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Antimycin binds to a small subunit of the ubiquinol : cytochrome *c* oxidoreductase

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Bovine heart ubiquinol : cytochrome *c* oxidoreductase in Triton X-100 is split with guanidine into a number of fractions. A new method for measuring antimycin binding is developed using extraction with pentanol of the reversibly bound antimycin. By this method and the normal titration method, antimycin-binding capacity is found in a fraction containing a small subunit with a molecular mass of about 12000. This polypeptide was associated with cytochrome c_1 but is probably not the 'hinge protein'. Fractions that contain cytochrome *b* did not show binding by the pentanol-extraction method.

Introduction

Antimycin is an inhibitor of the respiratory chain, binding to ubiquinol : cytochrome *c* oxidoreductase (cytochrome bc_1 complex, complex III) [1], but it is not yet known to which of the about ten subunits in this enzyme it binds. Since antimycin induces changes in the absorption and EPR spectrum of cytochrome *b* [2] it was generally assumed that the antimycin-binding site is on cytochrome *b*. In 1973 Das Gupta and Rieske [3], by using a radioactive antimycin derivative with a photoaffinity label, identified a small subunit as the antimycin-binding site, but later the same authors were doubtful of their interpretation [4]. In a more recent report [5] these authors propose, based on results with the same technique, the cytochrome c_1-associated polypeptide as the antimycin-binding polypeptide.

Genetic experiments with microorganisms also suggest that cytochrome *b* contains the antimycin-binding site. Cells have been described with a mutation in a gen for cytochrome *b*, in which the antimycin-binding capacity has been lost or impaired (for example, see Ref. 6). Recent experiments with mutants of mouse cells have also given similar results [7]. However, Chevillotte-Brivet et al. [8] found mutants in which the antimycin-binding sites are independent of cytochrome *b* synthesis.

Our approach was to split the cytochrome bc_1 complex and test the products for their antimycin-binding capacity. There are three main ways to do this: first that of Silman et al. [9], in which the complex is split with taurocholate, ammonium sulphate and mersalyl; second that used by König et al. [10], in which the complex is split with (deoxy)cholate, ammonium sulphate and 2-mercaptoethanol, and third the method introduced by Von Jagow et al. [11,12], in which the complex in Triton X-100 is split on a hydroxyapatite column with guanidine. The first and second methods were not useful because we found that antimycin-binding capacity was lost during the splitting procedure. Only by the third method could we obtain

Abbreviation: Mops, 4-morpholinepropanesulphonic acid.

fragments that still retained the capacity to bind antimycin.

The normal way of determining the antimycin-binding capacity of intact cytochrome bc_1 complex is to titrate the complex with antimycin, and measure the fluorescence [13]; the fluorescence of antimycin bound to the intact complex is quenched. However, since this quenching has not been observed when antimycin is bound to fragments of the complex, a new method had to be developed.

Materials and Methods

Bovine heart ubiquinol : cytochrome *c* oxidoreductase was isolated by the method of Yu et al. [14].

The enzyme was split into a cytochrome *b*-containing and a cytochrome c_1-containing fraction by a method which was in essence the same as that described by Von Jagow et al. [11]. All solutions are kept at 4°C unless indicated otherwise. The enzyme and the hydroxyapatite were mixed, incubated for 1 h with some stirring and poured into a column. The different fractions were obtained by eluting the column with the different buffers by the method of Penefsky [15] until the eluent was colourless. The fraction (A) obtained with the starting buffer given in Ref. 11 contained unbound material. The cytochrome *b*-containing fraction (B) was obtained with the guanidine-containing buffer (i) [11]. A cytochrome c_1-containing fraction (C) was obtained at room temperature with 0.4 M sodium phosphate (pH 7.2) and, unless otherwise indicated, 0.5% Triton X-100. By using these buffers we found higher yields than by using the buffers given in a modified method [12]. After measuring the cytochrome c_1 content, fraction C was dialysed against 10 mM sodium Mops (pH 7.2) and split by the method of Schägger and co-workers [11] except that the guanidine-split fraction was incubated with the hydroxyapatite for 1 h with some stirring before pouring it into a column, and the 4 fractions (D, E, F and G, respectively) were obtained by the method of Penefsky [15] with the four different buffers as in Ref. 12. Fraction D is the unbound material, fraction E the eluent with buffer II [12], fraction F that with buffer VI and fraction G that with buffer VII. For the elution of the last two fractions a little more than one column volume of buffer was needed. The fractions were dialysed against 10 mM potasisum Mops (pH 7.2).

