SHORT COMMUNICATIONS

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α-Substituted cystines as possible substrates for cystine reductase and L-amino acid oxidase

It seems imperative that an amino acid have an α-hydrogen atom present for it to act as a substrate for several enzymatic reactions[1-9].

ROMANO AND NICKERSON[10,11] have reported the existence of an enzyme in pea seeds and yeasts, catalyzing the reduction of cystine to cysteine by NADH. The authors were the first to call the enzyme cystine reductase (NADH:L-cystine oxidoreductase, EC 1.6.4.1). Unfortunately, further purifications have not been successful.

Because of the possibility that the α-hydrogen of cystine may not be involved in the reduction of cystine to cysteine by cystine reductase, a series of α-substituted cystines were investigated as possible substrates for the enzymatic reduction. The series of α-substituted cystines were also investigated as possible inhibitors of the enzyme L-amino acid oxidase where the α-hydrogen is known to be needed for the reaction. The results of these investigations are reported in this communication.

Cystine reductase was prepared from an acetone powder of baker's yeast (Fleischmann Co.) according to the procedure of ROMANO AND NICKERSON[11]. Protein content was determined by the Kjeldahl method[12]. In studying the kinetics of the cystine reductase system, the reaction vessel contained 2.5 ml of enzyme suspension (171 mg protein), 0.025 M phosphate buffer (pH 6.2) and concentrations of L-cystine, DL-cystine, or α-substituted DL-cystines[13] ranging from 0.4 to 1 mM. The cystines were dissolved in 0.5 M HCl and an equal volume of 0.5 M NaOH was added to neutralize the acid. The final volume of the reaction mixture was made up to 10 ml with deionized water. The controls contained 0.025 M phosphate buffer (pH 6.2) in place of the cystine. The enzyme was added last, the flasks tightly stoppered, and placed in a Dubnoff metabolic shaking incubator at 37 ± 0.5°. Flasks were removed at 0, 5, 10, 15, 20, 30, 60, and 90 min. The reaction was stopped by the addition of 0.5 ml of 10% (w/v) phospho-24-tungstic acid in 5% (v/v) HCl. The mixtures were centrifuged at 10 000 rev./min for 10 min and the clear yellow supernatant used for titration with *N*-bromosuccinimide[14]. All determinations were done in triplicate. The values for the control flasks were subtracted from the experimental values and the amount of cysteine produced from cystine determined. As reported earlier[11], there was sufficient endogenous NADH present in the crude, undialyzed enzyme preparation for the reaction. α-Substituted cystines and cysteines were determined by the method of THIBERT *et al.*[14]. α-Substituted cysteines were prepared from the appropriate α-substituted cystines by reduction with 1% sodium–mercury amalgam[15].

Oxygen consumption by L-amino acid oxidase (Sigma Chemical Co. Type I and Type IV) (L-amino acid:O_2 oxidoreductase (deaminating), EC 1.4.3.2) was followed using an oxygen electrode (Yellow Springs Instrument Co.). The electrode was standardized according to the method of DIXON AND KLEPPE[16]. For kinetic studies the reaction vessel contained 0.05 M pyrophosphate buffer (pH 8.0), 0.06 unit of L-amino acid oxidase (μmoles of L-leucine turned over per min, pH 7.8, 37°) and

concentrations of L-cystine or DL-cystine ranging from 0.1 to 2 mM. The total volume of the reaction mixture was 4 ml. All the solutions were air saturated at 25°. The enzyme was added last. The initial rate was found to be linear for at least the first 2 min. To test the α-substituted cystines as possible substrates the assays were carried out in exactly the same manner. Enzyme concentrations of 0.06 and 0.288 unit were used. The concentrations of the α-substituted DL-cystines were 0.1 M and 2 mM. Stock solutions of the α-substituted cystines were dissolved in 0.05 M pyrophosphate buffer (pH 8.0) by gentle heating.

TABLE I

ENZYMATIC AND POLAROGRAPHIC REDUCTION OF α-SUBSTITUTED CYSTINES

Amino acid	K_m *(mM)*	v_{max} *(μmin^{-1})*	*Reduction potential*[17] *(V)*
L-Cystine	0.9	0.74	—0.536
DL-Cystine	1.1	0.72	——
α-Methyl-DL-cystine	1.2	0.77	—0.514
α-n-Propyl-DL-cystine	1.4	0.83	—0.186
α-Isopropyl-DL-cystine	3.5	0.82	—0.370
α-Phenyl-DL-cystine	1.5	0.69	—0.184

The α-substituted cystines behaved as substrates in the cystine reductase system. Table I lists the apparent v_{max} and the K_m values from Lineweaver–Burk plots for L-cystine, DL-cystine, α-methyl-, α-*n*-propyl-, α-isopropyl-, and α-phenyl-DL-cystine obtained using an IBM 1620 computer by the method of least squares. The reaction was shown to obey first-order kinetics and the initial velocities were determined by use of the apparent first-order rate constants obtained and the initial concentration of substrate used. All determinations were done in triplicate. No correlation could be observed between reduction of these compounds enzymatically as compared to polarographically[17]. This type of correlation would be most difficult using the present system because the crude enzyme preparation probably contains more than one enzyme that could reduce the cystines[18]. It seems more likely that upon purification the reduction of these compounds could be attributed to a series of different, specific enzymes. The fact that cystine reductase has never been purified and only crude homogenates of yeast, pea seeds, wheat flour, or bacteria have been used in previous studies[10,11,19–22] throws some suspicion on the validity of the existence of a specific cystine reductase. At present it is possible to say only that whatever cystine reducing systems are present in the crude preparations, some are able to reduce substituted cystines to cysteines showing no dependency on the α-hydrogen.

α-Substituted cystines acted neither as substrates for L-amino acid oxidase nor as inhibitors for the oxidative deamination of L-cystine by L-amino acid oxidase. To test for the inhibitory effect of the α-substituted cystines assays were carried out with 0.1 mM L-cystine, 0.05 M pyrophosphate buffer (pH 8.0), 0.288 unit of L-amino acid oxidase and varying concentrations of each of the α-substituted DL-cystines. The ratio of concentrations of inhibitors to L-cystine was selected to give values of 0, 5, 10, 20, 30 and 50. The fact that no inhibition of the enzyme was found when

L-cystine was the substrate and the α-substituted cystines were added as inhibitors, indicates that the α position of cystine must be free from bulky group substitution in order to bind to the active site of the enzyme. The results are in agreement with previous investigations with other α-substituted amino acids as substrates for L-amino acid oxidase[1,23].

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