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Laboratory Exercises

Comparison of the Interaction of Cobalt Bovine Carbonic Anhydrase II with Acetazolamide and Methazolamide and the Reaction of Apoenzyme with Cobalt(II) Complexes of Acetazolamide and Methazolamide

SPECTROPHOTOMETRIC STUDY

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The metalloenzyme carbonic anhydrase (CA) is an attractive choice for a research-based bioinorganic laboratory course. In this project the interaction of cobalt bovine carbonic anhydrase II (CoBCAII) with acetazolamide and methazolamide and the reaction of apoenzyme with cobalt(II) complexes of acetazolamide and methazolamide is studied by UV-visible spectroscopy. Prior to this spectroscopic study students are given native BCAII, and they prepare apoBCAII and CoBCAII. A major aim is to provide experience in handling metalloproteins and in the study of metal complexes-protein interactions.

Keywords: Carbonic anhydrase, sulfonamide inhibitors, sulfonamide-CoCA interaction, cobalt(II) complexes-apoCA interaction, UV-visible spectroscopic study.

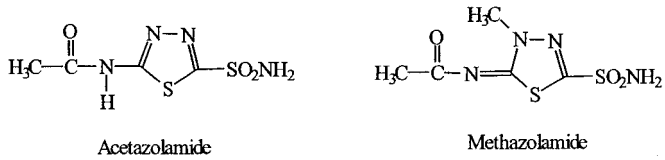
The ubiquitous enzyme carbonic anhydrase (CA)[1] is present in Archaea, prokaryotes, and eukaryotes. The comparison of sequences and crystal structures of the mammalian and plant enzymes demonstrates that they evolved independently, and the mammalian and plant enzymes have been designated the α- and β-class, respectively. An additional independently evolved γ-class was reported in 1994 for which phylogenetic analyses predict an ancient origin [1]. In higher vertebrates, including humans, 14 different CA isozymes have been described up to now. They are involved in the crucial physiological processes connected with respiration and transport of CO_2/bicarbonate, pH homeostasis, electrolyte secretion, and biosynthetic reactions such as lipogenesis, gluconeogenesis, and ureagenesis among others. In addition to the physiological reaction, the reversible hydration of carbon dioxide to bicarbonate, CAs also catalyze a variety of reactions such as aldehyde hydration and hydrolysis of carboxylic acid esters [2].

Human carbonic anhydrase II (HCAII) is a zinc enzyme predominantly found in red blood cells where it catalyzes the reaction [3, 4]:

$$CO_2 + H_2O \Longleftrightarrow HCO_3^- + H^+$$

With a turnover rate of 10^6/s at pH 9, 25 °C, it is one of the fastest enzymes known. The structure of HCAII has been refined at 2.0-Å resolution [5]. The active site cavity of HCAII has a conical shape. It is about 15 Å wide in entrance and penetrates about 15 Å into the middle of the molecule. The zinc ion, at the bottom of the cavity, is ligated to three histidyl residues (His-94, His-96, and His-114), and the fourth ligand is a water molecule (water 263). The zinc coordination is almost tetrahedral (see Fig. 1). CA has been widely investigated spectroscopically (using Co(II)-substituted enzyme), kinetically, and by x-ray crystallography. The research on CA includes the use of inhibitors of this enzyme.

Sulfonamides represent an important class of biologically active compounds with at least five different classes of pharmacological agents. The sulfonamides are well known inhibitors of zinc carbonic anhydrase enzyme and have many applications including use as diuretics, antiglaucoma agents, and anti-epileptic drugs among others [6]. Among the large number of sulfonamides, acetazolamide (H_2acm) and its derivative, methazolamide, (Hmacm) (Scheme 1) are used extensively.



SCHEME 1

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[1] The abbreviations used are: CA, carbonic anhydrase; CoBCAII, cobalt bovine carbonic anhydrase II; HCAII, human carbonic anhydrase II; H_2acm, acetazolamide; Hmacm, methazolamide; $Hacm^-$, monodeprotonated form of H_2acm; acm^{2-}, dideprotonated form of H_2acm.

