THE MECHANISM OF ACTION OF CARBOXYDISMUTASE*

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Carboxydismutase is inhibited by iodoacetamide, and the substrate RuDP protects the enzyme against this inhibition.[1] The magnitude of the rate of inhibition and the variation of this rate with pH suggested that the mercaptide form of a cysteine residue was alkylated by the inhibitor. A mechanism was proposed in which this mercaptide reacted with the carbonyl group of RuDP to give a thiohemiacetal. It was suggested that water was eliminated from this compound to give an enol which then reacted with enzyme-bound bicarbonate. Further evidence relevant to these proposals has now been obtained by spectrophotometric investigations and by use of the sulphydryl reagent DTNB.[2]

Experimental.—Enzyme: This was prepared and assayed as previously described.[1] *Buffers:* These were prepared from Trizma (Sigma Chemical Co., St. Louis) and redistilled hydrochloric acid. A stock solution (total tris, 1 *M*, pH 8.0) was freed from metal ions as previously described.[1] *RuDP:* RuDP was prepared and purified by the method of Rabin *et al.*[3] *Iodoacetamide 1-C^{14}:* This was purchased from Tracerlab (Waltham, Mass.) and was stated to have a specific activity of 1.25 mC/mM. *DTNB:* This was purchased from Aldrich Chemical Co., Inc., Milwaukee, Wisconsin. A stock solution (10 mM, pH 7.2) was prepared as follows. DTNB (39.6 mg, 0.1 mmole) was dissolved in 10 ml of a solution containing tris base (0.2 mmole) and tris/HCl buffer, (pH 7.2, 0.5 mmole with respect to total tris). The solution was centrifuged (500 × *g* for 10 min) before use. The stock was diluted with water to give a 1 mM solution.

Difference spectrophotometry: All measurements were made with a Cary Model 14 spectrophotometer with a 0–0.1, 0.1–0.2 slide wire. Split-compartment cells, as described by Yankeelov,[4] but with a total light path of 1.0 cm, were employed. These cells were manufactured to special order by Pyrocell Manufacturing Co., New York, New York. A solution of carboxydismutase in tris buffer (1 ml) was pipetted into the front compartment of both the reference and sample cells. A solution of RuDP in the same buffer (1 ml) was then pipetted into the rear compartments of the cells, and the base line recorded. The contents of the sample cell were then mixed by inversion, and the difference spectrum was recorded. Subsequently, the reference cell was inverted and the base line checked. Invariably no change in the base line was observed.

Reaction with DTNB: The reaction was started by the addition of DTNB solution (50 μl, 1 or 10 mM) to the enzyme in 0.95-ml solution containing tris buffer pH 8.0 to give a final concentration of 0.05 *M* with respect to total tris. The blank cell contained the same concentration of DTNB and buffer, and both cells had a total optical path of 1 cm. The reaction was followed by observing the change in absorbance at 410 mμ, using a Cary recording spectrophotometer. The molar extinction coefficient of the chromophore produced in the reaction with cysteine under these conditions was 14,000. Beer's law was found to be obeyed over the absorbance range observed in the enzyme experiments.

Preparation of alkylated enzyme: Carboxydismutase (2.7 ml, 3 mg/ml) was incubated in 0.4 *M* tris buffer (pH 8.0) with iodoacetamide (1 mM, final concentration) at 25° for 45 min. The solution was cooled to 0° and dialyzed by the method of Hospelhorn[5] against 0.4 *M* tris buffer (pH 8.0) at 2° for 3 hr. It was used in the spectrophotometric experiments without further purification.

Isolation and identification of C^{14}-carboxymethylcysteine from alkylated carboxydismutase: Carboxydismutase (2 ml, 5 mg/ml) in tris buffer (0.064 *M*, pH 8.0) was treated with iodoacetamide 1-C^{14} (0.37 mg, 2 μM) at 25° for 30 min. The reaction mixture was cooled to 0°, and dialyzed by the method of Hospelhorn[5] against deionized water taken to pH 8.0 by the addition of ammonia. After 1.6 l of dialyzing solution had flowed over the sac, the dialysate had no detectable radioactivity. The protein solution was freeze-dried, dissolved in redistilled HCl (6.0 *N*), deaerated, and

