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THE EXISTENCE OF HUMAN LIVER CYSTATHIONINE β-SYNTHASE IN MULTIPLE MOLECULAR FORMS

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Summary

1. Cystathionine β-synthase (L-serine hydro-lyase (adding homocysteine), EC 4.2.1.22) activity present in a fresh human liver supernatant prepared in 0.08 M Na_2HPO_4/K_2HPO_4 buffer, pH 6.5, was associated with two different molecular weight species (500 000 and 235 000 respectively) whereas in the corresponding supernatant fraction prepared in 0.2 M Tris · HCl buffer, pH 8.0, enzyme activity was associated with a species possessing a molecular weight of 130 000.

2. When fresh human liver supernatant fractions prepared in 0.08 M Na_2HPO_4/KH_2PO_4 buffer, pH 6.5 were maintained in the presence and absence of high concentrations of salt for prolonged periods, the enzyme species possessing a molecular weight of 235 000 disappeared to be replaced by species possessing molecular weights in the range 56 000—130 000. The enzyme species with the molecular weight of 500 000 did not alter appreciably under the same experimental conditions.

3. Human liver systathionine β-synthase can be partially purified by absorbtion on a column of DEAE-cellulose followed by gradient elution with increasing concentrations of salt. The resulting elution profiles were found to be dependent on the time scale of the experiment.

4. Relatively long preparation times yielded two distinct peaks of enzyme activity, peaks I and II respectively, whereas shorter preparation times resulted in the appearance of peak II only.

5. Peak I consisted of a mixture of molecular weight species (130 000—500 000) which were active enzymically, whereas peak II consisted mainly of a single active molecular weight species (500 000). Storage of both peaks in salt resulted in the appearance of lower molecular weight species.

Introduction

Previous reports on the preparation of human liver cystathionine β-synthase [L-serine hydro-lyase (adding homocysteine), EC 4.2.1.22], [1,2,3] have dealt

with relatively small scale preparations. For the purpose of performing structural studies on the purified enzyme, it became necessary to increase the amount of tissue being processed. As a result it became apparent that the nature of cystathionine synthase was sensitive to environmental conditions. This communication deals with the effects of varying experimental conditions on the nature of cystathionine β-synthase.

Materials and Methods

Chemicals

L-[U-^{14}C] Serine (spec. act. 10 Ci/mol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. DL-Homocysteine thiolactone HCl, pyridoxal 5'-phosphate and Dowex 50 (X8, 200—400 mesh, H^+form) ion exchange resin, were supplied by Sigma (London) Chemical Co. Ltd, Kingston, Surrey, U.K. Urease and Whatman DE52 DEAE-cellulose were obtained from British Drug Houses, Poole, Dorset, U.K. Sephadex G-200 and Dextran Blue were purchased from Pharmacia (U.K.) Ltd., London. Standard proteins for molecular weight determinations were obtained in kit form from the Boehringer Corp., (London) Ltd. Hollow fibre cartridges ($1 \cdot 10^4$ mol. wt. cut off) and XM-50 ultrafiltration membranes were supplied by the Amicon Corp., Lexington, Mass., U.S.A. All other chemicals used were of AnalaR grade.

Liver tissue

The starting material was postmortem samples of normal liver obtained from the victims of road accidents.

Assays

Cystathionine β-synthase activity was assayed essentially by the method of Mudd et al. [4]. One unit of enzyme activity is defined as the amount of enzyme producing 1 μmol cystathionine/h. Estimation of protein was performed according to Lowry et al. [5].

Determination of molecular weights

A column (1.5 cm × 90 cm) of Sephadex G-200 was equilibrated with either 0.08 M Na_2HPO_4/KH_2PO_4 buffer, pH 6.5 or with 0.2 M Tris · HCl buffer, pH 8.0 and calibrated in both systems using standard preparations of cytochrome C, myoglobin, pepsin, albumin, alcohol dehydrogenase, catalase and urease. The void volume of the column was determined with Blue Dextran and the inclusion volume with sodium dichromate. Samples (0.5 ml) of unknown molecular weight were applied in the appropriate phosphate and Tris buffers containing sucrose at a final concentration of 20%. Molecular weights were determined by reference to a standard graph according to the procedure of Andrews [6].

