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ELECTRON TRANSFER IN MONOMERIC FORMS OF BEEF AND CHICKEN HEART CYTOCHROME C OXIDASE

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ABSTRACT:

Beef heart cytochrome C Oxidase is dimeric in reconstituted membrane and in non ionic detergent at physiological pH (1, 2, 3, 4) raising the possibility that this aggregation state is a pre- requisite for enzymatic activity. A procedure for dissociating the enzyme in monomers is presented. This involves treating the protein with high concentration of triton x-100 at pH 8.5 or alkalination of the enzyme at pH 9.0 and above with addition of sodium hydroxide at temperature 4°C (5, 6). The electron transfer activity of the monomers is comparable to that of the dimer under identical assay condition. The beef heart cytochrome g oxidase monomer was found to heterogeneous in hydrodynamic studies, probably due to the dissociation of associated polypeptides including subunit III (7, 8). Sedimentation equilibrium (S) values indicated monomers molecular weight in range of 150,000 to 170,000 whereas chicken heart cytochrome c oxidase monomers MW 160,000 compare to 300,000 molecular weight of beef dimeric enzyme. Polypeptide composition of nomoners and dimer are identical as seven subunits.

Introduction

Cytochrome c oxidase (Ec 1.9.3) plays a central role in the aerobic metabolism of the obligative aerobes. It is terminal electron acceptor of redox chain in both bacteria and mitochondria (9, 10, 11). Cytochrome c oxidase is metallo-enzyme containing two haems and a3 and two copper atoms. The enzyme is embedded in the inner mitochondrial membrane (12) and catalyses the passage of electron from the single site redox protein cytochrome c to oxygen in multiple electron steps. The mechanism of this enzyme is subject of considerable research effort (for review see Brunori et al., 1980)[13].

It is generally agreed that cytochrome oxidase from fungal sources is a complex of seven or probably eight different polypeptides (14, L5). Cytochrome g oxidase isolated from mammalian sources contain at least 12 different polypeptides (16, 17, 18, 19).

There is now controversy about how many of polypeptides in cytochrome c oxidase preparation are true subunits of the enzyme. Our approach is to define the subunit composition of cytochrome c oxidase in terms of minimal structure able to transfer electrons from cytochrome c molecular oxygen and to couple this reaction to the generation of a portion gradient across the inner mitochondrial membrane. It appears that a complex of at least 8 different polypeptides is fully functional (Ludwig et al.)[20]. This does not rule out the possibility that additional polypeptides have a regulatory role.

Three of the sub units (I-III) are coded for and synthesized in the mitrochondria of all eukarytoes (21, 22, 23). The smaller sub units IV, V, VI VII-11e and VII - Ser (terminology for beef enzyme are made in cytoplasm (24). The minimum molecular weight of two haem, two copper complex containing one copy of each of the eight sub units can be ralculated. This value is 160,000 daltons. Though in literature complex containing one copy each of the 12 polypeptides that are considered to be sub units have molecular weight of 200,000. Some of the preparation of cytochrome oxidase with missing sub unit III have been reported in literature with complex containing other seven unit and molecular weight approximately 130,000. Molecular weight of beef heart cytochrome oxidase in non ionic detergents give value in the range 300,000 - 400,000 indicating that stable species from this source is admire (25, 26).

The studies of beef heart cytochrome c oxidase with other eukarytoes including Carnal, elasmo branch fish and enzyme from rat liver were reported to be monomeric under physiological condition though molecular weight was not mentioned. We have isolated cytochrome c oxidase from chicken hearts and various experiments was conducted related to its activity, electron transfer, sub units with molecular weight determination. Studies described here clarify the significance of the aggregation state in cytochrome oxidase activity. Monomeric cytochrome c oxidase from chicken hearts have been obtained which is functional in electron transfer. The physical characteristics including molecular weight alongwith sub units are comparable with beef cytochrome oxidase.

Materials and Methods

Cytochrome c oxidase preparation:

Beef heart and chicken heart cytochrome c oxidase were prepared following capable procedure (1972)[27] and Yonetani procedure (1960) respectively with little modification haem a to protein ratio was 9-10 =sole of haeme a (per)/mg of protein. Protein concentration was measured by the method of Lowry et al. (1951)[39] with bovine serum albumin as a standard.