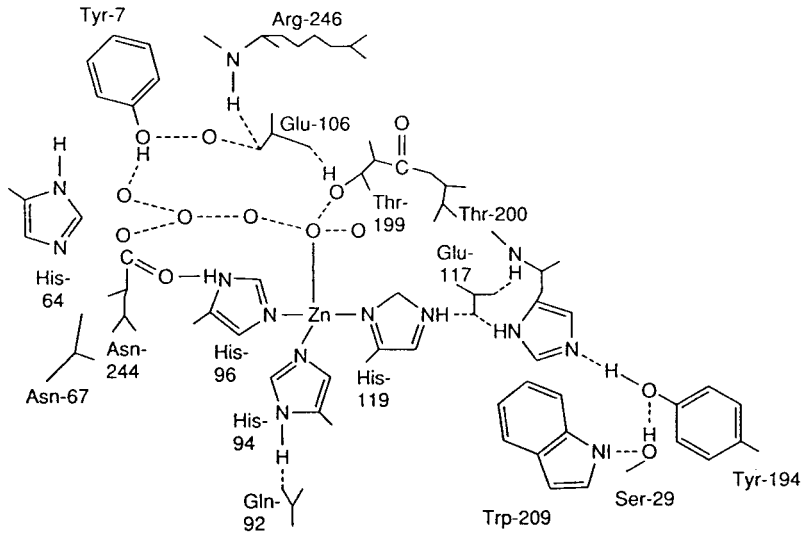

FIG. 1. **Active center of BCAII enzyme.**

It is known that the inhibition activity of sulfonamides is due to the coordination of the deprotonated sulfonamido group to the Zn(II) instead of the water molecule. The crystal structure of HCAII complex with H_2acm [7] shows that the Zn(II) ion is linked to the nitrogen atom of the deprotonated sulfonamido of acetazolamide and to three histidyl nitrogen atoms from the three histidine residues.

Our research group has synthesized and characterized numerous metal complexes of sulfonamides, particularly with H_2acm and Hmacm [8, 9]. H_2acm presents a diverse coordination behavior as ligand. It can act in a monodeprotonated form ($Hacm^-$) linking the metal ion through the nitrogen atom of the deprotonated sulfonamido group or through the thiadiazole nitrogen atom contiguous to the deprotonated acetamido moiety. Moreover, acetazolamide in its dideprotonated form (acm^{2-}) can behave as a bidentate ligand coordinating through both sulfonamido and thiadiazole nitrogen donor atoms. In the $[Zn(Hacm)_2(NH_3)_2]$ and $[Co(Hacm)_2(NH_3)_2]$ complexes acetazolamide presents a coordination behavior similar to that exhibited in the CA-acetazolamide complex [8].

Methazolamide only can interact with the metal ions through the nitrogen atom of the deprotonated sulfonamido group. The $[Zn(macm)_2(NH_3)_2]$ compound (Fig. 2) [9] can be considered the best simple structural model of the inhibition of zinc CA by methazolamide.

The proposed laboratory project for students in the last year of the bioinorganic area consists of:

1. Preparation of apoBCAII through dialysis using pyridine-2,6-dicarboxylic acid as chelating agent.
2. Preparation of CoBCAII by metalation of apoBCAII with Co(II).
3. Spectroscopic study of the CoBCAII at different pH values.
4. Spectroscopic study of the interaction between CoBCAII and H_2acm and between CoBCAII and Hmacm.
5. Spectroscopic study of the interaction between apoBCAII and $[Co(Hacm)_2(NH_3)_2]$ and between ApoBCAII and $[Co(macm)_2(NH_3)_2]$.

Finally, conclusions deduced from the spectroscopic studies will be summarized.