Results

Preliminary experiments employing 2 kg quantities of liver as starting material, yielded results which were consistent with the fact that human liver cystathionine β-synthase could exist in varying forms. Since such a phenomenon

influences any decision relating to the natural state of the enzyme, further studies on the nature of the enzyme under defined conditions were undertaken.

The effect of pH on the nature of cystathionine synthase in fresh tissue samples

The nature of cystathionine β-synthase activity in a crude tissue preparation was examined by preparing a 37.5% (w/v) suspension of human liver (obtained as soon as possible after death, 12 h) in 0.08 M Na_2HPO_4/KH_2PO_4 buffer, pH 6.5 and applying 5 ml of a clear supernatant fraction, obtained by centrifuging the suspension for 1 h at 14 000 × *g*, to a column (2 cm × 90 cm) of Sephadex G-200 equilibrated with the above phosphate buffer. Column development was achieved with starting buffer at a flow rate of 10 ml/h, the eluate being collected in 5 ml fractions. Fig. 1 illustrates the corresponding elution profile, from which it can be seen that enzyme activity was associated with two distinct molecular forms. The molecular weight of each form, determined by chromatography on a calibrated column of Sephadex G-200 was 500 000 and 235 000 respectively.

The effect of change of pH on the nature of the enzyme activity was determined by repeating the above experiment in an identical manner except that the phosphate buffer was replaced by 0.2 M Tris · HCl buffer, pH 8.0 (Fig. 1). The molecular weight of the predominant enzyme species was 130 000.

Effect of salt and ageing on enzyme activity

The effect of ageing and salt on the nature of cystathionine β-synthase activity was examined by maintaining portions of supernatant, prepared as previously described in 0.08 M Na_2HPO_4/KH_2PO_4 buffer, pH 6.5 at 4°C, in the

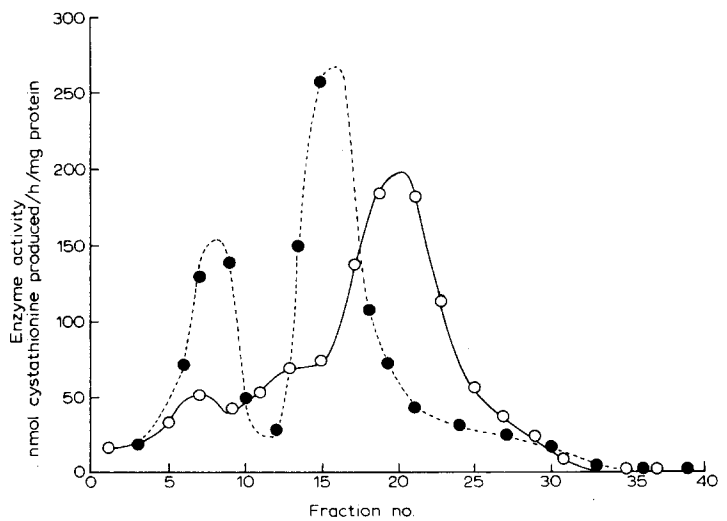

Fig. 1. Effect of pH on the nature of cystathionine β-synthase activity in a preparation of human liver supernatant. Samples of human liver supernatant (5 ml) prepared in either 0.08 M Na_2HPO_4/KH_2PO_4 buffer, pH 6.5 or 0.2 M Tris · HCl buffer, pH 8.0, were applied to a column (2 cm × 90 cm) of G-200 Sephadex previously equilibrated with starting buffer. Elution was performed using the appropriate buffer system. Eluates were collected in 5 ml fractions. ●- - - - -●, enzyme activity in supernatant prepared at pH 6.5; ○———○, enzyme activity in supernatant prepared at pH 8.0.

