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# Effect of Bovine Serum Albumin on Redox and Ligand Exchange Reactions Involving Aquacobalamin

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This paper is dedicated to prof. Rudi van Eldik on the occasion of his 75th birthday. His works on vitamin $B_{12}$ have been a source of inspiration for us

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*Bovine serum albumin (BSA) is capable of binding aquacobalamin ($H_2OCbl$) by two different modes, viz. (i) via hydrogen bonding or π-π interactions without substitution of water molecule, or (ii) via water substitution to give amino BSA–Cbl(III) complex. In this work, we showed that the first type of complex exhibits the same reactivity toward ascorbic acid and thiocyanate as $H_2OCbl$. The amino BSA–Cbl(III) complex is substantially less reactive toward ascorbic acid, thiocyanate and sulfite than $H_2OCbl$. Using sulfite, we showed that ligand exchange in this complex proceeds via two pathways, i.e. via (i) a direct substitution of BSA by sulfite species or (ii) a slow dissociation of BSA from the complex followed by rapid sulfite binding by Cbl(III).*

**Keywords**: Cobalamin, serum albumin, ascorbic acid, redox reactions, ligand exchange.

# Влияние бычьего сывороточного альбумина на редокс реакции и лигандный обмен с участием аквакобаламина

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*Бычий сывороточный альбумин (БСА) способен связывать аквакобаламин ($H_2OCbl$) двумя способами: (i) за счет образования водородных связей или π-π взаимодействия без замещения молекулы воды или (ii) в результате замещения воды и образования аминокомплекса. В этой работе показано, что комплекс первого типа обладает такой же реакционной способностью по отношению к аскорбиновой кислоте и тиоцианату, как и $H_2OCbl$. Аминокомплекс БСА–Cbl(III) является намного менее реакционноспособным по отношению к аскорбиновой кислоте, тиоцианату и сульфиту, чем $H_2OCbl$. С использованием сульфита показано, что лигандный обмен в этом комплексе протекает по двум маршрутам: через (i) прямое замещение БСА сульфитом или (ii) медленную диссоциацию БСА из комплекса, сопровождающуюся быстрым связыванием сульфита кобаламином(III).*

**Ключевые слова**: Кобаламины, сывороточный альбумин, аскорбиновая кислота, редокс реакции, лигандный обмен.

## Introduction

Human serum albumin (HSA) is the most abundant protein in blood plasma.[1,2] Bovine serum albumin (BSA) is frequently used as a model of HSA due to close structural resemblance.[3] Serum albumins bind and transport numerous endo- and exogenous molecules.[4,5] Serum albumins are capable of binding at several specific sites[6] and transferring fatty acids,[7–9] steroids,[10,11] bilirubin,[12,13] carotenoids,[14] porphyrins,[15–17] and other compounds. They are also capable of binding drugs and drug-like molecules.[18]

Serum albumins are capable of reacting with cobalamins (Cbls; Figure 1).[19–25] Cyano- (CNCbl) and aqua- ($H_2OCbl$) Cbls act as quenchers of HSA and BSA fluorescence, *i.e.* in the case of CNCbl and HSA the quenching occurs *via* the dynamic mechanism,[23] CNCbl quenches BSA fluorescence *via* the static or combined mechanism,[24,25] whereas the static mechanism was reported for $H_2OCbl$ and BSA.[22] It is reported that complexation of BSA with CNCbl leads to structural changes in protein molecule.[25] HSA tightly binds several hydrophobic Cbl derivatives to give hybrid materials capable of catalyzing carbon-skeleton rearrangement upon light irradiation.[26,27]

