Biokemistri Vol. 9, No. 2, December, 1999
Printed in Nigeria

0795-8080/99 $3.00 + 0.00

BKR 9939/9208

STUDIES ON CYTOCHROME c: CYTOCHROME c OXIDASE COMPLEX

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(Received March 31, 1999)

ABSTRACT: In this study, an attempt has been made to determine the stoichiometry of the electrostatic complex between horse heart ferricytochrome c and cytochrome c oxidase using gel filtration chromatography. The conditions under which this complex may be formed or dissociated were studied. The oxidase was, however, first isolated and purified from bovine heart according to the method of Yonetani (1961).

Being the terminal enzyme in the electron transport chain, cytochrome c oxidase accepts four electrons from the single electron donor, ferrocytochrome c, to effect the four electron reduction of dioxygen to water in a catalytic cycle. A requisite step for the proton translocation and mitochondrial energy conservation carried out by this enzyme is, therefore, the formation of a complex (if only transient under some conditions) between the two proteins, that is competent to transfer electrons.

The experiments reported in this study indicate that under conditions of low ionic strength, the enzyme formed a tight 1:1 electrostatic complex with cytochrome c (Kd ~ 10 nM) (i.e. one molecule of cytochrome c per functional unit of cytochrome c oxidase). This contrasts with the findings of other workers that each functional unit of cytochrome c oxidase contains two binding sites for cytochrome c.

The electrostatic complex between cytochrome c and oxidase is a tight and stable one over a fairly wide pH range, at low ionic strength (< 20 mM) which is far lower than that which is reported as physiological (~ 100 mM). It may be that extremely small local variations in ionic strength, in the area of the binding sites on one or both proteins, brought about for example during proton translocation by cytochrome c oxidase, or exclusion of surface water molecules during "docking" may have dramatic effects *in vivo* on the affinity of cytochrome c for cytochrome c oxidase.

Key Words: Cytochrome c; Cytochrome c oxidase; Electron transport chain; Electrostatic complex.

INTRODUCTION

Protein-protein interaction play a critical role in biological signal and energy transducing systems. In the terminal reaction of respiratory electron transfer, for example, four electrons are transferred to the redox centres (Cu_A, cytochromes a, a_3 and Cu_B) of the membrane-bound cytochrome c oxidase (where they are used to reduce molecular oxygen) via the transient formation of protein-protein complex between the nzyme and the soluble protein

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cytochrome c (Lynch and Copeland, 1992). The interaction between the two proteins is largely electrostatic (Michel and Bosshard, 1984; Hildebrandt and Stockburger, 1989; Bushnell *et al.*, 1990; Voet and Voet, 1995). The binding of cytochrome c to cytochrome c oxidase, which precedes electron transfer, occurs most likely in the vicinity of the Cu centre (Hildebrandt *et al.*, 1993; Gennis and Ferguson-Miller, 1995). The binding domain is constituted by negatively charged amino acid side chains (Blenkinsop, 1993; Hildebrandt *et al.*, 1993) so that an electrostatic complex is formed with cytochrome c via electrostatic attractions with the positively charged lysyl residues around the exposed haem crevice (Bushnell *et al.*, 1990; Lynch and Copeland, 1992; Voet and Voet, 1995).

Each functional unit of cytochrome c oxidase has been reported to contain two binding sites for cytochrome c (Ferguson-Miller *et al.*, 1976; 1978), high and low affinity sites, with approximate dissociation constants of 10^{-8} M and 10^{-6} M respectively. The binding of both the oxidised and reduced forms of cytochrome c to the oxidase are reported to have almost identical dissociation constants (Capaldu *et al.*, 1982). Under conditions of low ionic strength, the enzyme is believed to form a tight 1:1 electrostatic complex with cytochrome c. It is generally agreed that this complex involves the high affinity binding site for cytochrome c, located on subunit II and in close proximity to the Cu centre (Briggs and Capaldi, 1978).

This was, however, disputed by Azzi and Muller (1990). The existence of a second, low affinity, binding site for cytochrome c on oxidase, is still the subject of much controversy. The biphasic steady state kinetics of the enzyme observed at low ionic strength has at times been interpreted as due to the negative cooperativity between the two binding sites on the oxidase molecule with differing affinities for ferrocytochrome c (Ferguson-Miller *et al.*, 1978; Garber and Margoliash, 1990). However, Antalis and Palmer (1982) have also proposed that this biphasic kinetic may be as a result of steady state oscillation in the enzyme with a single binding site for ferrocytochrome c.