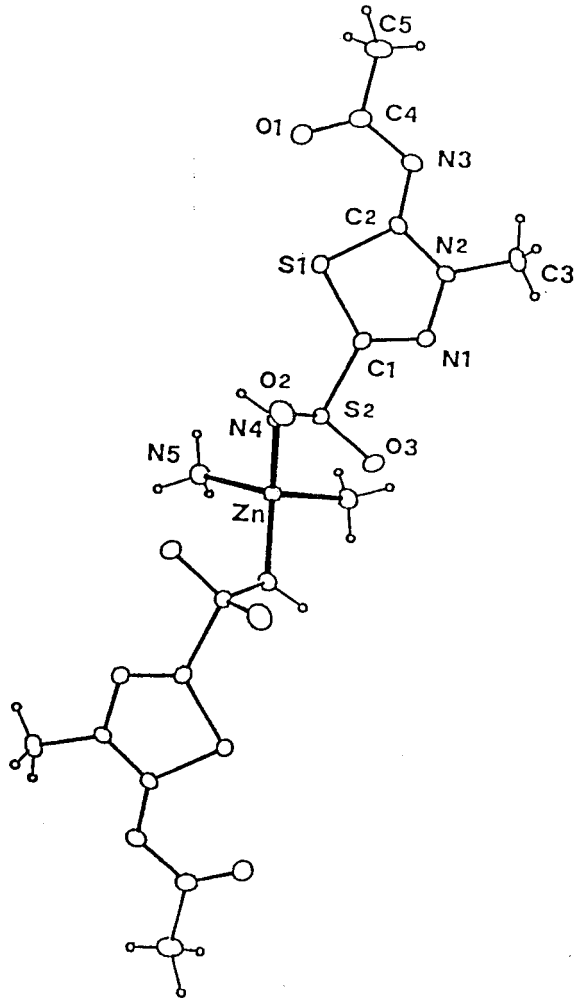

FIG. 2. **Crystal structure of $[Zn(macm)_2(NH_3)_2]$.**

MATERIALS AND METHODS

Equipment and Reagents—Bovine carbonic anhydrase II, acetazolamide, and methazolamide were from Sigma. All the other chemicals were AR grade and were supplied by Sigma. The laboratory equipment used in this class includes the following: a balance, a centrifuge, Eppendorf tubes, dialysis membrane, micropipettes, beakers, volumetric flasks, and UV quartz cuvettes.

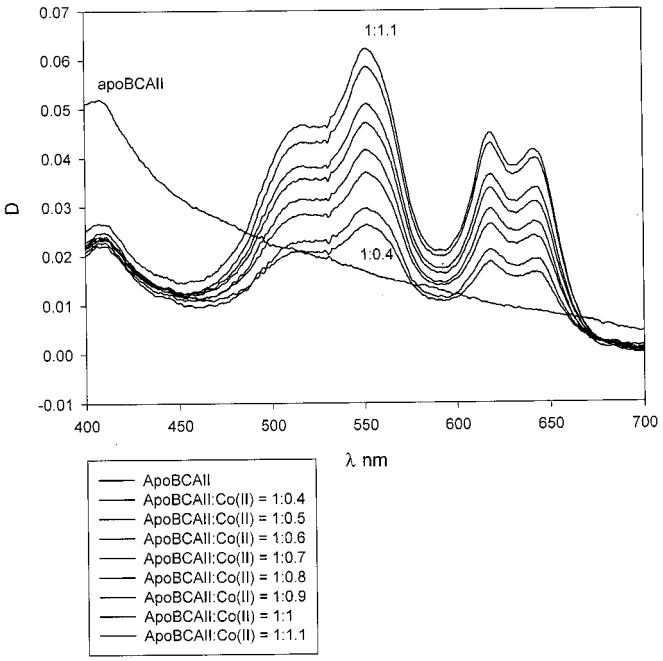

FIG. 3. **Spectroscopic titrations of apoBCAII with Co(II) at pH = 7.8.** *D* represents optical density in this figure and in Figs. 4–10.

Electronic spectra in the UV-visible region were recorded on a Shimadzu UV-240 double beam spectrophotometer.

Demetalation of ZnBCAII—A solution of 0.3 g of ZnBCAII in 20 ml of 0.2 M KH_2PO_4 buffer containing 50 mM pyridine-2,6-dicarboxylic acid at pH = 6.9 was transferred into a dialysis membrane kept in a large vessel that contained 800 ml of 0.2 M KH_2PO_4 buffer and 50 mM pyridine-2,6-dicarboxylic acid at pH = 6.9. Dialysis was performed with slow stirring at 4 °C. Dialysis was continued under these conditions for 3 days with two changes of buffer per day. Then the enzyme was dialyzed against 5 mM HEPES containing 100 mM NaCl at pH = 6.9 for 2 days with two changes of buffer per day followed by additional dialysis against 5 mM HEPES (pH = 6.9) for another 2 days with changes every 2–3 h. Partial denaturation of the enzyme was observed after dialysis. The suspension was centrifuged, and the supernatant containing apoBCAII was collected and kept at 4 °C.

Apoenzyme Concentration—The apoenzyme concentration was determined spectrophotometrically at 280 nm ($\epsilon = 4 \times 10^4$ M^{-1} cm^{-1}). Samples for determining the apoenzyme concentration were prepared by diluting 40 μl of the apoBCAII solution in 1 ml of 5 mM HEPES buffer at pH = 6.9 (adjusted with NaOH).

