counteranion, periodic purification by pyrolysis under a dynamic vacuum[20] is required. The purity of the salt was most conveniently monitored by iodometric titration. Typical titers using n-$Bu_4N^+I^-$ in CH_2Cl_2 were 98% of theory for freshly purified material and ~85% (based on $O_2^{\bullet+}SbF_6^-$) for salt which was 6 months old.

The utility of **1** in organic cation radical chemistry is, of course, not limited to cases where the cation radical salts are isolable. The reagent may also provide important clues to the intrinsic reactivity of cation radicals which cannot be directly detected. For example, oxidations of simple trialkylamines with **1** do not give solutions of stable cation radical salts, but the results do suggest the their intermediacy.[21] The extreme oxidizing power[22] of $O_2^{\bullet+}$ salts will undoubtedly prompt other applications.

Acknowledgment. We are grateful to R. W. Kreilick, S, P. Greiner, and D. L. Rowlands for their assistance in recording EPR spectra and to J. A. Kampmeier for his valuable encouragement. Support was provided by the Research Corporation and by the Camille and Henry Dreyfus Foundation for research and by the National Science Foundation for an instrumentation grant (CHE78-03089).

(20) McKee, D. E.; Bartlett, N. *Inorg. Chem.* **1973**, *12*, 2738.
(21) Dinnocenzo, J. P.; Rowland, R. G., manuscript in preparation.
(22) An approximate calculation reveals that the reduction of $O_2^{\bullet+}$ is exothermic by ca. 5.3 V vs. SCE! This was calculated by using an ionization potential for O_2 of 12.08 eV[6b] and Miller's empirical equation (Miller, L. L.; Nordblom, G. D.; Mayeda, E. A. *J. Org. Chem.* **1972**, *37*, 916). The $Ag/AgNO_3/CH_3CN$ electrode potential so obtained was converted to the SCE electrode potential by adding 0.34 V.

Observation of the Visible Absorption Spectrum of Cobalt(II)-Carbonic Anhydrase III during Catalytic Hydration of CO_2

C. K. Tu and D. N. Silverman*

Departments of Pharmacology and Biochemistry University of Florida, Gainesville, Florida 32610

Received March 31, 1986

We have prepared Co(II)-substituted carbonic anhydrase III from bovine skeletal muscle and observed its visible absorption spectrum during catalysis of CO_2 hydration using a stopped-flow spectrophotometer. The optical spectrum of the homologous Co(II)-substituted carbonic anhydrase from red cells (isozyme II) shows a variability depending on pH and the ligands of the metal and is a sensitive measure of the status of the active center.[1] The spectrum of the cobalt-bound hydroxide form has distinctive features with maxima near 620 and 640 nm; for isozyme II the spectra of the cobalt-bound water form and the HCO_3^- complex are similar and rather featureless.[1] The spectrum of the cobalt-bound water form of carbonic anhydrase III has not yet been observed since this isozyme is believed to have a pK_a for the metal-bound water below 5,[2-4] a region in which the protein denatures. We report here changes in the visible spectrum of Co(II)-carbonic anhydrase III after mixing with CO_2 which are consistent with the accumulation of the cobalt-bound water form of this isozyme during the catalysis. This is the first observation of a spectral property of the active center of carbonic anhydrase during the catalytic progress curve for CO_2 hydration and is

