CHARACTERIZATION OF [^{3}H]NoHOQnO BINDING TO PURIFIED COMPLEX III

P. RICCIO, A. BOBBA and E. QUAGLIARIELLO

Istituto di Chimica Biologica, Facoltà di Scienze, Università di Bari, and *Centro di Studio sui Mitocondri e Metabolismo Energetico, Consiglio Nazionale delle Ricerche, Via Amendola 165/A, 70126 Bari, Italy*

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1. Introduction

Complex III, ubiquinol–cytochrome *c* reductase (EC 1.10.2.2), is a membranous multiprotein complex of the mitochondrial respiratory chain [1,2].

Although many inhibitors of complex III are now known, 2 are most frequently used as a tool to elucidate the electron transfer mechanism in this region of the respiratory chain, namely antimycin [3] and the 2-*n*-alkyl derivatives of 4-hydroxyquinoline *N*-oxide [4,5].

It is not yet well understood whether these 2 inhibitors bind to the same site [4,6–8] or to different sites [9–11], and whether there are other binding sites for each [3,9,12,13].

We decided to tackle this problem by studying the binding of 3-^{3}H-2-*n*-Nonyl-4-hydroxyquinoline *N*-oxide ([^{3}H]NoHOQnO) to the already well-characterized complex III, purified by the fast Triton–hydroxyapatite procedure [14–17]. Since the inhibitors are highly lipophilic, thus having a great affinity for the membranous lipidic phase, it offers the advantage of working on a membrane free model.

This paper reports on the purification and characterization of the [^{3}H]NoHOQnO-loaded complex III. This binding is specifically recognized by NoHOQnO, antimycin and by 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT). The results obtained by these competition studies are discussed below.

2. Materials and methods

Beef heart mitochondria were prepared according to [18]. [^{3}H]NoHOQnO was synthesized by Dr H. Günther as in [5].

NoHOQnO (Research Plus Laboratories) and UHDBT were generous gifts from Professor B. L. Trumpower. [^{3}H]Antimycin was kindly donated by Professor G. von Jagow. Antimycin was obtained from Boehringer, hydroxyapatite (HTP) from Bio-Rad Labs and Triton X-100 from Sigma.

NoHOQnO, antimycin, cytochrome *b* and protein concentrations were determined as in [5]. Protein content was also routinely determined by the Bio-Rad protein assay. A modified Lowry [19] procedure was used for the purified complex III fractions.

Equilibrium dialysis experiments were carried out with the apparatus of Diachema (Zürich) for 6–30 h at 4°C. The purified [^{3}H]NoHOQnO–complex III was added to a 200 μl half-cell, the antagonists to the opposite half-cell. Cellulose dialysis tubing, obtained from Serva, were boiled in 0.2 $SnCl_2$ g/l and washed several times with water.

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS–PAGE) was done essentially according to [20], as described in [21].

3. Results

[^{3}H]NoHOQnO is a highly hydrophobic compound. For the solubilization and the purification of its binding site in an intact state, it is necessary to maintain detergent concentration as low as possible [22]. This was obtained by adding sucrose to the extraction medium and by lowering the Triton concentration during the chromatographic and dialysis steps.

The purification procedure is summarized in fig.1. After the solubilization of the particles, ~70% of the protein fraction, 80% of cytochrome *b* and 85% of radioactivity were recovered in the Triton extract.

Complex III was bound in a batch procedure to

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BEEF HEART MITOCHONDRIA

1. ADDITION OF ^{3}h -NoHOQnO
2. CENTRIFUGATION, 60' AT 160,000 x g

[^{3}H]-NoHOQnO - BHM

1. NEGATIVE EXTRACTION WITH 1,65% TRITRON, 200 mM NaCl, 20 mM MOPS, pH 7.2, AT 15 MG PROTEIN/ML, 15' AT 4°C
2. CENTRIFUGATION, 60' AT 160.000 X G

[^{3}H]- NoHOQnO - SUBMITOCHONDRIAL PARTICLES

1. EXTRACTION WITH 3% TRITON X-100, 11%, SUCROSE; 220 mM NaCl, 20 mM MOPS, pH 7.2, AT 10 MG PROTEIN, 15' AT 4°C.
2. CENTRIFUGATION,20' AT 160.000 X G.

