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PURIFICATION AND PROPERTIES OF MERCAPTOPYRUVATE SULFUR TRANSFERASE OF *ESCHERICHIA COLI*

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SUMMARY

The enzyme which catalyzes the conversion of mercaptopyruvate to pyruvate and elemental sulfur has been isolated from *Escherichia coli* as a single entity. Mercaptopyruvate sulfurtransferase (β-mercaptopyruvate:cyanide sulfurtransferase, EC 2.8.1.2) was indicated to be homogeneous from ultracentrifuge data and from elution patterns of gel filtration. The molecular weight was determined by sedimentation equilibrium ultracentrifugation to be 23 800; the sedimentation coefficient, $s^{\circ}_{20,w}$, was 2.48. Other constants indicated a typical globular protein. An isoelectric point was found at pH 4.2 by electrophoresis on cellulose acetate. The absorption spectrum revealed no prosthetic groups on the enzyme. Zinc was detected in ratio of 1 atom/mole and copper, presumably a contaminant, at approximately 0.5 mole/mole. The metals were not removed by extensive dialysis against 10^{-3} M EDTA. In solutions of low ionic strength, the enzyme dissociated into two active fragments of approximately 12 000 molecular weight. Urea inactivated the enzyme but the effect was reversed by dialysis, dilution, or electrophoresis. The enzyme was stabilized in 0.8 M KCl.

Kinetic studies indicated mercaptopyruvate sulfurtransferase was inhibited by the accumulation of persulfide sulfur unless an acceptor was present in the system. Apparently the transfer of persulfide sulfur from the enzyme to acceptors is not enzyme catalyzed. The optimum pH for the formation of persulfide sulfur, pH 9.3–9.6, was raised somewhat by the presence of cyanide. The enzyme was inhibited by the other product of the reaction, pyruvate. Pyruvate reacted with the substrate also. There was no indication of a function of associated metals, zinc or copper, in the mechanism of catalysis.

INTRODUCTION

Mercaptopyruvate sulfurtransferase (β-mercaptopyruvate: cyanide sulfurtransferase, EC 2.8.1.2) has been found in mammalian systems and in microorganisms[1–5]. In the presence of cyanide, the enzyme resembles rhodanese, (thiosulfate: cyanide sulfur-

Abbreviation: Bis, 2-amino-2-methyl-1,3-propanediol diamine.

transferase, EC 2.8.1.1) in its action, thiocyanate being formed. The initial products of the enzyme catalysis, pyruvate and elemental sulfur, were first described by MEISTER *et al.*[1]. The precipitation of free sulfur in the incubation medium does not occur in the presence of mercaptoethanol[1], cyanide[2], sulfite, or sulfinates[6]. The sulfur is transferred to the above acceptors and appears as mercaptoethanol persulfide, thiocyanate, thiosulfate, or thiosulfonates, respectively. In the absence of an acceptor, the sulfur remains bound to the enzyme, or to protein in the medium, probably as a persulfide[7]. A similar mercaptopyruvate sulfurtransferase has been somewhat purified from rat liver by FANSHIER AND KUN[8] who characterized it as a copper protein. Subsequently the enzyme has been isolated free of copper[9].

The present paper reports the isolation of a mercaptopyruvate sulfurtransferase from *Echerichia coli* in a highly purified form. Its chemical and physical properties have been determined and a kinetic study of the reaction has led to some observations on the mechanism of its catalytic action.

MATERIALS

Crystalline bovine serum albumin was obtained from Pentex, DEAE-cellulose, medium mesh, from Sigma, Sephadex G-100 from Pharmacia, cellulose acetate strips (PhoroSlide) from Millipore, and Silicone antifoam AF emulsion from Dow-Corning. Mercaptopyruvate was prepared as the ammonium salt according to the method of KUN[10] as modified by HYLIN AND WOOD[7]. Other chemicals were obtained from various commercial sources.

METHODS

Enzyme assays during isolation procedures were more convenient when thiocyanate was determined; the rate of pyruvate formation was higher and provided greater insight into the nature of the catalysis.

Enzyme assays—thiocyanate formation. For routine isolation procedures the incubation mixture contained 225 μmoles of 2-amino-2-methyl-1,3-propanediol diamine HCl buffer (Bis buffer), pH 9.55, 15 μmoles of ammonium mercaptopyruvate, 12 μmoles of KCN, 120 μg of bovine serum albumin, and an appropriate amount of enzyme in a total volume of 0.75 ml. The enzyme solution was diluted in 0.5 M Bis buffer containing 0.15 mg of bovine serum albumin per ml. The incubation was carried out for 10 min at 37° and was stopped by addition of 1 ml of 38% formaldehyde. GOLDSTEIN'S[11] reagent, 4 ml, was added and the absorbance was determined in a Zeiss PMQ II spectrophotometer at 460 nm after 30 sec. The readings were corrected with a blank prepared as above but with the enzyme added after the formaldehyde. Values were read from a standard curve.

In absence of albumin the activity of the enzyme was much lower. Addition of mercaptoethanol to the assay medium increased the activity 2- to 3-fold.