Extinction coefficient used for determining the concentration of both beef and chicken heart cytochrome c oxidase were those reported by Fonetani (1961)[30]. The extinction coefficient for cytochrome oxidase at 280 am was determined independently for each preparation and was found to be 210 nM^{l} cm^{1} per haem a for beef heart enzyme and 230 mM^{-1} cm^{-1} per haeme a for chicken enzyme.

Gel filtration and Triton x-100 binding:

A column (1 x 25 cm) containing Sephacryl S200 was used to measure the amount of Triton x-100 bound to cytochrome c oxidase (1-2 mg) were loaded into the column equilibrated with 0.1% Triton x-100, 200 mM NaCl and 10 mM Tris HCl buffer at pH 83 or pH 7.4. Samples were collected from column and their absorbance measured at 280 nm and 420 nm on a Backman Due 7 spectrophotometer.

Gel filtration was also done using same column using sepharose 4B, 6B in 0.1 M phosphate Buffer at various pH values, pH 7.4 to pH 10.0 Monomers of chicken cytochrome c oxidase were collected at pH 9.5 prior to activity measurement as well denaturalization for S.D.S. Zel electrophoresis (Alizai M.N. Thesis 1984)[31]

Sodium dodecyl sulfat (SDS) Gel electrophoresis:

S.D.S. polyacrylamide gels were prepared as described Fuller et al. (1981)[32]. 20-30 ug of cytochrome c oxidase denatured in 8M urea, 5% SDS, 2% 2. mercaptoethanol and 0.25 m Tris HCI, pH 6.2 for 30 minutes at room temperature. Samples were electrophoreses on slab gels (10 x 14 cm), stained and destined as described by Downer et al. (1976).[33] Channels were photographed or scanned at 560 nm on Gel-ford spectrophotometer with linear scanning attachment.

***Activity measurements**:*

Activity was measured polarographically by using oxygen electrode or spectrophotometrically using cytochrome c oxidase substrate of the oxidase. In oxygen electrode arylazide cytochrome c oxidase complex was assayed in Tween 80, 10 mM Sodium ascorbate and 1 mM TMPD (N.N.N.N-Tramethylphenylene diamin dihydrochloride). The concentration of cytochrome $_{c}$ in assay was 30 UM and that of cytochrome p oxidase, 100-125 nM. Fraction collected from the Sephacryl S200 column *were* assayed in 50 mM sodium sulphate, pH 7.4 containing 0.1% Lysophosphortidyl cholin or 0.5% Tween 80.

Analytical Ultra-Centrifugation:

Sedimentation velocity and sedimentation equilibrium analyses were performed on the Backman Spinco Model E analytical ultracentrifuge equipped with photo electric scanned. Double sector cells were used with an-D rotor and sample were scanned at 420 nm. Protein molecular weight was determined by the method of Tan-ford (1974).

Results and Discussion

Both oxidases (i.e.) Beef and chicken heart cytochrome g oxidase have been characterized by absorption spectra which are typical as reported by Yonetani (1960)[28] Fig. l show chicken cytochrome g oxidase spectra. The reduced *enzyme* has soret peak at 445 nm and clearly defined peak centered at 605 nm. The carbon monoxide adduct of the reduced enzyme is readily formed by passing gaseous CO through solution of the reduced enzyme and its spectrum, typical of cytochrome aa_3-Co is also given in the figure 1. The ratio of absorbance of reduced to the oxidised derivative in region (at 605 nm) is 2.1 for bovine enzyme and 1.8 for the chicken enzyme. These values may reflect intrinsic properties of oxidases or the presence of a fraction of the total haeme which is irreducible by dithionite as described by Gibson et al. (1965) for the bovine enzyme. Both enzymes isolated from chicken and beef heart has Copper and Iron with 1: 1.2 ratio. A long wave length absorption band central at approx. 830 nm was a feature of the oxidized spectra of chicken oxidase and may be presence of copper. Comparative study of bovine oxidase spectra produced in this laboratory and at a given by other authors in literature is given Table-I which is self-explanatory. Identical results for chicken cytochrome g oxidase were obtained.