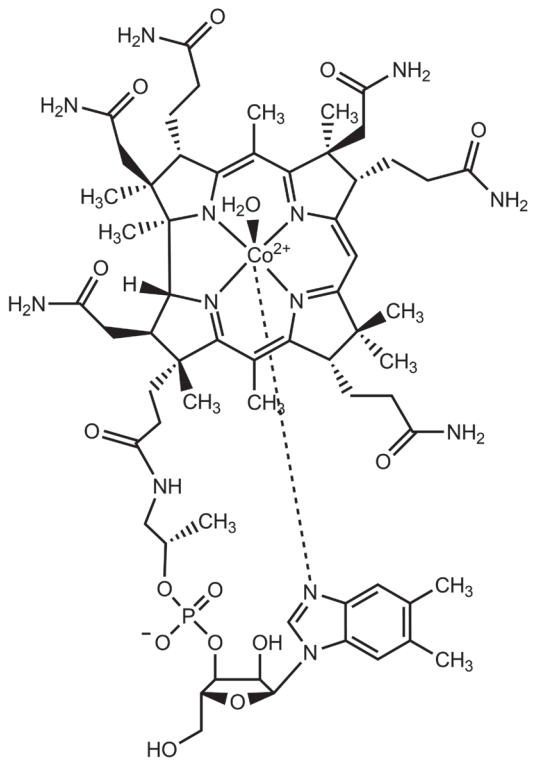


**Figure 1.** Structure of aquacobalamin.

Earlier, we showed that the reaction between $H_2OCbl$ and native BSA includes two steps.[22] The first step proceeds rapidly and generates a complex *via* hydrogen bonding or π-π interactions without substitution of water molecule (hereafter, 'the labile complex'). In the course of the second step, relatively slow substitution of water molecule occurs and the amino complex between Co(III) and one of BSA lysine residues is formed. The formation of amino BSA–Cbl(III) complex does not proceed stoichiometrically, and its fraction co-exists in solution with the labile complex. The interaction of $H_2OCbl$ and BSA does not affect the formation of methemealbumin, a complex between Fe(III)-heme and BSA, indicating involvement of different binding sites of BSA in reactions with these tetrapyrroles. Reduction of BSA changes the reaction mechanism: BSA forms with Co(III) thiolate complex, which is further decomposed to Co(II)-species.

$H_2OCbl$ is a relatively reactive complex, *i.e.* it can be involved in numerous ligand exchange[28–30] and redox reactions.[30] However, it remains unclear how binding to serum albumins affects its reactivity. This work provides data on reactions of $H_2OCbl$ in the absence and in the presence of BSA.

## Experimental

Hydroxocobalamin hydrochloride (Sigma-Aldrich; HOCbl; ≥96 %), bovine serum albumin (Sigma-Aldrich; heat shock fraction, p*H* 5.2; ≥96 %), ascorbic acid (Sigma-Aldrich; AA; ≥99 %), sodium sulfite (Sigma-Aldrich; ≥98 %), potassium thiocyanate (Sigma-Aldrich; 99 %) were used without additional purification. Oxygen-free argon was used to deoxygenate reagent solutions.

Phosphate buffer solutions (0.1 M) were used to maintain p*H* during the measurements. The p*H* values of solutions were determined using Multitest IPL-103 p*H*-meter (SEMICO) equipped with ESK-10601/7 electrode (Izmeritelnaya tekhnika) filled by 3.0 M KCl solution. The electrode was preliminarily calibrated using standard buffer solutions (p*H* 1.65–12.45).

Concentrations of Cbl stock solutions were determined using UV-Vis spectroscopy *via* a conversion of Cbl to its dicyano-form (extinction coefficient is 30400 $M^{-1}\cdot cm^{-1}$ at 368 nm[31]).

Ultraviolet-visible (UV-Vis) spectra were recorded on a cryothermostated (± 0.1 °C) Cary 50 UV-Vis spectrophotometer in quartz cells.

Equilibrium constants ($K$) were calculated using Eq. (1).[32]

$$A = \frac{A_0 + A_\infty K[\mathrm{L}]}{1 + K[\mathrm{L}]}. \quad (1)$$

where [L] is the ligand concentration in solution, M; $A$ is absorbance at the monitoring wavelength for the complex at a particular ligand concentration; $A_0$ is the absorbance for the starting complex; $A_\infty$ is the absorbance for the final complex.

Experimental data were analyzed using Origin 7.5 software.

## Results and Discussion

### *Effect of BSA on the Reaction between $H_2OCbl$ and Ascorbic Acid*

It is well-known that $H_2OCbl$ can be efficiently reduced to Co(II)-form (Cbl(II)) by AA (Figure 2A).[33–35] We found that preliminary incubation of $H_2OCbl$ with BSA substantially decreases the yield of Cbl(II) in the reaction with AA, *i.e.* the UV-Vis spectrum of the product includes peaks at 358 and 537 nm (Figure 2B), which are not typical to Cbl(II) and $H_2OCbl$. Addition of BSA to Cbl(II) does not change its UV-Vis spectrum.

