THE SYNTHESIS OF METHIONINE BY ENZYMIC TRANSMETHYLATION*

VI. STUDIES OF THE HOMOGENEITY, SHAPE PROPERTIES AND MONOMER FUNCTIONALITY OF THETIN–HOMOCYSTEINE METHYLPHERASE

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SUMMARY

It has been demonstrated, by means of moving partition cell ultracentrifugation, that the sedimentation coefficients of the enzymic activity of thetin–homocysteine methylpherase and of the total protein present in the system are the same over a wide range of degrees of polymerization. This observation is believed to be a highly sensitive criterion of homogeneity, and one which should be applicable to a number of other protein systems which may be studied at different degrees of aggregation.

Hydrodynamic studies of monomer and polymer preparations have shown that the shape of each of the components of THMenz polymer preparations is very close to that of the monomer. Direct observation by electron microscopy has confirmed this result. In accordance with this concept, it has been shown by application of the STOCKMAYER statistical theory of polymerization to the THMenz system that there are three functional groups on each monomer residue which can form intermolecular disulfide bonds in the polymerization reaction and that therefore the polymer molecules are three dimensional aggregates as opposed to linear ones.

INTRODUCTION

Earlier papers in this series have reported on the purification and properties of the enzyme THMenz[1–4], an enzyme which catalyzes the methylation of homocysteine to methionine by dimethylacetothetin. The purified protein behaves as a homogeneous substance in the ultracentrifuge, during free boundary electrophoresis and chromatographically, and is able to undergo an unusual, reversible, depolymerization. Since depolymerization can be effected only with agents which are known to cleave disulfide bonds and since repolymerization of the monomer can be prevented by blocking its sulfhydryl groups with a number of suitable reagents, it has been concluded that

Abbreviations: THMenz, thetin–homocysteine methylpherase; DEAE, diethylaminoethyl; DPNH, reduced diphosphopyridine nucleotide.

* Twenty-second paper in a series on enzymic mechanisms in transmethylation.

the reversible polymerization is a result of the formation of intermolecular disulfide bonds involving the participation of particularly reactive sulfhydryl groups.

It has been estimated that the purified THMenz represents about 1% of the protein of horse liver; its preferred substrate, dimethylacetothetin, is not known to occur in nature, and therefore there is some uncertainty as to the physiological role of this protein. Moreover, while THMenz can utilize betaine as a methyl donor at a slow rate, a more specific betaine homocysteine methylpherase has recently been purified in this laboratory from horse liver[5]. It became important, therefore, to obtain further information on the state of purity of THMenz in order to be able to design studies which might provide evidence on the physiological role of this interesting protein. A detailed comparison of the properties of the enzyme in its monomeric and polymerized states has also been carried out in order to gain further insight into the mechanism and significance of the polymerization process.

The results of the experiments reported in this paper confirm fully the earlier findings and provide strong evidence that THMenz is, within reasonable limits, a homogeneous protein and that both the polymerization and enzymic activity are properties of the same molecular species. Furthermore, it has been possible to deduce that three of the functional groups (presumably sulfhydryl) of each molecule are capable of taking part in the polymerization reaction to produce a highly cross-linked and symmetrical polymer molecule whose shape is very similar to that of the closely spherical monomer.

MATERIAL AND METHODS

THMenz was prepared as described previously[1] with the modifications introduced in a more recent publication from this laboratory[2]. The enzyme preparation was carried through the ammonium sulfate step, and then further purified with DEAE cellulose columns as described in the accompanying paper[4].

Protein concentrations were determined routinely from absorption measurements at 280 mμ measured in a Beckman DU spectrophotometer. The extinction coefficient used was 13.0 for a solution containing 1 g of protein in 100 ml of solution. This value was obtained from dry weight measurements and was found to be essentially independent of the degree of polymerization of the protein.

Viscosity measurements were performed with a capillary viscometer in a water bath at 20° ± 0.02° with an outflow time of 103 sec for water. Sedimentation experiments were performed in a Spinco Model E Analytical Ultracentrifuge, and all sedimentation coefficients were converted to water at 20°, assuming a partial specific volume of 0.75 for the protein[2]. Area measurements were made from tracings of enlarged schlieren patterns. Overlapping peaks were resolved into individual gaussian components (see ref. 6). All physical measurements were performed with the protein dissolved in 0.05 *M* potassium phosphate buffer, pH 7.4, unless otherwise specified.

Enzyme activity measurements were performed as previously described, with dimethylacetothetin serving as methyl donor[1].

