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THE FORMATION AND PROPERTIES OF COBALT (III) CARBONIC ANHYDRASE

HADASSAH SHINAR and GIL NAVON

Department of Chemistry, Tel-Aviv University, Tel-Aviv (Israel)

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Summary

Cobalt (III) bovine carbonic anhydrase B was prepared by the oxidation of the corresponding cobalt (II) enzyme with hydrogen peroxide. The cobalt (III) enzyme was found to be inactive towards the hydration of carbon dioxide and the hydrolysis of *p*-nitrophenyl acetate. The oxidation reaction is considerably slowed down in the presence of various anion and sulfonamide inhibitors of carbonic anhydrase. The oxidation products have characteristic absorption spectra indicating direct binding of the anion inhibitors to the metal ion in the cobalt (III) carbonic anhydrase.

Carbonic anhydrase (EC 4.2.1.1) contains one firmly bound zinc atom which is essential for the catalytic activity [1]. The zinc atom can be replaced by other transition metal ions, and with some of them part of the enzymatic activity is retained [2]. Cobalt (II)-substituted carbonic anhydrase is unique in its relatively high activity; 50% toward CO_2 hydration and 97% toward the synthetic substrate *p*-nitrophenyl acetate [3], as compared to the native zinc enzyme. In addition, cobalt (II) carbonic anhydrase has a distinct spectrum in the visible range which is very sensitive to the binding of inhibitors and pH variations. These two properties make the cobalt (II) enzyme useful in probing the active site.

The chemical and physical properties of cobalt (III) compounds have been studied in great detail [4]. One important difference between the properties of cobalt (II) and cobalt (III) compounds is that the rate of ligand exchange is much slower for the latter systems. Thus a possible residual activity of the cobalt (III) enzyme might mean that the first coordination

sphere of the metal ion is not involved in the enzymatic activity. This was recently shown by Kang and Storm [5] to be the case for the esterase activity of carboxypeptidase A.

When hydrogen peroxide was added to a solution of cobalt (II) carbonic anhydrase (prepared by the method of Lindskog and Malmstrom [6] from bovine carbonic anhydrase B (Seravac)) in 0.05 M Tris—sulfate buffer, pH 7.5, an immediate change was observed in the optical absorption spectrum of the cobalt enzyme in the visible region. This spectral change was accompanied by a decrease in the enzymatic activity. At the stage when no further spectral changes could be observed, no enzymatic activity towards either carbon dioxide hydration or *p*-nitrophenyl acetate hydrolysis was detected. Experiments with the zinc enzyme under similar conditions (see below) did not result in any loss of enzymatic activity.

The rate and the extent of the spectral change and the loss of activity of the cobalt enzyme was dependent on the concentration of hydrogen peroxide. In most of our experiments the enzyme concentration was about $2 \cdot 10^{-4}$ M. Under such conditions at least 10-fold excess of hydrogen peroxide was required to completely abolish the enzyme activity, and to cause a complete disappearance of the characteristic doublet at 618 and 640 nm. However, hydrogen peroxide concentrations exceeding about $4 \cdot 10^{-3}$ M led to an appearance of a yellow color which showed up in the spectrum as a continuous absorption, increasing toward the ultraviolet. Therefore, the concentration of the hydrogen peroxide in most of our experiments was $3 \cdot 10^{-3}$ M. It is interesting to note that this was not the case with the zinc enzyme. The only change was about one percent decrease in the absorbance at 270 nm, which may be due to some oxidation of tyrosine residues.

In the presence of the inhibitors, azide, perchlorate, cyanide, and *p*-toluene sulfonamide, the rate of oxidation was dramatically reduced. This is shown in Fig. 1. We also followed the disappearance of hydrogen peroxide in the various experiments, by the method of Hochanadel [7]. In control experiments, practically no disappearance of hydrogen peroxide was found in the presence of the Tris—sulfate buffer, the various inhibitors used in this work, and free Co^{2+}. In the presence of the zinc carbonic anhydrase the disappearance of hydrogen peroxide was very slow and was about 1% per hour. On the other hand, the disappearance of hydrogen peroxide in the presence of the cobalt enzyme with and without the inhibitors was found to be relatively fast and the amount of hydrogen peroxide which disappeared was greater than the amount needed for the oxidation of the cobalt (II) enzyme. The rate of the disappearance of hydrogen peroxide in the presence of the cobalt enzyme was not reproducible, but typically, about 50% of the hydrogen peroxide remained 1 h after the reaction was started. A possible hypothesis is that the cobalt (II) carbonic anhydrase catalyzes the decomposition of hydrogen peroxide, although other explanations cannot be excluded at this time.

It is well known that the spectrum of cobalt (II) carbonic anhydrase changes upon binding of anions and sulfonamides to the active site. Each