Polypeptides composition of cytochrome c oxidase

The polypeptides composition of bovine and chicken cytochrome c oxidase revealed by SDS polyacrylamide gradient gel electrophoresis are shown in Fig.2. These results are in agreement with Ludwig (1979[3]', Wilson et al. 1980[17]). We have identified peptides with the highest staining intensity after gel electrophoresis and numbered the sub units as were given by Ludwig (1979). As is shown in Fig.2 and Table 2. Overall submit composition of these enzymes are similar. Both have three high molecular weight polypeptide (I-III) and group of low molecular weight polypeptides (IV-VII). Sub unit I migrates as a broad diffuse band in both cases. Sub units II-III and III of chicken and beef oxidase migrate close to each other. It is evident that there are number of polypeptides constituents (named a, b, c) in beef oxidase by Ludwig 1979. These are shown among the low molecular weight. Sub unit III of chicken oxidase is light in the gel (Fig.2 A) than other enzyme beef dimeric (Fig. 2 C) and beef monomeric (Fig. 2 B) oxidases.

Ultracentrifuge Studies:

Sedimentation coefficient of different cytochrome c oxidases at a protein concentration of approximately 10 mg/ml. in 0.1M Sodium phosphate buffer containing 1% tween 80. Bovine oxidase gave a value of 11S which has been assigned by Love et al. (1970) for dimeric *form* of enzyme. Our data for chicken oxidase is somewhat different at physiological pH it shows 9S + 5S value which indicate in chicken oxidase monomers are present though sub units composition is the same. We have also found lower S value

at higher pH of the enzyme which confirm the fact that at higher pH, dimers dessociate to Monomers and Steady State Kinetic indicate that monomers are catalytically more active than dimers. Though in both cases, enzymes have two copper and two haems.

Steady State Measurements:

Cytochrome c oxidases were assayed polargraphically in both high and low ionic strength buffer using horse heart cytochrome c as substrate. Steady State Kinetics results are presented in Table 3 in terms of turn over numbers (i.e.) micromoles of cytochrome c per micro moles of oxidase per second. Comparative study showed that activity as turn over number at different pH of the enzymes are different. Obviously monomres showed more active value compare to the cytochrome $_c$ oxidase at physiological pH. These findings are very much in agreement with those of the other authors (37, 38) reported in literature. Carbon monixide binding of oxidase indicated that chicken oxidase at pH 10.5 and above b[i]rds twice the amount of carbon monoxide compare to physiological pH of the enzyme Fig. 3(A). Purified monomers binds same carbon monoxide units as enzyme of physiological pH (Fig. 38). These proves the fact that part of protein denature during alkalination of the enzyme and that denatured protein too binds carbon monoxide showing excess bindings of carbon monoxides.

The importance of dimer in cytochrome oxidase activity has been suggested in-directly by several studies and has been speculated upon in terms of control mechanism of electron transfer (39, 40, 41, 42). We have first to in isolated enzyme from avian species and comparative study was conducted with beef heart cytochrome oxidase as dimer and monomers purified through gel filtration.

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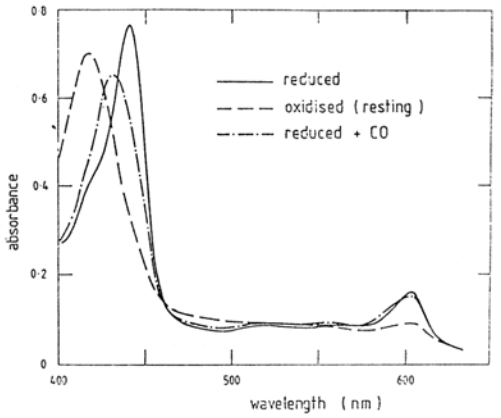

Fig. 1: Spectra of derivatives of chicken cytochrom c oxidase. Prepared oxidase was dissolved in 0,1 M sodium phosphate buffer pH 7.4 containing 1% Tween 80 at 20°C.

