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CoAS–SGLUTATHIONE AND GSSG REDUCTASES FROM RAT LIVER

TWO DISULFIDE OXIDOREDUCTASE ACTIVITIES IN ONE PROTEIN ENTITY

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SUMMARY

The characteristics of the NADPH-dependent CoAS–Sglutathione reductase from rat liver have been studied. In contrast to the enzyme obtained from yeast during the purification procedure using different types of column chromatography and isoelectric-focusing technique, the new reducing enzyme in rat liver was found to purify together with GSSG reductase. The data indicate that in liver both enzymatic activities belong to the same protein entity, since they cannot be separated by any of the above regular physical methods.

The CoAS–Sglutathione and GSSG reducing activities have a molecular weight of 42 500; a pI of 6.85 and do not reduce cystine, cystamine, pantethine or insulin.

On the other hand, an NADPH-dependent non-specific disulfide reductase also present in rat liver extracts can be easily separated and distinguished from the GSSG and CoAS–Sglutathione-reducing enzymes by ion-exchange column chromatography and isoelectric-focusing.

INTRODUCTION

In this work we wish to present data which indicate that the originally described NADPH-dependent CoAS–Sglutathione reductase (EC 1.6.4.6) in rat liver [1] is identical in its molecular weight and net charge, to GSSG reductase (EC 1.6.4.2) when these have been measured by gel filtration and isoelectric-focusing, respectively. This finding differs from a previous one done with yeast [2], where the new disulfide reducing enzyme could be partially separated by a physical method from the GSSG reductase.

Recently an NADPH-dependent non-specific disulfide reductase from rat liver was reported to reduce different disulfide compounds and to require a thermostable

Abbreviation: DTNB, 5′,5-dithio-bis-(2-nitrobenzoic acid).

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protein cofactor [3, 4]. Evidence which indicates that the GSSG and CoAS–Sglutathione reductases are different from the non-specific disulfide reductase is also presented.

MATERIALS AND METHODS

Substrates

CoAS–Sglutathione was prepared as previously described [5]; GSSG, cystamine, cystine, pantethine, bovine insulin and DTNB (5',5-dithio-bis-(2-nitrobenzoic acid)) were obtained from Sigma Chemical Co., and used without further purification.

Enzymatic assay

The conditions for the measurement of GSSG and CoAS–Sglutathione reducing activities have been established [2, 5]. However, due to the limited amounts of CoAS–Sglutathione as substrate, during the purification procedure, we used 75 nmoles in each assay.

For the measurement of the non-specific reductase, the assays were made against a blank that contained 5 mM Tris–HCl (pH 7.5), 1 mM EDTA, 350 nmoles of DTNB as substrate and the enzyme preparation in a final volume of 1.0 ml. The reaction was initiated by the addition of 100 nmoles of NADPH to the experimental cuvette. The absorbance change was followed at 412 nm; this corresponds to the maximal absorbance for reduced DTNB.

Enzymatic preparations

In order to facilitate comparison of results, the conditions described by Tietze [3] for the isolation of the non-specific disulfide reductase were followed in the initial steps of the preparation of CoAS–Sglutathione and GSSG reductases. Approximately 60 g of rat liver in each case were homogenized in 90 ml of 100 mM Tris–HCl (pH 7.5), 0.25 M sucrose and 1 mM EDTA. The homogenate was centrifuged at 105 000 $\times$ *g* for 45 min, and the supernatant precipitated with $(NH_4)_2SO_4$ at 40–65% saturation. The precipitate was collected by centrifugation at 15 000 $\times$ *g*, suspended in 10 ml of 5 mM Tris–HCl (pH 7.5) 1 mM EDTA and dialysed for 24 h against the same buffer. The precipitate that remained after dialysis was discarded by centrifugation. The material was applied to a DEAE-cellulose column (2.5 cm $\times$ 40 cm) equilibrated with a buffer of 5 mM Tris–HCl (pH 7.5) 1 mM EDTA and resolved with a linear gradient of NaCl that ranged from 0 to 0.5 M. The fractions that contained the GSSG and CoAS–Sglutathione reducing enzymes (Peak A in Fig. 1) were pooled, concentrated with Aquacide and applied to a column of Sephadex G150 (5 cm $\times$ 75 cm). The column was eluted with 2 l of 100 mM Tris–HCl (pH 7.5) and 1 mM EDTA. The material collected from the Sephadex column was dialysed against triple-distilled water for 24 h and processed with a calcium phosphate gel column according to Massey and Williams [6]. The column was eluted successively with 75 ml of distilled water, 75 ml of 50 mM of sodium phosphate buffer (pH 7.5) and 1 mM EDTA, and finally with 75 ml of 100 mM of the same buffer. The GSSG and CoAS–Sglutathione reducing enzymes appeared in this last elution fraction. The enzymatic material was concentrated and dialysed against distilled water before further purification by means of isoelectric-focusing.

