SOME IMMUNOCHEMICAL PROPERTIES OF *PSEUDOMONAS AERUGINOSA* CYTOCHROME OXIDASE (OR NITRATE REDUCTASE)

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1. Introduction

One of the terminal electron acceptors in the respiratory chain of *Pseudomonas aeruginosa*, Ps. cytochrome oxidase (Ps. cyt. *c* ox.), is an haem-protein of mol. wt 120 000 containing 4 iron atoms/hydrodynamic unit in the form of 2 haems *c* and 2 haems *d* [1,2]. In spite of the considerable amount of work done on this system [3,4] very little is understood of the catalytic mechanism and the protein binding sites. This enzyme is of interest because:

(i) It is a water-soluble protein which contains as cofactor only 1 kind of metal ion, experiencing very different environments;

(ii) It can use both O_2 and NO_2^- as reducible substrates and shares the role of terminal oxidase with another, membrane-bound enzyme, not yet purified.

The relative amounts of the two oxidases present in the cell can be strongly influenced by the choice of growth conditions [5].

A limitation to more extensive investigation of this system is imposed by the scarce quantities of the enzyme available with classical techniques [6].

Although immunochemical techniques could improve the cumbersome enzyme purification procedure and yield useful information regarding its structure and function, little attention has been paid to the immunological properties of Ps. cyt. *c* ox.

We have undertaken a study of immunochemical properties of Ps. cyt. *c* ox. with the aim of:

(i) Setting up a fast and easy-to-handle purification procedure based on immunoaffinity chromatography;

(ii) Investigating the possible modification of the enzymatic activity as a result of the complex formation with specific antibodies.

2. Materials and methods

2.1. *Purification of* Pseudomonas *cytochrome* c *oxidase*

This was done from *Pseudomonas aeruginosa* (strain NCTC 6750) according to [6]. The protein was >95% pure, as judged by spectrophotometry [7] and polyacrylamide gel isoelectrofocusing in the anpholine range 5–8. Enzyme and immunoglobulin concentrations were obtained by using the following extinction coefficients, respectively: $E_{411} = 282 \times 10^3$ $l \cdot mol^{-1} \cdot cm^{-1}$ for the oxidized form [2] and $E_{280} = 1.36\,l \cdot g^{-1} \cdot cm^{-1}$ [8].

2.2. *Production of antiserum*

Antibodies against Ps. cyt. *c* ox. were produced in a single adult New Zealand rabbit by injecting subcutaneously 1 ml of Ps. cyt. *c* ox. solution (0.8 mg/ml) in phosphate buffer 0.05 M (pH 7.4) plus 1 ml complete Freund adjuvant each week for 3 consecutive weeks. A 1.6 mg booster injection was delivered 10 days after the last inoculation. One week after the booster injection the rabbit was bled from the ear artery for 7 successive days (25 ml blood/day).

Abbreviations: Ps. cyt. *c* ox., *Pseudomonas* cytochrome *c* oxidase; PBS, phosphate buffered saline; NRS, normal rabbit serum

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2.3. *Purification of specific anti-Ps. ox. rabbit IgG*

IgG fraction was isolated from the total antiserum by precipitation in 40% saturated ammonium sulphate at pH 6.5 followed by ion-exchange chromatography on DEAE-cellulose according to [9].

From the total IgG fraction, specific antibodies were separated by affinity chromatography using a column (0.9 × 15 cm) packed with CNBr-activated Sepharose 4B (Pharmacia) containing immobilized Ps. cyt. *c* ox. Coupling of 15 mg Ps. cyt. *c* ox. was done as in [10]; thereafter the gel was washed by 0.05 M glycine–HCl buffer (pH 2.8) containing 2 M guanidine–HCl to remove unbound material, and equilibrated with PBS buffer (pH 7.2). Aliquots of 1 ml total IgG fraction in the same buffer (pH 7.2), were chromatographed at 2 ml/h flowrate. The total protein content of each aliquot was estimated by the biuret method to be 8 mg. After elution of 1 major peak, containing >50% of total protein (see fig.1), elution of the specific IgG was carried out with 10 ml 0.05 M glycine–HCl buffer (pH 2.8) containing 2 M guanidine–HCl at 20 ml/h flowrate.