-- Oxidised oxidase
-- dithionite reduced
-- dithionite reduced plus carbonmonoxide

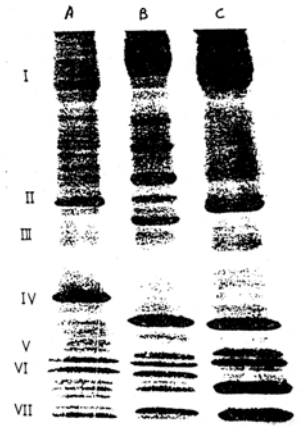

Fig. 2: Polyacrylamide gradient gel electrophoresis of cytochrome c oxdiase from chicken, beef and beef monomer.

Oxidases were run on the same gel (10-18% polyacrylamide gradient)

A- Chicken cytochrome c oxidase.
B- Bovine monomeric cytchrome oxidase
C- Bovine cytochrome c oxidase

Roman numerical indicate the position of cyctochrome c oxidase sub unit on the gel.

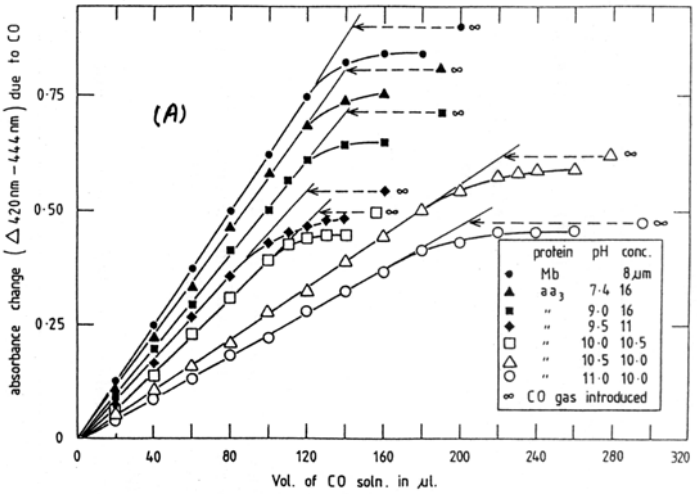

Fig. 3A: Absorption changes ΔA(420 - 444 nm) were ploted against the volume of carbonmonoxide solution. The spectral change were noted from the difference spectra (reduced carbonmonoxide minus reduced chicken oxidase at pH 7.4, ph 9.0, pH 9.5, pH 10.0, pH 10.5, pH 11.0. Figure shows the concentration of oxidase and at end saturation of reduced chicken oxidase with carbonmonoxide gas.

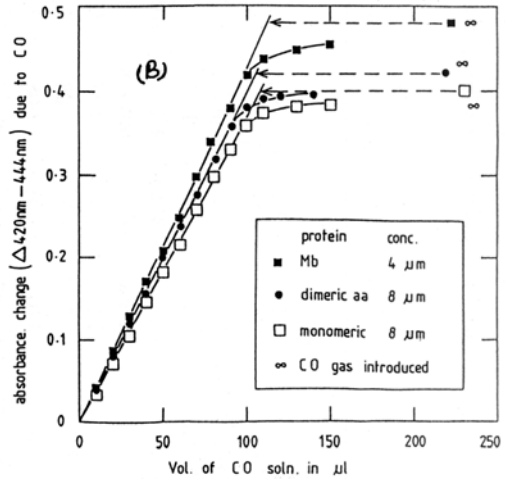

Fig. 3B: Absorption change ΔA (420-444 nm) due to carbonmonoxide were plotted against the volume of the carbonmonoxide solution in µLS. Figure is shown for dimeric chicken oxidase and monomeric form of chicken oxidase at the end is saturation with CO gas.