Cobalt(II) Metalation of ApoBCAII—The stoichiometric ratio between Co(II) and apoBCAII required to prepare CoBCAII was determined by spectrophotometric titrations of the freshly prepared apoBCAII in 5 mM HEPES at pH = 7.4 with a 0.01 M $CoCl_2$ aqueous solution. A few microliters of the Co(II) solution were added to 1 ml of apoBCAII to give increasing apoBCAII-Co(II) molar ratios (see Fig. 3). The spectra of apoBCAII-Co were corrected for dilution after each addition of the titrant. Absorbance increased gradually until the equivalence point, which corresponds to an apoBCAII-Co(II) stoichiometric ratio of 1:1. Successive additions of Co(II) did not modify the spectrum. From this equivalence point, the volume of the Co(II) solution needed to metalate the total amount of apoenzyme obtained after dialysis was determined. About 90–95% of the calculated volume was then added to the apoenzyme solution to avoid an excess of the metal ion. Addition of Co(II) must be carried out very slowly and with continuous stirring. Finally, the enzyme presented a pink color, typical of CoBCAII.

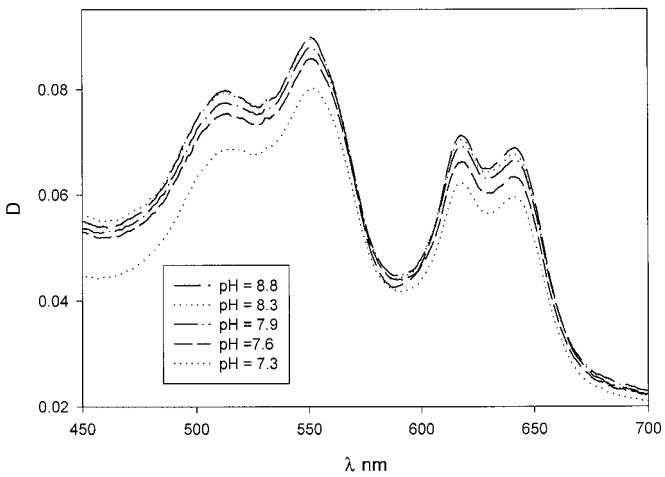

FIG. 4. **Spectra of CoBCAII solutions at different pH values (7.4, 7.6, 7.9, 8.3, and 8.8).**

Preparation of $[Co(Hacm)_2(NH_3)_2]$—2.20 g of H_2acm were added with stirring to a hot ethanolic solution (200 ml) of 10^{-2} M $CoCl_2 \cdot 6H_2O$. Then 9 ml of concentrated ammonia (11 M) was added dropwise. Immediately the solution became violet, and a solid of the same color was obtained. The product was filtered, washed with EtOH, and dried until a constant weight was reached [8].

Preparation of $[Co(macm)_2(NH_3)_2]$—To a solution of $CoCl_2 \cdot 6H_2O$ (10^{-2} M) and methazolamide (5×10^{-3} mol) in 100 ml of hot ethanol, 0.6 ml of concentrated ammonia was added dropwise with continuous stirring. The resulting violet precipitate was collected by filtration, washed with ethanol, and dried until a constant weight was reached [9].

Study of the Interaction between CoBCAII and Acetazolamide and Methazolamide—The binding of acetazolamide and methazolamide to CoBCAII was studied by spectrophotometric titrations in 5 mM buffer at pH = 8.5. A few microliters of 2.5×10^{-3} M sulfonamide solution were added to 1 ml of CoBCAII solution (1.1×10^{-4} M) to give CoBCAII-sulfonamide mixtures at molar ratios of 1:0.5, 1:1.0, 1:1.5, and 1:2. Spectra of enzyme-sulfonamide solutions were taken after each addition of sulfonamide and were corrected for dilution.

Study of the Interaction between ApoBCAII and the Cobalt Complexes—The interaction of $[Co(Hacm)_2(NH_3)_2]$ and $[Co(macm)_2(NH_3)_2]$ to apoBCAII was studied by spectrophotometric titrations in 5 mM buffer at pH = 8.5. A few microliters of 2.5×10^{-3} M complex solutions in DMSO were added to 1 ml of CoBCAII solution (1.6×10^{-4} M) to form CoBCAII-complex mixtures at molar ratio of 1:0.5, 1:1.0, 1:1.5, and 1:2. Difference spectra of apoenzyme-complex *versus* complex were taken after correction for dilution after each addition of complex.

