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STRUCTURE OF MATTER AND QUANTUM CHEMISTRY

Effect of Amino Acids on the Interaction between Cobalamin(II) and Dehydroascorbic Acid

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Abstract—The kinetics of the reaction between one-electron-reduced cobalamin (cobalamin(II), Cb(II)) and the two-electron-oxidized form of vitamin C (dehydroascorbic acid, DHA) with amino acids in an acidic medium is studied by conventional UV–Vis spectroscopy. It is shown that the oxidation of Cbl(II) by dehydroascorbic acid proceeds only in the presence of sulfur-containing amino acids (cysteine, acetylcysteine). A proposed reaction mechanism includes the step of amino acid coordination on the Co(II)-center through the sulfur atom, along with that of the interaction between this complex and DHA molecules, which results in the formation of ascorbyl radical and the corresponding Co(III) thiolate complex.

Keywords: reaction mechanism, kinetics, cobalamin, dehydroascorbic acid, ascorbic acid, thiols.
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INTRODUCTION

Cobalamins (vitamin B_{12}, Cbl) are the vitamins with the most complex structures. They are cobalt complexes with corrin macrocycle (equatorial ligands), 5,6-dimethylbenzimidazole nucleotide (lower axial ligand; DMBI), and different X groups (X = CN^-, H_2O, CH_3^-, and others; upper axial ligand) [1].

Pentacoordinated one-electron reduced cobalamin (cobalamin(II), Cbl(II)) is an important biological form of the complex [2, 3]. It is formed by the reactions between Cbl(III) and a variety of reducing agents: ascorbic acid [4], monosaccharides [5], formate [6], and others. Cobalamin(II) reacts at a high rate with such free radicals as NO [7], NO_2 [8], O_2^- [9], SO_2^- [10], and others.

Dehydroascorbic acid (DHA) is a product of the two-electron oxidation of ascorbic acid (HAA^-) or the one-electron oxidation of ascorbyl radical (AR^-). The reduction of DHA to HAA^- in vivo is performed by NADPH- [11, 12] and glutathione- [13–16] (GSH) dependent enzymes. It is known that thiols (cysteine, homocysteine, glutathione, and others [17, 18]), hydrogen sulfide [19], and *tris*(2-carboxyethyl)phosphine [20] are also able to reduce DHA.

Unfortunately, the kinetics of reactions of metal complexes with DHA remains poorly studied, in contrast to reactions involving ascorbic acid. It is known that DHA oxidizes nitrosylhemoglobin to methemoglobin and nitric oxide [21]. It was found in [22] that Cbl(II) does not react directly with DHA; however, the oxidation of Co(II) to Co(III) and the formation of HAA^- are observed in the presence of biological ligands (GSH, SCN^-).

In this work, we studied the kinetics of the reaction of cobalamin(II) in a weakly acidic medium with amino acids and the effect of the type of functional groups on the mechanism of the process.

EXPERIMENTAL

Hydroxocobalamin hydrochloride (Fluka; ⩾95%), L-cysteine (CySH), *N*-acetyl-L-cysteine (NACSH), L-methionine, L-asparagine, L-aspartic acid, L-glutamine, L-glutamic acid, L-tyrosine, L-serine (Sigma-Aldrich), and sodium borohydride were used without additional purification; other substances were of chemical grade. Argon was used to maintain anaerobic conditions. Cbl(II) was obtained via the anaerobic reduction of hydroxocobalamin with sodium borohydride. The excess of the reducing agent was removed by adding acetone [23].

DHA was synthesized from HAA^- according to the procedure reported in [24]. DHA concentrations were determined by its reduction with an excess of CySH in the pH range of 6.0 to 6.5 to HAA^-, the extinction coefficients of which are well known [25].

