Pakistan Journal of Pharmacology Vol.19,
No.2, July 2002, pp.39-45

OXIDATION OF FERROCYTOCHROM C BY CHICKEN HEART MITOCHONDRIA CYTOCHROME C OXIDASE

MUHAMMAD NISAR ALIZAI
P.C. S.I. R. Laboratories, Jamrud Road
P.O. Peshawar University 25120, Pakistan

ABSTRACT

Cytochrome c oxidase from a number of eukaryotic organisms have been isolated and thoroughly investigated in various laboratories. Chicken cytochrome c oxidase from heart mitochondria was isolated and study was conducted in view of its structural and functional properties. Spectral properties of chicken oxidase were found to be similar to that of bovin oxidase as standared. Both oxidases posses Cu and Fe atoms. Sedimentation equilibrium (S) values indicated chicken heart cytochrom C oxidase as 9+5s and beef oxidase as 11 S in dimeric form at physiological pH. Polypeptide composition of monomers and dimmers are identical.

INTRODUCTION

Cytochrom c oxidase, the terminal component of the respiratory chain in aerobic organism is one of the most extensively investigated enzyme. It is found in animals, yeast, algae and some of the bacteria and is responsible for catalyzing the reduction of oxygen to water.

The electrons are provided by reduction cytochrom c in the following over all reaction.

4 cytochrom $C^{+2} + 0_2 + 4H+$
=-------4 cytochrom C^{+3} $2H_20$

The free energy developed in oxygen reduction is used to promote oxidative phosphorylation and consequently becomes available as ATP to satisfy the energy requirement of the cell (Alizai *et al.,* 1990). In general cytochrome c oxidase from any species can be isolated from mitochondria or mitochondrial fragments by initial extraction of protein with surface-active agent followed by removal of contaminating detergent and protein. The common method used reported by Yonetani (1960) and other modified procedure were also cited in Literature (Capaldi *et al.,* 1982). The procedure differs in nature and amount of the detergent used.

The preparations obtained by different methods vary widely in activity. There is good reason to believe that oxidases isolated by many procedures are similar but not identical in relation to chemical and physical properties. All oxidases preparation contain a considerable amount of lipids mostly phospholipids. In most cases the phospholipids content is close to 20% but some methods remove the major part of the lipids during isolation of cytochrom c oxidase which show less activity (Gorden *et al.,* 1974). Data reported for chicken oxidase in this paper is similar if not identical to that already published for other Eukaryotic species (Georgevic *et al.,* 1983).

MATERIALS AND METHODS

Preparation of cytocbrome c oxidase

Beef and Chicken hearts cytochrome c oxidase were prepared by Capaldi procedure (1972) modified by Alizai M.N. (1998), to get purified cytochrome c oxidase. Ammonium sulphate percentage altered to get an active and more pure chicken oxidase which had haem a to protein ratio approximately 9 to 10 nmole of haema/mg of protein. Protein was determined by lowery method (1951). Analysis of the purified enzyme had shown that it contain two types of haem as haem a and a_3 despite the fact that only one particular

heam with unique structure haem a can be isolated from acetone treatment (Alizai M.N., 1990). Metal analysis of the chicken oxidase indicated that iron to copper ratio is in unity. It is widely reported/believed that both copper and Iron are essential components of cytochrome c oxidase. EPR of chicken oxidase showed that only half of copper is detectable called CuB which is coupled to haem of cytochrome a_3 resulting in less signals of EPR from both copper and Iron like beef oxidase as reported by Hayshi *et al.* (1977).

Determination of triton X-100 binding to Cytochrome

Gel filtration Technique was used for the determination of chicken oxidase molecular wt. A column (1*25 cm) containing Sephacryl S200 was used. Samples of cytochrom c oxidase (1-2 mg) were loaded onto the column equilibrated with 0.1% Triton. X-100, 200mM NaCl and 10mM Tris Hcl Buffer at pH8.5 or pH7.4. Fractions were collected from column and their absorbance was measured at 280nm and 420nm on a Backman Du 7 spectrophotometer.

