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DOPAMINE-β-HYDROXYLASE: EVIDENCE FOR BINUCLEAR COPPER SITES

Ninian J. Blackburn[a], Howard S. Mason[b] and Peter F. Knowles[c]

a Department of Chemistry
University of Manchester Institute of Science & Technology
Manchester, U.K.

b Department of Biochemistry
University of Oregon Health Sciences Centre
Portland Oregon, U.S.A.

c Astbury Department of Biophysics
University of Leeds
Leeds, U.K.

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SUMMARY: Copper has been progressively removed from dopamine-β-hydroxylase by incubation with chelex resin. The relationship between the activity (A) and the copper/protein ratio (r) has been shown to be of the form $A \propto r^2$ which implies that the enzyme catalysed reaction is second order with respect to protein bound copper. These results support a model for the catalytic mechanism involving binuclear copper sites rather than the four copper atoms in the enzyme acting independently.

Dopamine-β-hydroxylase (E.C.1. 14.2.1.; 3,4-dihydroxyphenylethylamine, ascorbate: oxygen oxidoreductase (hydroxylating)), is a copper containing mixed function oxidase which catalyses the oxidative hydroxylation of dopamine to noradrenalin with the associated four electron reduction of oxygen to water and is thus a candidate for a binuclear active site[1]. The native enzyme (mol wt 290,000) contains four polypeptide chains (mol wt 77,000) which are cross-linked in pairs by disulphide bridges to form identical dimeric sub-units[2,3] and requires four coppers per tetramer[4] for full activity. Friedman and Kaufman[5] demonstrated the existence of a reduced intermediate capable of the aerobic hydroxylation of one substrate molecule per two coppers oxidised from Cu^+ to Cu^{2+}. When combined with the results of steady state kinetic measurements[6], these studies led to the postulation of a "ping-pong" mechanism (see ref. 7 for nomenclature).

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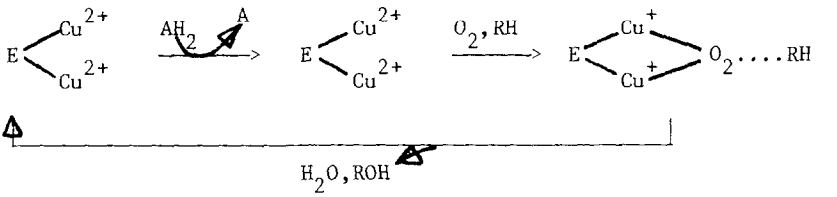

In this scheme AH_2 and A are the reduced and oxidised forms of the supplementary substrate (ascorbate) while RH and ROH represent the amine substrate (dopamine) and the hydroxylated product (noradrenaline) respectively. Ljones *et al*[8,9] have challenged the above mechanism in favour of one involving one-electron transfers via single copper atoms at the active site; this mechanism implies that the copper sites are acting independently.

In this paper we present evidence to support the view that the four copper sites in dopamine-β-hydroxylase do not act independently. A model for the catalytic cycle is proposed in which the enzyme contains magnetically isolated mononuclear copper atoms in the oxidised form but undergoes a conformational change to a binuclear (interacting) cuprous form upon reduction with ascorbate.

MATERIALS AND METHODS

Ascorbic acid, tyramine-HCl, α-methyl-D-mannoside and bovine serum albumin were obtained from Sigma Chemical Co. Catalase was obtained as a crystalline suspension from Boerhinger, Mannheim. Chelating resin for copper removal from the enzyme was Chelex 100, 200-400 mesh (Bio-Rad), while for copper removal from buffers, Dowex Chelating resin, dry mesh 80-100 (Sigma) was used. All other chemicals were purchased from BDH and were Analar grade quality.

Enzyme preparation: Dopamine-β-hydroxylase was isolated from bovine adrenal medulla essentially according to the method of Ljones *et al.*(10), except that Triton-X100 was omitted from the extraction buffer to prevent solubilisation of membrane bound enzymes (11). A second con-A Sepharose column was often necessary to obtain a completely homogeneous preparation. The purified protein was stored sterile at 4°C and retained full activity under these conditions for at least one year. The enzyme preparation was judged homogeneous by the criterion of SDS polyacrylamide gel electrophoresis (12). The specific activity defined as μ-moles product produced per minute per mg (assay method (a) below) was ∿31.