The aims of this study therefore, are:

1. To establish the conditions under which the electrostatic complex may be formed and dissociated.

2. To determine the stoichiometry of electrostatic complex formation between cytochrome c and cytochrome c oxidase. This may give further insight as to whether a second binding site for cytochrome c on cytochrome c oxidase exist.

3. To determine the possible variation of stoichiometry with pH.

MATERIALS AND METHODS

Isolation and purification of bovine heart cytochrome c. oxidase (Yonetani, 1961):

Fresh beef heart was purchased from a nearby abbattoir, Barbars of Cock's Clark, Chemsford, U.K. and transported to the laboratory in ice. The bovine hearts, together with all reagents and buffers used, which were usually prepared fresh, were kept at 4°C throughout the preparation. All chemicals and reagents used were of analytical grades except cholic acid (sodium salt) which was purchased from Sigma. Absorbance readings and spectra were determined at 20°C using Philips PU 8730 UV/Visible scanning spectrophotometer interfaced with a video monitor and recorder. All centrifugations were carried out at 4°C using either a Mistral 6000 centrifuge (1750 g), or a Sorvall RS28C ultra-centrifuge fitted with a GSA rotor arm (17500 g).

Buffers

Sodium phosphate buffers were made from a mixture of sodium salts to give desired pH and concentrations. Where necessary, pHs were adjusted with few aliquots of either

ammonium hydroxide or acetic acid solutions. All buffers were stored at 4°C prior to use. Measurements of pH were taken on a Philips PW9409 digital pH meter.

Fresh bovine hearts were cut into 1 cm3 portions while removing as much fat and connective tissue as possible in the process. The cubes were minced with ice and then washed with cold water in a muslin bag so as to remove the blood; the mixture was then blended with ice in 20mM phosphate buffer (pH 7.4) for six minutes in bursts of three minutes (approximately 1 litre of buffer to 1 kg of beef heart). The suspension was centrifuged at 3000 rpm for twenty minutes. The precipitate was resuspended in the cold buffer (ratio 1:4 v/v) and reblended for three minutes and centrifuged as above. The resulting supernatants were then pooled and the precipitate discarded. Acetic acid (1 M) was used to adjust the pH of the combined supernatants to 5.5 and was then stirred for thirty minutes to allow the mitochondria and submitochondria particles precipitate. The resulting mixture was centrifuged at 3000 rpm for twenty minutes. The supernatant was discarded while the precipitate which contained mitochondrial fractions was resuspended in cold distilled water at 4°C and centrifuged at 3000 rpm for twenty minutes.

This was repeated if necessary so as to wash the mitochondrial particles. The clean mitochondrial particles were recovered and resuspended in 20 mM phosphate buffer containing 20% sodium cholate. The solution was left overnight at 4°C in the cold room with slow stirring so as to disaggregate mitochondrial proteins from the membrane.

Solid ammonium sulphate was added to the above solution to give 25% saturation (144 g per litre of solution) and the pH of the resulting solution was adjusted to 7.4 using aqueous ammonia and stirred for thirty minutes. Solid ammonium sulphate (AMS) was further added to bring the solution to 35% saturation. This was allowed to sit in ice for twenty minutes and then centrifuged at 13,000 rpm for thirty minutes. The precipitate which contained mitochondrial membrane debris was discarded and the supernatant kept. The oxidised and dithione reduced absorption spectra between 400 and 600 nm of aliquots portion of the supernatant were taken to verify the spectral composition of the sample. The occurrence of absorption peaks at 445 and 605 nm confirmed the presence of cytochrome c oxidase in the reduced form in the supernatant (Yonetani, 1961).

The cycle was repeated in order to obtain as pure an oxidase preparation as possible as judged by optical spectroscopy. An indication of the oxidase purity can be obtained from the ratio of the absorbance at 440 and 420 nm for the sodium dithionite reduced enzyme. The absorbance ratio should be greater than 2.2, although a ratio of 2.0 is acceptable (Yonetani, 1961). The final, purified enzyme, was dissolved in the minimum amount of 20 nM sodium phosphate buffer pH 7.4 containing 0.1% w/v Tween 80 and stored in liquid nitrogen at -70°C until required.