Gel filtration was also done using from same column of sepharose 4B, 6B in 0.1M phosphate Buffer at various pH values pH 7.4 to pH 10.0. Monomers of chicken cytochrom c oxidase were collected at pH 9.5 prior to activity measurement and then denaturalized for S.D.S gel electrophoresis (Alizai M. Nisar, 1984).

Determination of molecular weight by S.D.S

S.D.S polyacrylamide gels were prepared as described by Fuller *et al* (1981). Twenty to thirty micro grams of cytochrom c oxidase denatured in 8M urea, 5% SDS, 2% 2. mercaptoethanol and 0.25 M Tris Hel, pH 6.2 for 30 minutes at room temperature. Samples were electrophoresed on slab gels (10 * 14 cm), stained and destained as described by Downer *et al* (1976). Channels were photographed or scanned at 560nm on Gelford spectrophotometer with linear scanning attachment.

Activity measurements

Activity of the enzyme was measured polarographically by using oxygen electrode or spectrophotometrically using eytochrom substrate of the oxidase. In oxygen electrode arylazide cytochrom c-minus cytochrom c oxidase complex was assayed in Tween 80, 10 mM Sodium ascorbate and 1mM TMPD (N.N.N.N — tetramethylphenylene diamin dihydrochloride). The concentration of cytochrom c as substrate in assay was 30 micro molar and that of cytochrom oxidase as enzyme was approximately 100-125um.

Fraction collected from the Sephacryl S200 column were assayed in 50 milimolar sodium sulphate, pH 7.4 containing 0.1% Lysophosphotidyl cholin or 0.5% Tween 80.

Ultra-Centrifuge

Ultra-Centrifuge was used to determine the value of the enzyme. Sedimentation velocity and sedimentation equilibrium analyses were performed on the Backman Spnco Model E analytical ultracentrifuge equipped with photoelectric scanner. Double sector cells were used with an-D rotor and samples were scanned at 420 nm. Protein molecular weight was determined by transparent method of Tanford (1974).

RESULTS AND DISCUSSION

Both oxidases i.e. Beef and Chicken heart c oxidases have been characterized by absorption spectra. Fig. 1(A) shows chicken cytochrom c oxidase spectra. The reduced enzyme has soret peak at 445 nm and also clearly defined oxidized centered at 605nm. 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 cytochrom aa_3 —CO is also given in the figure 1(A). The ratio of absorbance of reduced to the oxidased derivative in region (at 605nm) 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 fraction of the total haem which is irreducible

Table
pH dependent S value of bovine and chicken cytocrome c oxidases

	pH 7.4	pH 8.5	pH 9.0	pH 9.5	pH 10	pH 10.5	pH 11.0
Experimental I							
S value of beef oxidase	11.0 S	-	7.6 S	7.0S	6.5 S	5.6 S	3.8S
S value of chicken oxidase	9.0 S	-	7.0 S	5.5S	5.0 S	-	3.6S
Experimental H							
S value of beef oxidase	1.0 S	8.2S	6.8 S	6.0S	5.6 S	4.9 S	3.8S
S value of chicken oxidase	9.0+5.2S	8.4S	-	5.5S	5.0 S	5.0 S	3.6S
Experimental III							
S value of beef oxidase	11.0 S	8.8S	7.6 S	7.0S	5.9 S	5.0S	3.9S
S value of chicken oxidase	9.0 S	8.1S	7.0 S	-	5.6 S	5.0 S	-
Average for beef oxidase	11.0 S	8.5S	7.33 S	7.0S	6.0 S	5.2 S	3.8S
Average for Chicken oxidase	9.0+5.2S	8.25S	7.0 S	5.5S	5.2 S	5.0 S	3.6S

Abbreviation: S means sedimentation coefficient value.

by dithionite as described by Gibson *et al* (1965) for the bovine enzyme. The spectra of chicken mitochome is given in Fig. 1(B). Both enzymes isolated from chicken and beef hearts have copper and Iron with 1: 1.2 ratio. A long wavelength absorption band entered at approx. 830 nm was feature of the oxidized spectra of chicken oxidase and showed the presence of copper.