Pyruvate determinations. The incubation mixture contained 225 μmoles of Bis buffer, pH 9.55, 15 μmoles of ammonium mercaptopyruvate, 120 μg of bovine serum albumin, 15 μmoles of mercaptoethanol, 37.5 μmoles of KCl, in a total volume of 0.75 ml. Incubation was carried out for 3 min (unless indicated otherwise) at 37°. The reaction was stopped by addition of 0.5 ml of 0.5 M $CdCl_2$.For preparation of a

blank, the enzyme was omitted during the incubation and added after the $CdCl_2$. The solution was allowed to stand for 10 min and was cleared by centrifugation. A 0.5-ml aliquot of the clear supernatant fluid was incubated for 5 min at 25° with 0.5 ml of a 0.1% 2,4-dinitrophenylhydrazine solution in 2 M HCl. Then 2.5 ml of 1.5 M NaOH were added. The solution was clarified by centrifugation; the supernatant fluid was measured in the Zeiss spectrophotometer at 435 nm 35 min after addition of the reagent. Values were corrected by means of a pyruvate standard which was carried through the same procedure.

Rhodanese determinations were carried out according to the method of SÖRBO[12] or of WOOD AND FIEDLER[2]. Protein was measured by the methods of WARBURG AND CHRISTIAN[13], LOWRY *et al.*[14], or by total nitrogen determination involving nesslerization of a Kjeldahl digest[15]. The conversion factor was calculated from the amino acid analysis to be 5.6 times the nitrogen value.

Amino acid analysis. Samples were dialyzed against 0.5% potassium acetate prior to hydrolysis for 24 and 48 h *in vacuo* at 105°. Analyses were performed on a Beckman-Spinco Model C Amino Acid analyzer. Cysteic acid was measured on sample oxidized with performic acid and tryptophan was measured spectrophometrically[16].

Analytical disc electrophoresis was carried out according to the procedures of DAVIS[17].

Sedimentation velocity experiments were performed at 20° in a Spinco Model E ultracentrifuge equipped with a temperature control unit and a phase plate as a schlieren diaphragm. The rotor speed for these experiments was maintianed at 56 100 rev./min. For sedimentation equilibrium experiments a Raleigh optical system was used with a synthetic boundary cell rotating at 15 700 rev./min at 20°. The photograph was taken at 42 h.

The absorption spectra were recorded with a Cary 14 Spectrophotometer.

Isoelectric point. For this determination, cellulose acetate membranes were employed in a PhoroSlide electrophoresis cell. The migration was carried out at 100 V and 0.7 mA for 20 min in 0.02 M citrate or phosphate buffer from pH 4.0 to pH 6.0. 1 μg of protein was applied. The strips were fixed for 10 min in 10% sulfosalicylic acid, then stained for 2 h in 0.25% Coomassi Blue and destained with 7% acetic acid.

Sulfhydryl group determinations. Enzyme which had been dialyzed for 36 h against several changes of 0.05 M potassium acetate saturated with nitrogen was analyzed for sulfhydryl groups by the method of ELLMAN[18].

Metal analysis. Analyses for iron, copper and zinc were performed with a Perkin–Elmer Atomic Absorption apparatus. The samples were first dialyzed for 36 h against three changes of 10^{-2} M EDTA. Values were calculated by comparison to standards.

Growth of microorganisms. E. coli B were grown on an L-broth medium[19] for 16–24 h under vigorous aeration. The cells were harvested with a Sharples centrifuge. The wet weight of the cells was approximately 5 g cells/l of medium. The cell paste was frozen and stored at −20° for several months without observable loss of enzymic activity. Subsequently, the cells were grown in a 300-l fermenter at the Biology Division of the Oak Ridge National Laboratory on a tryptone–yeast extract medium.

Enzyme purification

Crude extract. 468 g of the frozen cells were suspended in approximately 1500 ml of NaCl–KCl (0.5%/0.5%) solution and the suspension was centrifuged for 15 min at 8000 rev./min at 4° in a Sorvall centrifuge. The washing procedure was repeated; the washed pellet was resuspended in 950 ml of 66 mM cold potassium phosphate buffer, pH 7.0, containing 10^{-3} M EDTA. The suspension was stirred and cooled by an ice–salt mixture while the cells were broken with a Bronwill Biosonik sonicator operated at full power for 1 h. Cell debris was sedimented at 9500 rev./min for 40 min at 4° in a Sorvall centrifuge. The pellet, resuspended in 400 ml of cold phosphate buffer as above, was centrifuged for 40 min. The combined supernatant liquors totaled 1300 ml. The crude extract could be kept frozen at —20° for several weeks without loss of activity.

Protamine treatment. A 1% protamine sulfate solution was adjusted to pH 6.0 with 2 M Tris and was stored overnight at 4°. The protamine solution was added dropwise to the crude extract over a period of 30 min until the 280/260 nm ratio reached a value between 0.8 and 0.9. After addition of the protamine, the final solution was 40 to 50 mM in phosphate, pH 7.0. After standing for 2 h at 0°, the solution was centrifuged at 9000 rev./min for 10 min at 4°. A clear supernatant of 1840 ml was allowed to stand overnight at 0°. This preparation was subjected to DEAE-cellulose chromatography as described in Fig. 1. In some instances the eluate was run through the second DEAE-cellulose column in sequence.

The eluate from the chromatogram was concentrated by ultrafiltration with a UM-10 Amicon filter, or by vacuum dialysis, to a volume of 11 ml and then centrifuged to remove insoluble particles.

