Evidence for an Active Site Persulfide Residue in Rabbit Liver Aldehyde Oxidase*

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SUMMARY

The reduction of aldehyde oxidase (EC 1.2.3.1) by the substrate $N^1$-methylnicotinamide under anaerobic conditions is a biphasic reaction. In the present work we present evidence that this behavior is due to the presence, in standard preparations, of active and inactive enzyme, the former being responsible for the fast reaction and differing from the latter by the presence of a persulfide group. It is also demonstrated that the earlier reported inactivation of the enzyme by cyanide is due to cyanolysis of this persulfide group.

Recent work from this laboratory with milk xanthine oxidase has revealed the presence in standard preparations of functional and nonfunctional enzyme molecules differing only by the presence or absence of an active center persulfide residue (1, 2). It was shown that cyanolysis of this residue to yield free thiosulfinate was the basis of the inactivation by cyanide (1, 2). In view of the many similarities in properties already noted between xanthine oxidase and aldehyde oxidase (see Refs. 3 and 4 and literature quoted therein), including the irreversible inactivation of aldehyde oxidase by cyanide (5), we decided to investigate whether aldehyde oxidase also depended for its catalytic activity on an active site persulfide residue.

MATERIALS AND METHODS

The preparation of the enzyme, the assay conditions, and the sources of reagents were as described previously (6). Rapid reaction studies were made with a Gibson-Milnes stopped flow spectrophotometer (7).

Radioactive potassium cyanide was obtained from Amersham-Searle and had a specific activity of 1.01 mCi per mmole. All experiments were done in 0.05 M potassium phosphate, pH 7.8, containing $3 \times 10^{-4}$ M EDTA.

RESULTS AND DISCUSSION

Reduction of Aldehyde Oxidase by Substrate—The early observation that the anaerobic reduction of milk xanthine oxidase by xanthine was a biphasic reaction (8) and the subsequent finding that such bihasticity is due to the presence, in standard preparations, of inactive enzyme (2, 9, 10) coupled with the strong similarity between the two enzymes suggested a more detailed investigation of the reduction of aldehyde oxidase by the substrate $N^1$-methylnicotinamide. When the enzyme with a specific activity in the NMN$^1$-oxygen reductase assay of 2.2 was reduced under anaerobic conditions in the presence of 5 mM NMN and the bleaching of the absorbance at 450 nm was followed with a Cary model 17 recording spectrophotometer, a total 60% bleaching was observed, 83% of which was complete by the time the first reading could be made (40 s). When the same experiment was repeated with an enzyme having a specific activity of 0.03 no rapid decrease of the absorbance at 450 nm was observed but the enzyme was much more slowly reduced to the same extent (Fig. 1). This observation that enzyme with a very low specific activity was completely reduced by substrate led us to make a survey of the reduction of the enzyme using the stopped flow technique.

Enzyme with a specific activity of 2 and a concentration of $11.5 \times 10^{-9}$ M before mixing was carefully deoxygenated and mixed in the stopped flow apparatus with deoxygenated 10 mM NMN and the bleaching at 450 nm was recorded. From the analysis of the data it was clear that the reduction was proceeding in two distinct phases. When the experiment was carried out at different NMN concentrations it was found that the rate of reduction in the fast phase was dependent on NMN concentration and equal to 760 min$^{-1}$ extrapolated to infinite NMN concentration (Fig. 2) while the rate of reduction in the slow phase was not dependent on substrate concentration and, under these conditions, equal to 4 min$^{-1}$.

Fig. 3 shows the time course of the reduction of the enzyme in the fast phase in the presence of 5 mM NMN. From these results it is apparent that, apart from a small deviation at short times, the reduction is accurately represented by a first order plot. Such reactions, carried out at different wavelengths, were used to obtain the spectrum of the enzyme at the end of the fast phase of reduction (Fig. 4). The conclusion from this first set of experiments was that only 45% of the reduction was occurring in the fast phase.

When enzyme preparations of differing AFR values (6) were tested, it was found that it was only the extent of the reaction occurring in the fast phase which changed, not the rate of reaction. There was a linear correlation of the AFR value and the extent of reaction occurring in the fast phase (Fig. 5). On the other hand, a correlation was observed between the extent of reaction occurring in the slow phase and the AFR of the enzyme.

The abbreviation used is: NMN, $N^1$-methylnicotinamide.
Fig. 1 (left). Anaerobic reduction of aldehyde oxidase in the presence of 6 mM NMN. ○, results obtained with an enzyme with specific activity of 0.03; ●, results obtained with an enzyme of specific activity 2.2.

Fig. 2 (right). Plot of the reciprocal velocity of reduction occurring in the fast phase versus the reciprocal NMN concentration. The limiting rate of reduction obtained corresponds to a rate constant of 760 min⁻¹. The enzyme was 11.5 × 10⁻⁴ M with respect to FAD content and the experiment was carried out in 0.05 M potassium phosphate, pH 7.5, at 25°C.

Fig. 3. The fast phase of reduction of aldehyde oxidase with NMN in 0.05 M potassium phosphate, pH 7.8, at 25°C. Concentration of the reactants after mixing in the stopped flow apparatus: NMN, 5 mM; enzyme, 6.6 × 10⁻⁷ M with respect to FAD content. Left, change in absorbance after mixing (2-cm pathlength). Right, first order plot of the data.