RESULTS

Partition cell experiments

The development of the theory of the moving partition ultracentrifuge cell by YPHANTIS AND WAUGH[7], along with the commercial availability of the apparatus

which they designed, has provided a powerful tool for the study of substances of biological interest. This technique has been applied in the present work in order to decide whether the enzymic activity of THMenz is a property of the same molecular species as is the reversible polymerization, and also as an unusually sensitive criterion of purity.

Solutions of THMenz of varying degrees of polymerization, at protein concentrations in the range of 0.1–1 g/100 ml, were sedimented in the partition cell, at either 44,700 rev./min or 59,780 rev./min, for varying periods of time, so that somewhere between 25 % and 75 % of the total material had moved below the separation plate at the end of the experiment. Protein concentrations as well as enzymic activities were determined in both the top and the bottom compartments of the cell, as well as in the original solution, and weight average sedimentation coefficients were calculated for both the enzymically active species and the total protein[7]. It is important in using the partition cell that essentially quantitative recoveries of material are obtained, and all of the data reported satisfy this condition. It had been observed in some early experiments that erratic results may be obtained if the recoveries are appreciably less than 95 %. In order to insure against inactivation of the enzyme during the ultracentrifugal run, most experiments were performed at 5–10° in the presence of 0.005 *M* Versene.

The results obtained from these experiments are presented in Fig. 1. In the figure, the sedimentation coefficients obtained from activity measurements are plotted against those derived from measurements of total protein concentration. The line,

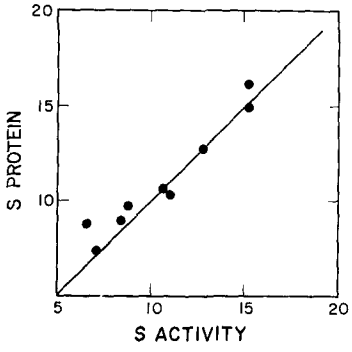

Fig. 1. Sedimentation coefficient ($s_{20,w}$) of THMenz activity as a function of the sedimentation coefficient of the total protein, for preparations of varying degrees of polymerization. ———, the expected distribution of the data for a homogeneous protein. The samples studied vary from monomer (S = 8) to preparations with a weight average degree of polymerization of 3 (S = 16).

with a slope of one and intersecting the origin represents the expected distribution of the data if the protein were indeed homogeneous. The experimental points conform quite closely to the expected distribution. For the sedimentation coefficients of the activity and of the protein to be the same by chance, at all degrees of polymerization, is an extremely unlikely occurrence. Such agreement must be interpreted as providing strong evidence for the identity of the enzymically active protein with the total protein present, and thus for the homogeneity of the preparation under consideration. The calculated sedimentation coefficients are sensitive to small errors in the concen-

tration measurements, and in the present work, it is conceivable that impurities present at a level of 5–10 % on a weight basis might not be detected.

Shape properties

In an earlier paper in this series[1], it was suggested, on the basis of ultracentrifugal measurements made at a single protein concentration, that the sedimentation coefficients of the various components of the polymer are in very close agreement with those calculated on the assumption that no shape or density change occurs during polymerization. Since these calculations carry important implications as to the geometry and configuration of the molecular species present in the polymeric system, it was considered of interest to study the concentration dependence of the sedimentation coefficients of the polymer, as well as of the isolated monomer. The results of these experiments are shown in Fig. 2. It is apparent that the rates of sedimentation of the monomer, dimer, trimer and tetramer are all independent of concentration in the range studied. This result is thought to be consistent with the highly symmetrical nature of the species in question[3]. It is also of interest to note that the sedimentation coefficient of the isolated monomer in the presence or the absence of the other components of the polymeric mixture is identical.

The assumption that no shape change occurs during the polymerization of the monomer was tested further by making viscosity measurements of a polymer preparation with a weight average sedimentation coefficient of 15 S and the monomer (S = 8). In Fig. 3 the specific viscosities of the monomer (open circles) and of the

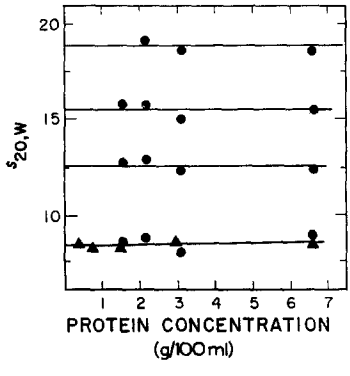

Fig. 2. Concentration dependence of the sedimentation coefficients of some of the components of THMenz polymer. The lines represent, reading from bottom to top, monomer, dimer, trimer, and tetramer. The triangles are data obtained with isolated monomer, while the other points were obtained by analysis of sedimentation patterns of polymeric mixtures.