Chromatography on Sephadex G-100. A Sephadex G-100 column (2.5 cm × 200

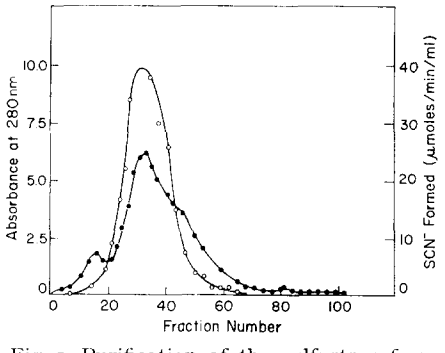
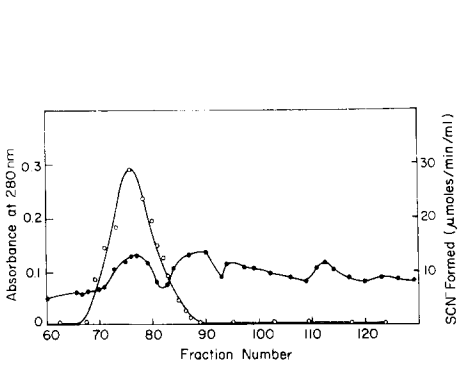


Fig. 1. Purification of the sulfurtransferase on DEAE-cellulose. A 30 cm × 5 cm DEAE-cellulose column was packed under a pressure of 5 lb and was equilibrated with 0.066 M phosphate buffer, pH 7.0, containing 10^{-3} M EDTA. The sample was applied on top of the column at a flow rate of 250 ml/h. After a preliminary washing with 50 ml of 66 mM phosphate, linear gradient elution (1 l of 0.066 M potassium phosphate, pH 7.0, EDTA 10^{-3} M and 1 l of 0.3 M potassium phosphate buffer, pH 7.0, containing 0.35 M KCl and 10^{-3} M EDTA) was applied. The enzyme activity appeared in Tubes 20–60 when 15-ml fractions were collected. The active fractions were combined, resulting in a volume of 580 ml. This was diluted with 2 vol. of cold water by dropwise addition over 20 min. The dilute sample was absorbed on a repacked DEAE-cellulose column as described above. Elution was carried out with a linear gradient (0.1 M phosphate buffer, pH 7.0, 10^{-3} M EDTA, 750 ml and 0.3 M phosphate buffer, 0.35 M KCl, pH 7.0, 10^{-3} M EDTA, 750 ml). 15-ml fractions were collected. The activity emerged as shown in the figure. ○—○, enzyme activity; ●—●, absorbance at 280 nm.



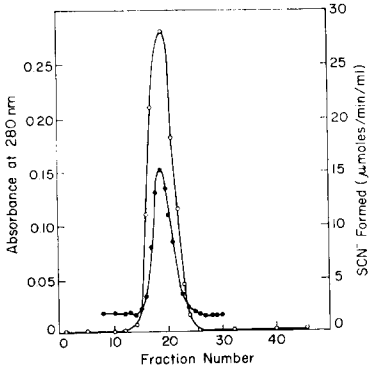


Fig. 2. Purification of the sulfurtransferase by electrophoresis on 18.5% polyacrylamide gel. A Canalco preparative gel electrophoresis assembly was used. The height of the separator gel was 5.5 cm, the height of the spacer gel was 2.5 cm. The separator gel was prepared from 4 parts of 37% acrylamide, being 0.08% in bisacrylamide, 1 part of 3.0 M Tris–HCl, pH 8.6, being 0.025 M in tetramethylenediamine, 2 parts water, and 1 part of 0.125 M ammonium persulfate. The spacer gel was prepared from 1 part of 0.5 M Tris–HCl, pH 7, being 0.05 M tetramethylenediamine, 1 parts of 14% acrylamide, being 0.25% in bisacrylamide, 1 part of 0.125 M ammonium persulfate and 4 parts of water. In order to remove excess of persulfate and some unpolymerized monomer, 12 h after polymerization the electrode chambers were filled with 0.375 M Tris–HCl buffer, pH 8.6, containing $1.6 \cdot 10^{-2}$ M mercaptoethanol and preliminary electrophoresis was carried out for 3 h at 10 mA. The concentrate of the Sephadex G-100 eluate (see METHODS) was dialyzed against 3 mM Tris buffer, pH 7.9, containing 10^{-3} M EDTA, $5 \cdot 10^{-3}$ M mercaptoethanol, and 35% sucrose for 1 h. A trace of bromophenol blue was added as a "tracker dye". The sample was then layered on top of the spacer gel and the electrophoresis was started at a current of 6 mA with 0.2 M glycine–Tris buffer, pH 8.35, in the chambers. After the sample was concentrated (after approximately 8 h) the electrophoresis was continued at 10 mA. When the tracker dye had reached the bottom of the separator gel, elution was started and 7.5-ml fractions were collected every 5 min, the elution buffer being 0.375 M in Tris–HCl, pH 8.0, and 10^{-3} M EDTA. The collection tubes were charged with 1 ml of 3.75 M KCl solution in 0.05 M Tris–HCl, pH 7.3, and 10^{-3} M EDTA. 7.5-ml fractions were collected. The activity appeared in a single peak as shown in the figure. ○—○, enzyme activity; ●—●, absorbance at 280 nm.

Fig. 3. Final purification of the sulfurtransferase on Sephadex G-100. The concentrate of the previous stage was applied to the bottom of a 95 cm × 2.5 cm Sephadex G-100 column equilibrated with 0.8 M KCl which was $2 \cdot 10^{-3}$ M in EDTA and $5 \cdot 10^{-3}$ M in mercaptoethanol. Elution was carried out with the same buffer, 9.5-ml fractions being collected. After a forerun of 256 ml, the enzyme was eluted as shown in the figure. ○—○, enzyme activity; ●—●, absorbance at 280 nm.

cm) was equilibrated with 0.05 M Tris–HCl, pH 7.3, containing 0.8 M KCl and $5 \cdot 10^{-4}$ M EDTA. The sample (11 ml) was applied to the bottom of the column at an effluent rate of 10 ml/15 min; 7.5-ml fractions were collected. The activity emerged as a symmetrical peak after an effluent volume of about 465 ml. The tubes containing the enzyme were pooled discarding 15% of the total activity in the least active fractions and the eluate was concentrated by ultrafiltration from 98 to 5 ml. At this step, the enzyme was fairly stable and could be stored for at least 3 weeks at 0° without loss of activity.

