrolysate were separated on columns of Dowex-50 according to the methods of MOORE AND STEIN[11] and were assayed quantitatively by the ninhydrin method[12].

The amount of nitrogen in chymotrypsinogen was determined on a sample which had been freed of all small ions by passage over a column of mixed-bed resin, Amberlite IR-120 on the H^+-cycle and Amberlite IRA-400 on the OH^--cycle. Repeated dry-weight and Kjeldahl nitrogen determinations on the isoionic protein gave an average value of 16.5% nitrogen. There was no ash, and no counter-ion corrections were necessary. The specific extinction coefficient at 282 mμ, determined on the isoionic protein was found to be 20.0 as compared to 20.6 previously reported[13].

Results of calculations leading to the molecular weight of chymotrypsinogen are presented in Table I. Serine, cystine, methionine, and tryptophane, which are present in the protein, are omitted from the Table because they are destroyed or largely transformed during the hydrolysis. Three amino acids were found to increase signif-

TABLE I

MOLECULAR WEIGHT OF α-CHYMOTRYPSINOGEN CALCULATED FROM AMINO ACID ANALYSES

Amino acid	*(1) g Amino acid/100 g protein**	*(2) Minimum molecular weight*	*(3) Assumed number of residues/mole*	*(4) Molecular weight*	*(5) Calculated number of residues/mole*
Aspartic acid	11.56	1151	22	25330	21.8
Threonine	10.89**	1094	23	25158	23.0
Glutamic acid	8.32***	1768	14	24752	14.1
Proline	4.00§	2878	9	25904	8.7
Glycine	6.97	1075	23	24736	23.3
Alanine	7.71	1156	22	25421	21.8
Valine	10.45§	1121	22	24663	22.4
Isoleucine	5.17§	2537	10	25371	9.9
Leucine	9.85	1332	19	25299	18.9
Tyrosine	2.95	6142	4	24568	4.0
Phenylalanine	4.29	3850	7	26954§§	6.5
Histidine	1.17	13265	2	26520§§	1.9
Lysine	7.72	1894	13	24617	13.2
Arginine	2.77	6289	4	25155	4.0

Mean molecular weight = 25081; Mean deviation = 336, 1.3%; Standard deviation = 398, 1.6%.

* Average of six analyses, two runs each at 24, 36, and 48 hours of hydrolysis, unless otherwise indicated.

** Data for 12, 24, 36 and 48 hours extrapolated to zero time.

*** Corrected for 3% loss of glutamic acid on the column[11].

§ Average of two runs at 72 hours of hydrolysis.

§§ Excluded from the calculation of the mean molecular weight.

* Detailed results of the amino acid analysis of chymotrypsinogen will be published elsewhere.

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icantly in the time from 24 to 72 hours, proline by 8%, valine by 12%, and isoleucine by 17%. Therefore, the values given in Table I for these three amino acids are the averages of two runs at 72 hours. On the other hand, threonine decreased by 7% during the time from 12 to 48 hours, and the data were extrapolated to zero time to give the tabulated value. Glutamic acid has been corrected by 3% for losses on the column[11]. The amounts of the remaining amino acids did not vary significantly between 24 and 72 hours of hydrolysis; therefore, the values given in Table I for these amino acids are averages of six analyses, two runs each at 24, 36 and 48 hours.

Minimum molecular weights (column 2) were calculated from the analytical data for each amino acid. It was possible to choose integral numbers of residues (column 3) such that each calculated molecular weight lay close to 25,000 (column 4). The only exceptions are histidine and phenylalanine. The deviation of histidine can be explained by the relatively large error introduced into the calculation of the small amount of amino acid owing to the uncertainty in the determination of the base line of the chromatograms. The deviation of phenylalanine remains unexplained. Molecular weights based on histidine and phenylalanine have been excluded from the final calculation of the mean.

Data for twelve amino acids give for chymotrypsinogen a mean molecular weight of 25,100. The low mean deviation and the comparable standard deviation lend credibility to the molecular weight calculated by this method.

LIGHT-SCATTERING MEASUREMENTS

Method

Light-scattering measurements were made in a tris(hydroxymethylamino)methane-HCl buffer of pH 8 and ionic strength 0.1 M, containing $4 \cdot 10^{-5} M$ di*iso*propylphosphofluoridate to suppress any residual proteolytic activity. Measurements were made at chymotrypsinogen concentrations of 0.830, 2.60, 5.27 and 7.19 mg per ml as determined by ultraviolet absorptions at 282 mμ.

