Dioldehydrase: New Observations Concerning the Effect of Oxygen, Nitrous Oxide and Carbon Monoxide

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Dioldehydrase (DL-1,2-propanediol hydrolase, E. C. 4.2.1.28) apoenzyme exists in an oxidized and a reduced modification due to the presence of disulfide- or sulfhydryl groups in the vicinity of the coenzyme binding site. Both modifications yield active holoenzymes which differ with respect to their sensitivity to O_2, N_2O and CO.

Nitrous oxide, N_2O, specifically oxidizes nucleophilic Co(I) derivatives of vitamin B_{12} and related model compounds and was used successfully to detect the presence of the Co(I) nucleophile derived from coenzyme B_{12} in functional dioldehydrase holoenzyme [1, 2]. A similar effect of N_2O was also demonstrated in a catalytic model system, in which a cobaloxime(I) nucleophile functions as the catalyst for the conversion of 2-bromoethanol to acetaldehyde [2]. The kinetic behavior of the model system and dioldehydrase holoenzyme are virtually identical, suggesting that N_2O in both cases specifically oxidizes the Co(I)-derivatives present. However, other authors have since studied the effect of N_2O on dioldehydrase and were unable to obtain similarly high degress of inactivation. FINLAY, *et al.* [3], for example, reportedly observed 15 % diminution of dioldehydrase activity in contrast to 75–85% inactivation reported by us [2]. The authors of ref. 3 furthermore quote results of FUKUI, who observed no inhibition at all with his dioldehydrase preparations. This contradictory evidence has been resolved on the basis of experiments to be reported in this communication. It is suggested that dioldehydrase holoenzyme exists in two modifications, of which only one is N_2O sensitive. We also include parallel observations with O_2, which is known to inactivate the enzyme-coenzyme complex giving rise to hydroxocobalamin [4]. We will furthermore show that the N_2O sensitive modification of dioldehydrase holoenzyme is inactivated by CO.

Our study of the N_2O effect on dioldehydrase indicated striking variations of the degree of N_2O sensitivity with the source and age of the enzyme preparations. Freshly dissolved solutions of apoenzyme assayed immediately usually showed the highest sensitivity to N_2O; the N_2O effect was occasionally absent in aged, but otherwise active enzyme preparations. We subsequently found that dioldehydrase apoenzyme is converted into an N_2O insensitive modification on exposure to air. Typical results and experimental conditions obtained with a commercial preparation of dioldehydrase are described below:

Two vials of dioldehydrase [5] were dissolved in 2.2 ml of Buffer E [6, 7] under complete exclusion of air, using pure argon as the protecting gas. After 30 min of standing at 25 °C this dioldehydrase solution was assayed for activity in rubber serum capped reaction tubes of 25 ml capacity, which contained pure Ar, N_2O [8], CO, and O_2, respectively, all at 1 atm of partial pressure. The reaction tubes were filled successively with 0.2 ml of 0.2 F pH 8.0 potassium phosphate buffer solution, 0.4 ml of boiled, deionized H_2O, 0.2 ml of an aqueous solution containing 16 μg of coenzyme B_{12}, and 0.1 ml of apoenzyme solution. All operations involving the coenzyme were performed in dimmed light; the reaction tubes were covered with aluminium foil to eliminate light-inactivation of the coenzyme. Aliquots of the reaction solutions (0.2 ml) were withdrawn after 10, 20, 30, and 40 min of incubation at 37 °C and assayed for propionaldehyde by the standard spectrophotometric procedure. The results of a typical experiment are shown graphically in Fig. 1 A. With this enzyme preparation the observed inactivation by N_2O, CO, and O_2 was 38, 48, and 56 % relative to the run under argon. Storage of an aliquot of the apoenzyme solution *under*

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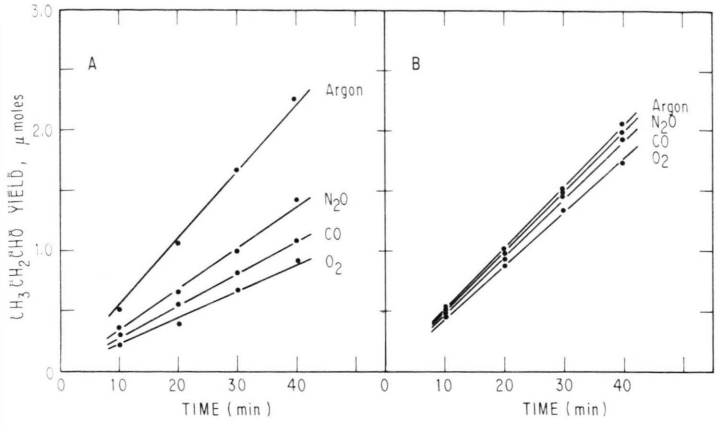

Fig. 1. Effects of N_2O, CO and O_2 on dioldehydrase holoenzyme. A: Apoenzyme dissolved under argon; B: apoenzyme stored under air for 24 hours at 5°C. Experimental conditions as described in the text.

argon for 24 hours at 5 °C caused a 4 % loss of dioldehydrase activity and diminished the inhibition by N_2O, CO, and O_2 to 20, 25, and 52.2 %, respectively. Storage of the same enzyme solution *under air* for 24 hours at 5 °C caused *virtual disappearance* of N_2O and CO inactivation, while causing only a 7 % loss of total dioldehydrase activity (Fig. 1 B). The degree of inactivation by the three gases varied with the enzyme preparation, but oxygen was invariably more effective than CO and N_2O.