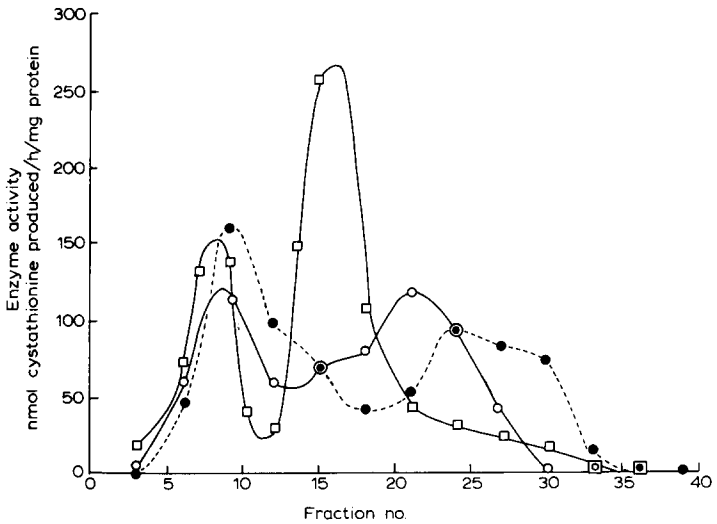

Fig. 2. Effect of storage in presence and absence of salt on nature of cystathionine β-synthase activity in human liver supernatant preparation. Human liver supernatant was prepared in 0.08 M Na_2HPO_4/KH_2PO_4 buffer, pH 6.5 and samples were maintained in the presence and absence of 0.3 M KCl at 4°C for 1 week. Portions (5 ml) of these aged preparations were then subjected to gel filtration with starting buffer on G-200 Sephadex (2 cm × 90 cm). Eluates were collected in 5 ml fractions. □———□, elution profile of fresh supernatant, ○———○, elution profile of aged supernatant; ●- - - - -●, elution profile of supernatant aged in presence of 0.3 M KCl.

absence and presence of KCl (final concentration 0.3 M) for two weeks. Sodium azide (0.01%) was included as a preservative. Gel filtration on Sephadex G-200 was performed as described previously. Fig. 2 shows the corresponding elution profiles. Changes in elution profiles resulted when storage was performed in the presence and absence of salt. Cystathionine β-synthase activity possessing a molecular weight of 500 000 was still identifiable in both systems, whereas the form with molecular weight 235 000 had disappeared, to be replaced by smaller enzymically active proteins, ranging in molecular weight from 56 000—130 000.

The significance of the changes observed in the above experiments, particularly those involving prolonged storage, could be questionable, since proteolytic activity which may be associated with crude tissue preparations could bring about alterations in the native structures. In order to remove this objection, similar experiments were performed using partially purified enzyme preparations.

Partial purification of human liver cystathionine synthase

Human liver (600 g) was suspended at 4°C in sufficient 0.08 M Na_2HPO_4/KH_2PO_4 buffer, pH 6.5 in a Waring Blender, to yield a 37.5% (w/v) suspension which was then centrifuged for 1 h at 14 000 × *g*. The clear supernatant which resulted was filtered through a nylon gauze (140 μm mesh) to remove fat, adjusted if necessary to pH 6.5 with 2 N NaOH and applied to a column (12 cm × 15 cm) of DEAE-cellulose equilibrated with 0.08 M Na_2HPO_4/KH_2PO_4 buffer, pH 6.5. After washing with starting buffer (2 l) until the eluate was

