OXIDATIVE DEAMINATION OF D-HOMOCYSTEINESULFINIC ACID

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1. Introduction

Homocysteinesulfinic acid (HCS) has been prepared by Jollès-Bergeret [1], who also demonstrated that it is deaminated by liver glutamate dehydrogenase (L-glutamate:NAD(P) oxidoreductase, deaminating EC 1.4.1.3) with production of ammonia and an α-ketosulfinic acid [2], and is decarboxylated by a crude preparation of glutamate decarboxylase from *Clostridium welchii* with production of homohypotaurine [3]. These results confirm the suggestion that, because of the great similarity in structure of glutamic acid and HCS – in the latter SO_2H replaces a carboxyl group – these compounds may be good substrates for the same enzyme.

Jollès-Bergeret further reports [2] that D-homocysteinesulfinic acid is not oxidized by hog kidney D-aminoacid oxidase (D-amino acid:oxygen oxidoreductase, deaminating EC 1.4.3.3). This results may be an obvious consequence of the fact that dicarboxylic amino acids are not affected by D-amino acid oxidase, but are substrates for the D-aspartate oxidase (D-aspartate:oxygen oxidoreductase, deaminating EC 1.4.3.1) [4–7].

We have therefore investigated the oxidative deamination of D-HCS by D-aspartate oxidase, to have further indications that enzymes active on glutamate also may be active on HCS.

It may be emphasized that D-aspartate oxidase is active on D-cysteinesulfinic acid [8], which shows the same analogy with aspartic acid that HCS shows with glutamic acid.

Abbreviations:
HCS : homocysteinesulfinic acid
2,4-DNPH: 2,4-dinitrophenylhydrazone

2. Methods

DL- and L-HCS were prepared from DL- and L-homocystine (Fluka) according to Jollès-Bergeret [1]. D-Aspartic and D-glutamic acid were obtained from Fluka; FAD from Sigma Chemical Co.; catalase from Boehringer and Soehne. D-Aspartate oxidase was a partially purified apoenzyme preparation obtained from beef kidney as recently described [9]. Preparations at the 5th or 6th step of the purification procedure were used throughout this work.

Oxygen consumption was determined either in a Warburg apparatus, or polarographically with a vibrating platinum electrode (Oxygraph G.M.E.) [10].

For ammonia determination Warburg vessels with two side arms were used, one of which contained 0.2 ml 10 N H_2SO_4. The acid was put in at the desired times to stop the reaction and to fix ammonia, and the vessels were then allowed to stand for 12 hr at room temperature in order to permit complete absorption of the ammonia present in the gas phase. The acidified reaction mixture was finally centrifuged, and the ammonium sulfate was determined on aliquots of the supernatant by nesslerization. Blanks were performed by acidifying the reaction mixture at zero time, and standard curves were prepared with ammonium sulfate. Details of the incubation conditions are reported in the legends to the figures.

Ketoacids were quantitatively determined according to Friedeman and Haugen [11], or after paper chromatography according to Cavallini and Mondovì [12]. Analyses for sulfites were performed with *p*-rosaniline as described by Sorbo [13]. Sulfates were detected by addition of $BaCl_2$ to acidified reaction mixtures after having removed proteins by addition of TCA. Amino acid analyses were performed in a

Beckman Spinco Unichrom amino acid analyzer, on the long column eluted with 0.1 M citric acid–0.2 M NaCl [14].

3. Results

Fig. 1 shows the time course of oxygen consumption and of ammonia and ketoacid production when DL-HCS is incubated with D-aspartate oxidase. Assuming that only the D-form is converted, half a mole of oxygen is consumed per mole of substrate (catalase added), and one mole of ammonia is produced. About one mole of ketoacid is also produced. It must be noted that α-ketobutyric acid was used as standard for quantitative determination of ketoacids, and it is possible that the 2,4-DNPH of the ketoacid arising from HCS would show a different extinction value.

No production of sulfite or sulfate was observed during or at the end of the reaction. No CO_2 was evolved. The enzyme was completely inactive with L-HCS, indicating that the D-form is oxidized. Amino acid analyses showed that at the end of the reaction half the amount of the DL-HCS incubated has disappeared and that no homocysteic acid was produced.

Paper chromatographic analyses of the 2,4-DNPH obtained during and at the end of the reaction showed the presence of a spot with the following R_f values in different solvents (the R_f values obtained for the 2,4-DNPH of α-ketobutyric acid are shown in brackets): butanol saturated with water 0.1 (0.53); butanol saturated with 3% ammonia 0.1 (0.65); butanol–ethanol–water (4:1:5, upper phase) 0.27 (0.55); butanol–acetic acid–water (4:1:5, upper phase) 0.41 (0.85). Sometimes a second minor spot with higher R_f values was obtained, which showed the same spectrum when eluted in 1 N NaOH, suggesting isomerization of 2,4-DNPH [15].