RESULTS AND DISCUSSION

The visible spectra of CoBCAII at the pH range from 7.4 to 8.8 (Fig. 4) with maxima at 515, 550, 620, and 640 nm are characteristic of a tetrahedral environment around Co(II) [10]. The transitions observed in these spectra correspond to the following two species involved in the acid-base equilibrium for CoBCAII at this pH range):

SCHEME 2

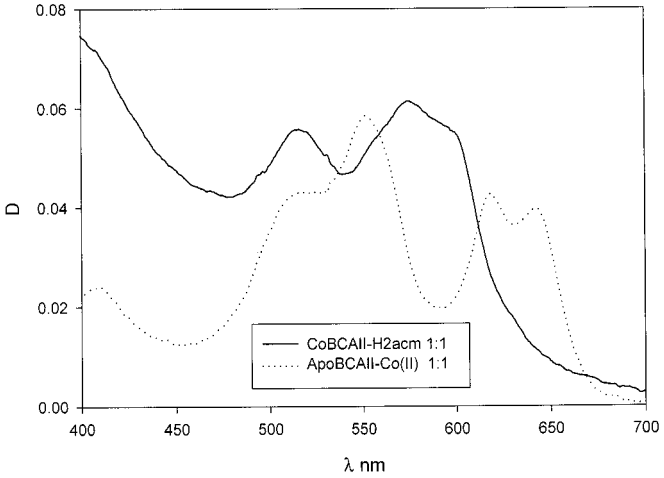

FIG. 5. **Spectra of apoBCAII-Co(II) 1:1 and CoBCAII-H_2acm 1:1.**

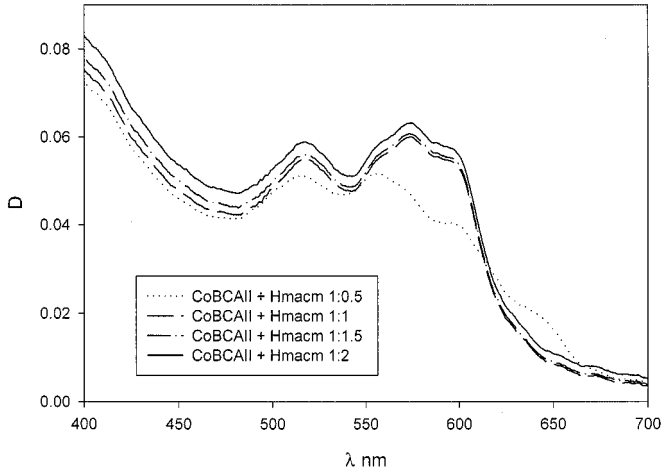

FIG. 7. **Spectra of CoBCAII-Hmacm at 1:0.5, 1:1, 1:1.5, and 1:2 molar ratios.**

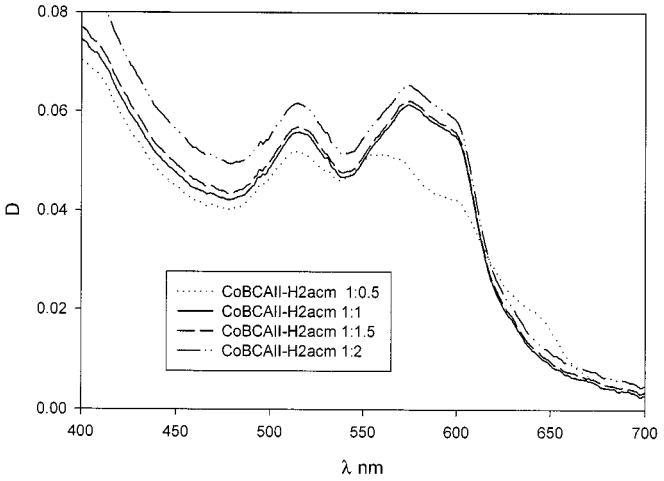

FIG. 6. **Spectra of CoBCAII-H_2acm at 1:0.5, 1:1, 1:1.5, and 1:2 molar ratios.**

