THE SYNTHESIS OF METHIONINE BY ENZYMIC TRANSMETHYLATION*

V. CHROMATOGRAPHIC ANALYSIS OF THETIN–HOMOCYSTEINE METHYLPHERASE ON MODIFIED CELLULOSE COLUMNS

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SUMMARY

The chromatographic behavior of the enzyme thetin–homocysteine methylpherase has been examined on both DEAE-cellulose and CM-cellulose columns. The enzyme is not held on DEAE-cellulose, but this adsorbent has been found suitable for a final purification step. After DEAE-cellulose treatment, thetin–homocysteine methylpherase behaves as a single component during chromatography on CM-cellulose columns. It has been shown that there is no chromatographic difference between the monomer and the polymeric form of the enzyme. On the other hand, chromatographic evidence has been presented for the formation of a number of mixed disulfides of thetin–homocysteine methylpherase and various low molecular weight thiols. Such mixed disulfides often exhibit markedly altered chromatographic behavior and may be of some general significance.

INTRODUCTION

The enzyme THMenz has been purified from horse liver to the point where conventional protein fractionation techniques are not capable of effecting further changes in the composition of the preparation[1]. In view of the fact that THMenz accounts for 1 % of the protein of horse liver, it was considered of interest to study the chromatographic behavior of the preparation in order to obtain further information on the state of purity of the enzyme. It had been found that THMenz can, under very mild conditions, undergo a reversible polymerization reaction. The aggregates formed are unusual in that they are linked by disulfide bonds and not simply by secondary valence forces[1,2]. It was therefore also of interest to study the chromatographic behavior of the polymeric** form of the protein in comparison with that of the monomer.

Abbreviations: THMenz, thetin–homocysteine methylpherase; DEAE, diethylaminoethyl; CM, carboxymethyl.

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

** The polymeric form of THMenz invariably consists of a mixture of molecular species ranging from monomer to aggregates containing 50 or more monomer units. The composition of the mixture with respect to any one component is defined by the degree of polymerization in the manner predicted for a trifunctional monomer by the statistical theory of Stockmayer[4].

Protein chromatography on the modified cellulose columns of PETERSON AND SOBER[3] has found wide application in recent years. In the present study, both DEAE-cellulose and CM-cellulose columns were employed. Conditions suitable for the chromatographic analysis of THMenz were found with CM-cellulose as the absorbent, and no evidence of chromatographic heterogeneity of the unmodified enzyme was found. Furthermore, it has been found that monomer and polymer preparations of the enzyme behave identically during chromatographic analysis. During the course of these studies, a series of observations were made of the chromatographic behavior of mixed disulfides of THMenz with low molecular weight thiols. In some cases, such mixed disulfides were found to have markedly altered chromatographic behavior. It is believed that such results may be of some general significance.

MATERIALS AND METHODS

THMenz was prepared as previously described[1], with the modifications introduced in a more recent publication from this laboratory[2]. Electrophoresis had been previously used after the ammonium sulfate step to remove a small amount of contaminating, non-protein material. It has now been found to be more convenient to remove this substance by means of treatment with DEAE cellulose as described in the present communication. Enzyme activity was measured as previously described[1], with dimethylacetothetin serving as the methyl donor. Protein concentrations were determined by measuring the absorbance at 280 mμ in a Beckman model DU spectrophotometer using 13.0 as the extinction coefficient for a 1 % solution[4].

DEAE cellulose was obtained from Eastman Organic Chemicals and washed according to the directions of PETERSON AND SOBER[3]. CM cellulose was prepared from Solka-Floc according to PETERSON AND SOBER, except that the cellulose powder was not sieved before use. All other reagents were commercial products of the highest available purity.