The spectrum of the spot eluted from the chromatograms with 1 N NaOH is reported in fig. 2. It is similar

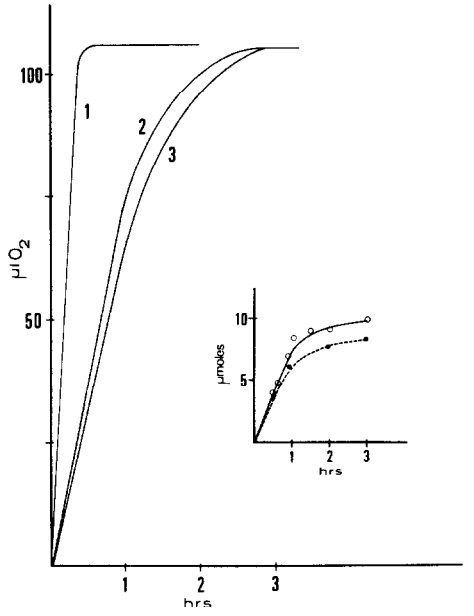

Fig. 1. Oxygen consumption curves (Warburg apparatus) for D-aspartate (curve 1), DL-HCS (curve 2) and D-glutamate (curve 3). Experimental conditions as follows: enzyme proteins (specific activity 0.7 μmole D-aspartate oxidized/min/mg protein [9]), 1.7 mg; FAD 15 μg; catalase 50 μg; pyrophosphate pH 8.3 50 μmoles; substrate: 10 μmoles D-aspartate or D-glutamate, 20 μmoles DL-HCS. Final volume 1.5 ml. Temp. 38°. Gas phase: air. *Inset*: μmoles ammonia (full line) and ketoacid (broken line) produced from 20 μmoles DL-HCS. α-Ketobutyric acid was used as standard for quantitative ketoacid determinations.

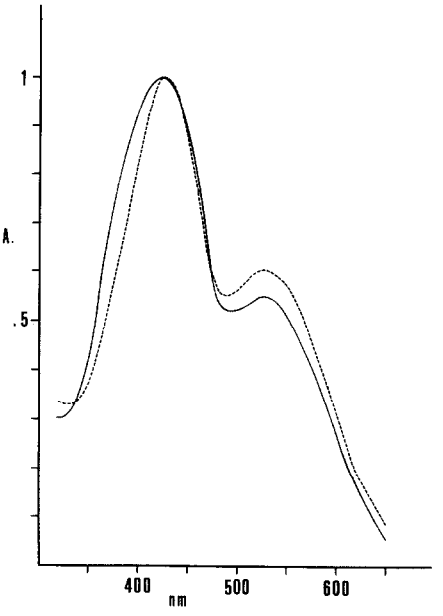

Fig. 2. Optical spectra of the 2,4-DNPH of the ketoacid arising from HCS (full line) and of the α-ketobutyric acid (broken line), eluted from paper chromatograms with 1N NaOH. The A values are reported as ratios $A_\lambda : A_{\lambda max}$.

to that of the 2,4-DNPH of α-ketobutyric acid, showing two maxima at 430 and 525 nm. The extinctions at the two maxima are however different: the ratio A_{525}:A_{430} is 0.55 for the 2,4-DNPH of the ketoacid arising from HCS, and 0.61 for the 2,4-DNPH of α-ketobutyric acid. The ratio of 0.55 and also the R_f values of the 2,4-DNPH of the ketoacid arising from HCS are instead very similar to those of 2,4-DNPH of α-ketoglutaric acid [16].

The expected ketoacid arising by oxidative deamination from HCS is α-keto-γ-sulfinyl-butyric acid. Therefore some tests were performed to detect the sulfinic group in the 2,4-DNPH separated by chromatography. After spraying the chromatograms with a solution of KI in 2 N HCl, iodine is liberated in the correspondence of the yellow spot of the phenylhydrazone. This reaction is given by compounds having R–SO_2H, R–SO_2S–R or R–SO_2SH groups [17]. After spraying the chromatograms with a 10% solution of $FeCl_3$ a positive reaction is also obtained (a rusty spot on a yellow background). This reaction is specific for sulfinic compounds [17].

When the solution of the 2,4-DNPH is oxidized by addition of H_2O_2 and ammonium molybdate before the chromatography, the yellow spot does not react with KI nor with $FeCl_3$, indicating that the SO_2H group has been oxidized to SO_3H. On the other hand the oxidation does not cause variations in the R_f values nor in the spectrum of the spot eluted in NaOH.

We have also determined the K_m for HCS. The initial reaction rate was determined from the oxygen consumption detected polarographically. The other incubation conditions were as described for the experiments reported in fig. 1, with the exception that an enzyme preparation with a specific activtiy of 2.1 was used. The value obtained was 5.3×10^{-4} M. The K_m for D-aspartate and D-glutamate are respectively 4.5×10^{-3} and 4×10^{-4} M [9]. The ratio V_{max} D-glutamate: V_{max} D-HCS was 0.69.

4. Discussion

The results obtained clearly demonstrate that D-HCS is a good substrate for kidney D-aspartate oxidase. This enzyme is active on D-aspartate, D-glutamate and D-α-aminoadipate, in decreasing order of reaction rate [5, 9]. D-Cysteinesulfinic and D-HCS acids are sulfur analogues of aspartic and glutamic acids, and are both acted upon by the enzyme.

It must be recalled that pyruvate was detected as a product of D-cysteinesulfinate deamination [8], indicating a splitting off of sulfur from sulfinyl-pyruvate, in analogy to the reaction occurring during transamination of L-cysteinesulfinate [18]. The ketoacid arising from HCS, the α-keto-γ-butyric acid, instead remains undegraded.

From the present results, from those previously obtained on the oxidative deamination of D-cysteinesulfinate [8], and from the results reported by Jollès-Bergeret studying the activity of glutamate dehydrogenase [2] and glutamate decarboxylase on HCS [3], it may be concluded that the substitution of an SO_2H group for the ω-carboxyl group does not appreciably affect the specificity of enzymes acting upon dicarboxylic amino acids.

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