TRITON EXTRACT

1. ADDITION OF HYDROXYAPATITE, 1.1—1.5 ML HTP/ML EXTRACT
2. PACKING INTO A COLUMN
3. ELUTION WITH 0.4 M PHOSPHATE

[^{3}H]-NoHOQnO-COMPLEX III

1. DIALYSIS vs 0.05% TRITON X-100, 10 mM MOPS, 30 mM NaCl (3 HOURS)
2. CENTRIFUGATION, 20' AT 160.000 X G

DIALYSIS SUPERNATANT

TO EQUILIBRIUM DIALYSIS

Fig.1. Summary of the purification of [^{3}H]NoHOQnO complex III. Preparation time: ~2 h, starting from the extraction of the [^{3}H]NoHOQnO-loaded submitochondrial particles. Enrichment, 10–11-fold.

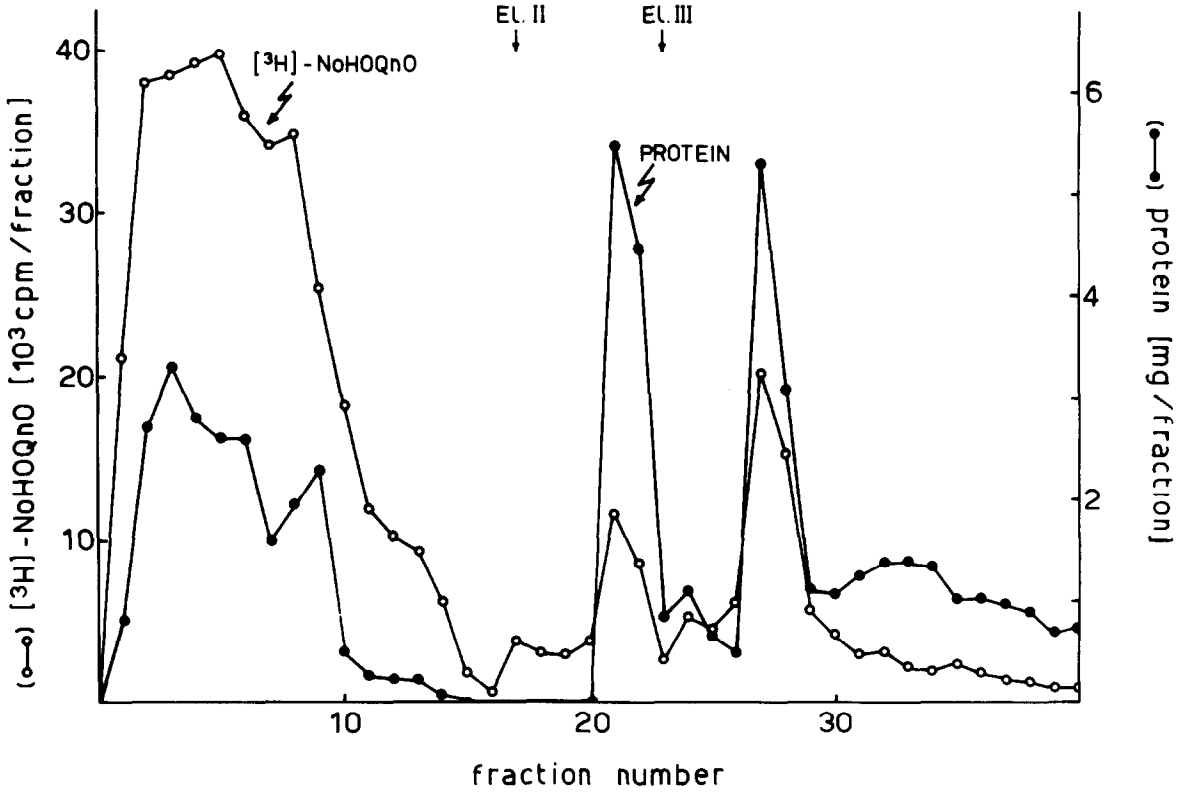

Fig.2. Eluate profile of a hydroxyapatite column: eluent I, equilibrating medium as in text; eluent II, 0.05% Triton X-100, 10 mM MOPS, 150 mM phosphate (pH 7.2); eluent III, 0.05% Triton X-100, 10 mM MOPS, 400 mM phosphate (pH 7.2); 3.75 ml/fraction, 60 ml/h. Protein content was determined by the Bio-Rad assay. Fraction 27 contains 3.7 μM cytochrome *b*, 0.53 mg protein/ml (Lowry), 5420 cpm/ml.