The yield of Cbl(II) strongly depends on incubation time of $H_2OCbl$ with BSA (Figure 3). The yield of Cbl(II) and kinetic curve profile coincide for experiments in the

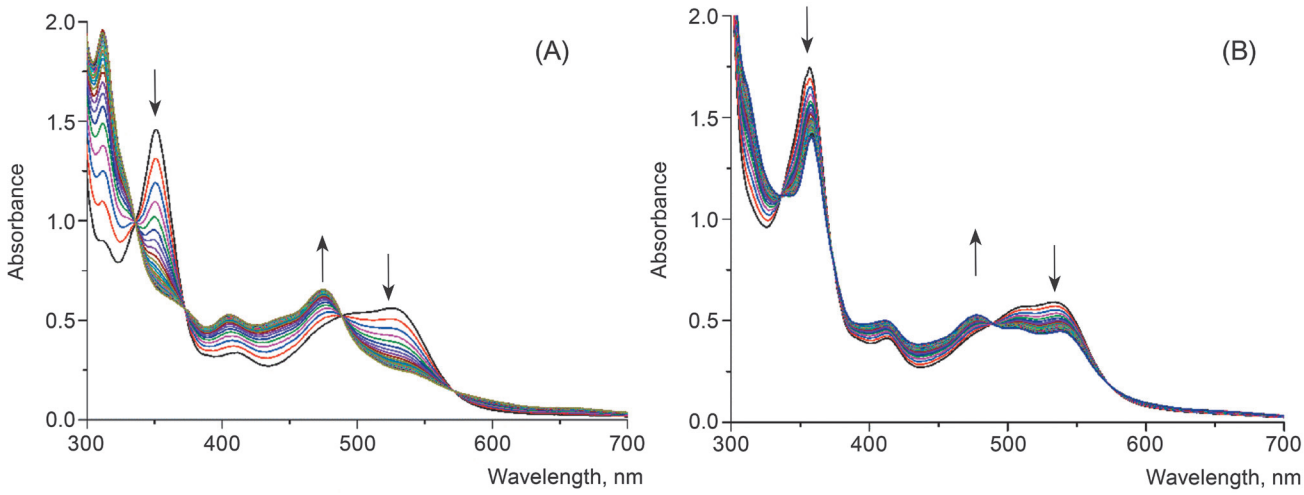



**Figure 2.** UV-Vis spectra of the reaction of $H_2OCbl$ ($7.6\cdot10^{-5}$ M) with AA ($7.5\cdot10^{-4}$ M) in the absence (A) and in the presence (B) of BSA ($4.0\cdot10^{-4}$ M) at p*H* 7.0, 25.0 °C. The mixture of $H_2OCbl$ with BSA was incubated for 1 h prior adding AA.

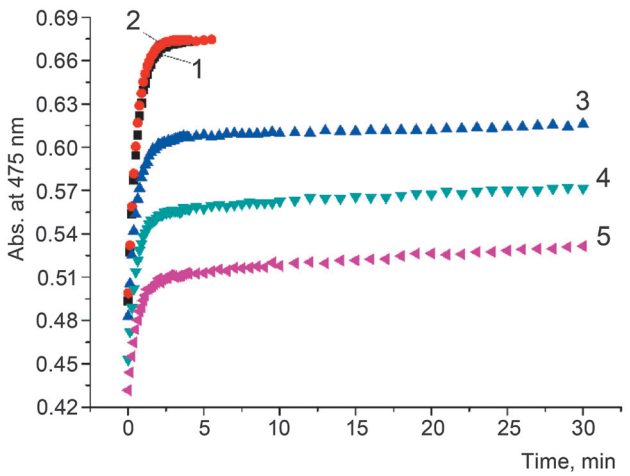



**Figure 3.** Time-course curves of the reaction between $H_2OCbl$ ($7.6\cdot10^{-5}$ M) and AA ($7.5\cdot10^{-4}$ M) in the absence (1) and in the presence of BSA ($4.0\cdot10^{-4}$ M) at p*H* 7.0, 25.0 °C. Incubation time of $H_2OCbl$ with BSA is 0 (2), 15 (3), 30 (4) and 60 (5) min.