Kinetic measurements were performed anaerobically in sealed quartz cuvettes 1 cm thick on a Cary 50 spectrophotometer equipped with cryothermostat. The reaction rate was controlled at 372 nm (for DHA reduction by cobalamin(II) with NACSH and CySH)

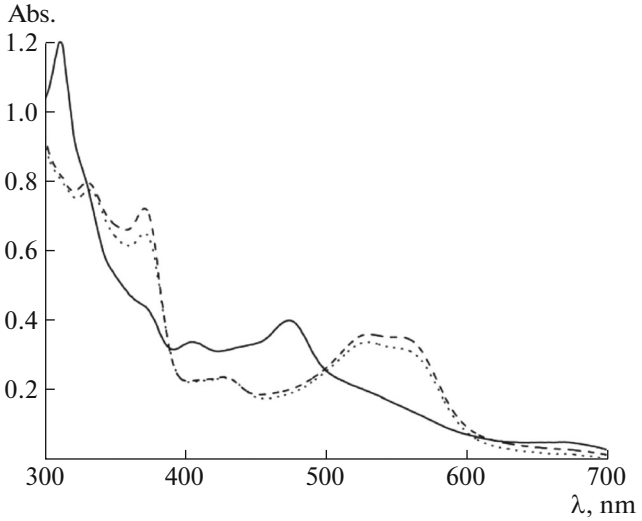


Fig. 1. UV–Vis spectrum of Cbl(II) (solid line) and product of the oxidation of Cbl(II) by dehydroascorbic acid in the presence of CySH (dashed line) and NACSH (dotted line); $[Cbl(II)]_0 = 5 \times 10^{-5}$ mol/L; [DHA] = 1×10^{-3} mol/L; [RSH] = 1×10^{-3} mol/L; pH 5.0; 25°C.

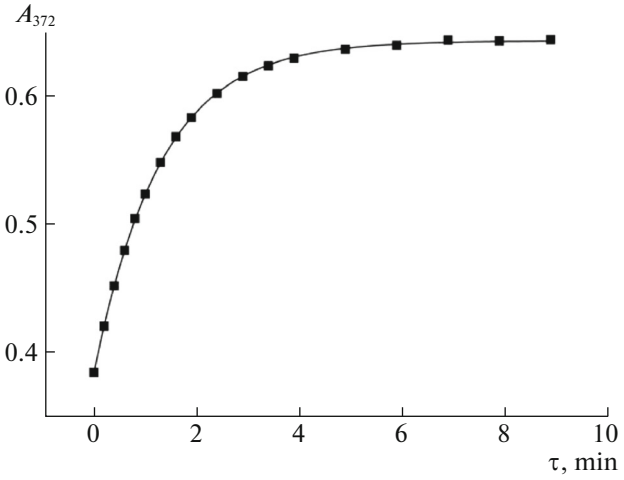


Fig. 2. Typical kinetic curve of the reaction between Cbl(II) and DHA in the presence of CySH (dots) and its fitting to the exponent equation (line); $[Cbl(II)]_0 = 5 \times 10^{-5}$ mol/L; [DHA] = 1.7×10^{-3} mol/L; [CySH] = 6×10^{-4} mol/L; pH 5.47; 25°C.

and 265 nm (for DHA reduction by thiol amino acids). The data were analyzed using the Origin 7.5 program.

RESULTS AND DISCUSSION

It is known that Cbl(II) does not react with DHA in the pH range of 1.5 to 7.0 [22]. The reaction was not investigated at higher pH values because of the instability of DHA [26]. It was found that there was no interaction between Cbl(II) and DHA in the presence of such amino acids as glutamine, asparagine, glutamic and aspartic acids, tyrosine, serine, and methionine. With CySH and NACSH, the growth of the maxima at 372 and 558 nm and their reduction at 312 and 474 nm are observed in the UV–Vis spectrum (Fig. 1). The UV–Vis spectra of the reaction products correspond to Cbl(III) complexes with cysteine [27] and acetylcysteine [28]. A similar reaction proceeds when GSH is present in the system [22].

The kinetics of the reaction between Cbl(II) and an excess of DHA in the presence of CySH and NACSH excesses was studied. A typical kinetic curve of the process is described by a first order equation (Fig. 2). The dependence of the observed rate constant (k_{obs}) on [DHA] is linear (Fig. 3), indicating the first order of the reaction with respect to the oxidant. In addition, there is a positive intercept on the *Y*-axis. The reaction rate depends on [RSH]: the calculated slopes (k') of the k_{obs} dependences on [DHA] increase linearly along with [RSH] (Fig. 4).

