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Specific Features of the Reaction of L-Cysteine with Pyridoxal and *N*-Pyridoxylidene-β-alanine

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Abstract—The mechanisms of condensation of L-cysteine, L-methionine, and L-serine with pyridoxal and of transaldimination of *N*-pyridoxylidene-β-alanine with L-cysteine were studied by the kinetic method. Unlike methionine and serine, the condensation of cysteine with pyridoxal and transaldimination with *N*-pyridoxylidene-β-alanine involves intermediate formation of stable product having a thiazolidine ring. Its structure was determined by elemental analysis, UV, and IR spectroscopy, and quantum-chemical calculations. The thiazolidine fragment in the pyridoxal condensation product with L-cysteine is turned through an angle of ~90° with respect to the pyridine ring plane due to mutual repulsion of the negatively charged oxygen atom in the *ortho* position of the pyridine ring and sulfur and nitrogen atoms in the thiazolidine ring.

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Serine, methionine, and cysteine are structural fragments of many biopolymers, peptides, proteins, and enzymes. Physicochemical and biological properties of biopolymers are determined by the polymer chain structure, amino acid sequence, number of amino acids, and substituent nature. Side chains are largely responsible for the formation of biopolymer surface and for many physical and chemical properties of proteins, peptides, and enzymes.

Studies on the chemical and biological roles of side chains in proteins, peptides, and enzymes in various biochemical processes involve serious difficulties related to fast and sometimes nonselective enzymatic reactions and diversity of chemical transformations and their products. Therefore, this global problem is solved using as models amino acids and coenzymes that are active structural fragments of enzymes. The role of side-chain groups in amino acid fragments is likely to be determined not only by their hydrophilic and hydrophobic properties, but also by steric factors, acid–base and redox properties, and paths of chemical transformations.

We previously studied [1–5] kinetics and mechanism of the reactions of pyridoxal and pyridoxal 5′-phosphate with various amino acids and found:

(1) α-Amino acids (α-alanine, glycine, leucine, phenylalanine, tryptophan, arginine, etc.) react with coenzymes to give the corresponding Schiff bases which, after tautomerization into quinoid form, undergo various chemical transformations, depending on their structure and reaction conditions (for instance, transamination, decarboxylation, side chain cleavage, transaldimination, etc.).

(2) β- and γ-Amino acids (β-alanine, L-lysine) give rise to stable Schiff bases which do not undergo further chemical transformations.

(3) L-Arginine reacts with pyridoxal at the α-amino group; the guadinine fragment is likely to participate in the formation of urea, regeneration of ornithine, and chemical transformations with liberation of NO.

(4) L-Proline with pyridoxal does not form Schiff base; presumably, it affects the mode of polypeptide chain packing.

(5) Unlike L-α-alanine, the reaction of pyridoxal with D-α-alanine is accompanied by decarboxylation of the latter with formation of pyridoxamine, pyruvate, or ethanal.

It was very interesting to study under comparable conditions the kinetics and mechanism of the reaction of pyridoxal with such amino acids as cysteine, serine, and methionine which have structurally similar substituents {CH_2SH in cysteine, CH_2OH in serine,

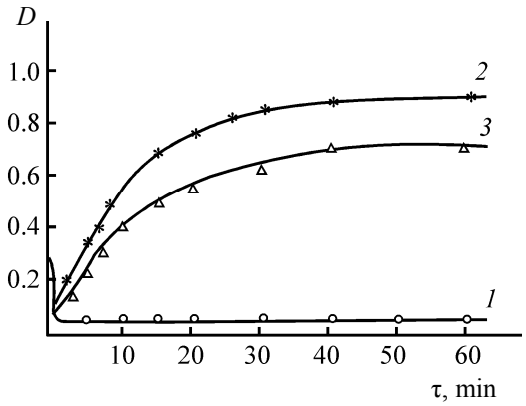


Fig. 1. Change of the optical density of mixtures of 0.01 M solutions of (*1*) L-cysteine, (*2*) L-methionine, and (*3*) L-serine with pyridoxal hydrochloride in 70% alcoholic–aqueous buffer (λ 430 nm, pH 6.8, 15°C).