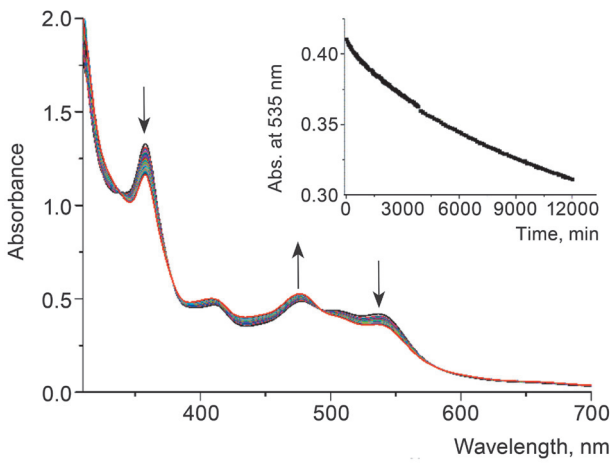



**Figure 4.** UV-Vis spectra of the second step of the reaction between $H_2OCbl$ ($7.6\cdot10^{-5}$ M) incubated with BSA ($4.0\cdot10^{-4}$ M) for 1 h and AA ($4.5\cdot10^{-3}$ M) at p*H* 7.0, 25.0 °C. Insert: a time-course curve of the reaction.

absence and in the presence of BSA, where BSA and AA are simultaneously added to Cbl(II), whereas the yield of Cbl(II) becomes lower upon increasing incubation time.

These observations can be explained by the slow formation of the amino complex between Cbl(III) and BSA,[22] which is relatively inert toward reduction by AA, whereas the labile complex between BSA and $H_2OCbl$ formed within mixing time has virtually the same reactivity as free $H_2OCbl$. Despite the interaction between BSA and AA occurs *via* static mechanism (*viz*, *via* complexation with equilibrium constant $K = 2.3\cdot10^4$ $M^{-1}$ at 22.9 °C),[36] this process cannot account for the significant decrease in reaction rate between $H_2OCbl$ and AA in the presence of BSA, because a major AA fraction exists in unbound state under experimental conditions.

Further, we examined whether the amino complex between BSA and Cbl(III) can be reduced by higher quantities of AA. The slow decay of absorbance at 535 nm indicating the reduction of the BSA-Cbl(III) complex is observed, although the reaction does not complete after 3 hours (Figure 4).

### *Effect of BSA on the Reaction of $H_2OCbl$ with Thiocyanate and Sulfite*

Next, we checked whether BSA can affect binding properties of $H_2OCbl$ toward thiocyanate and sulfite. $H_2OCbl$ is capable of reacting with $SCN^-$ to give mixture of N- and S-bound complexes[37,38] ($K = 1.1\cdot10^3$,[39] $1.4\cdot10^3$[40] $M^{-1}$, 25.0 °C) and almost stoichiometrically forms S-bound sulfitocobalamin.[41,42]

For binding $SCN^-$ by $H_2OCbl$, $K = (1.4 \pm 0.1)\cdot10^3$ $M^{-1}$ (25.0 °C) was obtained that coincides with literature data.[40] In the case of $H_2OCbl$ incubated in the presence of BSA