The effect of air on dioldehydrase apoenzyme suggests the presence of sulfhydryl groups in the vicinity of the coenzyme binding site. This interpretation is in accord with independent conclusions reached by Toraya *et al.* [9]; dioldehydrase activity has previously also been shown to be diminished in the presence of SH blocking reagents such as *p*-mercuribenzoate [10]. Addition of this reagent at the beginning of the reaction leads to almost complete inhibition. After completion of the lag period (20 min), no inhibition is observed [11]. These findings suggest that SH groups are involved in coenzyme binding and sensitive to the blocking reagent. Dioldehydrase apoenzyme stored under argon and dissolved anaerobically reacts with Ellman's Reagent [12], indicating the presence of free sulfhydryl groups. Exposure of the apoenzyme to air indicated a 37 % diminution of the amount of SH groups relative to the argon-stored apoenzyme. Since dioldehydrase is normally isolated and purified without consideration of its oxygen sensitivity, it is now understood why the degree of inactivation by the gases employed is subject to considerable variation and dependent on the time and mode of storage of the apoenzyme. Most of the dioldehydrase preparations accessible to us showed the presence of some of the reduced form. However, since subsequent purification of the apoenzyme is usually performed aerobically, it is clear that this will cause further diminution of the amount of reduced form present; this is why Finlay *et al.* [3] only observed 15 % inactivation of holoenzyme by N_2O, and Fukui none at all. Attempts to reduce the oxidized apoenzyme back to the reduced form have been partially successful using excess dihydrolipoic acid as the reductant in the presence of traces of selenide or aquocobinamide as catalysts, followed by anaerobic dialysis to remove the thiol and catalysts for disulfide reduction. The partial reduction of apoenzyme was indicated by the increase of oxygen sensitivity from 18 to 37 %, and the reappearance of 9 and 7 % of the CO and N_2O inactivation, respectively [13].

Oxygen is presumably the strongest inactivator because of its small molecular size and its ability to oxidize enzyme-bound coenzyme to the Co(III) state. Nitrous oxide inactivates to a lesser degree presumably because of its larger size and because it can only react with the Co(I) nucleophile, oxidizing it to Co(II) rather than Co(III).[14] The inactivation by both O_2 and N_2O is irreversible and must occur during the first 10 min of incubation, since the rates of propionaldehyde formation after inactivation are essentially constant. The inactivating effect of CO was invariably found to be greater than that of N_2O. It is postulated that CO forms a complex with enzyme-bound corrin Co(I) nucleophile; Co(I) derivatives of cobaloximes form CO adducts in solution [15]. Although an analogous adduct with vitamin B_{12s} has not yet been observed in solution, it could form in the enzyme. Attempts to reactivate CO-inhibited holoenzyme, *e. g.*, by light-irradiation, thus far have been unsuccessful.

It appears that the SH groups in the vicinity of the coenzyme binding site are unessential for catalytic activity, just as is apparently the case in ribonucleotide reductase from *Lactobacillus leichmannii* [16]. Oxidation of the SH groups to disulfide does not lower the overall activity of the holoenzyme significantly but appears to protect the active site against inactivation by gaseous oxidizing- or blocking reagents by way of a conformational change of the apoenzyme in the vicinity of the coenzyme active site.

The inactivation of *reduced* dioldehydrase holoenzyme by N_2O confirms the presence of the Co(I) nucleophile in functional holoenzyme. Since N_2O oxidizes Co(I) to Co(II), Co(II)-corrin derivatives are probably not involved in the actual process of enzymatic catalysis. These observations reaffirm the mechanism of dioldehydrase action proposed by us[17], which to date is the only mechanism supported by nonenzymatic model experiments.

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[1] *Abbreviations:* Dioldehydrase is DL-1,2-propanediol hydrolase, E. C. 4. 2. 1. 28; coenzyme B_{12} is α-(5,6-dimethylbenzimidazolyl)-Co-5'deoxyadenonsylcobamide. Cobaloximes are derivatives of bis (dimethylglyoximato)-cobalt.

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[6] Buffer E is a 0.01 F solution of K_2HPO_4 containing 20 ml of DL-1,2-propanediol per L[7]. The addition of bovine serum albumin to Buffer E for stabilization of dioldehydrase is occasionally recommended, but none was added in the exerpriments reported here.

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[8] The N_2O was a commercial product (Linde) of 99+% purity containing traces of water and air as only contaminants. Traces of oxygen from both N_2O and CO were removed by passage of the gases through alkaline pyrogallol solution.

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[11] All dioldehydrase assays in ref. 10 were performed areobically.

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[13] Exposure of this partically reduced apoenzyme to air again diminishes the O_2 inactivation and causes disappearance of the N_2O and CO effects. Addition of 0.1 ml of 0.1 F sodium arsenite solution partially protects the apoenzyme against reoxidation under the experimental conditions outlined in the text.

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