heated in a sealed tube at 105° for 16 hr. The protein hydrolysate was freeze-dried, and the HCl removed over solid KOH in a dessicator. Water (25 μl) was added, and samples (5 μl) were subjected to paper electrophoresis in pyridine/acetate buffer (at 78 volts per cm, 90 ma, and 0° for 45 min) as described by Rohr and Bassham.[6] The paper was dried and a radioautograph prepared in the usual manner. Essentially all of the radioactivity was found in a single spot in a position corresponding to S-carboxymethylcysteine. Spots were eluted with water, and the eluents were freeze-dried. The identification was confirmed by cochromatography on paper with authentic S-carboxymethylcysteine in semistench.[8] A sample was oxidized with performic acid at −10°, as described by Hirs,[7] and the product identified as S-carboxymethylcysteine sulphone by cochromatography in the same solvent system.

Results and Discussion.—Reaction with DTNB: The progress curves of the reaction of carboxydismutase with DTNB, in the presence and absence of RuDP, are shown in Figure 1. In the absence of RuDP, the fast primary reaction is followed by a secondary and nearly linear production of chromophore. Extrapolation of this secondary production to zero time gave an equivalent weight of 131,000 for this particular enzyme preparation. However, the equivalent weight determined

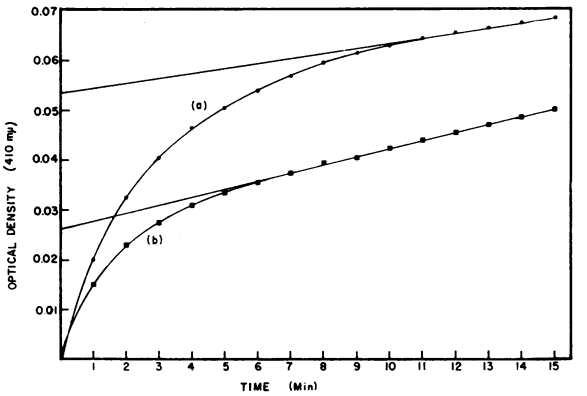

FIG. 1.—Progress curves of the reaction of carboxydismutase (0.5 mg/ml) with DTNB (50 μM) at 25° and pH 8.0 (50 mM, tris/HCl). Curve (*a*), control, no additions; curve (*b*), plus RuDP (1.5 mM).

in this way varied from one preparation to another. The catalytic activity of the enzyme was completely destroyed after incubation with DTNB for 30 min under these conditions. In the presence of RuDP, the initial rate of production of chromophore is considerably reduced, but the secondary rate of production is changed very little. The number of sulphydryl groups blocked by RuDP corresponds to an equivalent weight of 257,000. The value of this equivalent weight does not change from one enzyme preparation to another. On the basis of a molecular weight of approximately 500,000, there would be two catalytic sites per molecule.[9] Neither $MgCl_2$ (1 mM) nor $NaHCO_3$ (1 mM), alone or together, had significant effect on the reaction with DTNB. These results are consistent with our previous experiments using iodoacetamide,[1] and suggest that SH groups interact with RuDP but not with Mg^{2+} ions or bicarbonate.

The production of chromophore in the presence of RuDP is pseudo-first-order, as shown by the log plot (*x*) in Figure 2. The difference in the ordinate values of progress curves (*a*) and (*b*) of Figure 1 presumably gives the progress curve for the

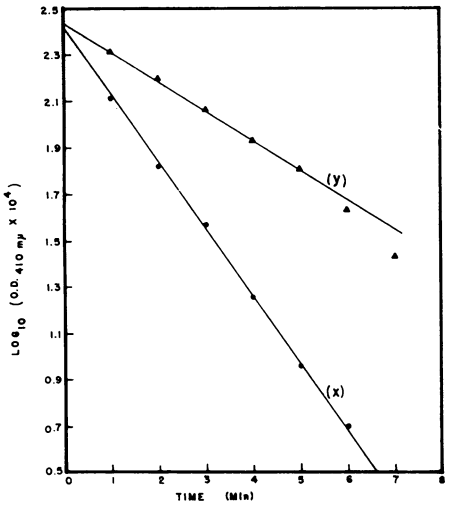

FIG. 2.—Log plots of data shown in Fig. 1. The ordinate of curve (*y*) is the difference between the ordinate values of curves (*a*) and (*b*) in Fig. 1. The ordinate of curve (*x*) is the difference between the ordinate values of the extrapolation of curve (*b*) and the curve itself.

reaction of DTNB with the SH concerned with the catalytic function. This reaction is also pseudo-first-order, as shown by the log plot (*y*) in Figure 2.