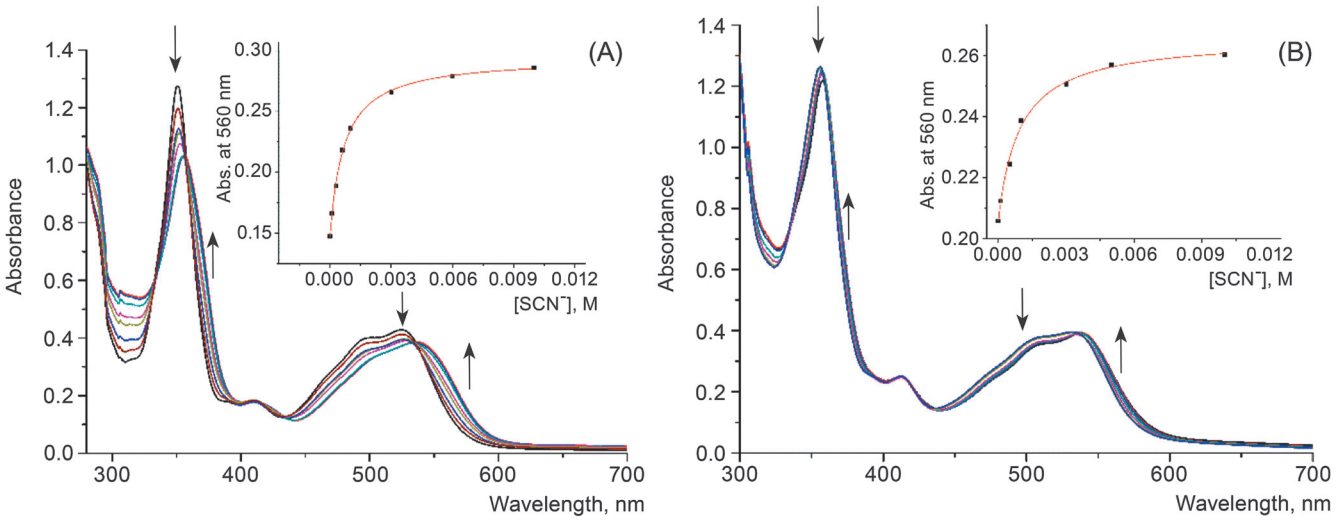



**Figure 5.** UV-Vis spectra collected in the course of $H_2OCbl$ ($5.0\cdot10^{-5}$ M) titration by $SCN^-$ in the absence (A) and in the presence (B) of BSA ($4.0\cdot10^{-4}$ M) at p*H* 7.0, 25.0 °C. The mixture of $H_2OCbl$ with BSA was incubated for 1 h prior adding $SCN^-$. Inserts: plots of absorbance at 560 nm *versus* [$SCN^-$] fitted to Eq. (1).

for 1 h, $K = (1.1 \pm 0.1)\cdot10^3$ $M^{-1}$ (25.0 °C) was determined that is close to equilibrium constant for free $H_2OCbl$, although in the presence of BSA UV-Vis spectral changes were less pronounced than for free $H_2OCbl$ (Figure 5). Formation of thiocyanato-Cbl(III) in these reactions proceeds rapidly, whereas prolonged incubation of $H_2OCbl$/BSA mixture with higher [$SCN^-$] is accompanied by further changes in UV-Vis spectrum, *i.e.* a slight decay of absorbance at 358 nm is observed (Figure 6). However, we were unable to study this reaction due to its low rate. Probably, in the course of the rapid step, the labile complex of $H_2OCbl$ with BSA reacts with $SCN^-$ similarly to free $H_2OCbl$. Further, the fraction of amino BSA–Cbl(III) complex is slowly transformed to thiocyanato-Cbl(III).

To study reactivity of amino BSA–Cbl(III) complex in ligand-exchange reactions, we used sulfite, which possesses higher affinity toward Cbl(III) than thiocyanate. UV-Vis spectra of the reaction between an excess of sulfite and amino BSA–Cbl(III) complex is provided in Figure 7A, *i.e.* decrease in absorbance at 358 nm is observed. The spectrum of the product coincides with that of sulfito-Cbl(III).[43] A typical kinetic curve of the reaction (Figure 7A) is well described by exponential equation indicating first order with respect to amino BSA–Cbl(III) complex. Dependence of observed rate constant ($k_{obs.}$) *versus* sulfite concentration is linear indicating first order with respect to sulfite and exhibits a positive intercept (Figure 7B), which can be explained by the parallel pathway of sulfito-Cbl(III) formation, *i.e.* sulfito-Cbl(III) can be formed *via* substitution of BSA in amino BSA–Cbl(III) complex by sulfite (which is ascribed to the slope of concentration dependence in Figure 7B) or *via* slow BSA dissociation from BSA–Cbl(III) and further rapid sulfite binding by Cbl(III) (which is ascribed to intercept of concentration dependence in Figure 7B; Scheme 1).

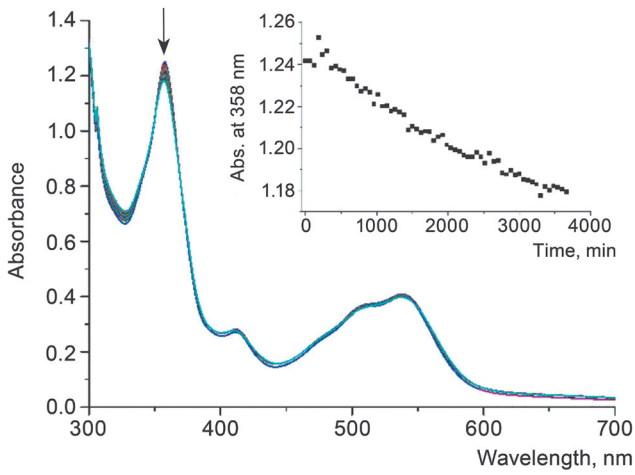



**Figure 6.** UV-Vis spectra of the second step of the reaction between $H_2OCbl$ ($5.0\cdot10^{-5}$ M) incubated with BSA ($4.0\cdot10^{-4}$ M) for 1 h and $SCN^-$ ($8.0\cdot10^{-2}$ M) at p*H* 7.0, 25.0 °C. Insert: a time-course curve of the reaction.