Electrophoresis on polyacrylamide gel was done as described in Fig. 2. The major portion of the elution peak from the electrophoresis was selected to eliminate a closely following protein. The combined fractions were concentrated by ultrafiltration to 5 ml. *Sephadex G-100 gel chromatography* as described in Fig. 3 was the final step. The enzyme emerged as a single symmetrical peak which had constant

TABLE I

PURIFICATION OF MERCAPTOPYRUVATE SULFURTRANSFERASE FROM *E. coli*

Stage	*Total protein (mg)*	*Specific activity (μmoles/mg per min)*	*Yield (%)*
Crude extract	41 000	0.65	100
Protamine precipitation	22 500	0.98	83
DEAE-cellulose-I	1 690	5.8	37
DEAE-cellulose-II	825	9.8	31
Sephadex G-100	63	87	18
Gel electrophoresis	5.4	525	11
Sephadex G-100	5.0	540	10

specific activity throughout the active fractions. The combined fractions were concentrated to 1.5 ml by ultrafiltration. This preparation was purified 830-fold compared to the first crude extract. The protocol of purification procedures together with yields and activities at the several stages is summarized in Table I.

RESULTS

When assayed at pH 9.6 in the presence of 0.02 M mercaptoethanol and 0.05 M KCl, the specific activity of the purified enzyme was 540 μmoles of thiocyanate formed per mg of protein per min at 37° and 1880 μmoles of pyruvate. In absence of mercaptoethanol and KCl, the specific activity was considerably lower. The presence of a stabilizing protein colloid such as bovine serum albumin was necessary also. The turnover number in terms of pyruvate at optimal conditions was 750 per min.

The enzyme migrated as a single symmetrical peak in the ultracentrifuge.

Stability

The enzyme was markedly stabilized during purification and storage by the presence of monovalent cations. Maximal stability was obtained when the purification and storage were carried out at pH 6.7–7.5 in the presence of 0.8 M KCl and $2 \cdot 10^{-3}$ M mercaptoethanol. No appreciable loss of activity was observed during the first 10 days of storage at 4°. The activity dropped slowly over 30 days to a level of 40–50% of the initial value. Dilute solutions of the enzyme could be stabilized by the addition of 0.15 mg of bovine serum albumin per ml. The albumin was routinely added to assays mixtures. Enzymatic activity in the assay medium was undiminished after 1 h at 37° and after at least 6 h at 0°. Four-fifths of the activity was lost when the albumin was omitted. Gelatin and heated albumin solution were equally as effective in stabilizing the enzyme activity.

When the eluate of the first DEAE-cellulose column (Fig. 1) was dialyzed for 16 h against 0.033 M phosphate buffer, pH 7.0, containing 10^{-3} M EDTA, considerable loss of activity occurred. When this dialysate was rechromatographed on DEAE-cellulose, two enzymically active fractions were obtained (Fig. 4). The molecular weights were estimated by gel filtration on Sephadex G-100 to be approximately 11 000 and 22 000. If the time of dialysis were restricted to 2 h or less, only the dimer

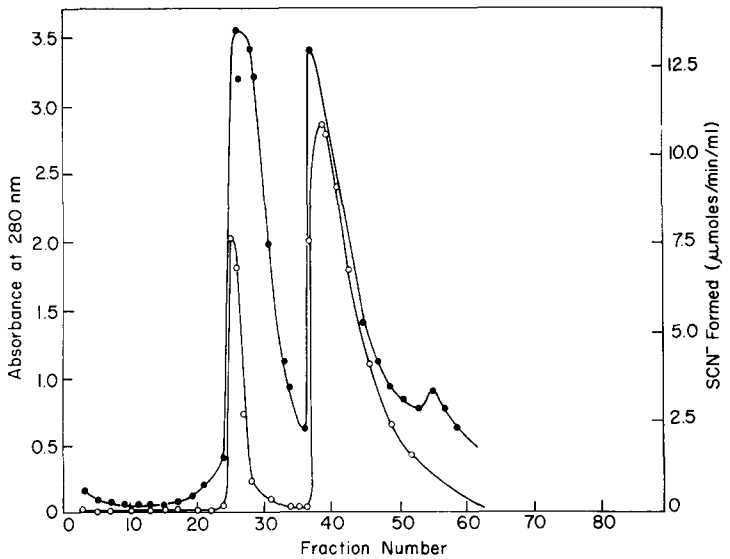


Fig. 4. Separation of two active forms of the mercaptopyruvate sulfurtransferase by DEAE-cellulose chromatography after extensive dialysis against weak salt solutions. The eluate from the first DEAE-cellulose column chromatography (Fig. 1) was dialyzed for 16 h against 33 mM phosphate buffer, pH 7.0, containing 10^{-3} M EDTA, applied to a DEAE-cellulose column (20 cm × 2.5 cm) equilibrated with the same buffer. The fractions eluted with a linear gradient between 33 mM and 0.3 M phosphate buffer, pH 7.0, containing 10^{-3} M EDTA. The active fractions of each peak were combined, concentrated by 60% ammonium sulfate precipitation, and the molecular weight was determined by gel filtration on a Sephadex G-100 column (95 cm × 2.5 cm) equilibrated with 1.7 M KCl in 0.05 M Tris buffer, pH 7.0, containing $5 \cdot 10^{-4}$ M EDTA. ○—○, enzyme activity; ●—●, absorbance at 280 nm. Lack of symmetry in the first peak (mol. wt. 11 000) results from a closely associated impurity which is later removed in the electrophoresis on acrylamide gel (see Fig. 2.)

was obtained. Increasing the ionic strength of the solution of KCl to 0.2 M caused partial restoration of lost enzyme activity.