To investigate the slow secondary reaction, experiments were conducted using a much higher concentration of DTNB. The results are shown in Figure 3. The reaction is very complex and variable from one enzyme preparation to another; for the enzyme preparation illustrated the production of chromophore becomes linear after approximately 1 hr, and extrapolation of this linear rate to zero time gives an equivalent weight of 34,000.

Spectrophotometric investigation of RuDP binding: The interaction of the enzyme with RuDP can be investigated spectrophotometrically, as shown by the difference spectra in Figure 4. The legend to this figure contains the relevant experimental details. A mixture of the enzyme and RuDP at pH 8.0, as measured against the sum of the separate components, shows two peaks of positive absorption at 268 and 288 mμ, and a negative peak at 298 mμ [Fig. 4 (*2*)]. The kinetics of the produc-

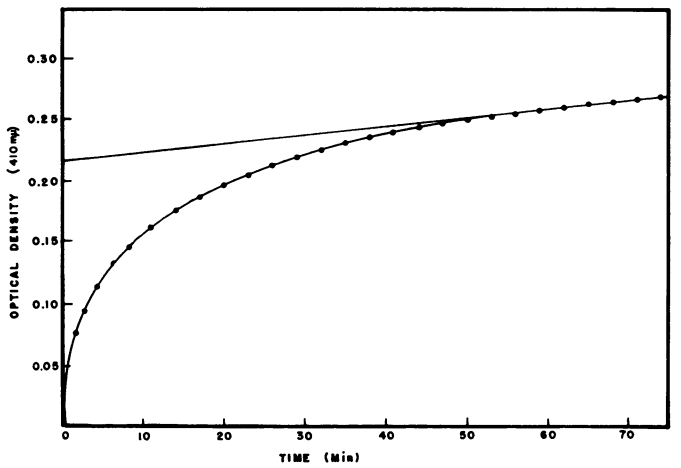

FIG. 3.—Progress curve of the reaction of carboxydismutase (0.53 mg/ml) with DTNB (1 mM) at 25° and pH 8.0 (50 mM, tris/HCl).

tion of the difference spectrum are too fast to follow, indicating that the interaction of the enzyme with RuDP is very rapid and almost certainly not rate-limiting in the catalytic reaction. The spectrum disappears on addition of sodium bicarbonate, provided that magnesium ions are also present; and the kinetics of this process