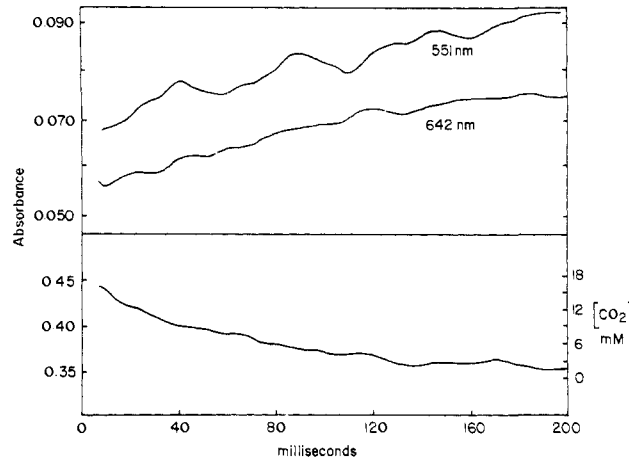


Figure 1. (Top) Absorbance change at 642 and 551 nm as a function of time after mixing Co(II)-substituted bovine carbonic anhydrase III and CO_2. Initial pH after mixing was 7.5 with the concentration of CO_2 and Co(II)-carbonic anhydrase at 0.017 and 1.4×10^{-4} M, respectively. (The estimated contamination by zinc-containing carbonic anhydrase III was 1.5×10^{-5} M.) Hepes sulfate was present at 100 mM with Na_2SO_4 at 15 mM. The path length of our optical cell was 2 cm. (Bottom) Absorbance change at 559 nm as a function of time after mixing of solutions identical with those described above except the enzyme solution contained phenol red. The concentration of this indicator after mixing was 7.5×10^{-6} M. The ordinate also shows the concentration of CO_2 calculated from the known initial value and rate of change of absorbance of indicator (see ref 11).

compatible with the hypothesis of a rate-contributing proton transfer in step 4 of eq 2. This hypothesis is also based on solvent

$$\gg ZnOH^- + CO_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \gg Zn(OH^-)CO_2 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \gg ZnHCO_3^- \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \gg ZnOH_2 + HCO_3^- \quad (1)$$

$$\gg ZnOH_2 \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \gg ZnOH^- + H^+ \quad (2)$$

hydrogen isotope effects and inhibition of the catalysis[5,6] and is analogous to an hypothesis for the catalytic mechanism of carbonic anhydrase II which is believed to proceed with a maximum velocity limited by a proton-transfer step.[1,7] In its most fundamental representation, the rate-contributing proton transfer is in step 4 of eq 2, the step in which the zinc-bound hydroxide at the active site is regenerated following a hydration sequence.

Carbonic anhydrase III was obtained from bovine flank steak by gel filtration and anion-exchange chromatography.[8] Apoenzyme was prepared by addition of the chelator 2-carboxy-1,10-phenanthroline to a solution of enzyme followed by dialysis against excess $CoCl_2$ as described by Engberg and Lindskog.[4] The total concentration of carbonic anhydrase III (both the zinc and cobalt forms) was estimated at 280 nm by using $\epsilon = 6.4 \times 10^4$ M^{-1} cm^{-1} and the concentration of cobalt(II)-substituted carbonic anhydrase III was estimated at 640 nm and pH 7.0 by using $\epsilon = 270$ M^{-1} cm^{-1}.[4] By this comparison our sample of isozyme III was determined to be 90-93% in the cobalt form. Saturated solutions of CO_2 (33.8 mM at 25 °C)[9] were prepared by bubbling CO_2 gas into water. The progress curve and initial rate measurements were carried out on a Durrum-Gibson (D-110) stopped-flow spectrophotometer equipped with a Nicolet Explorer (Model 206) digital oscilloscope interfaced with an IBM XT

(1) Lindskog, S. In *Zinc Enzymes*; Spiro, T. E., Ed.; Wiley: New York, 1983; p 78.
(2) Engberg, P.; Millqvist, E.; Pohl, G.; Lindskog, S. *Arch. Biochem. Biophys.* **1985**, *241*, 628.
(3) Tu, C. K.; Sanyal, G.; Wynns, G. C.; Silverman, D. N. *J. Biol. Chem.* **1983**, *258*, 8867.
(4) Engberg, P.; Lindskog, S. *FEBS Lett.* **1984**, *170*, 326.
(5) Kararli, T.; Silverman, D. N. *J. Biol. Chem.* **1985**, *260*, 3484.
(6) Kararli, T.; Silverman, D. N. *Ann. N. Y. Acad. Sci.* **1984**, *429*, 129.
(7) Silverman, D. N.; Vincent, S. H. *CRC Crit. Rev. Biochem.* **1983**, *14*, 207.
(8) Tu, C. K.; Thomas, H. G.; Wynns, G. C.; Silverman, D. N. *J. Biol. Chem.* **1986**, *261*, 10100.
(9) Pocker, Y.; Bjorkquist, D. W. *J. Am. Chem. Soc.* **1977**, *99*, 6537.