When an analytical polyacrylamide gel electrophoresis was carried out on the purified enzyme at pH 8.6, two sharp bands were separated, most material being in the faster migrating band. At pH 7.0, however, only one broad band was obtained. Both bands which separated at pH 8.6 had enzyme activity. This suggested that the enzyme was breaking down partially into subunits. In the preparative gel electrophoresis (Fig. 2) only one active fraction was observed.

The enzyme was inactivated by dialysis against 8 M urea for 72 h. When analytical polyacrylamide gel electrophoresis was done in the presence of 8 M urea, the dialysate showed only one protein band. The activity of the enzyme could be partially restored by extensive dialysis against 0.8 M KCl or gel electrophoresis. In the latter case, two active bands were obtained. When the enzyme was dialyzed against $5 \cdot 10^{-3}$ M Tris–HCl buffer, pH 6.5, and then was subjected to preparative electrophoresis as described above, one enzymatically active band was obtained. Without this dialysis procedure, two enzyme bands were obtained.

The stability of mercaptopyruvate in the assay media was questioned because an odor of H_2S could often be detected. Quantitative measurements, however, showed that incubation of 20 μmoles of mercaptopyruvate in Bis buffer, pH 9.5, for 10

min at 37° produced less than 2% of S^{2-}. Likewise, the enzyme, pyruvate, and thiocyanate were stable under the assay conditions. Incubation of mercaptopyruvate in the assay medium for 30 min at 50° produced only a 1% conversion to persulfides.

General properties

The absorption spectrum of the enzyme in 0.8 M KCl, 0.05 M Tris–HCl buffer, pH 7.5, showed a maximum of absorbance only at 280 nm with a small shoulder at 290–293 nm. A solution of 1 mg of enzyme per ml in a 1-cm cuvette exhibited an absorbance of 0.93 at 280 nm. The isoelectric point as determined on cellulose acetate

TABLE II

AMINO ACID COMPOSITION OF MERCAPTOPYRUVATE SULFURTRANSFERASE

The determinations were done in a Spinco Amino Acid Analyzer excepting for tryptophan which was determined photometrically. Ammonia nitrogen was 34 moles. Amino acid values were corrected to zero hydrolysis time for threonine and serine.

Amino acid residue	*Calculated equiv./23 800 g*	*Nearest integer*
Lysine	8.64	9
Histidine	5.66	6
Arginine	9.69	10
Aspartic acid	21.45	21
Threonine	9.83	10
Serine	14.60	15
Glutamic acid	26.52	27
Proline	12.06	12
Glycine	20.26	20
Alanine	22.35	22
Valine	16.69	17
Methionine	3.43	3
Leucine	19.25	19
Isoleucine	9.53	10
Tyrosine	3.87	4
Phenylalanine	7.15	7
Tryptophan	5.51	6
Cysteine	1.94	2
	Total	220

strips in the PhoroSlide apparatus was 4.2. At constant current, the distance the protein migrated, when plotted against the pH, yielded a straight line.

While two cysteine residues were found by amino acid analyses, only one sulfhydryl group per mole was detected by the reaction with 5,5′-dithiobis-(2-nitrobenzoic acid). Determinations were carried out on enzyme prepared in the presence of mercaptoethanol which was then dialyzed out under nitrogen protection.

Metal analysis on an active enzyme preparation which had been dialyzed against 10^{-3} M EDTA for 36 h showed no detectable iron, approximately 0.5 mole of copper, and 1 mole of zinc per mole of protein.

The amino acid analysis is shown in Table II and represents the composition of the dimer.

The enzyme was not inactivated by 10^{-3} M iodoacetate, sodium azide, *p*-hydroxymercurisulfonate, *N*-ethylmaleimide or 5,5′-dithiobis-(2-nitrobenzoic acid)

when incubated in Tris–HCl buffer, pH 7.5, containing 0.15 mg of bovine serum albumin per ml, for 30 min.

The presence of monovalent cations (KCl, K_2SO_4, Na_2SO_4, 0.02 M) increased enzymic activity approximately 70%, while $2 \cdot 10^{-5}$ M $CdCl_2$, $5 \cdot 10^{-4}$ M arsenite and 10^{-5} M copper acetate incubated at pH 9.5 did not alter the activity. EDTA, $5 \cdot 10^{-2}$ M, and diethyldithiocarbamate, dithiooxamide, or dithiobisurea, all at 10^{-4} M, were not inhibitors.

Hydrodynamic properties

When the enzyme was examined in the ultracentrifuge, it sedimented as expected for a single homogeneous entity. Since the enzyme lost its activity rapidly in solutions of low ionic strength and produced two bands on gel electrophoresis or DEAE-cellulose column chromatography, all centrifugations were done in 0.05 M Tris–HCl buffer, pH 7, containing 0.8 M KCl, 10^{-3} M EDTA, and $2 \cdot 10^{-3}$ M mercaptoethanol. Sedimentation coefficients of 2.22, 2.48 and 2.34 were found at protein concentrations of 1.25, 2.5 and 4.0 mg of protein per ml at 20°. A corrected value for water was $s^\circ_{20,w} = 2.48$. A partial specific volume of 0.728 ml/g was estimated from amino acid analysis data.