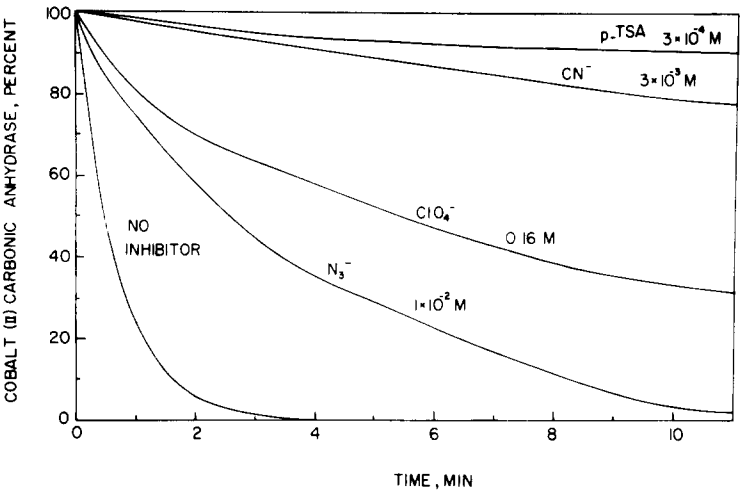

Fig. 1. The time course of the oxidation of cobalt (II) carbonic anhydrase by hydrogen peroxide in the presence of various inhibitors The concentration of the enzyme was $2.2 \cdot 10^{-4}$ M, and of the Tris—sulfate buffer 0.05 M, pH 7.5. The visible absorbance of the enzyme was followed immediately after the addition of 14-fold excess of hydrogen peroxide until no further change was observed. In the case of the *p*-toluene sulfonamide (*p*-TSA), no final spectrum could be reached and the curve was calculated on the assumption that the spectrum of the oxidized enzyme is identical to that of the free cobalt (II) enzyme.

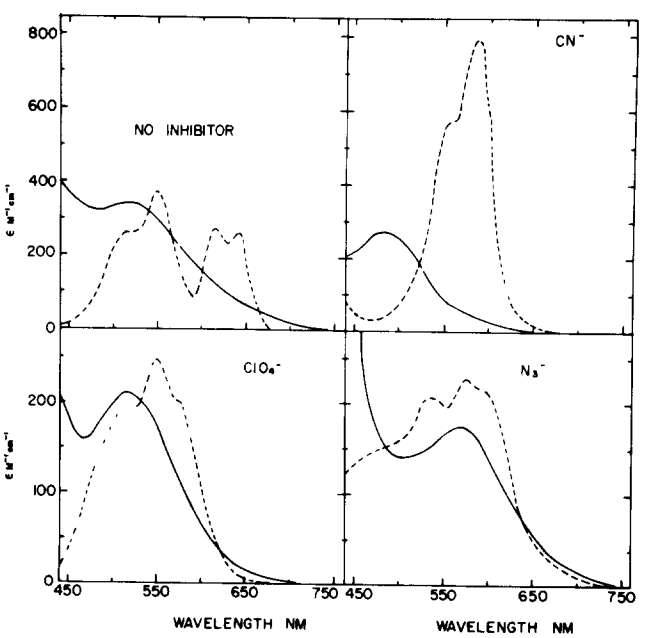

Fig. 2. Spectra of various cobalt (II) (- - - -) and cobalt (III) (——) bovine carbonic anhydrase B inhibitor complexes.

cobalt (II) carbonic anhydrase—inhibitor complex has a characteristic spectrum. This is shown for the cobalt (II) enzyme and its azide, cyanide, and perchlorate complexes by the dashed lines in Fig. 2; this figure also includes the spectra of the oxidized species. It is clear from the figure, that each cobalt (III) carbonic anhydrase—anion complex has a distinct spectrum in the visible range.

As mentioned above, the rates of ligand exchange in cobalt (III) complexes are usually very slow. We have used this property to characterize the cobalt (III) carbonic anhydrase. For instance, when the product of the cobalt (II)-enzyme oxidation in the presence of azide ions, (which we assign as cobalt (III) carbonic anhydrase—N_3^- complex) was dialyzed for 36 h against three changes of 100-fold volume excess, 0.05 M Tris—sulfate buffer, pH 7.5, no change could be observed in the spectrum of the solution. On the other hand, cobalt (II) carbonic anhydrase—N_3^- complex, under similar dialysis conditions lost all of its bound azide ions, and the spectrum obtained was identical with that of free cobalt (II) carbonic anhydrase.

Upon the addition of an anion like perchlorate, azide, or cyanide to a solution containing cobalt (III) carbonic anhydrase, a gradual spectral change was observed, with a half life of several hours. This is in contrast to the immediate binding of these anions to cobalt (II) carbonic anhydrase. It is interesting to note that the spectrum of the final product was identical with that of the oxidation product of the corresponding cobalt (II) carbonic anhydrase—anion complexes. Thus it is possible to prepare the cobalt (III) carbonic anhydrase-anion complexes either by oxidation of the corresponding cobalt (II)—carbonic anhydrase complexes, or by binding the anions to the free cobalt (III)—carbonic anhydrase.

This observation, together with the fact that the rate of oxidation in the presence of the anions is comparable to the rate of the binding of the anions to cobalt (III) enzyme, raises the question whether the oxidation of cobalt (II) carbonic anhydrase—anion complexes is carried out directly, or through a dissociation to the free cobalt (II) enzyme, its oxidation, and then the anion binding to the oxidation product. Experiments to answer this question, as well as other questions concerning the properties and the formation of cobalt (III) carbonic anhydrase, are now under way. It may be remarked at this point that just the fact that anions inhibit the oxidation of the cobalt (II) ion in the active site, corroborates previous conclusions about a direct binding of the anions to the metal ion [8—10]. Furthermore, it may indicate that the oxidation mechanism involves a direct binding of the oxidizing agent to the cobalt ion.

The lack of enzymatic activity of cobalt (III) carbonic anhydrase is consistent with the generally accepted view that the metal ion in the active site of the enzyme is actively involved in catalysis, through substitution in its first coordination sphere. It is hoped, that the cobalt (III) derivative, though not active, will provide a useful probe for the structure of the active site.

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