0002-7863/86/1508-6065$01.50/0

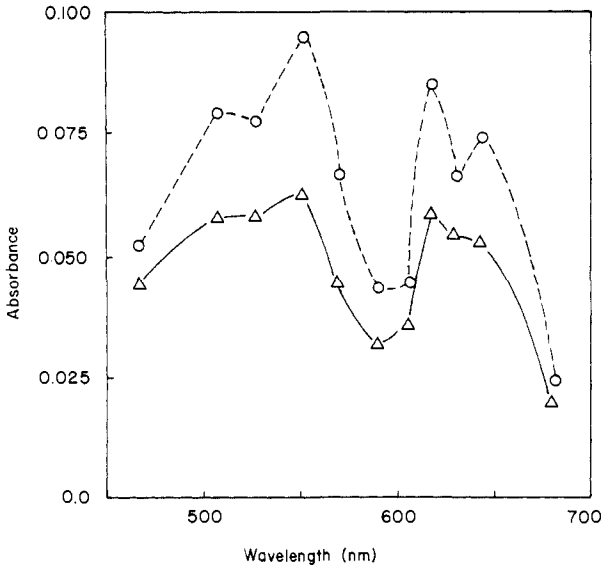


Figure 2. Absorbances at (△) 8 ms and (○) greater than 200 ms after the mixing of Co(II)-substituted bovine carbonic anhydrase III and CO_2. Conditions were the same as described in Figure 1 (Top). Lines are estimates of the spectra based on the 11 wavelengths measured; lines were not determined experimentally.

computer. One drive syringe contained CO_2, and the second contained enzyme and buffer and in some cases indicator. The status of the cobalt at the active center was determined from the absorbances at 551 and 642 nm in the absence of indicator; the progress curve and initial velocity for CO_2 hydration were determined by the rate of change of absorbance of phenol red at 559 nm by methods described earlier.[10,11]

Figure 1 (top) shows the absorbances at 551 and 642 nm as a function of time after mixing of Co(II)-isozyme III wtih CO_2. After the dead-time of our instrument, ~5 ms, we were able to observe a decreased absorbance resulting from the status of cobalt at steady state. As the hydration reaction proceeded to equilibrium, the absorbances at 642 and 551 nm approached their values at chemical equilibrium. To correlate this with the catalysis, we present in Figure 1 (bottom) the absorbance of the pH indicator phenol red and the calculated CO_2 concentration during the progress curve in a solution identical with that of Figure 1 (top) except for the addition of phenol red at 7.5×10^{-6} M. By initial velocity measurements at pH 7.5 using the method of ref 10, 11, we determined for the hydration of CO_2 catalyzed by bovine Co(II)-carbonic anhydrase III (data not shown) $k_{cat}^{CO_2} = (1.3 \pm 0.1) \times 10^3\ s^{-1}$ and $k_{cat}^{CO_2}/K_m^{CO_2} = (1.4 \pm 0.1) \times 10^5\ M^{-1}\ s^{-1}$. The analogous values for catalysis by the native zinc-containing bovine isozyme III are $3 \times 10^3\ s^{-1}$ and $3 \times 10^5\ M^{-1}\ s^{-1}$.[2,8]

The experiments of Figure 1 were repeated at 10 other wavelengths with results shown in Figure 2. The points at times greater than 200 ms represent the spectrum at equilibrium which is similar to the spectrum of Engberg and Lindskog.[4] Although we have not observed the spectrum of Co(II)-isozyme III fully in its low pH form, we anticipate that it will be similar to the corresponding spectrum for isozyme II. Oxygen-18 exchange between CO_2 and water catalyzed by Co(II)-carbonic anhydrase III (by the method of ref 12) showed no substrate or product inhibition at pH 7.5 up to a concentration of 80 mM for the sum of all species of CO_2. This suggests equilibrium dissociation constants too large to have significant enzyme-substrate or enzyme-product complexes at equilibrium under the conditions of this study. Our sample of Co(II)-carbonic anhydrase III had as large as 10% contamination by the native zinc-containing carbonic anhydrase III, the latter of which has steady-state rate constants larger than those for Co(II)-substituted isozyme III. This means our experimental values of $k_{cat}^{CO_2}$ and $k_{cat}^{CO_2}/K_m^{CO_2}$ can be considerably greater than the true values. The usual chelating agents have not proved useful in preparing the apoenzyme of isozyme III,[4] indicating that isozyme III has a greater affinity for zinc than does isozymes I and II.