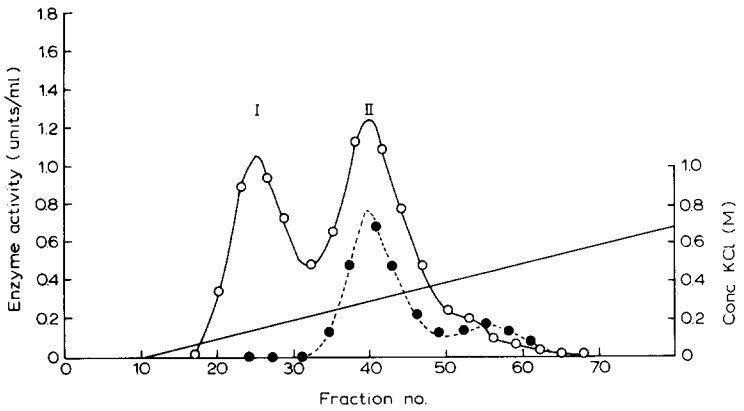

Fig. 3. Effect of time on gradient elution pattern of cystathionine β-synthase eluted from a column of DEAE-cellulose. Columns (4.5 cm × 20 cm) were run in 0.08 M Na_2HPO_4/KH_2PO_4 buffer, pH 6.5, employing a linear KCl gradient (0—0.7 M). The enzyme sample applied to the column consisted of a preparation obtained by the stepwise elution of a supernatant fraction from an identical column. Salt was removed by dialysis or hollow fibre treatment prior to application to column. Eluates were collected in 10 ml fractions. ○———○, enzyme preparation subjected to conventional dialysis, volume applied to column, 2 l, protein concentration 6.4 mg/ml; ●- - - - -●, enzyme preparation subjected to hollow fibre treatment, volume applied to column 400 ml; at protein concentration 28.4 mg/ml.

free from protein, cystathionine β-synthase activity was eluted by washing with starting buffer containing KCl at a final concentration of 0.3 M (2 l). The eluate was then dialysed for 24 h against two changes of starting buffer (total vol. 20 l) and the resulting material applied to a column of DEAE-cellulose (4.5 cm × 20 cm) equilibrated as previously described. After washing with starting buffer until the eluate was free from protein, the protein having enzyme activity was eluted employing a linear gradient of KCl (0—0.7 M) in 0.08 M Na_2HPO_4/KH_2PO_4 buffer, pH 6.5. Fig. 3 shows the elution profile obtained.

The above preparation was repeated in an identical manner, with the exception that the dialysis step was performed using an Amicon Hollow Fibre cartridge system (mol. wt. cut off 10 000) which was used to dialyse and concentrate the preparation (final vol. 400 ml). The total time taken for this step was 6 h. After applying the concentrate to a column of DEAE-cellulose, enzyme activity was eluted using a KCl gradient as described previously (Fig. 3). One major peak of activity was detected which corresponded in mobility to peak II of the previous preparation.

Analysis of peaks I and II by Sephadex G-200 chromatography

Peaks I and II were examined further by chromatography on a column (5 cm × 80 cm) of Sephadex G-200, equilibrated previously with 0.08 M Na_2HPO_4/KH_2PO_4 buffer, pH 6.5. Eluates from DEAE-cellulose corresponding to peaks I and II respectively were combined and concentrated separately to 30 ml using a Diaflo Ultrafiltration apparatus fitted with an XM·50 membrane (Amicon Corp.), before applying to the column of Sephadex G-200. Column development was performed using starting buffer at a flow rate of 20 ml/h, the eluate being collected in 10 ml fractions. Fig. 4 shows the eluation profiles of peaks I and II respectively. Peak I yielded a mixture of proteins of differing molecular