The centrifugal light-scattering cells and modified Brice instrument previously described by DANDLIKER AND KRAUT[14] were used throughout. These were calibrated by the "Ludox" method, the cell constants being obtained by extrapolation to zero turbidity. The calibration was carried out at the four mercury-arc wavelengths of 3650 A, 4047 A, 4358 A, and 5461 A, and scattering measurements were subsequently performed at all four wavelengths for each concentration of chymotrypsinogen. Clarification of the solutions was achieved by centrifugation of the cells for one hour at approximately 44,000 $\times$ g in the Spinco swinging-bucket rotor SB-1 and confirmed by observation of the ratios of scattering intensities at 25.8° to 90°, and at 45° to 135°. No preliminary clarification was found to be required. All data were corrected for solvent scattering (about 50–100% greater than that of water), the correction amounting to no more than 36% in the least favorable case. Depolarization was negligible; hence, no depolarization correction was required.

Values for dn/dc were obtained on an isoionic chymotrypsinogen solution with the Phoenix differential refractometer. It was felt that the data for the salt-free protein solution would be more reliable than those obtained with one which had been dialyzed against the buffer, since the latter procedure introduces the possibility of error owing to differential salt concentrations arising from the Donnan effect. Direct

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measurements could be made conveniently only for 4358 A and 5461 A, the dn/dc values for 3650 A and 4047 A being obtained by linear extrapolation of a plot of dn/dc *versus* λ^{-2} [15]. The results, in order of increasing wavelength, were 0.204, 0.198, 0.194 and 0.185 $ml \cdot g^{-1}$. These values were used to compute K for each wavelength in equation (1) below.

Results

The data for each wavelength were plotted as $Kc/R(90)$ versus concentration and extrapolated to $c = 0$ to obtain $1/M$ in accordance with the well-known relation[16]

$$Kc/R(90) = 1/M + 2Bc \qquad (1)$$

In order of increasing wavelength, the intercepts corresponded to molecular weights of 26,300, 25,100, 25,400 and 27,600 giving a mean of 26,100 ± 1,000. All four plots showed slightly negative slopes, the average value for B being $-(1.8 \pm 0.7) \times 10^{-4}$ moles ml/g^{-2}. Negative values for B are rather unusual in ordinary protein systems. It may be interpreted as indicating the presence of a monomer-dimer equilibrium[10,13,17], but the equilibrium must lie well to the left, since the effect is small.

SEDIMENTATION RATE – DIFFUSION

Methods

Diffusion measurements were carried out at 1° C in a moving boundary electrophoresis apparatus made by Frank Pearson Associates, equipped with the Rayleigh interference optical system[18]. Diffusion took place in a Tiselius electrophoresis cell made of quartz, containing reference windows as described by LONGSWORTH[19]. Diffusion was allowed to proceed for periods of time up to approximately 100,000 seconds. Diffusion constants were calculated by the method outlined by LONGSWORTH[19].

Sedimentation rate measurements were carried out as previously described[10]; the values used herein for calculations of molecular weights are those already reported[10].

Results

The results of diffusion measurements of chymotrypsinogen in a pH 3.0 buffer containing 0.02M glycine, 0.08M NaCl and sufficient HCl to bring the system to the desired pH, are summarized in Table II. For each experiment, the diffusion constant was calculated by extrapolation of the straight line, obtained by least square calculations, when the apparent diffusion constant was plotted against the reciprocal time of diffusion. The "zero time correction", given by the slope of the line, was usually of the order of 10^2 sec.

Table II includes also the results of a second series of diffusion measurements carried out in a pH 7.5 phosphate buffer, ionic strength 0.1, the same buffer employed in sedimentation equilibrium measurements. It will be noted that the value obtained upon extrapolation to zero protein concentration, $D_{20,w}$, is somewhat higher at the higher pH, *i.e.*, $9.48 \cdot 10^{-7}$ cm^2 sec^{-1}, as compared to $9.01 \cdot 10^{-7}$ at pH 3.0.

Molecular weights were calculated from these data with the equation[20]:

$$M = \frac{RTs}{D(1 - V\varrho)} \qquad (2)$$

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TABLE II

DIFFUSION MEASUREMENTS OF α-CHYMOTRYPSINOGEN

A. pH 3.0 (0.02 M glycine, 0.08 M NaCl, + HCl)		*B. pH 7.5 0.0308 M K_2HPO_4, 0.0072 M KH_2PO_4)*	
c_0 g/100 ml	$D_{20,w} \times 10^7$ cm^2 sec^{-1}	c_0 g/100 ml	$D_{20,w} \times 10^7$ cm^2 sec^{-1}
10.08	9.146	9.30	8.792
7.40	9.169	9.10	8.842
6.28	9.228	8.46	8.868
3.69	9.046	5.56	9.025
2.71	9.039	1.96	9.382
2.02	8.999		
	$D_{20,w} = 9.009$ $c \to 0$		$D_{20,w} = 9.480$ $c \to 0$

where M is the gram molecular weight, R the gas constant, T the absolute temperature, V the partial specific volume of the solute (taken as 0.721[13]), ϱ the density of the solution, and s is the sedimentation constant, extrapolated to zero protein concentration. The sedimentation constants are those previously reported, except that they have been corrected herein for adiabatic cooling of the rotor of $-0.9°$ C[21], giving values of $s_{20,w} = 2.49\ S$ at pH 3.0, and $s_{20\,w} = 2.58\ S$ at pH 7.5. The corresponding values for M are 24,400 at pH 3.0 and 24,000 at pH 7.5, respectively.