Prior to chromatographic analysis, the enzyme preparations were dialyzed *versus* 1000 volumes of a buffer solution of the same composition as the starting buffer to be employed in the subsequent chromatography. In general, dialysis for a period of 4 h at 2° was found to be satisfactory, although a number of experiments were performed which involved longer dialysis. The enzyme preparations used were either polymer preparations, or monomer prepared as follows: the protein was first depolymerized by treating a 1-% solution at pH 7.4 with 2 mg/ml of homocysteine for 15 min at 37° and then dialyzed for 4 h in the buffer to be used in the subsequent chromatography, which also included 0.005 *M* thioglycollate. Under these conditions, no appreciable amounts of mixed disulfides were found on chromatography. In experiments designed to demonstrate the occurrence of mixed disulfides of the protein with the thiol of the buffer, dialysis was carried out for periods of at least 12 h.

The columns were packed with the adsorbent after titration to the proper pH and equilibration with the starting buffer. Packing was accomplished by gravity followed by the application of a pressure of seven pounds/in^2. All experiments were performed at 2°, and constant volume fractions were collected with the aid of a GME fraction collector.

RESULTS

Many proteins with isoelectric points in the neutral or acidic pH range have been successfully studied by chromatography on DEAE cellulose[5,6]. Since it has been shown that THMenz exhibits an isoelectric point of 6.4 in 0.05 *M* phosphate buffer[1], it was felt that this protein too should be amenable to chromatographic analysis using DEAE cellulose as the adsorbent. It was found, however, that THMenz is not adsorbed on DEAE cellulose under any conditions and that the activity may be quantitatively recovered as a sharp band emerging at the column front (Fig. 1). The chromatogram illustrated in Fig. 1 also shows that a second peak of u.v. absorbing material is eluted at a higher ionic strength. This material corresponds to the relatively acidic electrophoretic impurity observed by DURELL *et al.*[1] to be present in the purified enzyme preparation and is non-protein in nature. In the light of this observation THMenz preparations were routinely purified by passage through a DEAE column before further studies were carried out. The experiments reported in this paper were performed with such material unless otherwise stated. The chromatographic behavior of the enzyme was found to be unchanged after passage through DEAE. It will be seen below, however, that the chromatographic behavior of the protein can be greatly altered by chemical modification with certain thiols, and that some mixed disulfides of THMenz may be readily chromatographed on DEAE cellulose.

In view of the fact that the unmodified protein is not held on DEAE cellulose columns, the behavior of the enzyme was studied on columns of CM cellulose. It was found that the enzyme was readily adsorbed onto such columns and quanti-

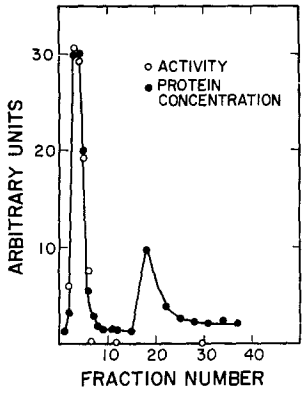

Fig. 1. The elution pattern of THMenz on DEAE cellulose. The starting solvent was 0.005 *M* in both phosphate and thioglycollate, and 0.001 *M* in Versene at pH 6.8. At Fraction 11, the buffer was changed to 0.4 *M* phosphate, 0.4 *M* sodium chloride and 0.001 *M* Versene. Activity and protein concentrations are expressed in arbitrary units. The dimensions of the column were 1 × 15 cm and the fraction size 2 ml.

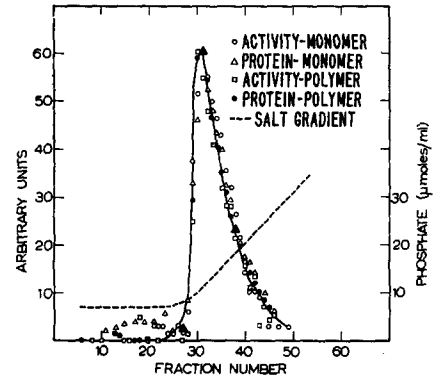

Fig. 2. Elution patterns of monomer and polymer on CM cellulose columns. The dimensions of the column were 1 × 15 cm, 10-mg samples were applied, and 2-ml fractions were collected. This figure is the superposition of two separate experiments performed under identical conditions. The activity and protein concentration figures are expressed in arbitrary units, normalized at the peak maximum. The initial buffer was 0.005 *M* in phosphate and 0.001 *M* in Versene at pH 6.1. In the case of the monomer, the buffer also included 0.005 *M* thioglycollate. The gradient was obtained by the addition of a buffer at pH 7.6 which is 0.1 *M* in phosphate to the starting buffer in a mixing chamber.