hydroxyapatite equilibrated with 0.05% Triton X-100, 20 mM morpholinopropane sulfonate (MOPS), 30 mM NaCl, at pH 7.2, then packed into a column. Free or so-called unspecific bound [^{3}H]NoHOQnO and excess Triton X-100 pass through the column (fig.2), whereas complex III precipitates in the column.

Phosphate application in concentrations normally used for the elution of complex III, 150–200 mM [14–16], does not permit recovery.

The [^{3}H]NoHOQnO–complex III is eluted only by means of 400 mM phosphate, to a purity of 6.6–7.0 μmol cytochrome *b*/g protein, in fractions 27 and 28.

In spite of the different elution molarity, eluted complex III is intact. It still contains cytochromes *b*

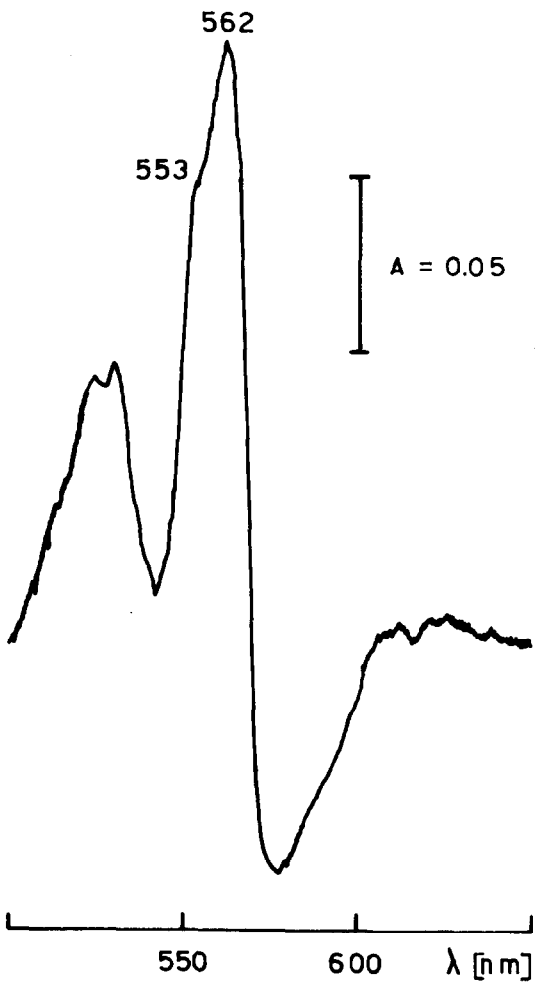

Fig.3. Difference absorbance spectrum of the complex III collected in fraction 27 (fig.2). Reduced by dithionite and oxidized by ferricyanide.