Immediately afterwards the column was re-equilibrated with buffer (pH 7.2) and subsequently used several times without any observable loss in efficiency.

The entire procedure was done at 4°C.

2.4. *Immunological assay*

Immunoelectrophoresis was performed on a thin layer of agar 1% in 0.05 M veronal buffer (pH 8.6) using an electric field across the plate of 20 mA for 2 h. Precipitin lines were obtained by anti-whole rabbit serum. NRS was used as a reference.

Immunodiffusion against Ps. cyt. *c* ox. was performed on 0.75% agarose in PBS buffer (pH 7.2) containing 0.1% Na-azide. The wells were 5 mm diam. and placed 4 mm apart from each other. IgG and Ps. cyt. *c* ox. are 0.8 mg/ml. The immunological reaction was for 24 h at room temperature.

2.5. *Enzymatic assay*

Spectrophotometric assays were performed at 25°C monitoring the oxidation of reduced *Pseudomonas* azurin at 625 nm in sodium acetate buffer 0.05 M (pH 5.3) [1]. The reduced substrate was prepared by addition of $Na_2S_2O_4$ and subsequent removal of byproducts by passage through a Sephadex G-25 column equilibrated with the same buffer.

Polarographic assays were carried out with a Clark oxygen electrode according to [6] using either TMPD or catalytic amounts of cyt. *c*-551 as an electron shuttle.

Before assays, Ps. cyt. *c* ox. in phosphate buffer 0.05 M (pH 7.4) was incubated for 15 min at 4°C with different amounts of total antiserum, or purified immunoglobulins.

3. Results

3.1. *Antigenicity of Ps. cyt. c ox.*

From the total serum of adult New Zealand rabbit immunized for Ps. cyt. *c* ox. the specific IgG fraction has been:

(i) Isolated by ion-exchange chromatography;
(ii) Identified by immunoelectrophoresis;
(iii) Purified by affinity chromatography using Ps. cyt. *c* ox. immobilized on Sepharose 4B.

Fig.1 reports the elution profile of the affinity chromatography. The total protein present in the 2 peaks has been estimated to be, respectively, 5.2 mg aspecific IgG (fractions 10–20) and 2.4 mg specific IgG (fractions 40–50) over a total 8 mg applied to the column. Specific IgG bound to the immobilized antigen (fractions 40–50) were eluted by breaking the immunocomplex with 2 M guanidine–HCl, at pH 2.8.

Immunodiffusion has been used to check the immunological properties of the 2 peaks, and the insert indicates that peak 2 which represents a substantial amount (≃30%) of the total IgG present in the serum, pertains to specific antibodies.

3.2. *Influence of the immunocomplex formation on the enzymatic activity*

Fig.2 shows the influence of the formation of the immunocomplex with specific IgG on the enzymatic activity of Ps. cyt. *c* ox. assayed using either azurin or ascorbate as oxidizable substrates. The rate of oxidation of azurin appears to be inhibited by addition of increasing amounts of specific IgG to the reaction mixture. At high IgG concentration, the effect levels off to a rate which is ~50% of that characteristic of the oxidase (fig.2A). Under similar conditions, the rate of oxidation of ascorbate is not affected by the addition of IgG even in large excess (fig.2B).

Table 1 reports the rate of O_2 consumption catalyzed by the native Ps. cyt. *c* ox. and the immunocomplex, before and after addition of cyt *c*-551 as an