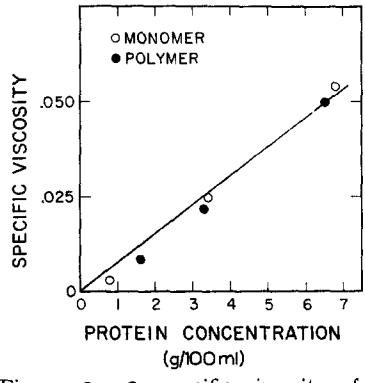

Fig. 3. ○—○, specific viscosity of THMenz monomer; ●—●, specific viscosity of THMenz polymer as a function of protein concentration.

polymer (closed circles) are presented as a function of protein concentration. It is apparent that there is little, if any, change in viscosity accompanying the interconversion of monomer and polymer. These data support the concept that the shape of the polymeric components is indeed similar to that of the monomer. Furthermore, the low intrinsic viscosity observed here agrees with calculations based on the diffusion of the monomer[3] in suggesting that the THMenz molecule in solution is close to spherical in shape.

A more direct confirmation of the hypothesis under investigation was sought

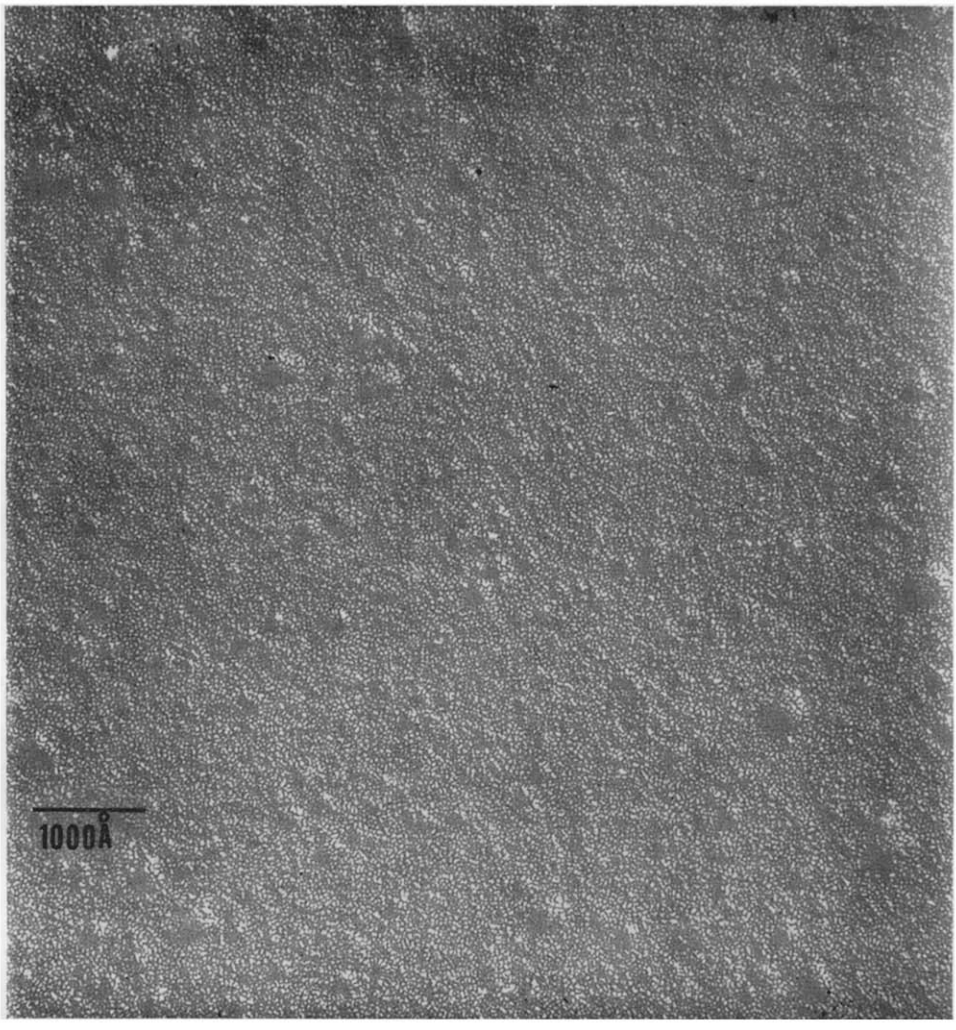

Fig. 4. Electron micrograph of a carbon replica of palladium shadowed THMenz polymer.