From data derived from sedimentation equilibrium centrifugation, the molecular weight was calculated to be 23 800. The diffusion coefficient calculated from the sedimentation constant and the molecular weight was $9.96 \cdot 10^{7}$ $cm^2 \cdot sec^{-1}$. The minimum frictional coefficient was calculated from the relationships between the above parameters as derived by TAKAGI AND TANFORD[20] to be $3.94 \cdot 10^{-8}$. A value of $f/f_0 = 1.07$ indicated a typical, globular protein. A molecular weight was estimated on a Sephadex G-100 column equilibrated with 0.05 M Tris–HCl buffer, pH 7.5, 0.8 M in KCl, 10^{-3} M in EDTA and $2 \cdot 10^{-3}$ M in mercaptoethanol. The results from three experiments revealed a value of 21 700 ± 830, which was in fair agreement with the value 23 800 obtained in the centrifuge. The molecular weight calculated from amino acid analyses was 23 983.

Kinetic studies

The activity of the enzyme was determined as a function of the pH in terms of thiocyanate and pyruvate formation (Fig. 5). In all cases, the enzyme exhibited a pH optimum in the range of 9.3–9.6.

The enzyme activity showed a typical temperature dependence. The activity diminished from a maximum between 45 and 50° to zero at 60°.

The activity of the enzyme was directly proportional to the amount of protein in the preparation and to the amount of substrate in the assay. As is shown in Fig. 6, the enzyme did not obey Michaelis–Menten kinetics in the absence of mercaptoethanol. However, in the presence of 0.02 M mercaptoethanol and 0.05 M KCl, the K_m value was $8.34 \cdot 10^{-3}$ M mercaptopyruvate when pyruvate was determined. In the presence of 0.02 M KCN, the K_m for thiocyanate formation was $1.25 \cdot 10^{-2}$ M mercaptopyruvate.

The initial rate of formation of thiocyanate was approximately one-third that of pyruvate (when cyanide was added to the latter assay, Fig. 7). Also, there was a distinct time lag in thiocyanate formation. Pyruvate (10^{-2} M) in the standard assay medium inhibited thiocyanate formation 17%, and $2 \cdot 10^{-2}$ M, 45%. Preincubation

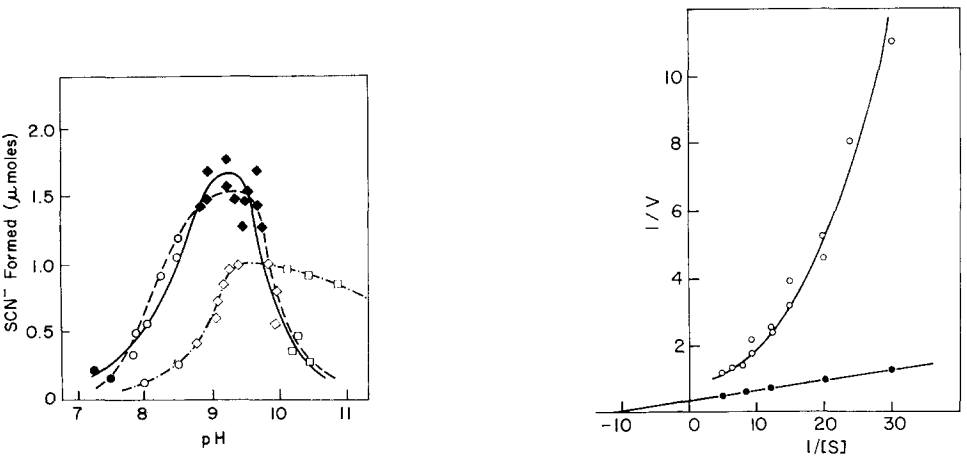


Fig. 5. pH optimum of sulfurtransferase. The complete system contained in 0.75 ml: mercaptopyruvate, 15 μmoles; enzyme, 0.3 μg; and buffers as designated in the graph: ●, sodium phosphate, 180 μmoles; ○, Tris–HCl, 225 μmoles; ◆, Bis–HCl, 225 μmoles; ◇, carbonate, 135 μmoles. Assays were done as described under METHODS. —·—, thiocyanate formation in the presence of 167 mM KCN with an incubation time of 10 min; — — —, pyruvate formation under the same conditions excepting for an incubation time of 5 min; ———, pyruvate formation with 0.02 M mercaptoethanol substituted for the KCN and an incubation period of 3 min.

Fig. 6. Effect of substrate concentration on pyruvate formation. Influence of mercaptoethanol on the Lineweaver–Burk plot. The enzyme assay medium was $5 \cdot 10^{-2}$ M in KCl. Pyruvate was determined. The enzyme concentration was 0.5 μg/ml and 0.15 mg of bovine serum albumin was added per ml. The time of incubation was 4 min at 37°. v was expressed as nmoles of pyruvate formed per μg of enzyme. The substrate concentration was expressed in mM mercaptopyruvate. ○, assays without mercaptoethanol; ●, assays with 0.02 M mercaptoethanol in the incubation mixture.