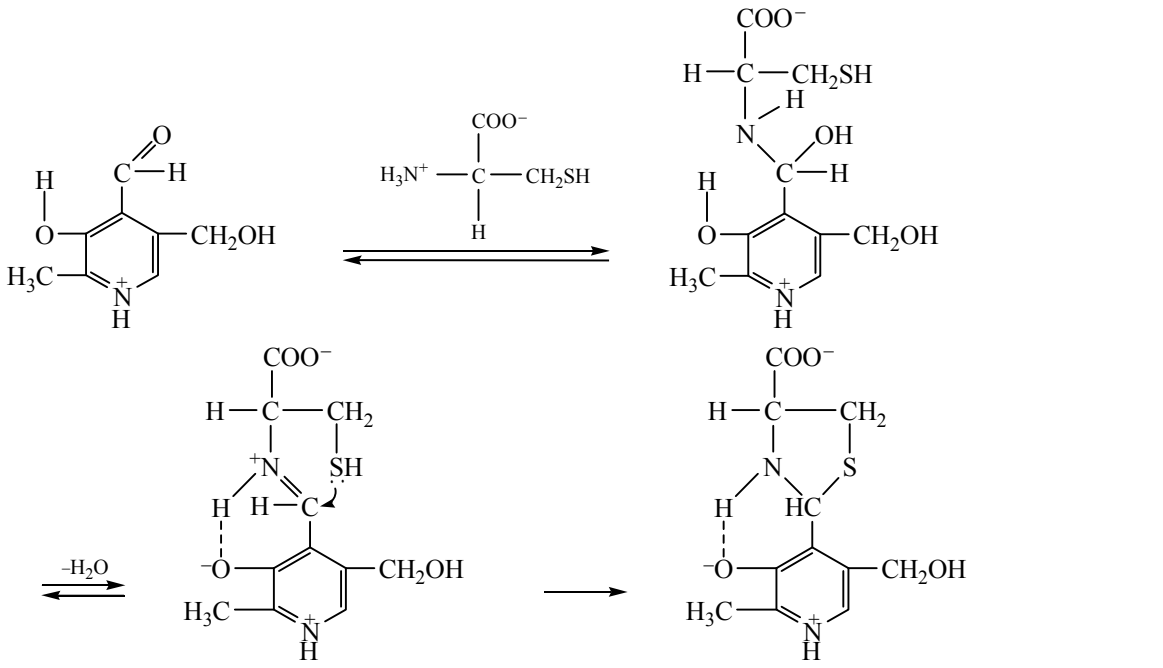
and $(CH_2)_2SCH_3$ in methionine] but different heteroatoms and are characterized by different acid–base properties and reactivities. The data in Fig. 1 show that the condensation of these amino acids with pyridoxal includes three kinetically distinguishable steps: (1) addition of α-amino acid to pyridoxal with formation of amino alcohol (which is accompanied by sharp reduction of the optical density); (2) dehydratation of amino alcohol with formation of Schiff base (increase of the optical density); and (3) transformation of Schiff base into quinoid form and (depending on the structure) subsequent transamination, decarboxylation, or cleavage of the side chain in the amino acid fragment (very slow step). The reaction of L-cysteine with pyridoxal occurred somewhat unexpectedly (Fig. 1, curve *1*). Instead of increase of the optical density with time (appearance of yellow color), as in the reactions with all (except for proline) the examined α-, β-, and ε-amino acids (alanine, phenylalanine, tryptophan, asparaginic and glutamic kislot, glycine, lysine, arginine, etc.), the optical density of the reaction mixture instantaneously dropped down to zero (λ 350, 420–430 nm) at the stage of amino alcohol dehydration and remained unchanged over a long time. The color of the reaction mixture did not change as well. Data were reported [6, 7] on the reaction of a cysteine derivative, L-penicillamine, with PLP-dependent enzymes. It was presumed that this reaction involves formation of stable thiazolidine ring via addition of the SH group at the C=N bond of the Schiff base. This thiazolidine derivative acts as convulsant and reduces the concentration of glutamate decarboxylase in brain. However, no direct proofs for the assumed structure were given.

Analysis of published [6] and our experimental data showed that in the reaction with pyridoxal L-cysteine is capable of undergoing ring closure to thiazolidine derivative due to the presence of two nucleophilic centers (α-NH_2 and γ-SH groups). Such unusual mechanism may be interpreted as follows: in the first step, fast addition of the α-amino group in L-cysteine at the carbonyl group of pyridoxal gives amino alcohol; in the second step, dehydration of the amino alcohol yields the corresponding Schiff base; and the third step involves nucleophilic attack by the SH group in the L-cysteine fragment at the azomethine C=N bond with formation of thiazolidine ring. According to the kinetic data and TLC, the third step is irreversible. Irreversible transformation of the Schiff base (λ_{max} 350, 420–430 nm) into cyclic thiazolidine derivative is accompanied by disappearance of absorption in the visible region.

In order to confirm the proposed mechanism for the condensation of L-cysteine with pyridoxal, we examined the kinetics of the reaction of L-methionine with the same coenzyme (Fig. 1, curve *2*). Unlike cysteine, the methionine molecule has a methylsulfanyl group instead of HS. The results of kinetic studies and calculation of the rate constants showed that methionine reacts with pyridoxal in a way similar to all other amino acids with formation of stable Schiff base with λ_{max} 350, 420–430 nm. Methylation of the SH group hampers cyclization. A probable scheme for the reaction of L-cysteine with pyridoxal is shown below.