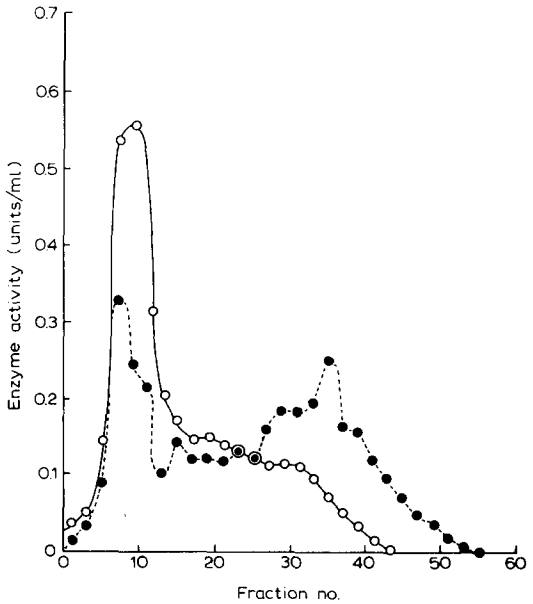

Fig. 4. Gel filtration of peaks I and II obtained from gradient elution on DEAE-cellulose. Preparations of peaks I and II (concentrated to 30 ml by membrane ultrafiltrations) were applied to a column of G-200 Sephadex (5 cm × 80 cm) equilibrated with 0.08 M Na_2HPO_4/KH_2PO_4 buffer, pH 6.5. Elution was achieved with the starting buffer at a flow rate of 20 ml/h. Eluates were collected in 10 ml fractions. ●- - - - -●, peak I from DEAE-cellulose; ○———○, peak II from DEAE-cellulose. Fraction 0 denotes end of void volume.

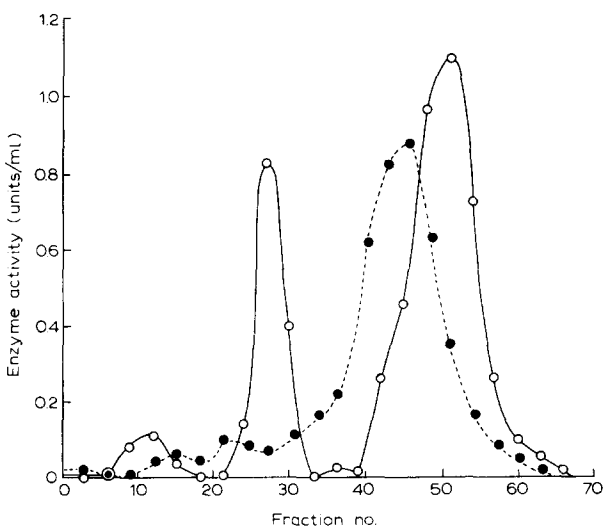

Fig. 5. Effect of storage in the presence of KCl on peaks I and II obtained from DEAE-cellulose chromatography. Peaks I and II were maintained in the presence of 0.08 M Na_2HPO_4/KH_2PO_4 buffer, pH 6.5, containing KCl at a final concentration of 0.3 M for 2 weeks at 4°C. Samples of each peak were then subjected to gel filtration on a column (5 cm × 80 cm) of Sephadex G-200. Eluates were collected in 10 ml fractions. ●- - - - -●, elution profile obtained from peak I; ○———○, elution profile obtained from peak II.

weights (130 000—500 000) which still possessed cystathionine β-synthase activity whereas peak II yielded one major active fraction (mol. wt. 500 000).

The above experiment was repeated in an identical manner except that both peaks were maintained in 0.08 M Na_2HPO_4/KH_2PO_4 buffer, pH 6.5 containing KCl at a final concentration of 0.3 M for 2 weeks at 4° C. Sodium azide (0.01%) was added as a preservative. Fig. 5 shows the corresponding elution profiles following chromatography on Sephadex G-200. It may be seen that the original elution profiles associated with peaks I and II have undergone changes such that now most of the enzyme activity appears in the form of lower molecular weight molecules. Storage of high molecular weight cystathionine β-synthase in salt for relatively long periods thus promotes the formation of lower molecular weight enzyme.