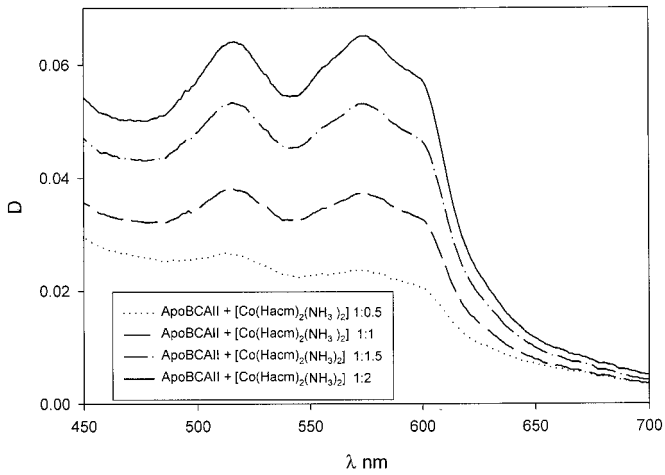

FIG. 8. **Spectra of apoBCAII-[$Co(Hacm)_2(NH_3)_2$] at 1:0.5, 1:1, 1:1.5, and 1:2 molar ratios.**

The interaction of the CoBCAII with H_2acm and Hmacm at different enzyme:sulfonamide ratios and at pH = 8.5 has been studied spectrophotometrically.

Fig. 5 shows the spectra of apoBCAII-Co(II) 1:1 and CoBCAII-H_2acm 1:1. The high intensity of the *d-d* bands in both spectra is indicative of Co(II) in a tetrahedral environment. This arrangement of the Co(II) in CoBCAII and in CoBCAII-Hacm suggests that one $Hacm^-$ anion substitutes for the OH^- anion in the active form of the CoBCAII enzyme. Consequently, when acetazolamide interacts with the enzyme, it losses its catalytic activity.

Fig. 6 presents the spectra of CoBCAII-H_2acm mixtures at enzyme:sulfonamide ratios of 1:0.5, 1:1.0, 1:1.5, and 1:2. The spectrum of CoBCAII-H_2acm 1:0.5 is similar to that of CoBCAII (see Fig. 3). The other spectra are different from that of CoBCAII. The fact that CoBCAII-H_2acm ratios less than 1:1 give similar spectra to that of CoBCAII together with the fact that CoBCAII-H_2acm 1:1 presents a different spectra to that of CoBCAII indicates that the formation of the CoBCAII-Hacm complex requires at least a molar ratio of 1:1. The spectra show that CoBCAII binds only one acetazolamide because ratios higher than 1:1 have similar optical densities.

Fig. 7 shows the spectra of CoBCAII-Hmacm mixtures at enzyme:sulfonamide ratios of 1:0.5, 1:1.0, 1:1.5, and 1:2. From these spectra similar conclusions to those above indicated for CoBCAII-acetazolamide can be inferred. The similar behavior exhibited by acetazolamide and methazolamide toward CoBCAII is due to both sulfonamides binding to the Co(II) ion through the nitrogen atom of the sulfonamidate group. The optical density of the maximum at $\lambda = 572$ nm in the spectrum of CoBCAII-acetazolamide 1:1 and in the spectrum of CoBCAII-methazolamide 1:1 has a similar value. This suggests that the interaction between CoBCAII and Hmacm is the same as that found between CoBCAII and H_2acm.

Fig. 8 shows the spectra of solutions containing apoBCAII and $[Co(Hacm)_2(NH_3)_2]$ at different molar ratios of 1:0.5, 1:1, 1:1.5, and 1:2. The pattern of these spectra is similar to that of CoBCAII-H_2acm (Fig. 6). It indicates that the interaction of $[Co(Hacm)_2(NH_3)_2]$ with the apoenzyme gives rise to the same arrangement around Co(II) as that in the interaction of CoBCAII with acetazolamide. Therefore, before linking the apoenzyme, the complex must dissociate partially leaving a $Co(Hacm)^+$ entity that directly interacts with apoBCAII:

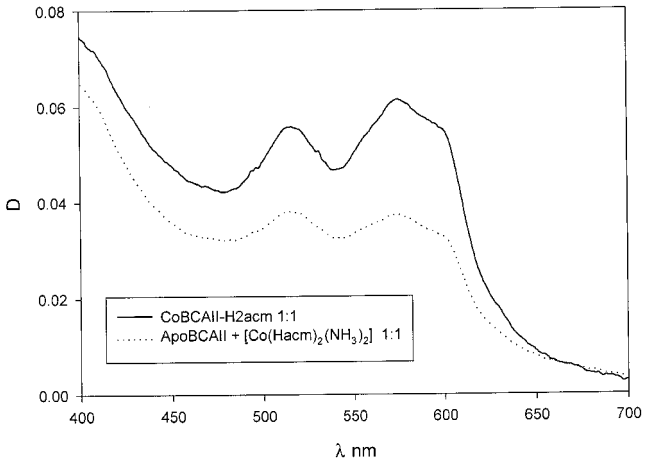

FIG. 9. **Spectra of CoBCAII-H_2acm 1:1 and apoBCAII-$[Co(Hacm)_2(NH_3)_2]$ 1:1.**

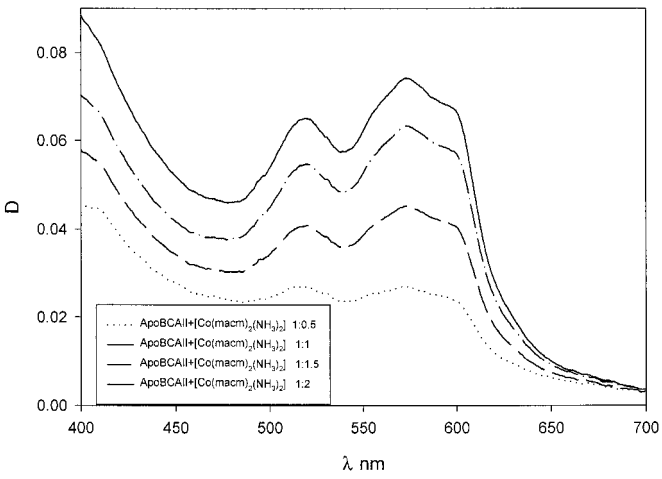

FIG. 10. **Spectra of apoBCAII-$Co(macm)_2(NH_3)_2$] at 1:0.5, 1:1, 1:1.5, and 1:2 molar ratios.**

$$[Co(Hacm)_2(NH_3)_2] \rightleftarrows Co(Hacm)^+ + Hacm^- + 2\ NH_3$$

$$apoBCAII + Co(Hacm)^+ \rightleftarrows CoBCAII\text{-}Hacm^-$$

Moreover, a comparison of the spectrum of CoBCAII-H_2acm 1:1 with that of apoBCAII-$[Co(Hacm)_2(NH_3)_2]$ 1:1 (Fig. 9) shows that the former has a higher optical density than the latter. This fact seems to suggest that the formation of CoBCAII-$Hacm^-$ takes place to a lesser extent when the interaction is between the ApoBCAII and the Co(II) complex.

The spectra for solutions containing apoBCAII and $[Co(macm)_2(NH_3)_2]$ at different molar ratios of 1:0.5, 1:1, 1:1.5 and 1:2 are shown in Fig. 10. The spectra are almost coincident with those of CoBCAII-Hmacm mixtures. As in the previous study with the cobalt complex of acetazolamide, a direct interaction of a $Co(macm)^+$ entity with the apoBCAII to give rise to the CoBCAII-$macm^-$ structure can be inferred:

$$[Co(macm)_2(NH_3)_2] \rightleftarrows Co(macm)^+ + macm^- + 2\ NH_3$$

$$apoBCAII + Co(macm)^+ \rightleftarrows CoBCAII\text{-}macm^-$$

As it has been found for the Co(II)-acetazolamide complex, the interaction of the $[Co(macm)_2(NH_3)_2]$ compound with the apoenzyme occurs to a lesser extent than the interaction of CoBCAII with methazolamide.

STUDENT BACKGROUNDS AND ACTIVITIES

The majority of the students taking this course are students of bioinorganic chemistry in the last year of their chemistry studies. Although they have taken other laboratory courses (inorganic, organic, and analytical laboratories), for many this is the first time performing bioinorganic chemistry, and they have little or no experience in techniques regarding the handling of metalloproteins.