Insofar as we failed to distinguish particular steps in the reaction of L-cysteine with pyridoxal and the more so to estimate their rates quantitatively even at low temperatures, we examined the kinetics and mechanism of transaldimination of amino acids. As initial components we selected L-cysteine and pyridoxal condensation product with β-alanine. It was presumed that the rate of the reaction of the corresponding Schiff base (*N*-pyridoxylidene-β-alanine) with L-cysteine should be much lower because of steric factors, so that we would be able to distinguish particular steps and estimate their rates.

In the reaction of *N*-pyridoxylidene-β-alanine with L-cysteine the optical density of the reaction mixture at λ 430 nm first sharply dropped down (over a period of 1 min) and then slowly decreased to zero. These findings are likely to confirm the proposed scheme for the reaction of pyridoxal with L-cysteine (Fig. 2). The first step is very fast addition of the α-NH_2 group in L-cysteine at the C=N bond of *N*-pyridoxylidene-β-alanine with formation of intermediate aminal. In the



second step simultaneous elimination of the β-alanine fragment and nucleophilic attack by the SH group on the azomethine C=N bond lead to formation of thiazolidine ring. The rate of the cyclization was estimated at k_2 = 0.205 min^{-1}). The product formed in the reactions of L-cysteine with pyridoxal and *N*-pyridoxylidene-β-alanine were identified by UV and IR spectroscopy, TLC, and elemental analysis in support of the proposed mechanism.

Quantum-chemical calculations on the product of the reaction of L-cysteine with pyridoxal with optimization of energetic and geometric parameters revealed that the thiazolidine fragment is turned through a dihedral angle of ~90° with respect to the pyridine ring plane, presumably as a result of mutual repulsion of the negatively charged oxygen atom in the *meta* position of the pyridoxal fragment and sulfur and nitrogen atoms in the thiazolidine ring.

It was also interesting to study the kinetics and mechanism of the reaction of L-serine with pyridoxal. Cysteine and serine differ from each other only by heteroatoms in the substituent on C^3. We tried to elucidate whether replacement of the sulfur atom by oxygen in going from cysteine to serine will lead to cyclization of the amino acid fragment. Analysis of published data and our kinetic curves (Fig. 1, curve *3*) in combination with TLC data showed that the reaction of serine with pyridoxal initially gives amino alcohol (very fast step) whose dehydration yields Schiff base, and the latter (after transformation into the quinoid form) undergoes aldol-type cleavage of the side chain with formation of glycine and formaldehyde (the optical density of the reaction mixture decreased with time). The formation of formaldehyde was confirmed by qualitative reaction with 2,4-dinitrophenylhydrazine, and glycine was detected using ninhydrin and by TLC. The absence of cyclization may be attributed to the lower basicity of the serine OH group as compared to the cysteine SH group. The rate constant of the amino alcohol dehydration step (k =