Activity determinations: Enzyme activities were determined using tyramine as substrate by one of two methods:

(a) conversion of the product octopamine to parahydroxybenzaldehyde by periodate oxidation (2).

(b) following the rate of oxygen consumption at 25°C using an oxygen sensitive electrode (Rank Bros., Cambridge, U.K.). Method (b) was used to determine the relationship between specific activity and copper to protein ratio and consequently both the electrode and assay reagents were pre-treated to remove traces of inorganic copper. Saturated KCl used to provide the liquid junction between the Pt cathode and Ag/AgCl anode was treated with chelating resin prior to fitting the teflon membrane. The electrode chamber was rinsed once with 2% nitric acid, several times with water, once with 10^{-3}M EDTA, again with water and finally was allowed to stir overnight in the presence of chelating resin. The cell stopper was similarly treated. A stock solution of 200 mM sodium acetate pH 5.07 was passed through a column of chelating resin. 0.170g tyramine-HCl and 0.090g ascorbate were dissolved in 50.0 ml of the treated buffer; 5.0g of chelating resin (washed to a constant pH of 5.07 with 200 mM sodium acetate) was added and the solution stirred at room temperature for thirty minutes. The resin was allowed to settle but the solution was not decanted. Catalase (2mg/ml) was also stored over chelating resin. Fumarate was not included in the assay reagents since no stimulation of activity was achieved by its addition. 4.0 ml of assay solution was pipetted (using plastic pipette tips) into the electrode chamber followed by 0.1 ml catalase solution. After a constant oxygen level was established, enzyme was added and the rate of oxygen consumption recorded. Control experiments in the absence of enzyme or with boiled enzyme samples were run to obtain the small contribution from the non-enzymatic rate of oxygen consumption The equilibrium oxygen concentration in air saturated buffer at 25.0°C was taken as 0.250 mM (Handbook of Chemistry and Physics, Chemical Rubber Co., 1963).

Protein was determined by the method of Lowry *et al.* (13) using bovine serum albumin as standard.

Total copper was determined by atomic absorption spectroscopy using either a Varian Techtron AA-5 or Pye-Unicam SP2900 spectrophotometer. Copper was also determined chemically by the method of Van der Bogard and Beinert (14) modified for the use of bathocuproin sulphonate. Agreement with the atomic absorption method was within 2%.

Copper removal. Initial copper removal experiments were carried out by incubating samples of native enzyme with 0.1g chelex per ml at 4°C for periods up to one week. Assay method (a) was used to measure the activity. Later studies used incubation at 10°C in the presence of 0.1g/ml chelex resin for periods up to 24 hours, with constant gentle rotation of the solution. Aliquots were removed for copper, protein and activity analysis at suitable time intervals. Activities were measured using the oxygen electrode (method b) (i) under copper-free conditions and (ii) after the addition of 1.0×10^{-7}M cupric nitrate. An enzyme sample with a very low copper to protein ratio was also prepared by dialysing native enzyme vs 50 mM KCN in 50 mM sodium phosphate buffer pH 6.0, followed by dialysis vs the same buffer in the absence of cyanide.

RESULTS

Oxygen Electrode Assay

The rate of oxygen uptake was a linear function of enzyme concentration over the range 0-5000 mg enzyme per ml assay. A slight induction period was observed in all runs and the subsequent linear portion of the trace extended over 65-70% of the reaction. The rate of octopamine production measured at

25°C by assay method (a) was compared with the rate of oxygen consumption under the same conditions and good agreement was obtained between the two assay methods.

Dependence of enzyme activity on the copper/protein ratio

The half time for copper removal from the enzyme at 4°C using chelex incubation was of the order of days. It was found that while the copper to protein ratio decreased to 0.35, the specific activity as assayed by method (a) did not decrease; similar results were obtained for samples where copper was removed by dialysis against potassium cyanide. When either of these copper depleted samples was assayed under strictly copper-free conditions using assay method (b) they were essentially inactive. Addition of six equivalents of Cu^{2+}/enzyme (4×10^{-8}M added Cu^{2+}) immediately restored full activity. Thus the high specific activities observed using assay method (a) indicate that apo-enzyme is rapidly and completely reconstituted during assay by trace amounts of copper ions.

Similar results have been obtained by Skotland and Ljones[4] who used the chelators bathocuproin disulphonate or EDTA to prepare apo dopamine-β-hydroxylase. The chelex method reported in the present paper has the advantage that it precludes the presence of soluble non-protein bound complexes of copper which might otherwise complicate interpretation of results.