The students have to set up all the experiments and make all the solutions required for these experiments. The project can be carried out conveniently in laboratory classes of 6 h each. The first stage was the preparation of apoBCAII by dialysis; this requires 7 days. This stage can be combined with the preparation of the cobalt(II) complexes and with a bibliographic search from library sources. The following stage is the metalation of apoBCAII to obtain CoBCAII. This work can be carried out in one practical session. The study of the interaction between CoBCAII and acetazolamide can be performed in one practical session. Another session will be devoted to the study of the interaction between CoBCAII and methazolamide. Finally, the time requirements for the study of the interaction between the cobalt(II) complexes and apoCAII are approximately two additional practical sessions. Additional class periods of ~12 h (4 h each) are needed for data manipulation, interpretation, and presentation of the results.

This laboratory project provides the students with experience in techniques regarding the handling of metalloproteins that students cannot easily obtain in the laboratory of first courses. The ultimate goal is to use the students' knowledge on coordination chemistry in the interpretation of metal complexes-protein interactions.

CONCLUSIONS

In this laboratory project the students prepare the CoBCAII by metalation of apoBCAII. They perform a spectroscopic study of the interaction of CoBCAII with acetazolamide and methazolamide, two potent inhibitors of CA enzyme. From this study the students are able to deduce that both sulfonamides interact with CoBCAII in the same way through the nitrogen atom of the deprotonated sulfonamido group. Finally, a spectroscopic study of the interaction of apoBCAII with $[Co(Hacm)_2(NH_3)_2]$ and $[Co(macm)_2(NH_3)_2]$ complexes is carried out. From this experiment the students can deduce that the interaction of $[Co(Hacm)_2(NH_3)_2]$ or $[Co(macm)_2(NH_3)_2]$ with the apoenzyme leads to the formation of the same species as that of the interaction of CoBCAII with acetazolamide and methazolamide, respectively. This conclusion is of special interest because usually the metal sulfonamide complexes are stronger inhibitors of carbonic anhydrase than the parent sulfonamides [11].

REFERENCES

[1] B. C. Tripp, K. Smith, J. G. Ferry (2001) Carbonic anhydrase. New insights for an ancient enzyme, *J. Biol. Chem.* **276**, 48615–48618.
[2] A. Scozzafava, C. T. Supuran (2001) Carbonic anhydrase and matrix

metalloproteinase inhibitors: sulfonylated amino acid hydroxamates with MMP inhibitory properties act as efficient inhibitors of CA isoenzymes I, II and IV, and *N*-hydroxysulfonamides inhibit both these zinc enzymes, *J. Med. Chem.* **44**, 1016–1026.

[3] D. Voet, J. G. Voet (1995) Biochemistry, 2nd ed., John Wiley & Sons, Inc., New York.

[4] L. Stryer (1995) Biochemistry, 4th ed., W. H. Freeman, Hampshire, UK.

[5] A. E. Eriksson, T. A. Jones, A. Liljas (1988) Refined structure of human carbonic anhydrase II at 2 Å resolution, *Proteins* **4**, 274–282.

[6] A. Casini, A. Scozzafava, F. Mincione, L. Menabuoni, M. A. Illies, C. T. Supuran (2000) Carbonic anhydrase inhibitors: water-soluble 4-sulfamoylphenylthioureas as topical intraocular pressure-lowering agents with long-lasting effects, *J. Med. Chem.* **43**, 4884–4892.

[7] J. Vidgren, A. Liljas, N. P. C. Walker (1990) Refined structure of the acetazolamide complex of human carbonic anhydrase II at 1.9 Å, *Int. J. Biol. Macromol.* **12**, 342–344.

[8] S. Ferrer, A. Jimenez, J. Borrás (1987) Synthesis and characterization of acetazolamide complexes of Co(II) and Zn(II), *Inorg. Chim. Acta* **129**, 103–106.

[9] G. Alzuet, J. Casanova, J. A. Ramírez, J. Borrás, O. Carugo (1995) Metal complexes of the carbonic anhydrase inhibitor methazolamide. Crystal structure of the $Zn(macm)_2(NH_3)_2$. Anticonvulsant properties of $Cu(macm)_2(NH_3)_3(H_2O)$, *J. Inorg. Biochem.* **57**, 219–234.

[10] I. Bertini, C. Luchinat, in I. Bertini, H. B. Gray, S. J. Lippard, J. S. Valentine, Eds. (1994) Bioinorganic Chemistry, University Science Books, Mill Valley, CA.

[11] G. Alzuet, S. Ferrer, J. Borrás, C. T. Supuran (1994) Complexes of heterocyclic sulfonamides. A class of potent, dual carbonic anhydrase inhibitors, *Roumanian Quarterly Rev.* **2**, 283–300.