Polypeptides composition of cytochrom c oxidase

The polypeptides composition of bovine and chicken cytochrom c oxidase revealed by SDS polyacuylamid gradient gel electrophoresis are shown in Figure 2.

These results are in agreement with Ludwig *et al* (1979) and with our own data for other species (Darley Usmar *et al* (1981), (Wilson *et a!* 1980). We have identified peptides with the highest staining intensity after gel electrophresis and numbered the subunits as were given by Ludwig *et al* (1979). Overall subunit composition of these enzymes are similar as evident from fig-2A. Both oxidases have three high molecular weight polypeptides (I-III) and group of low molecular weight polypeptides (IV-VII). Subunits I migrates as a broad diffuse band in both cases. Subunits 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 reported by Ludwig 1979. These are shown among the low molecular weight. Subunit III of chicken oxidase is light in the gel (Fig.2-A) than other enzyme beef dimeric (Fig.2-B).

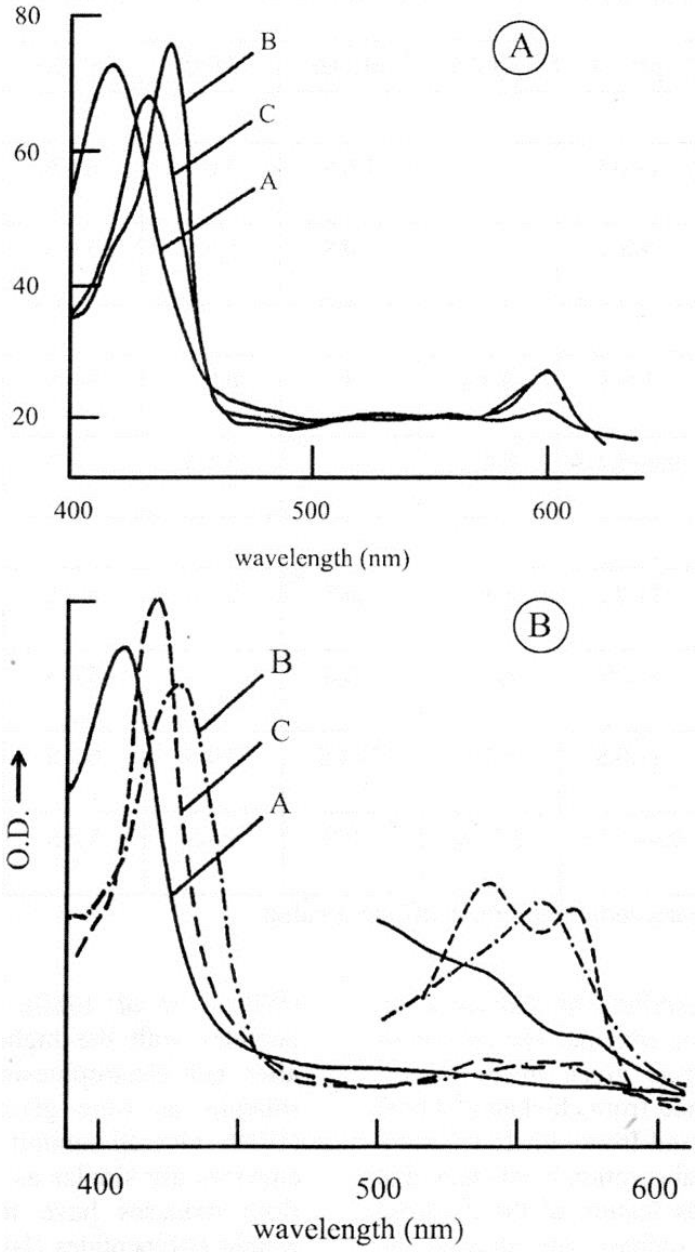

Figure 1(A): Spectra of chicken cytochrom c oxidase in 0.1 M sodium Phosphate buffer pH 7.4 containing 1% Tween 80.
(A) Oxidised spectrum (B) Sodium dithionite reduced spectrum (c) Sodium dithionite reduced plus carbonmonooxide.