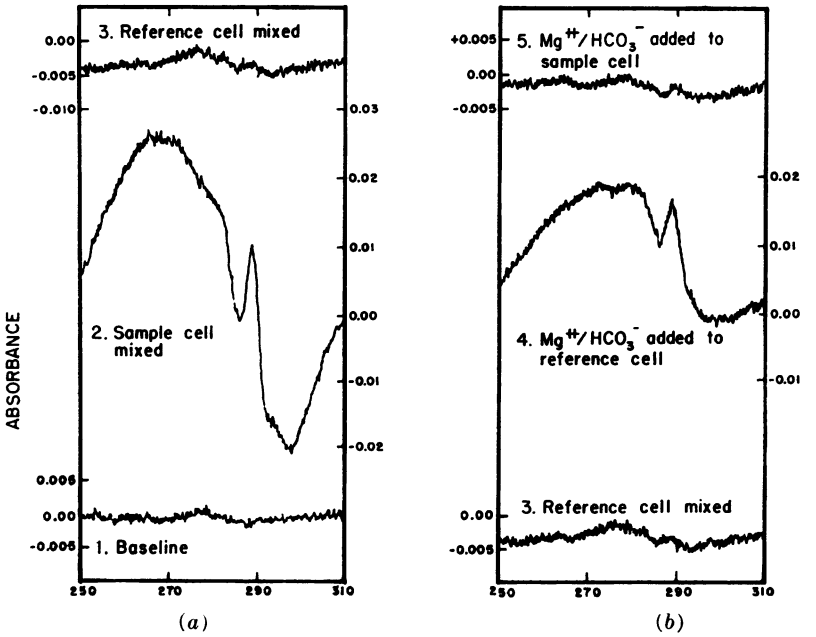

FIG. 4.—Difference spectrum of complex formed between carboxydismutase (1.21 mg/ml) and RuDP (0.4 mM) in tris/HCl buffer (0.3 *M*) at pH 8.0 and 25°, in a total volume of 1 ml and for a total light path of 1 cm. *1*, Base line; *2*, spectrum obtained after mixing sample cell; *3*, base line obtained after mixing reference cell; *4*, spectrum obtained 30 min after addition of $MgCl_2$ (50 μl, 0.2 *M*) and $NaHCO_3$ (50 μl, 0.5 *M*) to reference cell. NaCl (50 μl, 0.5 *M*) and $MgCl_2$ (50 μl, 0.2 *M*) were added to the sample cell; *5*, base line obtained 30 min after adding $NaHCO_3$ (50 μl, 0.5 *M*) to sample cell and NaCl (50 μl, 0.5 *M*) to reference cell.

have been followed at 268 mμ, and are shown in Figure 5. The difference spectrum of a mixture after reaction with $NaHCO_3$ is shown in Figure 4 (*4*). There are two absorption peaks, at 279 and 289 mμ, and the spectrum is not the same as Figure 4 (*2*). The most notable difference is the absence of the negative peak at 298 mμ. The differences between Figure 4 (*4*) and 4 (*2*) could be due either to the effect of Mg^{2+} on the enzyme-RuDP complex or to the absence of RuDP, which has a small absorption peak at 270 mμ, from the blank cell in Figure 4 (*4*).

The difference spectrum of a mixture of alkylated enzyme and RuDP against the sum of the separate components has also been measured, and the results are shown in Figure 6. No peaks at 268 or 298 mμ are observed, although a small peak at 288 mμ, similar in magnitude and shape to that obtained with the unalkylated enzyme, is found. This small peak might be taken to indicate that interactions between RuDP and the alkylated enzyme occur. The phosphate groups of the substrate are possible loci of such interactions. It is clear that the peaks at 268 and 298 mμ are due to interactions between RuDP and the sulphydryl at the catalytic site, and it remains to be investigated whether the chromophore is the enol intermediate previously suggested.

Analogous spectral changes to those observed in the interaction of the enzyme with RuDP can be observed in the reaction of cysteine with dihydroxyacetone phosphate at pH 8.0, as shown in Figure 7. This reaction is sufficiently slow that the kinetics of formation of the peak at 275 mμ can be followed spectrophotometrically,

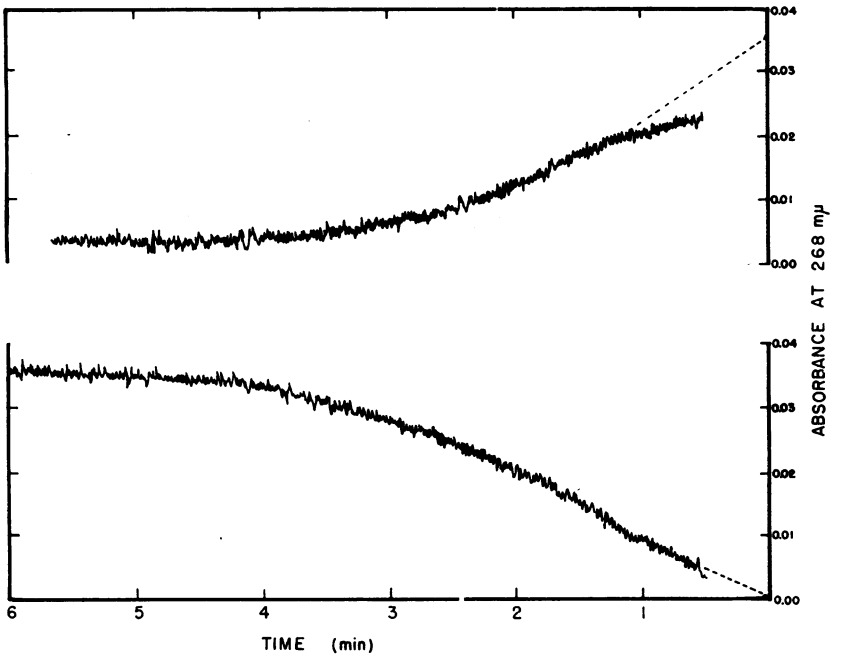

FIG. 5.—Kinetics of disappearance of chromophore on addition of $NaHCO_3$ and Mg^{2+} ions. The lower and upper curves represent the transitions from state 2 to state 3, and state 3 to state 4 (Fig. 4), respectively.