Determination of enzyme concentration:

To obtain the final concentration of the enzyme preparation an aliquot of the preparation was diluted 100 fold in a cuvette with 20 mM sodium phosphate buffer pH 7.4 containing 2% w/v sodium cholate. A reduced minus oxidised spectrum was taken between 400 and 650 nm wavelengths. The difference in absorbance between oxidised and dithionite reduce enzyme at 605 nm was recorded and concentration determined using the difference in extinction coefficient of 11 $mM^{-1}cm^{-1}$ (Abubakar, 1994), taking into consideration the dilution factor. This extinction coefficient yields the concentration of haem a present. The enzyme concentration is half the haem concentration.

Spectra of cytochrome c oxidase

The spectra of the enzyme was taken by adding a 30 uL aliquot of the purified enzyme to 1ml of 20 mM phosphate buffer pH 7.4 containing 2% cholate (10 µM total haem, final). The reduced spectrum was taken by reducing the enzyme with a few grains of sodium dithionite. The CO spectrum was taken by bubbling 10 ml of 1 atmosphere CO through a sodium dithionite reduced enzyme in the reaction cuvette.

Determination of the stoichiometry of electrostatic complex formation using cell filtration chromatography

Gel filtration chromatography using sephadex G50, (separation range 1,500-30,000 Da), was used to investigate the stoichiometry of electrostatic complex formation between horse heart cytochrome c and bovine, dimeric cytochrome c oxidase. Experiments were carried out over a range of pH values, between 7.5 and 10, which would encompass the alkaline transition of cytochrome c, but would not cause an appreciable loss of activity of cytochrome c oxidase.

Sephadex G50 was swollen and de-aerated by heating on a steam bath in low ionic strength buffer for two hours, allowed to cool to room temperature, and then packed into a column. A column (1.5 x 42 cm) was found to separate adequately the complex (which would be excluded from the gel matrix and emerge at the void volume) from the uncomplexed cytochrome c (which would enter the gel matrix and be retarded, thus emerging after the complex). The column was equilibrated with several column volumes of low ionic strength (10 mM Tris/acetate/0.3% Tween-80) buffer, pH 7.5.

The concentration of cytochrome c oxidase was determined as total haem after it was dialysed against the eluant buffer. A 1 ml portion, containing - 30 uM enzyme was mixed with a 3-4 fold excess of horse heart cytochrome c. This was calculated based on the assumption that 1:1 complex formation involved the binding of one molecule of cytochrome c to a functional unit of oxidase, containing half the total haem concentration of the native, dimeric enzyme. The sample was allowed to stand for five minutes at room temperature, before being loaded in to the column and eluted with the same low ionic strength buffer used to equilibrate the column.

One (1) ml fractions were collected and the protein located by measuring the absorbance of each fraction at 280 nm (due to aromatic amino acid residues in the proteins). Fractions with the highest absorbance at 280 nm were reduced with a few grains of sodium dithionite, and the spectrum recorded between 500 and 630 nm to determine whether the fraction contained the: oxidase only (peak at 605 nm), complex of the two (peaks at 550 and 605 nm) or cytochrome c only (peak at 550 nm).

The experiment was repeated at several pH values (between 8 and 10). At each pH the column was equilibrated with the same low ionic strength buffer as described above and equlibration checked by measuring the pH of the eluate. To avoid excessive denaturation of enzyme, it was not dialysed against buffer of pH greater than 9, instead, reliance was placed on thorough equlibration of the column and addition of cytochrome c in buffer at the eluant pH, to ensure pH adjustment to the required value.

At each pH value, the stoichiometry of the cytochrome c oxidase: cytochrome c complex was calculated, as molecules of cytochrome c bound per functional unit of oxidase, from the absorbance at 550 nm and 605 nm (after reduction by sodium dithionite), of the fraction containing the largest amount of electrostatic complex.

Dissociation of the electrostatic complex

A column (2.5 x 62 cm) containing sepadex G50, was equilibrated at room temperature with high ionic strength buffer, containing 0.2M NaCl or 0.15 M Na_2SO_4. A one (1) ml portion of electrostatic complex was applied to the column and eluted with the same high ionic strength buffer which had been used to equilibrate the column. Initial experiments using high ionic strength buffer containing > 0.15 M sodium sulphate caused precipitation of protein within the column, visible as a brown stationary band.

One (1) fractions were collected and those containing protein were identified by measuring the absorbance of each fraction at 280 nm. In both experiments using each of the high ionic strength buffers containing chloride or sulphate, fractions with maximum absorbance at 280 nm were reduced with sodium dithionite and the spectrum from 400-630 nm recorded to verify separation of the complex into oxidaseand cytochrome c. The activity of cytochromew c oxidase recovered was measured polarographically using Clarke type oxygen electrode.