Discussion

It is now apparent that human liver cystathionine β-synthase is capable of existing in quite distinct molecular forms which are also enzymically active. The precise nature of the enzyme obtained in any given preparation would depend upon the experimental conditions employed in the isolation, since alterations in salt concentration, pH and age are all capable of influencing the molecular status of the system. This phenomenon is not uncommon with enzymes which possess large molecular weights. Partial explanations for the conflicting reports that have appeared on human liver cystathionine β-synthase are now forthcoming from the present investigation. Quite different values for the molecular weights of human liver cystathionine β-synthase have appeared in the literature [1,2,3] ranging from 120 000—450 000. It is now possible to rationalize this situation when due account is taken of the experimental conditions. Different isolation condition were employed in each of the above preparations and it may be concluded that individual experiments contained an inbuilt bias for the production of specific molecular species.

Similar conclusions could be formulated to explain anomalous observations on the corresponding system isolated from rat liver. It is clear from the investigations of Brown and Gordon [7] and those of Kimura and Nakagawa [8] that the rat liver enzyme also exists in a number of different molecular states, presumably for the same reasons outlined for the human liver system. Again an examination of the rat liver preparations employed by these workers reveals the anticipated differences, in variables such as pH and ionic strength. The possibility thus exists that the conditions used in the isolation of the rat liver systems were directly responsible for the observed differences in the molecular properties.

The presence of two enzymically active proteins following DEAE-cellulose chromatography, yielding in one instance a species possessing mixed molecular weight forms, is not identical to the pattern of published observations [2], where both peaks were reported to yield species possessing molecular weights approximating to 250 000. These inconsistencies could be reconciled by differences in age of starting material, duration of experiment as well as differences in purification procedure. Previously it was suggested [2] that the two peaks of activity represented isoenzyme forms, but the possibility that they could arise

by a dissociation-reassociation phenomenon should not be discounted.

Rat liver cystathionine β-synthase is known to be composed of two non-identical subunits of molecular weight 52 000 and 73 000 respectively [9]. If a similar situation obtains with the corresponding human enzyme, then the various compound molecular species reported in this communication would correspond to an octamer, a tetramer and a dimer respectively.

Braunstein et al. [10] have concluded that cystathionine β-synthase and serine sulphydrase activities are properties of a single enzyme. This conclusion was based on a comparison between chicken liver serine sulphydrase and rat liver cystathionine β-synthase. Serine sulphydrase has been reported to possess molecular weights in the range 90 000—130 000 [11,12], whereas cystathionine β-synthase was assumed by Braunstein et al. [10] to possess a molecular weight of 250 000. The conclusions of these workers [10] should now be considered in the light of the observations made in the present investigation. What is called serine sulphydrase could be an activity that is associated with a part of the cystathionine β-synthase complex. The published procedures for the preparation of serine sulphydrase [12] would tend to favour the production of a low molecular weight product. Serine sulphydrase activity associated with protein possessing a high molecular weight was observed in this preparation [12] but was not commented on further. There is some evidence available to support the possibility that serine sulphydrase is a fragment of cystathionine β-synthase (Ansell, P. and Tudball, N., unpublished observations) but more information is required before unequivocal statements can be made.

It is interesting to speculate whether or not the existence of cystathionine β-synthase in multiple molecular forms has any physiological significance. Since the enzyme is operational at a junction point in a metabolic pathway, serving to valve the conversion of methionine to cysteine it is to be expected that it will be subject to rigid control. Some factors capable of affecting the levels of cystathionine β-synthase have been described [13]. These regulatory factors could be involved in altering the molecular complexity of the enzyme with consequent changes in activity. Kimura and Nakagawa [8] have also commented on this possibility for the rat liver system. Preliminary investigations seem to indicate that substrates for cystathionine β-synthase induce changes in the molecular weight of the human liver enzyme (Ansell, P. and Tudball, N., unpublished observations). In this context it is worth noting that sigmoid shaped progress curves have been observed for the rat liver system using homocysteine as substrate [14]. Too much conjecture in this direction would however, be unwise at this stage since the results of Kashiwamata and Greenberg [15] are not consistent with these findings [14]. Further investigations into the molecular properties of cystathionine β-synthase are currently in progress in order to provide answers to some of the questions posed by the present investigation.

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