Table 1
Bovine Oxidase

Spectras	pH 7.4	pH 9.5	pH 10.0	pH 10.5	pH 11	Gel Chromatography purified cyt. c oxidase		E. Stotz (1970)		Lemberg (1965)
						Dimers	Mono-mers	Dimers	Mono-mers	
$\gamma Fe^{+3}/\alpha Fe^{+3}$	9	5.6	6.2	10	5.9	6.6	6.7	-	-	-
$\gamma Fe^{+2}/\alpha Fe^{+2}$	5	4.18	4.46	6.62	3.9	4.3	4.5	4.84	3.48	-
γFe^{+2}-CO/αFe^{+2}-CO	4.46	3.9	4.6	6.71	4.8	3.93	4.4	-	-	-
$\gamma Fe^{+2}/\gamma Fe^{+2}$-Co	1.2	1.09	1.04	1.12	0.89	1.16	1.07	-	-	-
γFe^{+2}-CO	430 nm	430 nm	430 nm	420 nm	420 nm	430 nm	428 nm	430 nm	431 nm	434 nm
αFe^{+2}-CO	605 nm	605 nm	605 nm	604 nm	604 nm	605 nm	605 nm	605 nm	604 nm	604 nm
$\gamma Fe^{+2}/\alpha Fe^{+3}$	1.11	1.03	0.77	0.75	0.66	1.04	0.96	1.25	1.09	-
$\gamma Fe^{+2}/\alpha Fe^{+3}$	2	1.39	1.08	1.14	1.13	1.6	1.4	-	-	-
$\gamma Fe^{+2}/\alpha Fe^{+2}$-CO	1.07	1.03	1.08	1.14	1.2	1.06	1.04	-	-	-
Shoulder at 593 nm	visible	visible	visible	lost	lost	visible	visible	visible	lost	lost

Table 2
Apparent molecular weight of the sub unit of Cytochrome Oxidase (Bovine and Chicken) using different methods

Sub unit	Chicken Capaldi, R.A. procedure 1977	Bovine Capaldi, R.A. procedure 1977	Chicken Weber and Osborn method 1969	Bovine Weber & Osborn method 1969	Chicken Gradient gel V. Darley-Usmar (1980)	Bovine Gradient gel V. Darley-Usmar (1980)
I	38,100	35,300	36,500	34,100	37,920	35,650
II	22,700	22,500	25,400	24,100	21,800	22,500
III	21,500	21,300	21,700	21,000	21,350	21,100
IV	16,500	16,600	17,200	15,300	16,500	16,900
V	11,500	11,700	12,500	12,000	11,400	11,700
VI	8,700	7,800	8,200	6,100	8,970	7,600
VII	4,800	4,400	4,300	3,800	4,830	4,500
Total	123,800	119,600	125,800	116,400	122,970	120,000

Comparative molecular weight of sub units of chicken cytochrome c oxidase and bovine oxidase applied to different methods of gel electrophoresis.

Table 3
Bovine Oxidase

pH	K_m	V_{max} (μM cyt. c/sec)	T.N. (μM cyt. c/μM oxi/ec^{-1})
7.4	19.2	6.75×10^{-3}	22.5 sec^{-1}
9.0	13.1	4.89×10^{-3}	16.3 sec^{-1}
9.5	10	4.26×10^{-3}	14.2 sec^{-1}
9.7	22.7	6.81×10^{-3}	22.7 sec^{-1}
10.0	62.5	12×10^{-3}	40.3 sec^{-1}
10.5	20.8	2.50×10^{-3}	8.3 sec^{-1}
10.8	19.1	1.47×10^{-3}	4.9 sec^{-1}
oxidase			
dimeric	11.36	5.1×10^{-3}	17.0 sec^{-1}
monomeric	19.23	8.2×10^{-3}	27.55 sec^{-1}

Chicken Oxidase

pH	K_m	V_{max} (μM cyt. c/sec)	T.N. (μM cyt. c/μM oxi/sec^{-1})
7.4	40	3.9×10^{-3}	13
8.9	27	2.25×10^{-3}	7.5
9.2	70	3.63×10^{-3}	12.0
9.5	50	6.64×10^{-3}	22.1
9.9	62.5	12.25×10^{-3}	40.8
10.3	30	3.15×10^{-3}	10.5
10.5	20	2.1×10^{-3}	7
11.0	20	0.66×10^{-3}	2.2
dimeric oxidase	32	3.9×10^{-3}	13
monomeric oxidase	58	4.08×10^{-3}	23.6

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