Figure 1(B): Spectra of chicken mitochrom, purified through sepharose 4B gel. Chicken heart mitochrom was in 0.1 M sodium phosphate buffer pH 7.4 containing 1% Tween 80.
(A) Oxidised spectrum (B) Sodium dithionite reduced spectrum (C) Sodium diathionite reduced plus carbonmonoxide.
All spectra were taken at 20°C using Parkin Elmer 575 spectrophotometer with memorise baseline.

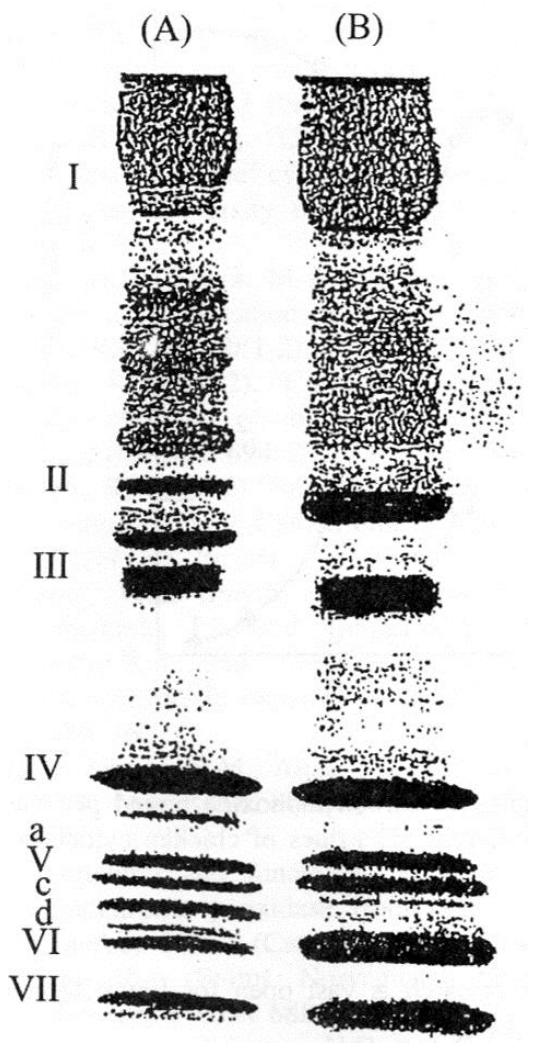

Figure 2: Polyacrylamide gradient gel electrophoresis of cytochrom c oxidase from chicken and beef hearts. Oxidases were run on the same gel (10-18%) polyacrylamide gradient).
(A) Chicken cytochrom c oxidase (B) Beef cytochrom c Rman numericals indicate the position of the subunits.

Most of the Bovine oxidase preparation reported in literature contain approximately 11 nm of haem per mg of protein. This corresponds to minimum molecular weight of approx. 90000 dalton or interms of functional unit contain two haems group and molecular weight 180000 dalton. This is considerably proportional to molecular weight detected by polypeptides determination through poly- acrylamide gel electrophoresis (Alizai M.N. 1984, 1998).

Ultracentrifuge Studies

Sedimentation coefficient of different cytochrom c oxidases at a protein concentration of approximately 10 mg/mL in 0.IM 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 from of enzyme. Our data for chicken oxidase is some what different at physiolgical pH it shows 9S+5 S value which indicates that in chicken oxidase monomers are also present though subunits composition is the same (Table). We have also found lower S value at higher pH of the enzyme, which confirm the fact that at higher pH, dimmers dissociate to monomers. Steady state kinetic indicates that monomers are catalytically more active than dimmers. Though in both cases, enzymes have two copper and two haems.