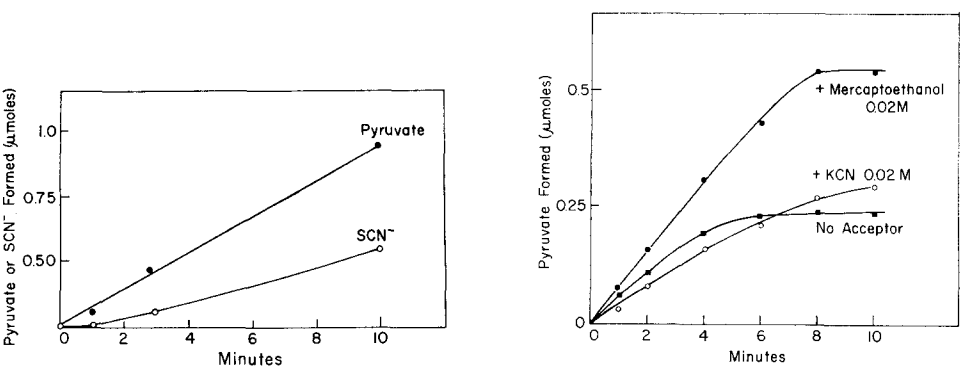


Fig. 7. Initial rates of thiocyanate and pyruvate formation. The reaction mixture contained 12 μmoles of KCN, 100 μmoles of Bis buffer, pH 9.55, 67.5 μg of bovine serum albumin, 15 μmoles of mercaptopyruvate and 0.2 μg of enzyme in a total volume of 0.75 ml. The incubation at 37° was stopped by addition of 1 ml of 38% formaldehyde for the thiocyanate determination or of 0.5 ml of 0.5 M $CdCl_2$ for the pyruvate determination. Determinations were carried out as described under METHODS. The rate of pyruvate formation was not altered by preincubation of the mixture for 5 min before adding the enzyme.

Fig. 8. Effect of sulfur acceptors on rate of pyruvate formation. The complete system contained 225 μmoles Bis buffer, pH 9.55, 67.5 μg bovine serum albumin, 15 μmoles mercaptopyruvate, 0.5 μg of enzyme and additions to bring the total volume to 0.75 ml. The additions were either water (■) KCN, 12 μmoles (○), or mercaptoethanol 15 μmoles (●). The pyruvate was determined as described under METHODS.

of mercaptopyruvate with an equimolar amount of pyruvate completely prevented its function as a substrate in the enzymic reaction even with the enhancing effect of mercaptoethanol. KCN reversed the inhibition about one-half while additional mercaptopyruvate was only slightly effective.

The effect of cyanide is seen in Fig. 8. Cyanide was inhibitory at short time intervals but at longer periods exerted a slight enhancement. However, when the enzyme was preincubated with $5 \cdot 10^{-2}$ M KCN at pH 7.5 for 1 h, nearly complete inactivation resulted. After preincubation with cyanide at pH 9.5 for 5 min, there was a slight inhibitory effect on the enzyme which was partially reversed by mercaptoethanol.

TABLE III

INHIBITION OF ENZYME ACTIVITY BY POLYSULFIDE SULFUR

Flowers of sulfur were shaken in 0.5 M Bis buffer, pH 9.6, containing 0.12 M mercaptoethanol for 30 min at 37°. After filtration, the amount of polysulfide sulfur was determined and aliquots were added to the enzyme assay medium with additional mercaptoethanol to make a total of 15 μmoles of sulfhydryl compound. Pyruvate was determined as described under METHODS. When cyanide was added to a similar preparation and thiocyanate formation was determined, the apparent inhibition of the enzymic reaction was 11%.

Ratio of mercaptopersulfide to persulfide	*Pyruvate formation (%)*
None	100
18	102
13	82
3.5	81
2.2	78
1.2	29

The enhancing effect of mercaptoethanol on the reaction was exerted relative to its level in the medium reaching a maximum only when approximately equal to the substrate concentration which saturated the enzyme. Higher concentrations of mercaptoethanol were inhibitory. Other sulfhydryl compounds were very slightly effective, *e.g.* cysteamine and thioglycollic acid. Mercaptosuccinic and mercaptopropionic acids had a slight inhibitory effect at $2 \cdot 10^{-2}$ M. Cysteine[7] was inhibitory. Likewise glutathione at 10^{-3} M inhibited the reaction 70%, cystine at 0.1 M, 75%.

Persulfides of mercaptoethanol had a marked inhibitory effect on enzymic pyruvate formation (Table III). The inhibitory effect on thiocyanate formation was somewhat less.

DISCUSSION

After the final stage of purification, the enzyme appeared to be free of any significant amount of impurities. The emergence of the enzyme from the final Sephadex G-100 gel in a symmetrical peak and the patterns of the sedimentation velocity studies indicated the sulfurtransferase to be highly purified. The enzyme contained approximately 0.5 mole of copper per 24 000 molecular weight and no iron but also 1.1 moles of zinc. The removal of copper from proteins is very difficult. In the present case, it was retained after 36 h of dialysis of the enzyme against 10^{-3} M EDTA.

VAN DEN HAMER *et al.*[9] experienced similar difficulties with the mercaptopyruvate sulfurtransferase from rat liver and erythrocytes. These investigators removed practically all the copper from the enzyme with sodium diethyldithiocarbamate and concluded the association of copper with the enzyme as reported by KUN AND FANSHIER[3] was an artefact. In the present study, incubation with sodium diethyldithiocarbamate did not inhibit the enzyme nor did EDTA. It thus appears that if a divalent metal ion is involved in the active form of the enzyme, it is tightly bound. It is of interest that 1 mole of zinc per monomer of 18 500 molecular weight has been reported by WESTLEY and co-workers[21] for rhodanese.

As observed by FANSHIER AND KUN[8] for the corresponding rat liver enzyme, the sulfurtransferase from *E. coli* is very unstable in solutions of low ionic strength. Like the rat liver enzyme it readily dissociates into monomers which lowers the activity. However, in solutions of about 0.8 M KCl, the enzyme monomer rapidly dimerizes.