by means of electron microscopy. Electron micrographs of a highly polymerized preparation of THMenz were kindly prepared by Dr. L. W. LABAW of these Institutes. An approximately 0.005-% solution of the protein was dried on a colloidion film, shadowed with palladium and then coated with carbon. A typical field of such a carbon replica is shown in Fig. 4. Because of the similarity in size of the monomer units and the grains representing the substrate background no useful conclusions may at present be drawn about the size and shape of the smaller units by means of this technique. It is apparent, however, that there is a large range of particle sizes, some of which particles correspond to very large molecular aggregates (of the order of 50–100 monomer residues). It may be noted that, as a first approximation, the shapes of all of the particles are the same and that they are also quite symmetrical. These observations are in good agreement with the results of the hydrodynamic measurements discussed above.

Monomer functionality

It has been observed that a concentrated solution of THMenz will gel on standing at 2° for a period of a few days. This behavior is interpreted to mean that at least three functional groups exist, per monomer unit, which are capable of forming intermolecular bonds[8]. A bifunctional monomer could give rise only to straight chain polymers which would not be expected to form gels.

This observation prompted an investigation of the distribution of the various species in preparations of varying degrees of polymerization in order to obtain more reliable data on monomer functionality. The statistical theory of STOCKMAYER[9] may be expressed as follows[10]:

$$Wn = \frac{(fn - n)!\, f}{(n - 1)!\,(fn - 2n + 2)!}\, p^{n-1}\,(1 - p)^{(fn - 2n + 2)}$$

where W_n is the weight fraction of n-mer, f is the functionality of the monomer and p represents the fraction of the functional groups which have reacted. This relationship is valid only if intramolecular reactions do not occur and if, at any stage, of the polymerization, all unreacted functional groups have an equal chance of reacting, no matter what size molecule they are attached to. The STOCKMAYER theory predicts that, for systems in which there are three or more functional groups on the monomer, no matter what the degree of polymerization, there will always be more n-mer than $(n + 1)$ - mer. On the other hand, if only two functional groups are present on the monomer, then at some degrees of polymerization the distribution of molecular species will be such that there will be less monomer than dimer. Since out of more than 50 polymer preparations, of varying degrees of polymerization, which have so far been examined in the ultracentrifuge none was found to have more dimer than monomer, it seemed likely on these grounds also that the monomer must carry at least 3 functional groups.

A more quantitative application of the STOCKMAYER theory was attempted after estimation of the weight fraction of monomer, dimer, trimer and tetramer from area measurements made on ultracentrifugal patterns of THMenz preparations of varying degrees of polymerization. These experiments were carried out in double sector ultracentrifuge cells containing solvent in one sector in order to obtain accurate base lines for the area measurements. The total amount of material present in a given cell was estimated, in area units, from schlieren patterns obtained at very early times during the run when the boundary had completely separated from the meniscus, but a plateau region was still present. All area measurements were corrected for radial dilution, but no attempt was made to apply JOHNSTON-OGSTEN corrections. As shown above, the rate of sedimentation of each of the species of interest is independent of concentration and therefore JOHNSTON-OGSTEN anomalies are not an important consideration in this system[11,12]. From such area measurements one obtains directly the value of W_1, W_2, W_3, and W_4. By substituting the value of W_1 so determined into the equation given above, and assuming a value for f of either 2, 3, or 4, one obtains the value of p which corresponds to the chosen functionality. Employing these corresponding values of p and f, one may calculate the theoretical distribution of components for each of the three functionalities chosen. A comparison of the measured distribution with those calculated, Fig. 5, has, in all cases, shown that the functionality of the monomer more closely approximates three than four, and cannot

be as low as two. It is apparent, however, that the experimental points deviate from the theoretical curve. Perhaps the most likely cause of this anomalous behavior is that a small percentage of the functional groups of the monomer are not free, but have been converted to mixed disulfides during the depolymerization process. That such a situation actually exists is not unlikely in view of the chromatographic evidence for mixed disulfide formation discussed in the accompanying paper[4].

It should be pointed out, however, that there is much more uncertainty about the area measurements of trimer and tetramer than those of monomer and dimer. This is due to the fact that the latter two components are present in greater amounts and are more completely separated from each other in the schlieren patterns. Thus, a part of the observed deviation may be an apparent one.