Steady State Measurements

Cytochrom c oxidases were assayed polargraphically in both high and low ionic strength buffer using horse heart cytochrom c as substrate. Steady state kinetics results are presented in terms of turn over number (i.e.) micromoles of cytochrom c per micromoles of oxidase per second. Comparative study showed that activity as turn over number at different pH of the enzymes are different. Obviously monomers showed more active value compare to the cytochrom c oxidase at pH 7.4 (Fig.3). These findings are very much in agreement with those of the other authors (Capaldi *et al.,* 1982) reported in literature. Carbon Monoxide binding of oxidase indicated that chicken oxidase at pH 10.5 and above binds twice the amount of carbon monoxide compare to physiological pH of the enzyme. Purified monomers binds same carbon monoxide units as enzyme of physiological pH. This proves the fact that part of protein denature of during alkalination of the enzyme and that denatured protein too binds carbon monoxide showing excess bindings of carbon monoxides. Computerized

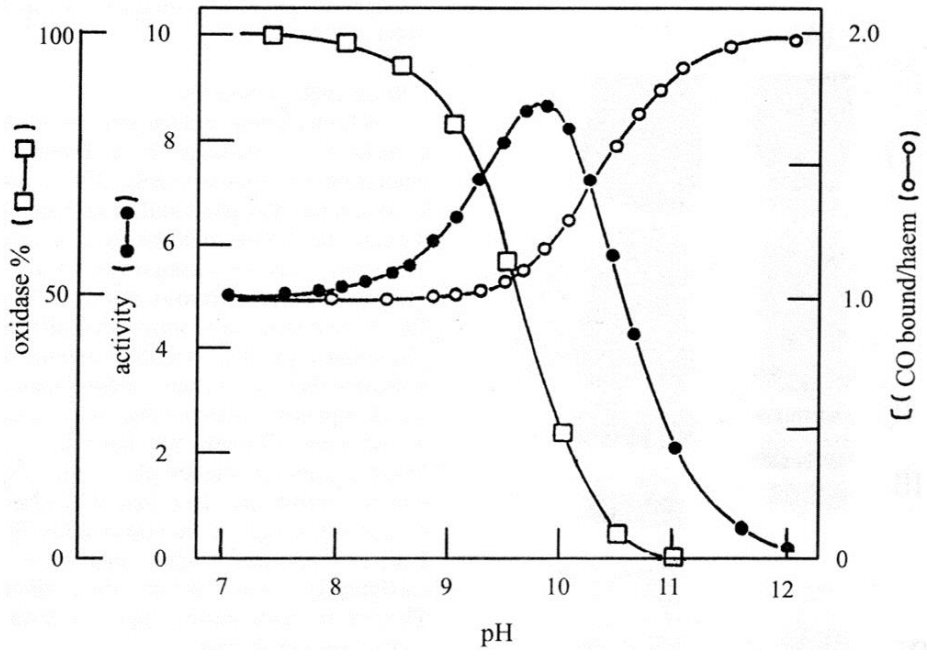

Figure 3: Figure constructed from computerized results for carbon monoxide bound per haem, catalytic activity and percent of oxidase present at different pH values of chicken cytochrom c oxidase.

data of the oxidase percentage, catalytic activity and carbon monoxide bound per haem is shown in Figure 3.

CONCLUSION

The importance of dimmer in cytochrom c oxidase activity has been suggested indirectly by several studies and has been speculated upon in terms of control mechanism of electron transfer (Tanaka *et al* 1979, Whart *et al* 1976). We had isolated enzyme from avian species and comparative study was conducted with beef heart cytochrom c oxidase as standard and chicken oxidase as dimmer and monomers were purified through gel filtration. Further investigation related to polypeptides composition, catalytic activity and carbon monoxide binding can be carried out on cytochrom c oxidase from other species. Area of research is vast open for future research workers.

ACKNOWLEDGEMENT

Author is grateful to Professor Dr. M.T. Wilson Department of Chemistry and Biological sciences university of ESSEX England (U.K.), and Dr. Winston L. Marajh, post doctor fellow, Colchester ESSEX for their constructive, discussion and suggestion during this research work.

REFERENCES

Al Ayash, A. and M.T. Wilson (1977). Structural and functional properties of oxidases *Biochemistry.* **56**B, 147.

Alizai M. N. and Wilson M. T. (1990). Structural enzymatic Properties of beef and chicken cytochrome c oxidases". *M. J. Sci. and Ind. Res.* **33**: 10.

Alizai M.N. and Wilson M.T. (1993). "pH dependent properties of cytochrome c oxidases" *Pak. J. Biochemistry* **27**: 103.