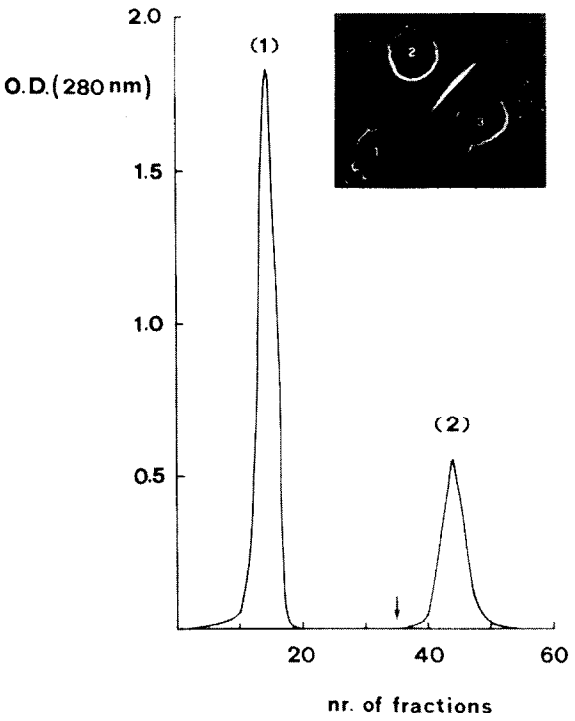

Fig.1. Purification of specific anti Ps. cyt. *c* ox. IgG by immunoaffinity chromatography. IgG fraction (8 mg/ml) 1 ml, from immune rabbit serum was chromatographed on a 0.9 × 15 cm column containing the Sepharose 4B–Ps. cyt. *c* ox. covalent complex (see section 2). The arrow denotes the point where 2 M guanidine–HCl in 0.05 M glycine–HCl buffer was added to the column. The A_{280} of each fraction (1 ml) was measured. The insert shows the antigenic activity of the peak (1) and the peak (2) tested by immunodiffusion (see section 2) against Ps. cyt. *c* ox.

Table 1
Effect of cyt *c*-551 on the O_2 consumption by Ps. cyt. *c* ox. and the Ps. cyt. *c* ox. immunocomplex

	O_2 consumption velocity (a.u.)	
	–cyt. *c*-551	+cyt. *c*-551
Ps. cyt. *c* ox. immunocomplex	0.21 ± 0.02	0.43
Ps. cyt. *c* ox.	0.20	0.59

The immunocomplex was obtained preincubating the native Ps. cyt. *c* ox. with specific IgG in the 1:20 (w/w) ratio. Experimental conditions: Ps. cyt. *c* ox. either native or in the immunocomplex form 0.1 μM; Cyt *c*-551 was 2 μM, and ascorbate 13 mM. All other conditions as in fig. 2B. Each value in the table is the av. ⩾2 independent experiments

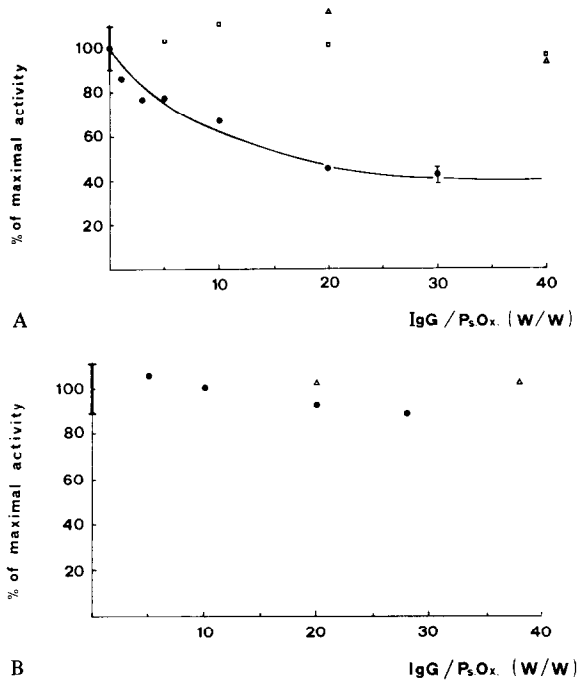

Fig.2. Inhibitory effect of various fractions of immune rabbit serum on the oxidase activity of Ps. cyt. *c* ox. The oxidation catalyzed by Ps. cyt. *c* ox. was observed using azurin (A) and ascorbate (B) as reducing substrates in the presence of variable amounts of immune rabbit serum (□), non-specific IgG (Δ) (fraction (1), fig.1) and specific IgG (●) (fraction (2), fig.1). Experimental conditions: (A) azurin oxidation was followed at 625 nm in acetate buffer 0.05 M (pH 5.3). Azurin and Ps. cyt. *c* ox. were 50 and 0.06 μM, respectively. Temp. 25°C; (B) O_2 consumption was followed polarographically by a Clark-type O_2 electrode in phosphate buffer 0.05 M (pH 6.5). Ascorbate was 13 mM, Ps. cyt. *c* ox. 0.06 μM. TMPD 0.2 mM was used as an electron shuttle. Temp. 30°C. Each point is av. ⩾3 independent measurements, and the bars represent maximum errors.

electron shuttle. In the absence of cyt. *c*-551, the rate of the process is the same for both assays. Addition of the specific macromolecular substrate, however, shows that the enhancement in rate is a factor of ≃3 with PsOx, and only a factor of ≃2 with the immunocomplex. The drop in rate (≃45%) as between the latter cases is similar to that shown above (see fig. 2A) in the oxidation of reduced azurin.