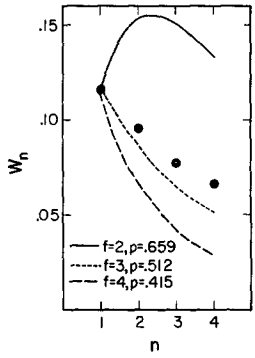

Fig. 5. The weight fraction of monomer, dimer, trimer and tetramer in a typical sample of THMenz polymer. The lines are theoretical distributions calculated as indicated in the text, for assumed functionalities of ———, 2; ·····, 3; – – – –, 4. The corresponding values of p employed were 0.659, 0.512, and 0.415 respectively.

DISCUSSION

The happy chance that THMenz can undergo a reversible polymerization reaction and also has an enzymic activity which is stable to changes in its state of aggregation enables the application of partition cell centrifugation as an unusually sensitive method of studying the homogeneity of this protein. The demonstration that the sedimentation coefficients of both the total protein present and of the enzymic activity are the same over a wide range of polymerization represents strong evidence for the homogeneity of THMenz. This conclusion is supported by the electrophoretic and sedimentation measurements which have been reported by DURELL *et al.*[1], and also by the behavior of the enzyme on CM columns[4]. It is apparent that any macromolecular system which can be studied at different degrees of aggregation or unfolding and for which two different properties of the molecule of interest can be independently measured is amenable to partition cell analysis as a criterion of homogeneity. Absolute purity is, however, a state which can only be approached in an asymptotic manner. There are indications that THMenz is not completely free of contaminating proteins. Thus, it has been found that THMenz preparations have DPNH oxidase activity, and it has been established, with the partition cell, that the THMenz and DPNH oxidase activities are functions of different protein species. It has been possible to calculate that the DPNH oxidase must be present in very small amounts in the

THMenz preparations, amounts which are outside the limits of detectability of the partition cell experiments described in this paper.

Perhaps the most rigorous criterion of homogeneity available in protein chemistry is that of constant solubility[13]. Attempts to apply this test to THMenz have not been successful, but it may be pointed out that the monomer undergoes partial polymerization during the prolonged equilibration periods required in solubility determinations. Thus, the number of components of such a system does not remain constant with time, and therefore the phase rule criterion of purity cannot be applied.

Throughout this paper it has been tacitly assumed that the mechanism of the polymerization process is one involving the oxidation of two sulfhydryl groups, each on a different molecule, to produce an intermolecular disulfide bond. Although there is a good deal of evidence which indicates that the primary bonds formed during the polymerization are disulfides[1-3], the means by which these intermolecular bonds form are more obscure. DURELL AND CANTONI have found a small difference between the sulfhydryl contents of monomer and polymer[2], which would indicate an oxidative mechanism, but some type of disulfide interchange reaction has not yet been ruled out. If a mechanism of the latter type were operative then the three functional groups would not be sulfhydryls, but rather particularly reactive disulfide groups. It is not inconceivable that the reaction may proceed by both of these mechanisms simultaneously. More detailed studies of the actual polymerization process are required to answer this question.

The conclusion that there are three functional groups on a monomer unit has been arrived at in an indirect manner, with the aid of reasonable, but only partially tested assumptions. Although the conditions which obtain during the polymerization of a number of small organic molecules have been shown to comply, to a first approximation, with the requirements of the STOCKMAYER theory[14], there have been no comparable studies with protein systems.

The physiological role of THMenz has long been of interest to this laboratory[1]. Since the enzyme has been shown to be essentially homogeneous, the calculation that it represents about 1 % of the total horse-liver proteins is strengthened. In view of the fact that a separate enzyme has recently been isolated from horse-liver preparations which also catalyzes the methylation of homocysteine to form methionine[5], and which accounts for about 50 % of the betaine-homocysteine methylpherase activity of horse-liver extracts, the large amounts of THMenz present in this tissue seems surprising. It is possible that the primary physiological role of this protein is related more closely to the reversible polymerization which it can undergo than to its enzymic activity. It is particularly interesting in this regard to note that the THMenz isolated from rat liver by FROMM AND NORDLIE[15] exhibits ultracentrifugal behavior which is analogous to that of the horse-liver enzyme[5]. The work of MAZIA and others[16] indicates that the protein of the mitotic apparatus may be capable of a reversible polymerization with the formation of intermolecular disulfide bonds, and that this process may play an important role in mitosis. A similar system may also be involved in the cell wall protein of yeast during the budding process[17].

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