RESULTS AND DISCUSSION

The stoichiometry of electrostatic complex formation

At each pH value, the separation of complex from excess cytochrome c was clearly visible by eye as a red-brown and red band on the gel filtration column. Typical spectra of dithionite-reduced protein fractions containing (a) cytochrome c only and (b) electrostatic complex are shown in Fig. 1.

From a standard spectrum of ferrocytochrome c it can be seen that the contribution of this protein to the absorbance of the complex at 605 nm was negligible (Fig. 1). Thus the amount of cytochrome c oxidase in the electrostatic complex (total haem), could be determined from the 605 nm absorbance of the dithionite-reduced fraction(s) which contained the complex, using the extinction coefficient 21 $mM^{-1}cm^{-1}$ (Blenkinsop, 1993). Cytochrome c content of the electrostatic complex was found from the absorbance of the dithionite-reduced fraction(s) at 550 nm. At this wavelength, the calculation was complicated by the contribution of both proteins to the absorbance. The contribution from oxidase was subtracted by using the concentration as determined at 605 nm, and an extinction coefficient of 7.2 $mM^{-1}cm^{-1}$ at 550 nm (from a standard spectrum).

The stoichiometry of the electrostatic complex, in terms of molecules of cytochrome c bound per functional unit of oxidase was found by halving the total haem concentration of the native, dimeric oxidase and comparing this with the cytochrome c concentration of the complex. A sample calculation of stoichiometry, in an experiment carried out at pH 9.3, ionic strength < 20 mM, is as follows:

Fraction number 19, reduced with sodium dithionite

A_{550} = 0.028 A_{605} = 0.029

[ε_{550} 27.6 (cyt.c), 7.2 (oxidase) $mM^{-1}cm^{-1}$;

ε_{605} 21 $mM^{-1}cm^{-1}$ (oxidase) all as total haem]

Concetration of dimeric oxidase (total haem) in the electrostatic complex,

= 0.029 /21 mM
= 0.0014 mM (1.4 μM)
= 0.7 μM per functional unit

A_{550} = 0.028 = ([concn. cyt.c] x 27.6) + (0.0014 x 7.2)

Therefore, concentration of cytochrome c in the electrostatic complex,

= (0.0028 - 0.01) / 27.6 mM
= 0.0007 mM
= 0.7 μM

Stoichiometry of complex at pH 9.3,

= 0.7 : 0.7
= 1 : 1

A summary of the variation with pH of the stoichiometry (dimer) of the electrostatic complex as determined by gel filtration chromatography at low ionic strength, is shown in tabular form in Table 1 and graphically in Fig. 2. It can be seen from Fig. 2, that even under conditions of low ionic strength, which should maximise electrostatic complex formation

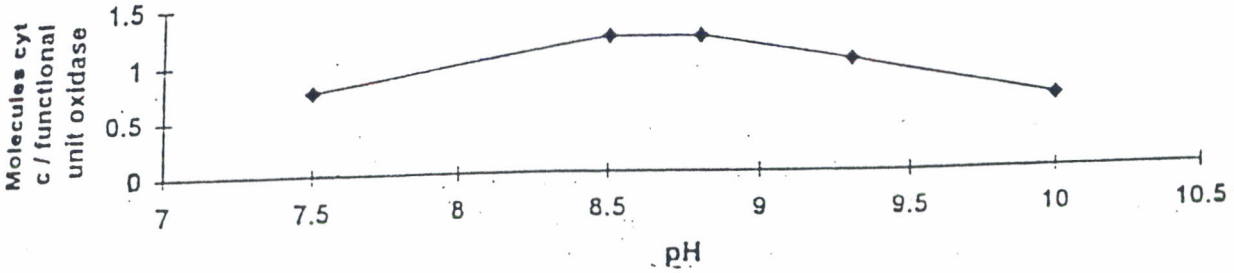

Fig. 2: Variation, with pH, of the stoichiometry* of the electrostatic complex formed between cytochrome c and cytochrome c oxidase at low ionic strength, expressed as molecules of cytochrome c bound per functional unit of cytochrome c oxidase. (* for explanation of the stoichiometry calculation, see text).

between the native, dimeric cytochrome c oxidase and cytochrome c, the stoichiometry does not approach 1 : 2, showing no evidence for two (2) binding sites on oxidase, even at low ionic strength and with an excess of cytochrome c present. It is possible that a second site with lower affinity may have a relatively higher dissociation rate so that dissociation takes place in the time required for elution of the column.