On the other hand, the rate of absorbance bleaching in the slow phase was dependent on the AFR value, increasing significantly as the AFR increased. This is evident from Fig. 1, where the half-times of reaction in the slow phase were less than 6 min and 20 min, respectively, for the more active and less active preparations. These results suggest that the reduction of nonfunctional enzyme proceeds primarily through reduced functional enzyme, rather than directly via substrate. The results of Fig. 5 are quite analogous to those found with xanthine oxidase (2, 9, 10) where the phenomenon of the biphasic reduction of the enzyme has been shown to be due to the presence of an active site persulfide group in functional enzyme, the remaining complement of oxidation-reduction groups, molybdenum, iron-sulfur centers, and flavin being intact in both types of enzyme molecules (2). These results suggest that the same situation exists with aldehyde oxidase, and that in this enzyme the rate-limiting step in catalysis is the reduction of the enzyme by substrate (mediated by the persulfide group). The data obtained are perfectly consistent with this hypothesis. Thus, from the results of Fig. 2, the limiting rate of the reduction of the enzyme by NMN was found to be 760 min⁻¹. It was found that this rate was independent of the AFR value of the enzyme; only the extent of the reduction occurring at this rate was affected by the AFR value. The enzyme used in the experiments of Fig. 2 had an observed turnover number in catalytic assays of 340 moles of NMN oxidized per min per mole of flavin. If indeed only 45% of this enzyme was in a functional state, the turnover number for fully functional enzyme would be expected to be 340/0.45, or 755 min⁻¹, a value in very good agreement with the rate of reduction actually measured in the fast phase of the enzyme bleaching.

Reaction of Aldehyde Oxidase with Oxygen—Fig. 6 shows the results of a study of the reaction rate of reduced enzyme with molecular oxygen. At all oxygen concentrations tested, 85 to 90% of the absorbance increase at 450 nm occurred with apparent first order kinetics; there was no variation in this pattern with enzyme of different AFR value. As shown in Fig. 6, the pseudo-first order rate constants are within experimental error directly proportional to oxygen concentration. From these results a second order rate constant of 3.2 × 10⁷ M⁻¹ min⁻¹ can be calculated.

Cyanalysis of Active Site Persulfide—The same enzyme as used in the experiment in Fig. 2 (specific activity of 2, turnover number of 340 min⁻¹, and showing in the stopped flow analysis 45% of total reduction in the fast phase) was incubated with radioactive cyanide and, after complete inactivation was reached, chromatographed on a Sephadex G-25 column (1.5 × 50 cm). The inactive enzyme was eluted at a volume of 23 ml and only 0.1 to 0.3 mole (depending on the time of incubation) of cyanide was incorporated into the protein per mole of enzyme-bound FAD. The unreacted cyanide was eluted at a volume of 50 ml and a third peak at a volume of 73 ml. The amount of radioactive thiocyanate released was 0.45 mole per mole of enzyme-bound FAD. Again, when enzyme showing 55% reduction in the fast phase was treated in the same way, 0.56 eq of thiocyanate per mole of enzyme-bound FAD were found in the eluate.

In order to extend the range of correlation and since only a 20% maximum difference was observed among different preparations (probably due to the fact that all manipulations and conditions during each step of the purification were rigorously standardized) the enzyme was also inactivated to different levels with [³⁵C]cyanide. During the inactivation by cyanide the activity and the change in the visible portion of the spectrum...
Fig. 4. Spectra of aldehyde oxidase at various states of oxidation and reduction in 0.05 M potassium phosphate, pH 7.8, at 25°C. Left Panel, Curve A, oxidized enzyme at a concentration of 2.9 X 10^{-4} M with respect to FAD content. Curve B, the same at the end of the fast phase of the reduction with NMN (determined with the stopped flow technique). The experimental values from which the curve was determined were obtained at 10-nm intervals. Curve C, the same again after complete reduction with NMN. Right Panel, difference spectra of the reduced enzyme. Curve A, oxidized enzyme minus fully reduced; Curve B, oxidized enzyme minus reduced at the end of the fast phase.

Fig. 5 (left). Correlation of the extent of reduction at 450 nm occurring in the fast phase of reduction by NMN versus the AFR value of the enzyme.

Fig. 6 (center). Plot of the reciprocal oxidation rates versus the reciprocal of the oxygen concentration. The enzyme was previously reduced with 4 moles of NMN per mole of enzyme-bound FAD, mixed in the stopped flow apparatus with buffer at different oxygen concentrations, and the reoxidation was followed at 450 nm. The enzyme concentration after mixing was 3.24 X 10^{-4} M with respect to the FAD content.

Fig. 7 (right). The extent of the extinction coefficient change at 320 nm during the reaction of aldehyde oxidase with cyanide correlated with the AFR of the enzyme. Enzyme concentration was determined using a $\varepsilon_{320}$ of 34,700.

From the analysis of the reduction of the enzyme at different levels of cyanide inactivation we found also a proportional relationship between the per cent of the bleaching at 450 nm occurring in the fast reaction and the amount of the persulfide present in the protein (Fig. 8). Since in all cases only 0.1 to 0.3 eq of cyanide were incorporated per mole of enzyme-bound FAD and the varying parameter was only the amount of thiocyanate released from the enzyme it is reasonable to conclude that the inactivation of the enzyme by cyanide is due to the cyanolysis of a persulfide group essential for the catalytic activity of the enzyme: $R-S-S^- + CN^- \rightarrow R-S^- + SCN^-$. It would be
Apart from subtle differences in substrate specificity, the two enzymes appear to have similar molecular weights, identical oxidation-reduction groups (molybdenum, iron-sulfur centers, and FAD) in the same proportions, similar spectra in both their native and deflavop forms (6), and, as now shown, both appear to contain an active center persulfide residue which is essential for catalysis.

REFERENCES

![Graph](image-url)
Evidence for an Active Site Persulfide Residue in Rabbit Liver Aldehyde Oxidase
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