DISCUSSION

The values for the molecular weight of chymotrypsinogen obtained in this study, summarized in Table III, are within the range of the estimates which have been more recently reported. These include a value of 25,000 obtained by light-scattering measurements[22], 24,000 ± 500 by osmotic pressure measurements[23], and 25,000 ± 800 by X-ray diffraction measurements[8]. The lower value of 22,000–23,000 calculated from sedimentation and diffusion constants, independently determined by two groups of investigators[13,24], will be considered below in conjunction with the present measurements of these constants.

The high degree of accuracy attending the determination of several amino acids which have been used in the calculation of minimum molecular weights, gives con-

TABLE III

MOLECULAR WEIGHT OF α-CHYMOTRYPSINOGEN

Method	*Molecular weight*
Amino acid analysis	25,081 ± 336
Light-scattering	26,100 ± 1000
Sedimentation rate – diffusion	
pH 3.0	24,400
pH 7.5	24,000
X-ray diffraction[8]	25,000 ± 800
Osmotic pressure[23]	24,000 ± 500

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siderable weight to the value of 25,100 as the true mean molecular weight of the protein. Moreover, this estimate is independent of any complications arising from the monomer-polymer equilibrium which under certain conditions exists in solutions of chymotrypsinogen[13,17].

Within the limits of experimental error, the values for the solution molecular weight (extrapolated to infinite dilution) obtained from light-scattering and sedimentation rate-diffusion measurements, all agree with the estimate of 25,000 derived from analysis of the solid protein. The present value calculated from extrapolated sedimentation and diffusion constants measured at pH 7.5 is somewhat higher than that calculated by SCHWERT[13] from measurements at pH 3.86. Differences exist with regard to the diffusion constants* determined at finite protein concentrations[13], but the value obtained by extrapolation to zero protein concentration happens to be the same in both sets of data. Although SCHWERT's data suggest that the sedimentation constants are practically independent of pH, within the range of pH 3.86 to 7.41, the present data show conclusively that at a more acid pH, *i.e.*, pH 3.0, both the diffusion and sedimentation constants are somewhat lower, though the calculated molecular weight is the same.

Measurements of the molecular weight of α-chymotrypsinogen by different methods of sedimentation equilibrium are in progress and will be presented in a future publication.

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SUMMARY

The molecular weight of crystalline α-chymotrypsinogen has been determined from amino acid analysis, light-scattering, and sedimentation-diffusion measurements. The values obtained by the use of these methods are, respectively, 25,100, 26,000 and 24,200. These values, together with the recently reported X-ray estimate of 25,000, converge toward 25,000 as the most probable molecular weight of α-chymotrypsinogen.

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* The diffusion constant reported by SMITH, BROWN AND LASKOWSKI[24] at pH 4.0 is significantly higher than that reported by SCHWERT[13], accounting for the lower molecular weight calculated by these authors.

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INHIBITION OF ENZYMIC TRANSAMINATION OF ASPARTIC ACID BY HYDROXYASPARTATE, 2,3-DIAMINOSUCCINATE AND 2,3-DIAMINOPROPIONATE*

MARIO GARCIA-HERNANDEZ AND ERNEST KUN**
Department of Biochemistry and Institute for Enzyme Research, University of Wisconsin, Madison, Wis. (U.S.A.)

It was reported from this laboratory (KUN AND GARCIA-HERNANDEZ[1], KUN[2]) that oxaloglycolic and diketosuccinic acids were among the main products of the enzymic oxidation of tartaric acid. One of the enzymic pathways by which these keto acids may be further metabolized is transamination. So far we have been able to show only one transamination, *viz.* the reaction of oxaloglycolate with glutamate, to yield hydroxyaspartate and α-ketoglutarate. This reaction is catalyzed by an aqueous extract of acetone powder of isolated pig kidney mitochondria. In the course of an independent investigation, SALLACH[3] also described this reaction, brought about by an enzyme preparation obtained from sheep brain. Diaminosuccinic and diaminopropionic acids were obtained only by chemical synthesis. It has been previously known (HASKELL *et al.*[4]) that diaminopropionic acid is a constituent of a tuberculostatic antibiotic isolated from certain actinomycetes, while hydroxyaspartic and diaminosuccinic acids inhibited bacterial growth (SHIVE AND MACOW[5], SUZUKI *et al.*[6,7]). One of the many possible mechanisms by which these uncommon amino acids may exert their inhibitory action is an interference with certain enzymic transaminations. In order to test this hypothesis we determined their effect on the transamination of aspartate and α-ketoglutarate.

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** Established Investigator of the American Heart Association Inc. Present address: The University of California Medical Center, Department of Pharmacology and Experimental Therapeutics, San Francisco 22, California.

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