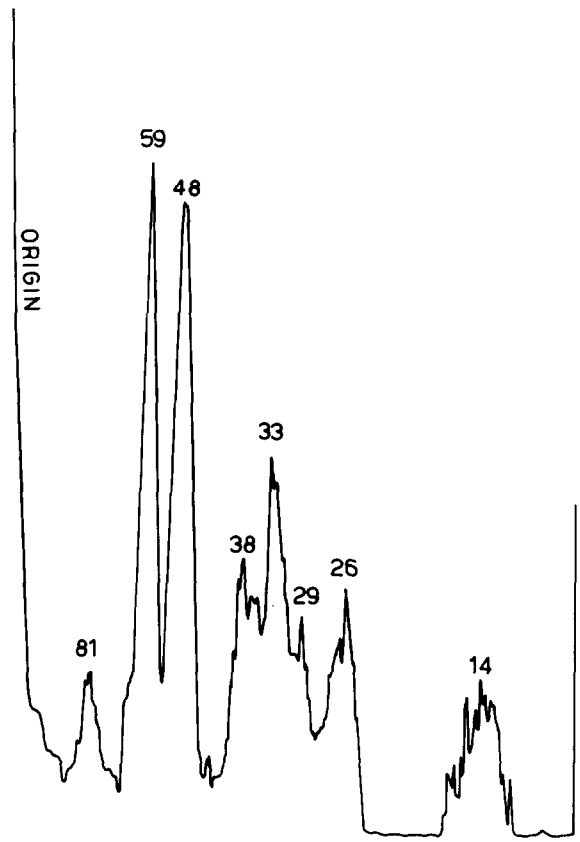

Fig.4. SDS–PAGE: polypeptide pattern of purified [^{3}H]-NoHOQnO–complex III.

and c_1 (fig.3) and the other polypeptides, the iron–sulfur protein included (fig.4).

Therefore, as determined by gel filtration, the [^{3}H]NoHOQnO–complex III has the same app. M_r as [^{3}H]antimycin–complex III, prepared in a similar way but eluted by 150 mM phosphate in the presence of higher Triton concentrations in the eluent (not shown).

Purified [^{3}H]NoHOQnO binding was further characterized by equilibrium dialysis studies (section 3.1).

3.1. *Interaction of other complex III ligands with [^{3}H]NoHOQnO binding*

The [^{3}H]NoHOQnO specific binding has been defined in submitochondrial particles as a saturable, antimycin-sensitive binding [3,4,23].

To test if the isolated [^{3}H]NoHOQnO binding is specific the [^{3}H]NoHOQnO–complex III was submitted to equilibrium dialysis in the presence of either NoHOQnO, antimycin or UHDBT.