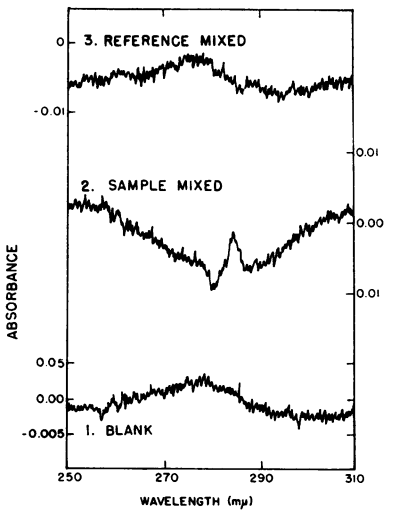

FIG. 6.—Difference spectrum produced in interaction of carboxymethyl-carboxydismutase (1.4 mg, ml) and RuDP (0.4 mM) in tris/HCl buffer (0.4 *M*) at pH 8.0 and 25°, in a total volume of 2 ml and for a total light path of 1 cm. *1*, Base line; *2*, spectrum obtained by mixing sample cell; *3*, base line obtained by mixing reference cell.

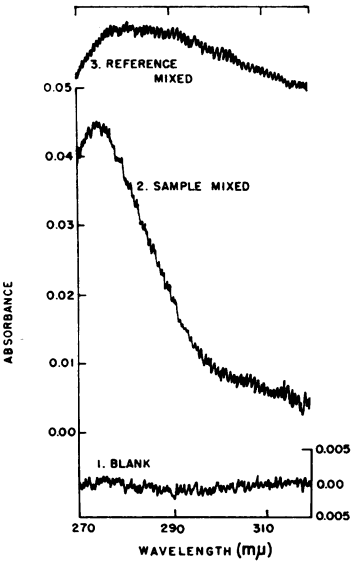

FIG. 7.—Difference spectrum produced in interaction of cysteine and dihydroxyacetone phosphate (each 5 mM) in tris/HCl buffer (0.4 *M*) at pH 8.0 and 25°. *1*, Base line; *2*, spectrum obtained on mixing sample cell: *3*, base line obtained by mixing reference cell.

as shown in Figure 7. The origin of these spectral changes is not certain, and further detailed investigations are required.

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The following abbreviations are used: Tris—Tris (hydroxymethyl) aminomethane; RuDP—Ribulose-1,5-diphosphate; DTNB—5,5'-Dithiobis-(2-nitrobenzoic acid).

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*ACTIVE AND INACTIVE REGIONS OF NUCLEAR CHROMATIN AS REVEALED BY ELECTRON MICROSCOPE AUTORADIOGRAPHY**

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Since RNA synthesis in the cell nucleus is a DNA-dependent process, an autoradiograph which shows the sites of RNA synthesis also indicates where the active DNA is located. The electron microscopic autoradiographs of thymus nuclei presented in this paper demonstrate that the DNA active in RNA synthesis is mainly in the diffuse, extended chromatin, rather than in the condensed, compact masses of chromatin. Since most of the DNA of thymus nuclei is present in the condensed masses of chromatin, these observations show that most of the DNA is inactive in promoting RNA synthesis.

Previous experiments on isolated thymus nuclei have shown that most of their DNA is inactive in RNA synthesis, for it was found that removing the DNA which is readily hydrolyzed by pancreatic DNAase (and this is 75–80% of the total DNA) does not diminish the rate of RNA synthesis. The remaining DNA, however, is essential for RNA synthesis, and the RNA being made in isolated thymus nuclei is largely of the "messenger" type.[1, 2] The earlier experiments on thymus nuclei and those reported in this paper are in line with the point of view, now widely accepted, that in differentiated cells of higher organisms many of the genes present are inactive.

The direct autoradiographic demonstration that RNA synthesis in intact nuclei is more active in diffuse than in condensed chromatin confirms the results of recent experiments[3] on isolated thymus nuclei in which chromatin from masses of nuclei