The fractions corresponding to the non-specific reductase (Peak B in Fig. 1) measured with DTNB as substrate, were processed with a Sephadex column. The enzymatic material was precipitated with 60% $(NH_4)_2SO_4$ and stored at 2–4 °C for later use.

Isoelectric-focusing technique

The method developed by Vesterberg and Svenson [7] was followed for the separation and determination of the isoelectric point of the disulfide reducing activities in the conditions previously specified [2].

Protein measurement

The protein content of the various fractions was estimated by the method of Lowry et al. [8] with bovine serum albumin as standard.

Cofactor preparation

The thermostable protein cofactor reported by Tietze [4] to be necessary for the non-specific reductase activity was prepared and assayed with the various reducing activities, in the presence of several disulfides as substrates.

Molecular weight estimation

The method of Andrews [9] was followed for the estimation of the molecular weight of proteins by gel filtration. A column of Sephadex G-100, particle size 40–120 μm (2.5 × 65 cms) with 5 mM Tris–HCl (pH 7.5) 1 mM EDTA was used. The following standards were utilized: transferrin, serum albumin, hemoglobin, ovalbumin and cytochrome *c*.

RESULTS

During the purification by a DEAE-cellulose column, the GSSG and CoAS–Sglutathione-reducing enzymes obtained from rat liver extracts eluted together and kept a similar ratio of activity as in the previous step in which these enzymes were precipitated with $(NH_4)_2SO_4$ (Table I and Fig. 1, Peak A). On the other hand, the non-specific disulfide reductase followed a different pattern of elution and thus was separated from the other two reducing activities (Fig. 1, Peak B). Thereafter, the two fractions (A and B) were studied separately: the material from Peak A was passed first through a column of Sephadex G-150 and later on through a calcium phosphate gel column. The non-specific reductase was purified only to the step of chromatography in a Sephadex G-150 column (Table I). As a final step, the fractions from Peaks A and B were purified by means of isoelectric-focusing.

The GSSG and CoAS–Sglutathione-reducing enzymes (from Peak A) were purified 385 and 289 times, respectively, while the non-specific reductase (from Peak B) only 68 times. A summary of the results appears in Table I.

The determination of the activity of the three enzymes as a function of pH is shown in Fig. 2. CoAS–Sglutathione-reducing enzyme has an optimum pH of 5.75; the GSSG reductase of 7.0 and the non-specific reductase of 7.5.

The apparent Michaelis constant for the CoAS–Sglutathione-reducing enzyme from liver at a fixed value of NADPH (110 nmoles) at pH 5.75, was calculated by

TABLE I

PURIFICATION OF THE GSSG REDUCTASE, CoAS–SGLUTATIONE REDUCTASE AND THE NON-SPECIFIC REDUCTASE FROM RAT LIVER

Step	GSSG reductase			CoAS–Sglutathione reductase			Non-specific reductase		
	Total protein (mg)	Specific activity (munits)	Purification (-fold)	Specific activity (munits)	Purification (-fold)	Ratio GSSG red. / CoAS–Sglutathione red.	Total protein (mg)	Specific activity (munits)	Purification (-fold)
40–65% $(NH_4)_2SO_4$	7800	41.2	1	4.5	1	9.1	7800	27.2	1
DEAE-Cellulose	650	500	12.1	55	12.2	9.0	500	150	5.5
Sephadex G-150	62.2	1 770	43.0	182	40.4	9.7	15.4	720	26.4
Calcium phosphate	12.6	4 050	98.3	454	100	9.0	—	—	—
Polyampholine (pH range 3–10)	0.6	15 870	385	1,301	289	12.2	2.18	1852	68