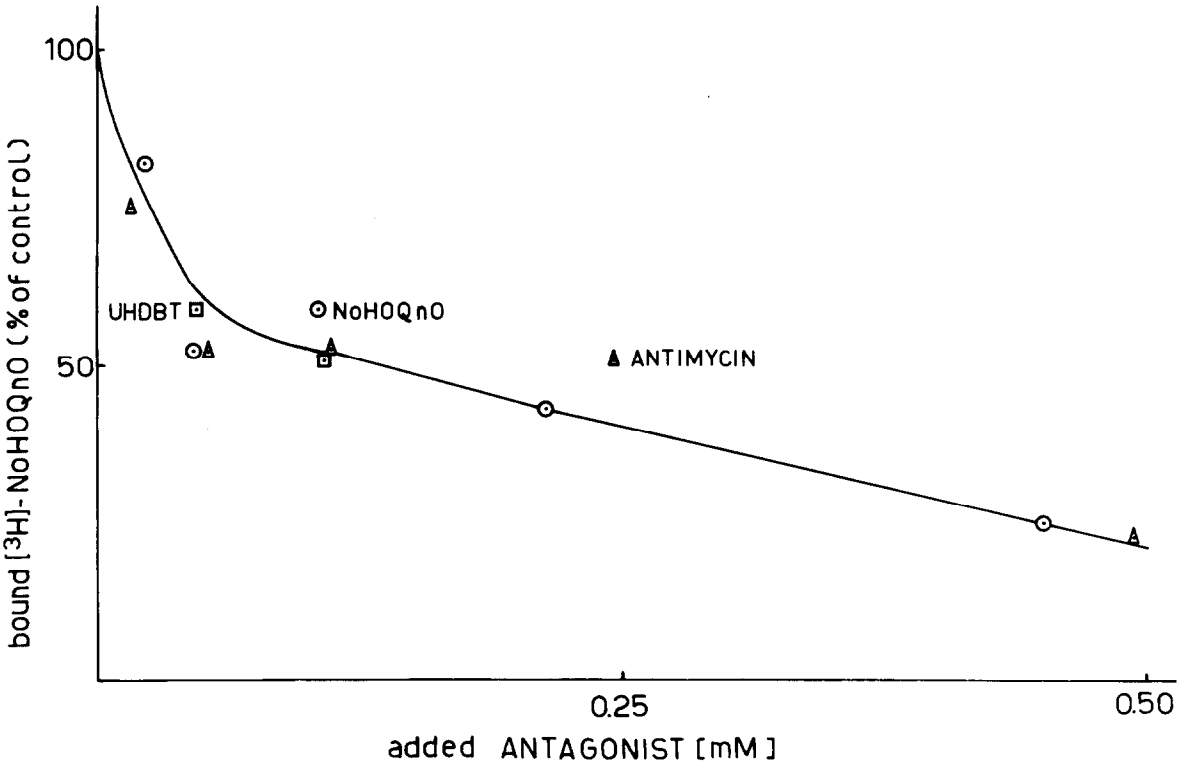

Fig.5. Demonstration of ligand interaction with the [^{3}H]NoHOQnO–complex III as determined by equilibrium dialysis for 15–30 h at 4°C. 100% corresponds to 51% binding in the control. Dialysis medium: 0.05% Triton X-100, 10 mM MOPS, 30 mM NaCl (pH 7.2). Free [^{3}H]NoHOQnO reached the equilibrium in 3–4 h. In a typical experiment the protein sample contained 0.74 nmol cytochrome *b*, 0.11 mg protein, 1084 cpm, in 200 μl. Ligands were added in ethanolic solutions to the opposite half-cell. Ethanol final concentration did not exceed 4.5%.

Fig.5 shows the displacement of bound [^{3}H]-NoHOQnO as the dependence of [^{3}H]NoHOQnO binding on the concentration of the added ligands. Rather than the expected difference in curves, a single curve can be drawn which is roughly valid for the action of all 3 ligands. However, the removal of bound [^{3}H]NoHOQnO should be ascribed to a specific interaction of the added ligands with the [^{3}H]NoHOQnO binding site, since the unrelated lipophilic compound rotenone, added to 46 μM, does not remove any bound [^{3}H]NoHOQnO (not shown).

4. Discussion

The [^{3}H]NoHOQnO specific binding has been isolated in its intact and functional state. No other [^{3}H]-NoHOQnO binding could be detected.

The similar effectiveness of the 3 ligands, NoHOQnO, antimycin, and UHDBT, in removing bound [^{3}H]-NoHOQnO has to be explained since antimycin ($K_{d,s} = 10^{-11}$–10^{-13} M) [3] was expected to display [^{3}H]NoHOQnO much better than NoHOQnO ($K_d = 5 \times 10^{-9}$ M) [5] and UHDBT should not have interacted with the [^{3}H]NoHOQnO binding site.

These results can be best explained by the assumption of different binding sites for NoHOQnO and antimycin. If so, the question arises as to the location of the NoHOQnO binding site.

[^{3}H]NoHOQnO bound to the purified complex III is displaced from 2 different types of inhibitors such as antimycin and UHDBT: antimycin binds to cytochrome *b* [24] near the heme center b_{562} [6,25,26]; whereas UHDBT apparently binds to the iron–sulfur protein [27,28].

However, a structural interaction between the iron–sulfur protein and cytochrome *b* has been proposed in [29] and therefore it is known that the

Rieske iron–sulfur protein can be split from complex III in the presence of antimycin [14,30].

The displacement of bound [^{3}H]NoHOQnO by antimycin could be explained by negative allosteric interaction between 2 different binding sites [10], whereas the [^{3}H]NoHOQnO displacement by UHDBT should be ascribed to either proximity or identity of the [^{3}H]NoHOQnO and UHDBT binding sites.

We can assume that the NoHOQnO binding site is located either on a cytochrome *b* region near the association site of the Rieske iron–sulfur protein, or on the Rieske protein itself. In other words the binding site should be near the reacting sites of $QH^{\cdot}_{o}$, the non-diffusible ubisemiquinone of complex III [28].

References

[1] Rieske, J. S. (1976) Biochim. Biophys. Acta 456, 195–247.