The enzymatic activity of Ps. cyt. *c* ox. has also been tested in the presence of the same amounts (on a total protein basis) of aspecific IgG (peak 1) and total serum, and in both cases no effects were observed (fig.2). This is in line with the conclusion

that the substantial inhibition observed with the macromolecular substrates in the presence of the specific IgG fraction is not due to aspecific interactions.

From the data in fig.2A, 10–20 antibody binding sites/Ps. cyt. *c* ox. molecule may be estimated. This is in fair agreement with ~1 site individual specificity/ 5×10^3 mol. wt in globular proteins, as obtained [11] for bovine serum albumin.

3.3. *Spectroscopic properties of the immunocomplex*

Formation of the immunocomplex following the mixing of two solutions containing Ps. cyt. *c* ox. and specific IgG in a 1:1 molar ratio was detected by differential spectroscopy. In spite of the increase in light scattering, due to the slow formation of larger molecular aggregates, a small but definitive ΔA_{280} was detected. This perturbation may be related to unmasking of buried tyrosines in the formation of the immunocomplex, as indicated by studies carried out using horse radish peroxidase (Zito et al. unpublished). No significant change in the absorption region characteristic of the heme moiety was observed. Neither the time-dependent increase in turbidity nor the ΔA_{280} were observed in the identical experiments carried on with the aspecific IgG fraction.

4. Discussion

The above data indicate that the quantity of specific antibodies which can be purified from the serum of the rabbit inoculated with Ps. cyt. *c* ox. is fairly high as compared to the commonly reported values [11]. This result on the antigenicity of this enzyme, which is perfectly reasonable in view of the evolutionary distance between *Pseudomonas* and rabbit, seems promising in order to set up an improved version of the purification procedure, based on immunoaffinity chromatography.

The effect of specific antibodies on the catalytic properties of the oxidase is very clear and intriguing. The drop in the rate of oxidation of the macromolecular partner, either azurin or cyt *c*-551, increases with the increase in the concentration of the antibody present in the incubation mixture. The maximal effect is observed with a stoichiometric ratio of ~10–20 antibody molecules/enzyme molecule.

Two mechanisms have been proposed to interpret the effect of antibodies of the enzymatic activity of an antigen reviewed ([12]):

(i) A direct mechanism involving steric hindrance or electrostatic effects between the antibody and the substrate;

(ii) An indirect (or allosteric) mechanism involving the (de)stabilization, induced by the reaction with antibodies, of some conformation of the enzyme particularly (un)efficient in performing the catalytic activity.

Although with Ps. cyt. *c* ox. it is not possible to discriminate between the 2 alternatives, the following observations appear relevant to the point in question:

(i) Only when the electron donor is a macromolecule the oxidase activity of Ps. cyt. *c* ox. is depressed (see table 1 and fig.2). This fact may be taken as an indication that steric hindrance to the approach of the macromolecular substrate to the oxidase is the basis for the inhibition of the enzymatic activity (see table 1 and fig.2).

(ii) The absorbance changes observed by differential spectroscopy involve exclusively a perturbation of the ultraviolet region and are not reflected into changes at the level of the characteristic bands of heme *c* and *d*.

The latter point, however, taken together with the fact that entrance of electrons into the enzyme takes place through heme *c*, does not necessarily exclude that the formation of the immunocomplex involves regions of the oxidase molecule stereochemically correlated to the pathway of electrons from azurin (or cyt. *c*-551) to heme *c* on the oxidase.

Pre-steady state investigations, as well as quantitative comparison of the dynamic aspects of the nitrate-reductase and oxidase activities, are necessary to have an insight in the molecular mechanism of the inhibition of activity reported here. A systematic work intended to answer these and other relevant points may provide a better understanding of the catalytic properties of *Pseudomonas* cytochrome *c* oxidase.

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