tatively eluted as a single peak by means of either sodium chloride or potassium phosphate gradients. The normalized results of two separate experiments with identical CM cellulose columns are shown in Fig. 2. In these experiments, the samples differed in that one was highly polymerized while the other was a monomer preparation. In the case of the monomer, the buffer also contained 0.005 *M* thioglycollate. It is readily seen that the two preparations exhibit identical chromatographic behavior, and that there is no evidence for the presence of appreciable amounts of extraneous material in the enzyme preparations. A number of similar experiments, under varying conditions, were performed in an unsuccessful attempt to resolve chromatographically the components of the polymeric mixture.

Mixed disulfides

Although THMenz normally behaves as a single substance on CM cellulose chromatography, it is possible to induce chromatographic heterogeneity by means of small changes in experimental procedure which result in readily reversible chemical modifications of the enzyme. THMenz polymer preparations exhibit only a low solubility in the dilute buffers employed in CM cellulose chromatography, but the solubility of the enzyme is appreciably enhanced by depolymerization of the protein. Accordingly, THMenz preparations are routinely depolymerized before preparative scale chromatography on CM-cellulose columns. When dialysis of the monomer against the column buffer containing 0.005 *M* thioglycollate is allowed to proceed overnight rather than the usual 4 h, chromatography on CM cellulose shows the presence of a number of components. This situation is illustrated in Fig. 3, in which three enzymically active components are seen to be present. In this chromatogram, the first peak also contains some inactive material, since this protein preparation had not previously been purified either electrophoretically or by DEAE chromatography. Each of the three components seen in the elution diagram was collected as an ammonium sulfate precipitate and depolymerized and dialyzed for the usual 4 h. On rechromatography, each of the three components behaved in a manner identical to that illustrated in Fig. 2. The material of the first peak was found to be resolved into a normally migrating active component, and an inactive one which still migrated with the column front. It is apparent therefore, that the heterogeneity seen in Fig. 3 is an artifactual result of the conditions of depolymerization and/or dialysis of the protein preparation. The slowest moving, and largest component of the elution diagram shown in Fig. 3 corresponds in its chromatographic behavior to unmodified protein. The two new peaks are held much less tightly onto the column, since they emerge prior to the beginning of the salt gradient. This behavior indicates that the protein emerging in the two fast peaks is more negatively charged than is the unmodified material. The ready restoration of normal chromatographic behavior to these materials by treatment with homocysteine followed by a short dialysis indicates that the modification observed may be due to mixed disulfide formation between the enzyme and thioglycollate in the buffer. In testing this hypothesis, it was observed that the conditions for obtaining appreciable amounts of chromatographic heterogeneity are twofold. In the first place, depolymerization must be effected with concentrations of homocysteine not higher than 3 mg/ml, and secondly, the subsequent dialysis *versus* buffer containing thioglycollate must be for a period of at least 10–12 h. Small variations in protein concentration did not affect these

results. When these conditions are met, appreciable amounts of fast moving materials are reproducibly obtained in CM cellulose chromatograms (Fig. 4). The figure also shows that if mercaptoethanol is used in the place of thioglycollate, no fast moving material is obtained, and that the same is true for mercaptoethylamine. With the

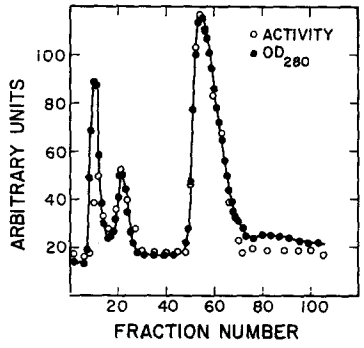

Fig. 3. Chromatography of THMenz on a preparative scale CM cellulose column. The sample size was 250 mg and the column dimensions 2.5 × 15 cm; 19-ml aliquots were collected. The initial solvent was 0.005 *M* in both phosphate and thioglycollate, and 0.001 *M* in Versene at pH 6.1. At fraction 35 gradient elution was begun with the initial solvent in the mixing chamber and the same buffer made 0.3 *M* in sodium chloride in the reservoir. Protein concentration and enzyme activities are expressed in arbitrary units normalized at fraction 54. See the text for discussion of this figure.