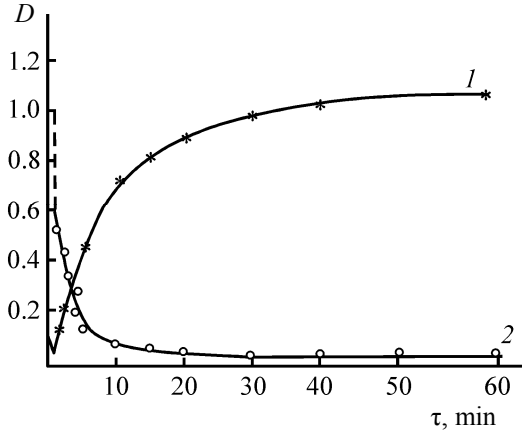
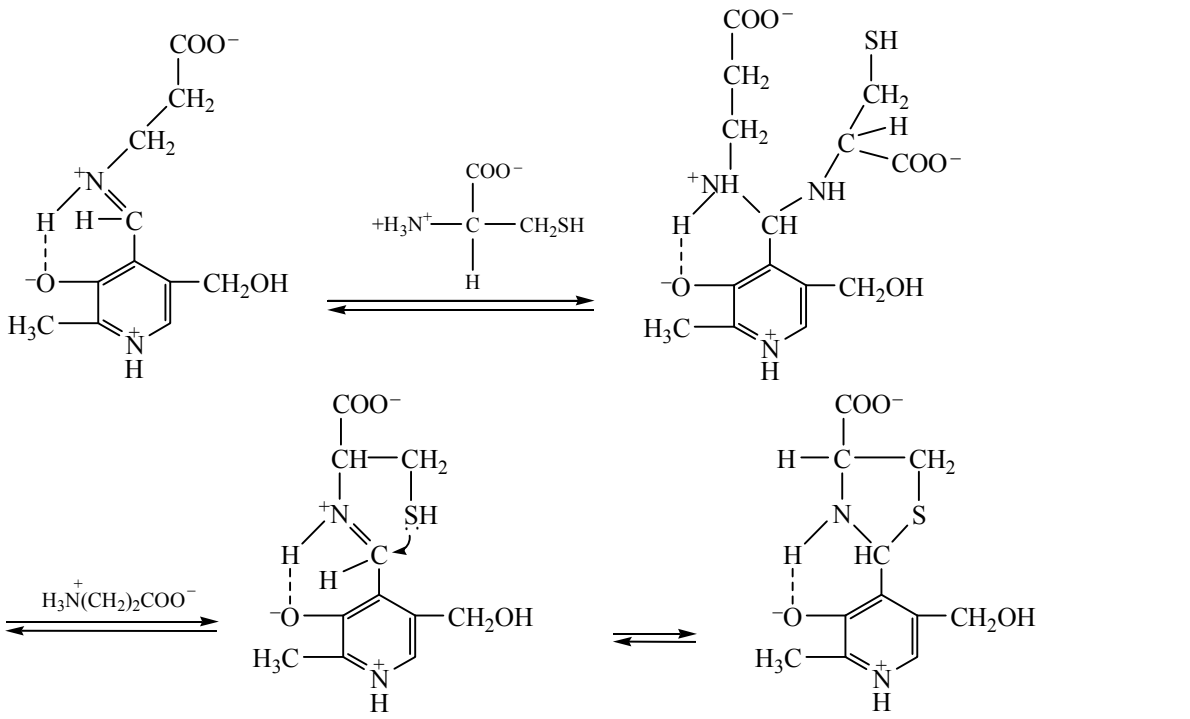


Fig. 2. Change of the optical density of mixtures of 0.01 M solutions of (*1*) pyridoxal hydrochloride and β-alanine and (*2*) *N*-pyridoxylidene-β-alanine and L-cysteine in 90% alcoholic–aqueous buffer (λ 430 nm, pH 6.6, 20°C).



0.0112 min^{-1}) in the condensation of serine with pyridoxal is lower than the corresponding rate constant for amino alcohol dehydration in the reaction of pyridoxal with methionine (k_2 = 0.0197 min^{-1}).

EXPERIMENTAL

Commercial pyridoxal hydrochloroide of chemically pure grade (Ferak Berlin, Hungary) and amino acids (Reanal, United Kingdom) were used. Buffer solutions were prepared according to standard procedures. The reaction mixtures were maintained at a constant temperature with an accuracy of was ±0.1°C using a UH-8 thermostat. Equimolar amounts of pyridoxal or *N*-pyridoxylidene-β-alanine and the corresponding amino acid were dissolved in aqueous–ethanolic buffers, and the solutions were kept for 30 min at a required temperature. The moment of mixing of reactant solutions was assumed to be the reaction onset. The kinetic measurements were performed on a Spektromom-204 spectrophotometer using 1.008-mm cells maintained at a constant temperature. Taking into account that the UV spectra of pyridoxal solutions depend on pH and solvent nature, the reference cell was charged with an equimolar solution of pyridoxal in the same solvent at the same pH value. The condensation and transaldimination rate constants were calculated using calibration curves with the aid of a computer program for reversible and irreversible reactions [8]. The condensation products were synthesized according to the procedure described in [2, 3]. The initial compounds and products were identified by elemental analysis, UV and IR spectroscopy, and chromatography.

2-(3-Hydroxy-5-hydroxymethyl-2-methylpyridin-4-yl)thiazolidine-4-carboxylic acid. A mixture of 0.103 g of pyridoxal hydrochloride, 0.072 g of L-cysteine, and 5 ml of 96% ethanol was heated for 20 min at 50°C until complete dissolution. The progress of the reaction was monitored by spectrophotometry (the absence of absorption in the visible region, λ 335–500 nm) and TLC. The mixture was evaporated at room temperature until a yellowish crystalline solid separated. Yield 0.128 g (74.8%). mp 144–145°C (vigorous decomposition with evolution of gaseous products). IR spectrum (KBr), ν, cm^{-1}: 1512, 1411 (C=O, COO^-), 504 (C–S); no absorption band at 1584 cm^{-1} (C=N) typical of the condensation product of pyridoxal with L-methionine was observed. Found, %: C 42.75; H 4.80; N 9.05. $C_{11}H_{15}N_2O_4S \cdot HCl$. Calculated, %: C 42.9; H 4.88; N 9.1.

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