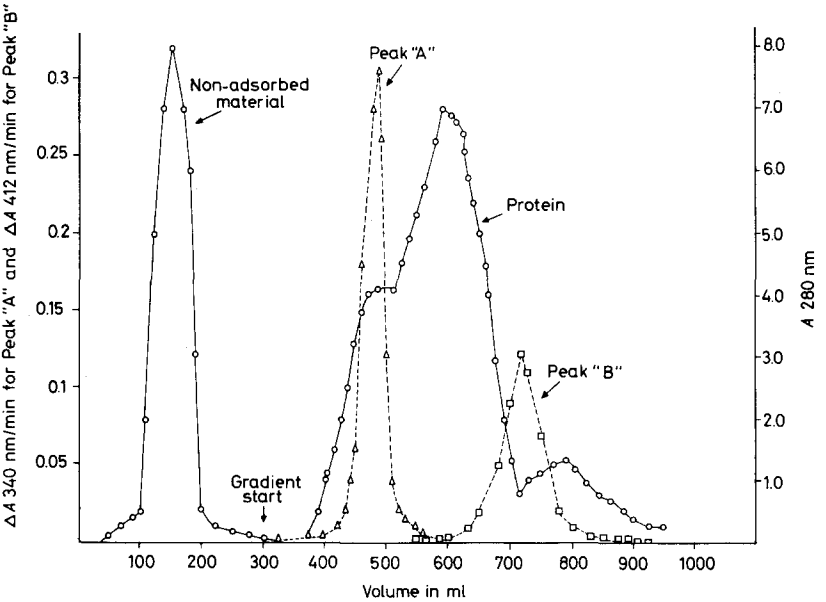

Fig. 1. Separation of 40–65% $(NH_4)_2SO_4$-precipitable material from rat liver by DEAE-cellulose column chromatography (2.5 cm × 40 cm) equilibrated with a buffer of 5 mM Tris–HCl (pH 7.5) 1 mM EDTA and resolved at a flux rate of 30 ml/h using a NaCl gradient from 0 to 0.5 M. Fractions of 5 ml were collected. The enzymatic activities were determined with 50-μl aliquots from each fraction as described in Materials and Methods. Peak A corresponds to GSSG and CoAS–Sglutathione reductases and Peak B to the nonspecific reductase. Due to limited amounts of CoAS–Sglutathione as substrate, Peak A was localized only by its GSSG reductase activity, but the ratio (GSSG-reductase)/(CoAS–Sglutathione reductase) = 9 was established in the pooled material from Peak A (see Table I).

plotting the reciprocal of initial velocities against substrate concentration according to the Lineweaver–Burk Method [10]. The value was $2.3 \cdot 10^{-4}$ M.

A molecular weight of 42 500 for GSSG and CoAS–Sglutathione reductases and of 47 000 for the non-specific reductase was found by gel filtration in Sephadex G-100, by the method of Andrews [9], (Fig. 3). Mize et al. [11] have reported, on the basis of sedimentation velocity and diffusion techniques, a molecular weight of 44 000 for GSSG reductase from rat liver, which is consistent with the results.

In order to determine the isoelectric point and homogeneity of Peaks A and B, they were mixed and subjected to isoelectric-focusing. The GSSG and CoAS–Sglutathione reducing enzymes showed an identical isoelectric point (Peak A in Fig. 4) and separated from the non-specific reductase, which appeared in a different fraction (Peak B in Fig. 4). It was found that the GSSG and the CoAS–Sglutathione reducing enzymes have a pI of 6.85, while that of the non-specific reductase is 5.35.

To increase the accuracy in the separation of GSSG and CoAS–Sglutathione reductases, a pH gradient between 5 and 8 was chosen (Fig. 5). The results indicate that even within this pH range, both the GSSG and CoAS–Sglutathione reducing enzymes remained in a single peak and the ratio of activities was constant between the two extremes of the maximum peak. A pI of 6.85 was found; the same result has been obtained in several experiments. It is worthwhile mentioning that when using this