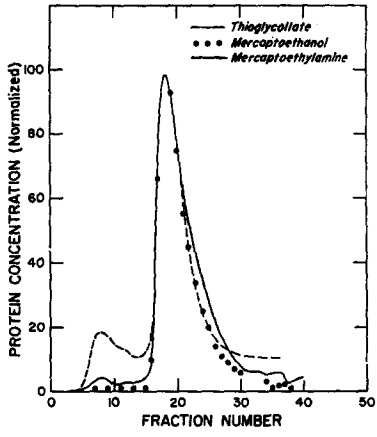

Fig. 4. The superimposed elution patterns of three separate experiments with 1 × 8 cm CM cellulose columns. The patterns shown are normalized and present only protein concentration data. Enzyme activities were found to superimpose well on these curves. 3-ml fractions were collected, under elution conditions identical to those described in Fig. 2 except that the starting buffer was 0.025 *M* in phosphate and that all solutions were 0.005 *M* in the appropriate thiol. See the text for further discussion of this figure.

latter thiol, however, there is a small but significant broadening of the protein band, indicating the presence of a new component which is eluted from CM cellulose columns at a slower rate than is the unmodified protein. The results of these experiments demonstrate that the chromatographic heterogeneity observed is the result of mixed disulfide formation, and further that the mixed disulfides contain the thiol of the buffer and not homocysteine. Mixed disulfides of the protein with the homocysteine used for depolymerization, although probably present, would not be expected to alter the chromatographic mobility of the protein, since at pH 6 there would be essentially no net change in the charge of the protein as a result of such a modification. For similar reasons, one should observe unchanged chromatographic characteristics when mercaptoethanol is employed in the buffer. Mixed disulfides of the protein with thioglycollate would, on the other hand, be expected to result in a more negatively charged protein at pH 6 and therefore account for the presence of fast moving components on CM cellulose columns, while mercaptoethylamine would be expected to have an effect in exactly the opposite direction.

Another kind of mixed disulfide, which can be demonstrated chromatographically, is that formed between THMenz and glutathione. If the enzyme is treated with

oxidized glutathione at 37° for 2 h, and then subjected to chromatographic analysis on DEAE cellulose columns, it is found that, in contradistinction to the untreated enzyme, the modified protein is held to the column and eluted as shown in Fig. 5.

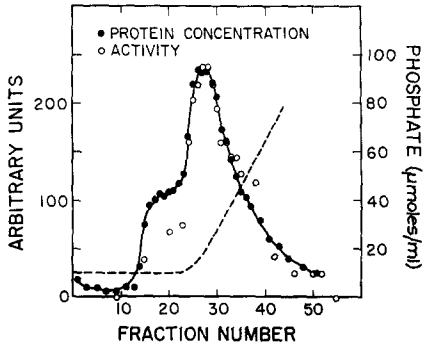

Fig. 5. Elution pattern of THMenz treated with oxidized glutathione on DEAE cellulose column. A 1 × 15 cm column was employed with conditions similar to those of Fig. 1, except that a phosphate gradient was applied instead of the second buffer. Sample size 10 mg, fraction size, 2 ml. Activity and protein concentrations are expressed in arbitrary units normalized at the peak maximum.

DISCUSSION

One of the major aims of this study was to gain information on the state of purity of THMenz. Chromatographic analysis of the enzyme on CM cellulose gives no indication of the presence of any major contaminants and is in agreement with the results presented earlier[1] and in the accompanying paper[4] in suggesting the essential homogeneity of the protein. The chromatographic heterogeneity seen under some conditions of treatment of the protein has been shown to be an artifactual one due to the formation of disulfide bonds between the protein and a thiol of net charge at pH 6. The potentially great sensitivity of chromatographic analysis is again illustrated here since the mixed disulfides observed cannot differ from the unmodified protein by very many charges. However, it is also apparent from the data presented here that alterations of the protein structure which do not affect the net charge may not be reflected in changed chromatographic behavior.