The effect of pH on the rate of reaction was studied. It was shown that with CySH and NACSH, the reaction rate grew along with pH, as was observed in the presence of GSH in [22]. The increase in the rate was apparently due to the growing concentration of thiolate anion (RS^-). It should be noted that the highest rate was observed for the reaction proceeding in the presence of GSH; the lowest one was for the reaction with NACSH. Microscopic pK_a values of the dissociation of the SH-group are 8.9 for GSH with the protonated amino group, 9.1 for GSH with the deprotonated amino group [29], 8.5 for CySH with the protonated amino group, 10.2 for CySH with deprotonated amino group [30], and 9.6 for NACSH [31] at 25°C. It seems that the negligible difference in the rates of the reaction proceeding with CySH and GSH was due to the similarity between the ionization constant values of

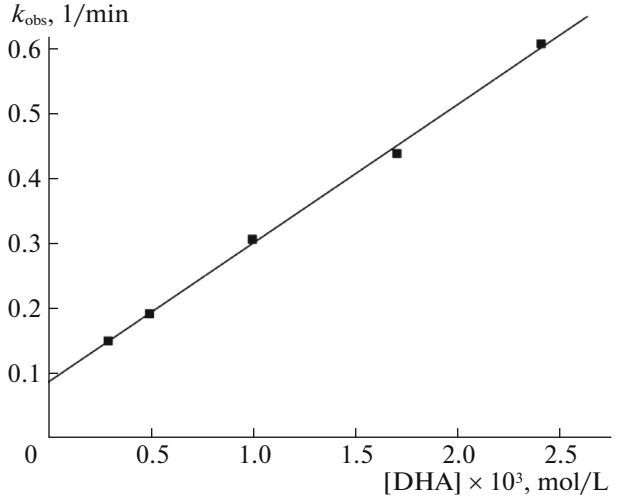


Fig. 3. Dependence of the observed reaction rate constant (k_{obs}) on [DHA]; $[Cbl(II)]_0 = 5 \times 10^{-5}$ mol/L; [CySH] = 3×10^{-4} mol/L; pH 5.47; 25°C.

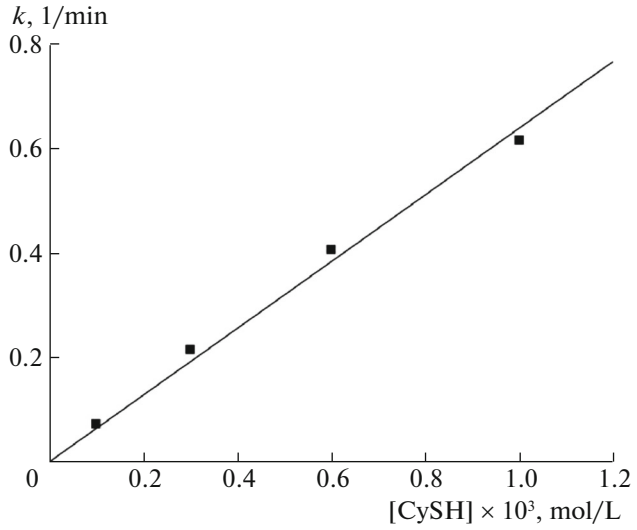


Fig. 4. Dependence of the observed reaction rate constant (k') on [CySH]; $[Cbl(II)]_0 = 5 \times 10^{-5}$ mol/L; pH 5.47; 25°C.

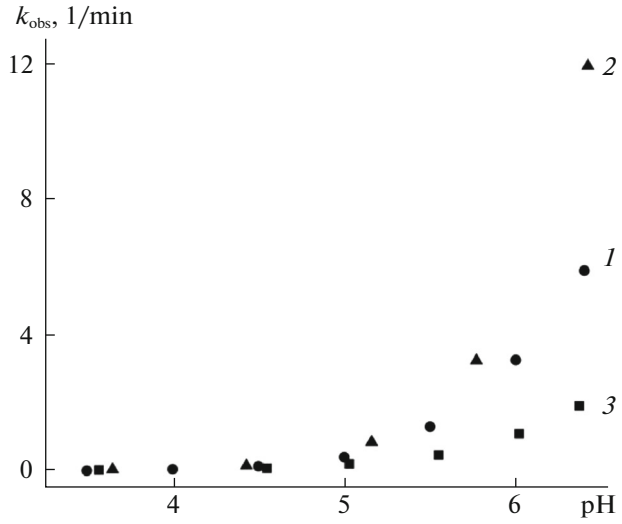


Fig. 5. Dependence of k_{obs} on pH for the reaction between Cbl(II) and DHA in the presence of (*1*) CySH, (*2*) GSH, and (*3*) NACSH; $[Cbl(II)]_0 = 5 \times 10^{-5}$ mol/L; [DHA] = 1×10^{-3} mol/L; [RSH] = 1×10^{-3} mol/L; 25°C.