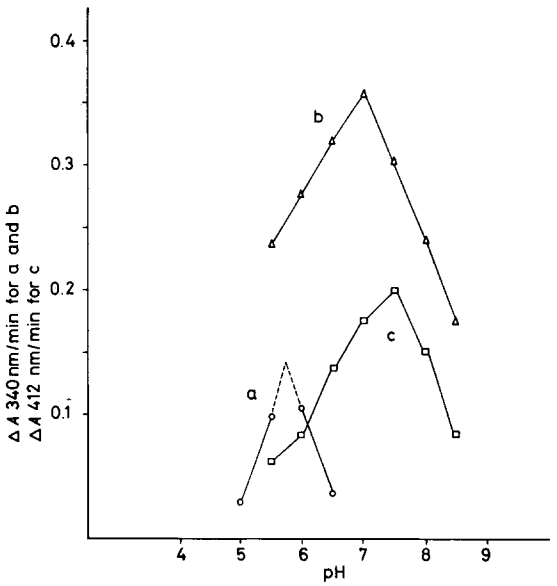

Fig. 2. Effect of the pH on GSSG and CoAS–Sglutathione reductases and the non-specific disulfide reductase activity. The incubation conditions were as follows: (a) for CoAS–Sglutathione reductase 100 mM Tris–HCl buffer, 1 mM EDTA; 110 nmoles of NADPH; 125 nmoles of CoAS–Sglutathione plus 100 μg of protein in a final volume of 1.0 ml. (b and c) for GSSG reductase activity and the non-specific reductase, 100 mM Tris–HCl buffer, 1 mM EDTA; 110 nmoles of NADPH; 350 nmoles of gluthathione or DTNB accordingly; plus 100 μg of protein in a final volume of 1.0 ml.

technique in a pH range between 5 and 8, two disulfide reducing activities appeared at a pI of 6.75 and 6.65 (see Fig. 5). Whether these two latter activities are isoenzymes of the reducing activity remains to be established.

The enzyme specificity in the two preparations obtained from the DEAE-cel-

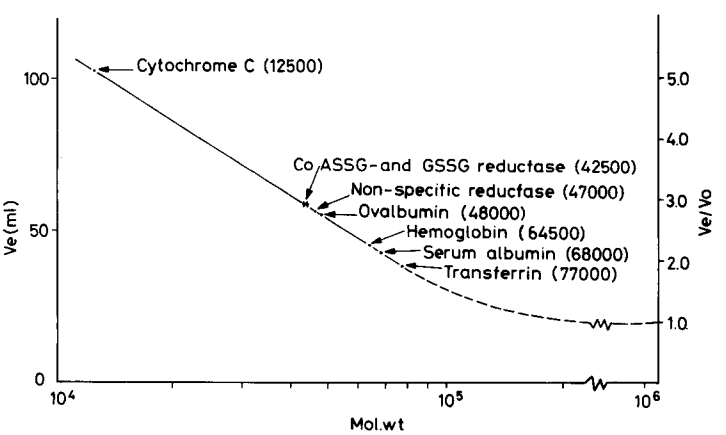

Fig. 3. Plot of elution volume V_e against log (molecular weight) for CoAS–Sglutathione reductase, GSSG reductase, non-specific reductase and various proteins used as standards. The results were obtained by the method of Andrews [9] under the conditions described in Materials and Methods.