Two active bands were obtained from a DEAE-cellulose column after the enzyme preparation had been dialyzed against dilute salt but only one was seen in the presence of urea or in solutions dialyzed against high salt concentration. Urea produced reversible inactivation. These observations are suggestive that the enzyme occurs as a dimer which is in equilibrium with monomers.

All centrifugation studies were performed in high salt concentration. When the data were arranged so that the log fringe displacement *versus* the square of the radius of rotation could be plotted, the resulting slightly curved line suggested that, during centrifugation at high protein concentration, some association phenomena may have taken place. Normalized values for the amino acid analysis show eight amino acids to occur in an odd number per dimer. This argues against the dimer dissociating into identical monomers.

The amino acid analysis revealed only two half-cystines per molecular weight of 23 800. Only one of these was accessible to sulfhydryl group reagents. The effects of inhibitors indicate the accessible sulfhydryl group is not necessary for function of the enzyme. This is in contrast to the rhodanese system[22]. However, the catalytic system of the mercaptopyruvate sulfurtransferase contains a sulfhydryl group contributed by the substrate. Hence, it might be expected that the action of mercuribenzoate, metals, and other reversible reactants for sulfhydryl groups would be inconclusive. *N*-Ethylmaleimide, iodoacetic acid and sodium azide had but little effect on the activity of the purified enzyme.

The enzyme was sensitive to oxygen, especially when additional copper was added[23]. Chelating agents for copper as noted above had no effect on enzyme activity. It should be noted that Mn^{2+} catalyzes decomposition of mercaptopyruvate to yield persulfide sulfur. Under conditions of the enzyme assay, 10^{-3} M Mn^{2+} was approximately equivalent to 0.7 unit of enzyme.

The pH optimum for pyruvate formation (Fig. 5) was 9.3–9.6 for the purified enzyme, which confirms our earlier report[23]. The addition of mercaptoethanol increased the velocity of the reaction but had no effect on the optimum pH values. When cyanide was added as a sulfur acceptor and thiocyanate was determined, the activity extended into the alkaline ranges. The shift represents the slower rate of thiocyanate formation (Fig. 7) and the pH effect on the rate of reaction of cyanide with persulfide sulfur when no catalyst was present[7].

The reaction catalyzed by mercaptopyruvate sulfurtransferase is the removal of sulfur from the substrate.

$$HSCH_2\text{–}\overset{\overset{O}{\|}}{C}\text{–}COOH \rightarrow CH_3\text{–}\overset{\overset{O}{\|}}{C}\text{–}COOH + S^0$$

Mercaptopyruvate is the only substrate known for this enzyme and free carboxyl, sulfhydryl and carbonyl groups are necessary for the reaction[24]. The substrate apparently attaches to the enzyme surface and pyruvate is rapidly released. An enzyme–sulfur complex that remains liberates free sulfur when acidified.

As shown by MEISTER *et al.*[1] for a liver extract at pH 7.4, the sulfur precipitates with protein. HYLIN AND WOOD[7] found the sulfur remains in solution as a persulfide in alkaline medium. In this form, the sulfur is available for transfer to various acceptors, *e.g.* cyanide to form thiocyanate[2], and sulfite to form thiosulfate[6]. The enzyme–sulfur complex can be transported by electrophoresis on paper and retains its ability to form thiocyanate when cyanide is added (J. W. HYLIN AND J. L. WOOD, unpublished results). Addition of persulfides caused a progressive inhibition of the enzymic production of pyruvate (Table III). Probably the attachment of the persulfide sulfur to the enzyme could stop the reaction if all the active centers were saturated. This does not occur because mercaptopyruvate accepts the sulfur to form a persulfide. This is noted by development of a characteristic yellow color in the incubation mixture which is dissipated by the addition of cyanide or mercaptoethanol. Mercaptopyruvate is relatively poor as a persulfide acceptor[7].

The inhibiting effect of pyruvate on the enzymic reaction was noted by MEISTER *et al.*[1] who observed the conversion of mercaptopyruvate to pyruvate was not quantitative except in the presence of mercaptoethanol. Repeated additions of enzyme to an incubation mixture had no effect. We have observed that preincubation of mercaptopyruvate with equimolar amounts of pyruvate completely eliminated substrate activity. It was apparent that the mercaptopyruvate was converted to other products possibly through formation of a thiomercaptol compound[26]. Pyruvate may act on the enzyme also since addition of more substrate failed to provide for the reaction at the expected level.

Fig. 8 shows the rate of pyruvate formation by the purified enzyme is increased by the presence of mercaptoethanol approximately 2-fold. The maximum effect of mercaptoethanol was seen at a concentration of 2.5 mM. A Lineweaver-Burk plot of the velocity–substrate activity (Fig. 5) shows the relationship is not linear unless mercaptoethanol is present. Fig. 7 shows pyruvate forms much faster than thiocyanate when cyanide is present as an acceptor. A time lag in thiocyanate formation has been observed consistently. Cyanide is slightly inhibitory initially but enhances the production of pyruvate above the control at seven minutes of incubation. Those findings support the conclusion that the transfer of sulfur from persulfide to cyanide is not an enzyme-catalyzed reaction, although the enzyme enhances the reaction of mercaptoethanol persulfide with cyanide at pH 9.6 to a slight degree. This is similar to the action of serum albumin on colloidal sulfur[25].

The formation of persulfide sulfur is a unique reaction of the sulfurtransferases of this class. The precipitation of free sulfur probably never occurs *in vivo* due to the presence of acceptors and of reducing agents such as sulfhydryl compounds which can convert the sulfur to sulfide ion.

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