These findings are of interest in postulating control mechanisms over the "docking" and "un-docking" of the proteins of electron transfer.

Table 1: Summary of results of gel filtration experiments to determine the variations with pH of the stoichiometry of the electrostatic complex, formed between cytochrome c and cytochrome c oxidase at low ionic strength (I = < 20mM).

pH	Stoichiometry-Dimer (mol cyt c/mol aa3
7.5	0.75 ± 0.05
8.5	1.20 ± 0.05
8.8	1.25 ± 0.05
9.3	1.00 ± 0.08
10	0.65 ± 0.05

The Table shows the number of moles of cytochrome c bound per mole of functional unit of oxidase (half the total haem concentration). The variation of stoichiometrey with pH was found to be statistically significant at $P < 0.05$.

Dissociation of the electrostatic complex

The dissociation of the electrostatic complex at the high ionic strength of the gel filtration column, which was clearly visible by eye, was verified spectroscopically as shown in Fig. 3.

The activity of the cytochrome c oxidase emerging from the column was assayed polarographically and found to be high, confirming the spectroscopic evidence of no apparent damage to the enzyme, even though the separation was carried out at room temperature (20°C). The time taken to separate the complex on the column was 30-40 minutes, and dialysis to remove the salt could be conveniently carried out at 4°C, all with no apparent loss of the enzyme activity. It was also confirmed that the complex was dissociated by both 0.2 M NaCl and 0.15 M Na_2SO_4, at pH 7.5 and 8.

The experiments reported in this study indicate that the electrostatic complex between cytochrome c and oxidase is a tight and stable one over a fairly wide pH range, but at an ionic strength (< 20mM) which is far lower than that which is reported as physiological (~ 100 mM) (Blenkinsop, 1993). It may be that extremely small local variations in ionic strength, in the area of the binding sites on one or both proteins, brought about for example during proton translocation by cytochrome c oxidase, or exclusion of surface water molecules during "docking" may have dramatic effects in vivo on the affinity of cytochrome c and cytochrome c oxidase for one another.

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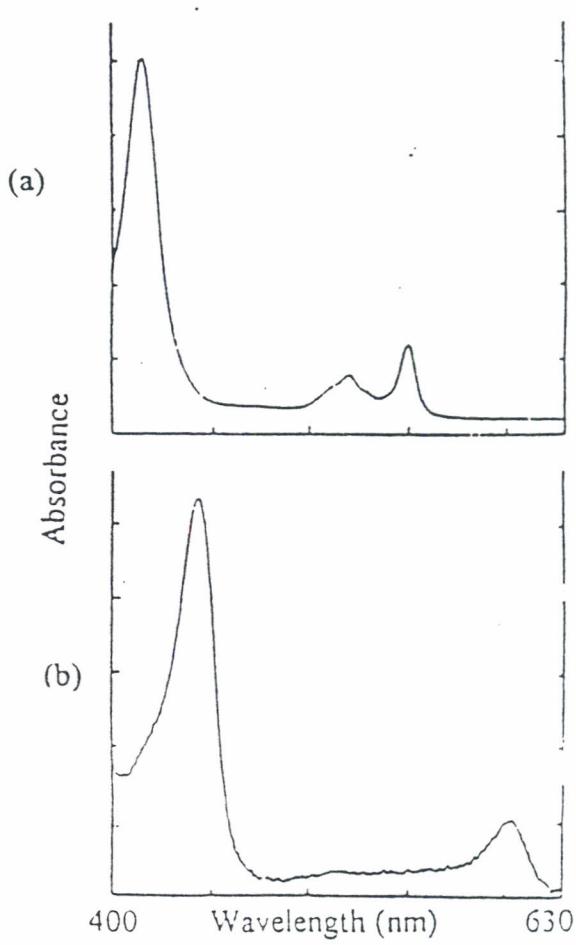

Fig. 3: Dithionite-reduced spectra of a) fraction containing cytochrome c and b) fraction containing cytochrome c oxidase obtained as a result of the dissociation of electrostatic complex at high ionic strength, and separation by gel filtration (column sephadex G50, eluted with 10 mM tris/acetate buffer + 0.3% Tween 80 + 0.2 M NaCl, pH 8).

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