## Conclusions

This study showed that two types of complexes of bovine serum albumin with aquacobalamin exhibit different reactivity in redox and ligand exchange reactions. A complex of $H_2OCbl$ and BSA rapidly formed without substitution of water molecule reacts with ascorbic acid and thiocyanate similarly to free $H_2OCbl$. The slowly generated amino BSA–Cbl(III) complex can be reduced to Cbl(II) by ascorbic acid at substantially lower rate than $H_2OCbl$. Ligand substitution reactions involving amino BSA–Cbl(III) proceed slowly as well. Using sulfite, we showed that substitution of BSA in this complex proceeds *via* two pathways, *i.e. via* a direct substitution of BSA by sulfite species or a slow dissociation of BSA from the complex followed by rapid sulfite binding by Cbl(III).

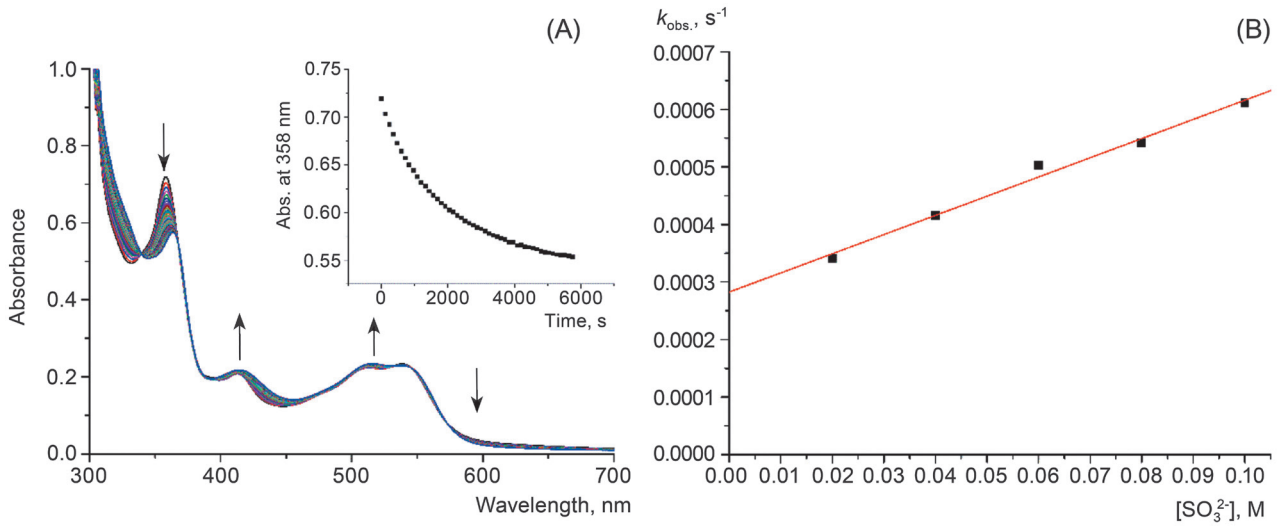
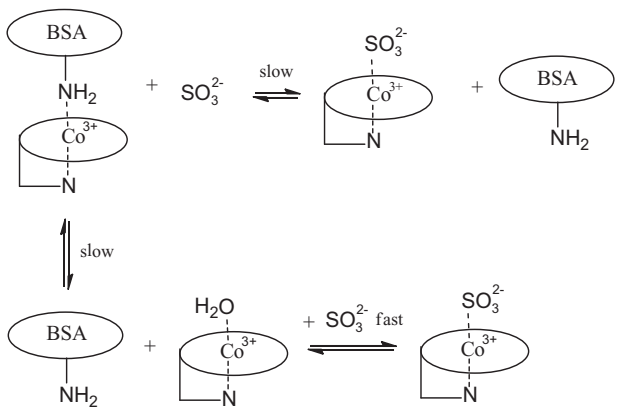



**Figure 7.** UV-Vis spectra of the second step of the reaction between $H_2OCbl$ ($5.0\cdot10^{-5}$ M) incubated with BSA ($4.0\cdot10^{-4}$ M) for 1 h and $SO_3^{2-}$ ($8.0\cdot10^{-2}$ M) at p*H* 7.0, 25.0 °C (A; the insert is a time-course curve of the reaction), and plot of $k_{obs.}$ *versus* [$SO_3^{2-}$] for the reaction (B).




**Scheme 1.** Mechanism of reaction between amino BSA-Cbl(III) complex and sulfite.

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