It is perhaps surprising in view of the acidic character of THMenz (isoelectric point of 6.4) that this protein is not held onto DEAE cellulose columns. This fact may be a reflection of an uneven distribution of charges on the surface of the protein so that more cationic groups are available for interaction with the cellulose than anionic ones, even though the latter predominate over the whole molecule. Alternately, it is also possible that the isoelectric point measured is low due to the binding of phosphate ions by the protein under the conditions of the mobility measurements. Such phenomena are known to sometimes cause large variations in the electrophoretic mobility of proteins[7]. DEAE cellulose chromatography has, however, proven to be a useful procedure for the removal of the last major contaminant known to be associated with THMenz, and has been so employed throughout the present work.

It is clear now, as the result of the work of a number of laboratories, that mixed disulfides may readily be isolated from thiol-disulfide systems[8,9]. Indeed, a number of measurements of the equilibrium constants of such systems indicates that they are of the order of 1 and that therefore high concentrations of such products may easily be formed under favorable conditions[10–12]. The results of the present study may be interpreted to mean that the mixed disulfides which were demonstrated on

CM cellulose chromatography are formed as the result of an interchange reaction between the thiol of the buffer and a mixed disulfide of the protein and homocysteine. Such a mechanism is indicated by the fact that the conditions of depolymerization of the polymer by homocysteine determine whether mixed disulfides with thioglycollate may be formed. A highly simplified scheme to illustrate what is believed to be the major pathway of mixed disulfide formation in this system is as follows:

$$\text{Protein–S–S–protein} + \text{RSH} \longrightarrow \text{protein–S–S–R} + \text{protein–SH} \quad (1)$$

$$\text{Protein–S–S–R} + \text{TSH} \longrightarrow \text{protein–S–S–T} + \text{RSH} \quad (2)$$

In the above scheme, RSH is homocysteine and TSH is thioglycollate; all protein species except protein–S–S–T, which represents the mixed disulfide with thioglycollate, migrate identically on CM-cellulose columns, for the reasons outlined above. The reactions outlined above represent only two of many which can take place. Others, such as the reaction of protein–SH with oxidized thioglycollate may be important routes for the formation of the mixed disulfides observed.

Recent studies by WHITE[13] indicate that, under certain conditions, impure thioglycollate preparations can effect a thiolation of proteins. It is believed that such a phenomenon cannot account for the results found in the present work, since the thiolation reaction results in amide bond formation between the thiol esters present in the thioglycollate and the protein. Such a reaction should not be found to be reversible by homocysteine treatment, whereas the mixed disulfides found in the present work are readily cleaved by such treatment. It is therefore felt that thiolation does not represent a major reaction pathway in this system.

The fact that the elution patterns of monomer and polymer preparations of THMenz on CM cellulose are identical is of particular interest in view of the demonstration that small alterations in the charge of the protein are reflected in large changes in chromatographic mobility. It seems apparent that the different forms of the enzyme must be very similar in net charge at pH 6. Thus, if the combination of monomer residues in the polymer molecules involves charged group interaction, this interaction must be such that equal numbers of cationic and anionic side chains are masked.

There is reason to believe that the alterations in chromatographic behavior induced by mixed disulfide formation may be a phenomenon of general significance. Many proteins are known to contain reasonably reactive sulfhydryl groups, and protein mixed disulfides have in the present study been shown to be formed under very mild conditions. During the course of purification, proteins are often exposed to thiols under conditions similar to those used in the present study, and it is not unreasonable to expect that many such protein systems contain appreciable concentrations of mixed disulfides. In view of this consideration, it should be borne in mind that the demonstration, by physical means, of the presence of a number of biologically active components in a protein system does not necessarily indicate that each of the active species represents a different protein. The ease with which a single protein can be induced to exhibit chromatographic heterogeneity as a result of relatively minor structural alterations is at the same time a demonstration of the effectiveness of cellulose column chromatography as an analytical tool and of the potential pitfalls involved when appropriate controls are not carried out or when the system under study is incompletely characterized.

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