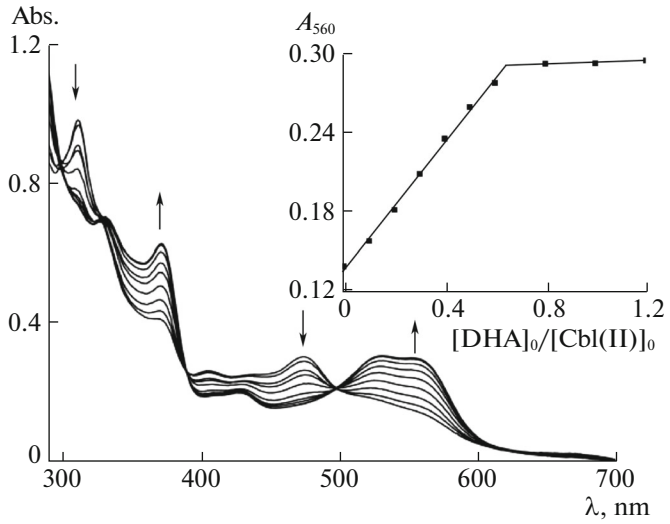


Fig. 6. Spectral changes observed during the titration of cobalamin(II) with dehydroascorbic acid. Insert: dependence of absorption at 560 nm on $[DHA]_0/[Cbl(II)]$; $[Cbl(II)]_0 = 5 \times 10^{-5}$ mol/L; $[CySH] = 1 \times 10^{-3}$ mol/L; pH 6.8; 25°C.

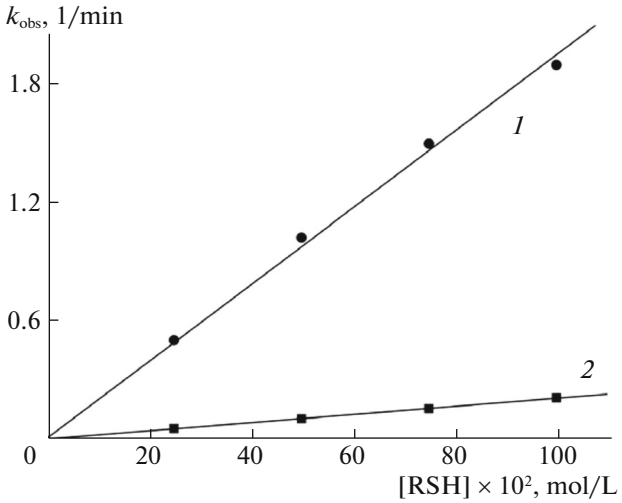


Fig. 7. Dependences of the observed rate constants (k'_{obs}) for the reduction of dehydroascorbic acid with (*1*) cysteine and (*2*) acetylcysteine on the concentration of these amino acids; $[DHA]_0 = 1 \times 10^{-5}$ mol/L; pH 6.45; 25°C.

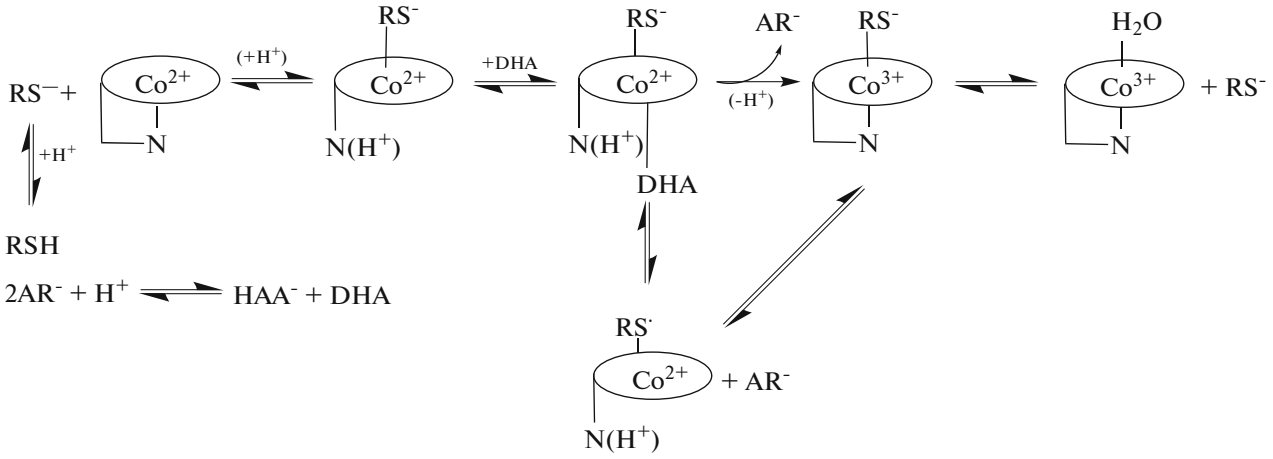
their thiol groups, while the higher value of the ionization constant of NACSH thiol group relative to other amino acids explains the lowest reaction rate for this system at the same pH value (Fig. 5).