We conclude that the hypothesis of a rate-contributing proton transfer in the maximum velocity of catalysis of CO_2 hydration involving the aqueous ligand of the metal in carbonic anhydrase III remains viable, for both the native and cobalt-substituted forms. The cobalt-bound water and cobalt-bound HCO_3^- forms of isozyme III, in analogy with isozyme II, are anticipated to have similar absorption spectra with weak absorbance at 640 nm. Therefore, these results cannot differentiate between a rate limitation by step 3 of eq 1 and step 4 of eq 2. It is known, however, that there is a significant H/D solvent isotope effect of 2.5 on the turnover number for CO_2 hydration catalyzed by isozyme III.[5,6] This suggests a change in bonding to hydrogen and favors step 4 of eq 2 as a rate-contributing event. Although the proton donor in this transfer is zinc- or cobalt-bound water, the proton acceptor is not known. Unlike carbonic anhydrase II, isozyme III is not enhanced in CO_2 hydration activity by buffers in solution,[5,6] indicating that proton transfer to buffer is not a rate-contributing event. Moreover, unlike carbonic anhydrase II, isozyme III does not have a nonliganded histidine at postion 64 to act as a proton shuttle residue. Residue 64 in bovine isozyme III is lysine.[13] The catalysis by carbonic anhydrase III is slow enough that water itself could serve as proton acceptor.

Acknowledgment. We thank George Wynns and Dr. Asok Sen for their expert assistance. This work was supported by a grant from the National Institutes of Health (GM 25154).

(10) Rowlett, R. S.; Silverman, D. N. *J. Am. Chem. Soc.* **1982**, *104*, 6737.
(11) Khalifah, R. G. *J. Biol. Chem.* **1971**, *246*, 2573.
(12) Silverman, D. N.; Tu, C. K.; Lindskog, S.; Wynns, G. C. *J. Am. Chem. Soc.* **1979**, *101*, 6734.
(13) Tashian, R. E.; Hewett-Emmett, D.; Stroup, S. K.; Goodman, M.; Yu, Y. S. L. In *Biophysics and Physiology of Carbon Dioxide*; Bauer, C., Gros, G., Bartels, H., Eds.; Springer-Verlag: Berlin, 1980; p 165.

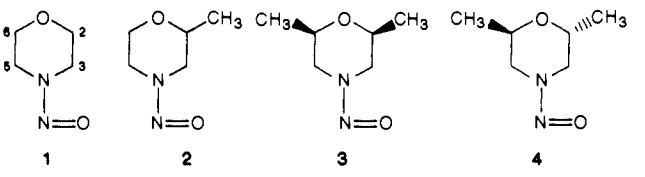
3-(2-Deoxy-β-D-erythropentofuranosyl)-6,7-dihydro-6,7-dihydroxyimidazo[1,2-*a*]purin-9(3*H*)-one, a Major Deoxyguanosine Adduct Formed from a Novel Diazo Hydroxide Product of α-Hydroxylation of the Carcinogen *N*-Nitrosomorpholine

George F. Palladino,*[1a,b] Fung-Lung Chung,[1c] and Stephen S. Hecht[1c]

Division of Chemical Carcinogenesis
Naylor Dana Institute for Disease Prevention
Valhalla, New York 10595

Received March 21, 1986

N-Nitrosomorpholine (**1**) and its methylated analogues **2**-**4** are an important group of remarkably potent and versatile carcinogens.[2] For example, in Syrian golden hamsters, **1** causes mainly



1 **2** **3** **4**

nasal cavity and tracheal tumors, **2** induces tumors of the nasal

(1) (a) Department of Chemistry, United States Military Academy, West Point, NY. (b) Visiting Scientist, Naylor Dana Institute for Disease Prevention, 1983-1984. (c) Division of Chemical Carcinogenesis, Naylor Dana Institute for Disease Prevention, Valhalla, NY. This is Paper No. 97 In *A Study of Chemical Carcinogenesis*.
(2) Preussmann, R.; Stewart, B. W. In *Chemical Carcinogens*; Searle, C. E., Ed.; ACS Monograph 182; American Chemical Society: Washington, DC, 1984; Vol. 2, 643-828.

0002-7863/86/1508-6066$01.50/0