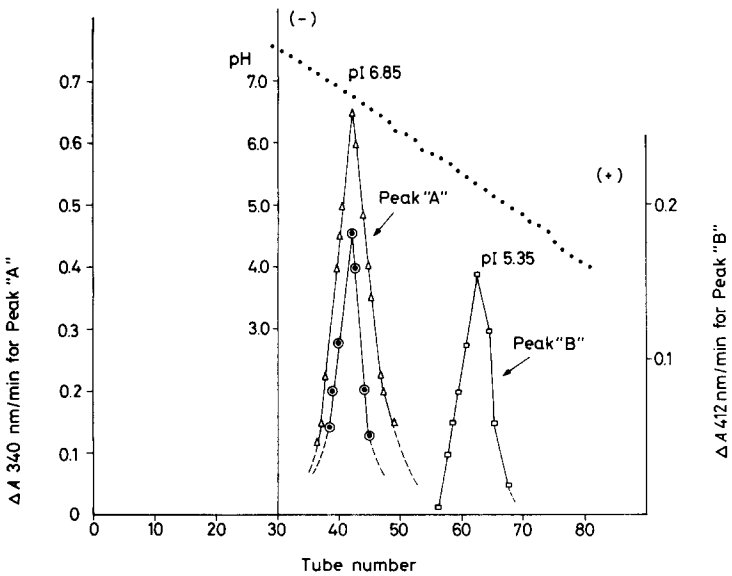

Fig. 4. Treatment of GSSG and CoAS–Sglutathione reductase (12.6 mg of protein from Peak A) and non-specific reductase (15.4 mg of protein from Peak B) by means of isoelectric-focusing [7], with an electrophoresis column (LKB 8100 of 110 ml of capacity), in a concentration gradient from 6 to 2% of carrier ampholytes within a pH range of 3 to 10. The initial charge of the column was 550 V, 6.5 mA. The experiment was carried out at 5° for 72 h. Fractions of 1.0 ml were collected. The enzymatic activities were determined with 10 μl GSSG reductase and 50 μl for CoAS–Sglutathione reductase, under optimal conditions. △—△, GSSG reductase, ⊙—⊙, CoAS–Sglutathione reductase.

lulose column (see Fig. 1 Peaks A and B) and further purified by Sephadex G-150, was measured with several disulfides as well as their dependence on the thermostable protein cofactor [4]. The enzymatic preparation from Peak A reduced GSSG, CoAS–Sglutathione and DTNB in that order of effectiveness. There was no effect of the protein cofactor on either of these activities (see Table II). The non-specific disulfide reductase (Peak B) reduced DTNB, cystine, GSSG, pantethine, insulin and cystamine. With this latter enzyme there was a marked enhancing effect of the protein cofactor with all the substrates assayed, except with DTNB where the cofactor decreased the reducing activity (see Table II).

DISCUSSION

During the various steps of purification, the GSSG reductase and the CoAS–Sglutathione reductase from rat liver maintained a close relationship in the ratio of activities and followed a parallel pattern of purification. On the other hand, the non-specific reductase was easily separated from the above enzymatic activities during the second step of purification with a DEAE-cellulose column. These findings, together with the studies on the specificity, indicate that the CoAS–Sglutathione reductase is on a distinct protein entity entirely different from the non-specific disulfide reductase studied by Tietze [4]. Indeed his studies suggested that this later activity may corre-

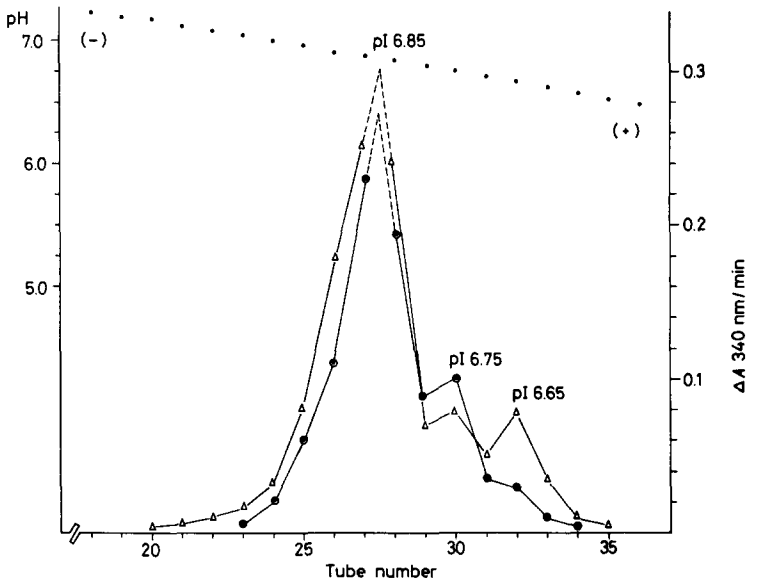

Fig. 5. Treatment of GSSG and CoAS–Sglutathione reductase (30 mg of protein obtained from the phosphate gel column step) by means of isoelectric-focusing [7] with an electrophoresis column (LKB 8100 of 110 ml of capacity) in a concentration gradient from 6 to 2% of carrier ampholytes within a pH range of 5 to 8. The initial charge of the column wsa 450 V, 6 mA. The experiment was carried out at 5° for 48 h. Fractions of 1.0 ml were collected. The enzymatic activities were determined with 20 μl GSSG reductase and 100 μl for CoAS–Sglutathione reductase under optimal conditions. △—△, GSSG reductase, ⦿—⦿, CoAS–Sglutathione reductase.