A series of samples of copper to protein ration in the range $2.0>r>0.4$ was then prepared from two separate preparations of enzyme and the activities measured using the oxygen electrode (i) under copper free conditions and (ii) after addition of excess Cu^{2+} to a final concentration of 1.0×10^{-7}M. The activity measured in (ii) was the maximum attainable and activities measured under copper-free conditions were normalised to this value. The results are presented in the figure and show a non-linear dependence of activity on the copper to protein ratio.

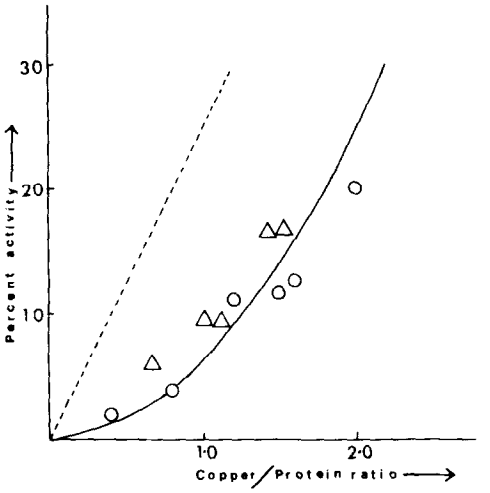

Fig. 1 The dependence of activity on the copper to protein ratio of dopamine-β-hydroxylase. Activities measured under copper-free conditions are expressed as a percentage of the maximum value obtained in the presence of excess copper. The open circles (O) and triangles (Δ) denote results from separate enzyme preparations. The solid curve represents the function $r^2/16$ (where r is the copper to protein ratio) and is equal to the mole fraction of dimeric subunits containing two copper atoms. The dashed line is the function r/4 and is equal to the mole fraction of monomeric subunits containing a single copper atom.

DISCUSSION

Skotland and Ljones[4] have shown that fully active dopamine-β-hydroxylase requires four coppers per tetramer but that the enzyme is also capable of binding copper non-specifically. Quantitative interpretation of the relationship between activity and copper to protein ratio is therefore dependent on establishing that only active site copper is present. The samples used to obtain the data presented in the table fulfil this requirement since

(i) they have been prepared from native enzyme containing only two coppers per tetramer by controlled copper removal rather than by reconstitution of apo enzyme samples. Samples having copper to protein ratios between 0 and 2 were chosen since this region is the most sensitive to detect whether the four copper centres operate independently in the catalytic mechanism or whether the *modus operandi* involves more complex participation by the copper centres;

(ii) the rate constants for copper removal on chelex are slow (half life of days at $4^{\circ}C$) which is characteristic of active site copper[4];

(iii) removal of copper from the enzyme using chelex 100 resin rather than a soluble chelator eliminates the possibility that the extracted copper might be contaminating the sample. In the figure, the percent activity is plotted against copper to protein ratio, r, in the range $0<r<2$. The dotted line is the function $r/4$, which, since the fully active enzyme contains 4 coppers/mole, represents the mole fraction of individual copper sites. The solid line is $r^2/16$ or the square of this function. It is apparent that in the range $0<r<2$ activity is proportional to the square of the mole fraction of individual copper sites, i.e. is second order in protein bound copper. This suggests that two coppers participate in each catalytic event and hence the four coppers of the fully active enzyme would be distributed among two active sites.

The presence of active sites composed of two copper atoms in the enzyme must be reconciled with the observations by Walker *et al*[15] and Ljones *et al*[16] that all four coppers in the fully oxidised enzyme are paramagnetic, implying that the coppers are mononuclear and separated by a distance of ≥ 7Å. A model consistent with these two sets of observations is to suppose that an interaction between the copper centres occurs in the reduced form, while in the oxidised form the copper sites are mononuclear and non-interacting. This mechanism, which is similar to that originally proposed by Friedman and Kaufman[5] would require that a change in tertiary structure occurs upon reduction so that the coppers are brought into an interacting conformation. The induction period noted in the oxygen electrode assays would be consistent with a conformational change.

Although our evidence for interaction is kinetic and thus gives no information on the structural relationship between the interacting cuprous ions, a binuclear cuprous cluster would be particularly attractive as the binding site for O_2, resembling established binuclear binding sites of other copper proteins which reduce oxygen to water[1].

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