Alizai M.N. (1984). "Comparative study of chicken and beef cytochrome c oxidases. Thesis University of ESSEX, England U.K."

Alizai M.N, Wilson M.T. (1998). Isolation and characterization of chicken oxidase. *Pak. Jr. Res.* **50**(1-2).

Capaldi, R.A (1982). "Comparative study of Cytoclirome c oxidase". *Biochem. Biopliys. Acta* **694**: 291.

Capaldi, R.A (1972) "Structural and sub unit composition of Cytoclirome c oxidase" *FEBS Lett.* **26**: 261.

Coughy, W.S. Smyth, G.A Keefee, D.H. Maskasky J.E. and Smith M. (1975). "The sub unit composition of beef cytochrome c oxidase" *J. Biol Chem.* **250**: 7602.

Darley Usmar, V.M., Aiizai M.N. Al Ayash A.I., Jones G.D., Sharpe, A. and Wilson M.T. (1981). "A comparison of the structural and functional properties of cytochrome c oxidase from beef (Bos Tauros) carnal (Camelus Dromedairus) and Rat (Rattus Norvegicus) *Comp. Biochem. Physioi* **68B**: 445 (U.K.)

Downer N.W., Robinson N.C. and Capaldi, R.A. (1976). "Characterization of Seventh subunit of Beef heart cytochrome c oxidase".

Draskin D.L. (1954). Physiological Review "Cytoclirome c oxidase" . **31**, 345 U.S.A.

Fuller, S.D., Darley Usmar V.M. and Capldi, R.A. (1981). Studies on Cytoclirome c oxidase interaction and binding" *Biochemistry.* **28**: 2930.

Georgevic, G., Darley Usmar V.M., Maltasts F. and Capaldi R.A. (1983). "Electron Transfer in monomeric forms of beef and shark heart cytochrome c oxidase" *Biochemistry.* **22**(6): 1317.

Gibson Q.H., Palmer G. and Wharton D.C. (1965) "The binding of Carbon monoxide by cytochrome c oxidase and ratio of cytochrome a and a_3". *J. Biol. Chem.* **240**: 11915.

Gorden M.S., Reynold J.A. (1974). Cytoclirome c oxidase and functional units" *J. Proc. Nat. Acad. Sci.* U.S.A. **71**: 3913.

Hayashi Q., Chance B. and Nicholls P. (1977). "Cytoclirome oxidase" Academic Press New York U.S.A., 479.

Lemberg. P. (1969). "Physiol Review". 49.

Lemberg. K and Barret. I. (1973). "Cytocbromes" Academic Press, U.K

Lowery 0.H., Rose-Brough N.J., Fan A.L. and Randall R.J. (1951). "Preparation purification and determination of protein". *Biol Chem.* **193**: 6328.

Ludwig B., Downer N.W. and Capaldi, K.A. (1979). `"labelling of Cytocbrome c oxidases Orientation of election Transfer complex in the inner mitochondrial membrane". *Biochemistry.* **18**: 1401.

Tanford C. Nazaki Y., Reynold, J.A. and Mikiuos S. (1974). "Cytocbrome c oxidase characterization and binding." *Biochemistry.* **13**: 2369.

Tanaka M., Haniu M., Yasunobu KT., Yu, C.A.Yu, L. Yau Huei W. and King T.E. (1979). "Amino acid sequence of sub unit V of Bovine heart cytocbrome e oxidase" *J. Biol. Chem.* **254**: 3879.

Wharton D.C. and Gibson Q.H. (1976). "Stoiciometery of Carbon monoxide binding to cytocbrome c oxidase".

Wilson M.T., Lalamarajl W., Darley Usm V.M., Bonuventura M. and Brunori M. (1980). "Structural and functional properties of cytochrome c oxidase isolated from sharks." *J. Biochem.* **255**: 272.

Yonetani T. (1960). "Studies oncytochrome c oxidase absolute and difference spectra" *J. Biol. Chem.* **235**: 845.

Yonetani T. (1961). "Studies on cytochrome c oxidase Improved preparation and some properties." *J. Biol. Chem.* **236**: 1680.