TABLE II

ENZYME SPECIFICITY AND EFFECT OF A THERMOSTABLE PROTEIN COFACTOR ON THE REDUCTION OF SEVERAL DISULFIDE COMPOUNDS

Peak A, contains the GSSG and CoAS–Sglutathione reducing activities passed first through a DEAE-cellulose column and further purified by filtration with a column of Sephadex G-150 (see Materials and Methods). Peak B, contains the non-specific reducing activity obtained as described (see Materials and Methods). In each experiment, where indicated, 140 μg of thermostable protein cofactor were added. The incubating conditions were: 100 μg of enzyme protein; 110 nmoles NADPH; 350 nmoles of substrate (except for CoAS–Sglutathione where 125 nmoles were used) and 100 mM Tris–HCl (pH 7.5) with 1 mM EDTA in a final volume of 1 ml. The buffer used for CoAS–Sglutathione reducing activity was of sodium phosphate (pH 5.75).

Substrate	Enzyme preparation (munits)			
	Peak A		Peak B	
	No cofactor	Cofactor	No cofactor	Cofactor
GSSG	1280.00	1280.0	12.8	29.00
CoAS–Sglutathione	96.6	100.0	0.64	1.28
DTNB	59.0	53.2	1500.00	1154.00
Cystine	1.28	1.28	13.58	41.8
Cystamine	2.58	2.58	3.22	58.06
Pantethine	1.93	1.93	6.44	25.8
Insulin	1.28	1.28	7.1	71.0

spond to the wellknown thioredoxine reductase originally described in *Escherichia coli* [12] and in rat hepatoma [13].

For a long time the GSSG reductase was considered to be a very specific enzyme [14]. Such is the case for CoAS–Sglutathione reductase of yeast, since it does not reduce substrates such as CoAS–SCys, GS–SCys and Cys–Cys [2]. Furthermore, Mannervick and Nise [15] established that for GSSG-reductase of yeast, the mixed disulfide of pantethine–glutathione is a bad substrate, although Smith [16] recently discovered that the di γ-glutathione cystine can be a substitute for GSSG as a substrate in the reaction that catalyses the GSSG reductase.

In the present work it has been possible to determine very clearly that both liver enzymes, GSSG reductase and CoAS–Sglutathione reductase, do not reduce disulfides such as cystine, cystamine, pantethine and insulin in a significant form; moreover, their activities are not enhanced by the protein cofactor.

It is interesting that CoAS–Sglutathione reductase and GSSG reductase reduce DTNB to a limited extent, however, this reduction amounts to about 5% of that observed with GSSG as substrate. On the other hand, DTNB is the most effective substrate for non-specific reductase. Clearly this is further evidence of the high specificity of CoAS–Sglutathione reductase and GSSG reductase.

Previous studies obtained with yeast GSSG reductase and CoAS–Sglutathione reductase [2] have indicated that both enzymes have an identical molecular weight ($108 \cdot 10^3$), but differ in their isoelectric points (pI of 5.9 for GSSG reductase and 5.1 for CoAS–Sglutathione reductase), which opens the future possibility of a total separation from each other in this material.

On the other hand, according to the present results, GSSG reductase and CoAS–Sglutathione reductase from rat liver may form part of the same protein entity, since both enzymes have not only an identical molecular weight (42 500), but also an identical isoelectric point (pI 6.85).

It should be pointed out that, so far as it is known to date, the isoelectric focusing technique is the best tool to determine the purity of proteins.

This evidence opens the question as to how in liver in a mechanistic sense, the two disulfide reducing activities reside in the same protein and as to what is the physiological significance of this finding. These questions will be explored in future studies.

NOTE ADDED IN PROOF (received March 1st, 1974)

During the printing of this paper we received a personal communication from Bengt Mannervik, University of Stockholm, Sweden, informing us that in his laboratory they have been unable to separate CoAS–SG-redactase either from commercial yeast GSSG-reductase, or in preparations from rat liver. We would, in this sense, like to mention that in yeast we could separate the two enzymes only when using the highest ampholyte concentration in the isoelectric focusing technique (6 to 2%). Mannervik reports to have used 1%. In our experiments on rat liver, as have been described in this paper, we used the best possible conditions to separate the two enzymes and have been unsuccesful.

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