To determine the stoichiometry of the reaction, we conducted the titration of cobalamin(II) with dehydroascorbic acid in the presence of CySH and NACSH (Fig. 6). It was found that the oxidation of 1 mol of Cbl(II) required 0.6 mol of DHA in the presence of CySH, and 0.4 mol of DHA in the presence of NACSH. This indicates the two-electron reduction of DHA and formation of ascorbic acid in the course of the reaction.

It is known that CySH, NACSH, and GSH are able to reduce DHA to HAA^- [17]. In this work, we investigated the kinetics of the reaction in the presence of CySH and NACSH. The reaction involving GSH was studied in [22]. It was found that the first order of the reaction with respect to DHA was observed with

an excess of the reducing agent (Fig. 7). The observed rate constant (k'_{obs}) depends linearly on [RSH], confirming the first order of the reaction with respect to RSH. However, the rate of the reaction of DHA with the abovementioned amino acids was considerably lower than that of the reduction of dehydroascorbic acid in the presence of both Cbl(II) and thiols (table).

Based on the obtained data, we propose the following scheme of the reduction of dehydroascorbic acid by cobalamin(II) in the presence of thiol amino acids:



The crucial step in this mechanism is the coordination of amino acids on the Co^{2+} center, which likely occurs through the sulfur atom. The binding of sulfur amino acids by cobalamin(II) was proved previously by electron paramagnetic resonance [32], while the coordination of the ligands in the upper position of cobalamin(II) through the nitrogen atom was not confirmed experimentally [33]. This could explain the need for thiol amino acids for the reaction to proceed. The presence of the methyl group in the methionine molecule prevents the formation of the Co(II)–methionine complex.

Coordination of the ligand in the upper position of cobalamin(II) weakens the Co^{2+}–N(DMBI) bond [32, 34]. It should be noted that the binding of ligands by the Co^{2+} center is a very fast process [35]. The reaction of the resulting Co^{2+}-thiolate complex with the DHA molecule probably leads to electron transfer and the formation of AR^- and the corresponding thiolate cobalamin(III) complex. AR^- is unstable in acidic and neutral media and rapidly disproportionates to HAA^- and DHA [36].

Let us consider the reasons for the origin of *Y*-intercepts on the concentration dependences (Fig. 3). The reverse reaction of the reduction of Cbl(III) thiolate complexes to Cbl(II) by thiol amino acids or HAA^- formed in the reaction proceeds much slower than the oxidation of Cbl(II) by the dehydroascorbic acid in the presence of CySH, NACSH, and GSH. The origin of the *Y*-intercepts (Fig. 3) is presumably due to reactions with free radicals (ascorbyl- and thiyl), since the oxidation of Co^{2+}–thiolate complex can proceed on both Co^{2+}-center and thiolate anions.

Comparison of the rates (r_{max} is the maximum reaction rate corresponding to the one at the initial point in time) of reactions for the reduction of dehydroascorbic acid by thiols and cobalamin(II) in the presence of thiols

RSH	r_{max}, mol/(L min)	
	DHA + RSH	Cbl(II) + DHA + RSH
CySH	1.95×10^{-8}	2.94×10^{-4}
NACSH	2.04×10^{-9}	9.58×10^{-5}
GSH	1.18×10^{-8}	5.96×10^{-4}

[DHA] = 1×10^{-3} mol/L; [RSH] = 1×10^{-3} mol/L; pH 6.45; 25°C; $[Cbl(II)]_0 = 5 \times 10^{-5}$ mol/L.

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