[2] Trumpower, B. L. and Katki, A. (1979) in: Membrane Proteins in Energy Transduction (Capaldi, R. A. ed) pp. 89–200, Marcel Dekker, New York.

[3] Slater, E. C. (1973) Biochim. Biophys. Acta 301, 129–154.

[4] Van Ark, G. and Berden, J. A. (1977) Biochim. Biophys. Acta 459, 119–137.

[5] Riccio, P., Günther, H. and Quagliariello, E. (1981) Eur. J. Biochem. 114, 153–157.

[6] Chance, B. (1958) J. Biol. Chem. 233, 1223–1229.

[7] Brandon, J. R., Brocklehurst, J. R. and Lee, C. P. (1972) Biochemistry 11, 1150–1154.

[8] Nijs, P. (1967) Biochim. Biophys. Acta 143, 454–461.

[9] Eisenbach, M. and Gutman, M. (1975) Eur. J. Biochem. 59, 223–230.

[10] Izzo, G., Guerrieri, F. and Papa, S. (1978) FEBS Lett. 93, 320–322.

[11] Doussière, J., Sainsard-Chanet, A. and Vignais, P. V. (1979) Biochim. Biophys. Acta 548, 236–252.

[12] Norling, B., Nelson, B. D., Nordenbrand, K. and Ernster, L. (1972) Biochim. Biophys. Acta 275, 18–22.

[13] Burger, G., Lang, B., Bandlow, W. and Kaudewitz, F. (1975) Biochim. Biophys. Acta 396, 187–201.

[14] Ricçio, P., Schägger, H., Engel, W. D. and Von Jagow, G. (1976) Biochim. Biophys. Acta 459, 250–262.

[15] Von Jagow, G., Schägger, H., Engel, W. D., Riccio, P., Kolb, H. J. and Klingenberg, M. (1978) Methods Enzymol. 53, 92–98.

[16] Engel, W. D., Schägger, H. and Von Jagow, G. (1980) Biochim. Biophys. Acta 592, 211–222.

[17] Riccio, P. (1981) in: Chromatography in Biochemistry, Medicine and Environmental Research (Frigerio, A. ed) Elsevier Scientific, Amsterdam, New York.

[18] Smith, A. L. (1967) Methods Enzymol. 10, 81–86.

[19] Helenius, A. and Simons, K. (1972) J. Biol. Chem. 247, 3656–3661.

[20] Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412.

[21] Riccio, P., Aquila, H. and Klingenberg, M. (1975) FEBS Lett. 56, 133–138.

[22] Riccio, P., De Cotiis, M. and Quagliariello, E. (1981) in: Vectorial Reactions in Electron and Ion Transport in Mitochondria and Bacteria (Palmieri, F. et al. eds) pp. 183–185, Elsevier Biomedical, Amsterdam, New York.

[23] Von Ark, G. (1980) Electron transfer through the ubiquinol:ferricytochrome *c* oxidoreductase segment of the mitochondrial respiratory chain, Rodopi, Amsterdam.

[24] Roberts, H., Smith, S. C., Marzuki, S. and Linnane, A. W. (1980) Arch. Biochem. Biophys. 200, 387–395.

[25] Berden, J. A. and Slater, E. C. (1972) Biochim. Biophys. Acta 256, 199–215.

[26] Dervartanian, D. V., Albracht, S. P. J., Berden, J. A., Van Gelder, B. F. and Slater, E. C. (1973) Biochim. Biophys. Acta 292, 496–501.

[27] Bowyer, H. R., Dutton, P. L., Prince, R. C. and Crofts, A. R. (1980) Biochim. Biophys. Acta 592, 445–460.

[28] Trumpower, B. L. (1981) J. Bioenerg. Biomembr. 13, 1–24.

[29] Crofts, A. R., Meinhardt, S. V. and Bowyer, J. R. (1981) in: Function of Quinones in Energy Conserving System (Trumpower, B. L. ed) Academic Press, New York.

[30] Rieske, J. S., Baum, H., Stoner, C. D. and Lipton, S. H. (1967) J. Biol. Chem. 242, 4854–4866.