Guanidine · HCl, obtained from Pierce or BDH, was recrystallised in ethanol, benzene and methanol [16].

The cytochrome *b* and cytochrome c_1 concentrations were determined from absorption-difference spectra of the dithionite-reduced (in the case of cytochrome c_1 in the presence of cytochrome *b* the ascorbate-reduced) minus the ferricyanide-oxidized preparations. For cytochrome *b* an absorption coefficient of 25.6 $mM^{-1} \cdot cm^{-1}$ for $A_{563\,nm} - A_{577\,nm}$ was used and for cytochrome c_1 an absorption coefficient of 20.1 $mM^{-1} \cdot cm^{-1}$ for $A_{553\,nm} - A_{539\,nm}$ [13]. The spectral measurements were carried out with an Aminco DW-2 spectrophotometer.

Antimycin A was obtained from the Nutritional Biochemical Corporation. Its concentration was determined from the absorbance at 320 nm using an absorption coefficient of 4.8 $mM^{-1} \cdot cm^{-1}$ [17].

Fluorescence was measured with a Perkin-Elmer fluorescence spectrophotometer MPF-2A. Titrations with antimycin were carried out by the method of Berden and Slater [13,18] using an excitation maximum of 355 nm and an emission maximum of 420 nm for antimycin.

Gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) was performed by the method described by Marres and Slater [19].

Gels were scanned at 550 nm after staining with Coomassie brilliant blue, with an automatic scanning attachment connected to a Zeiss spectrophotometer equipped with a linear scale expander.

For determining the amino acid composition, samples were hydrolyzed with 6 M HCl at 110°C and analyzed with a Beckman Multigraph M liquid chromatograph.

Results

The pentanol-extraction method for determination of antimycin-binding capacity

In each of a number of test tubes, 2 nmol antimycin and 2 nmol presumed antimycin-binding site were mixed together in a total volume of 0.5 ml, and extracted with 0.75 ml distilled pentanol. The extraction was carried out on a Vortex

mixer in the highest speed position, during different times up to 30 s. As reference samples without antimycin were also extracted; if the values between 2 and 30 s extraction were the same, only these two samples were sufficient. After the extraction the test tubes were centrifuged at low speed. Often a band of denatured protein appeared in the pentanol layer. When the band was so broad that there was too little free pentanol left to take a sample, the centrifugation was repeated at higher speed and for longer times. From each pentanol layer a sample of 0.2 ml was taken, diluted with 2 ml distilled ethanol and the fluorescence was measured at an emission maximum of 420 nm. The values of the fluorescence of the samples without antimycin were subtracted from the values of the samples with antimycin.

Fig. 1 shows the result of this method applied to water and the reductase. With water, already after 2 s extraction almost all the antimycin had gone into the pentanol layer. With the enzyme, less than half the amount of antimycin had gone into the pentanol layer and after about 20 s the same value is obtained as with longer extraction times. When the enzyme without antimycin was extracted and the antimycin was added afterwards to the pentanol layer, the same value was obtained. This shows that after 10–20 s all the antimycin has gone into the pentanol layer. When the method was applied to bovine serum albumin, no binding was found, which suggests that the method only gives a positive result if the dissociation constant for the complex with antimycin is less than $1.2 \cdot 10^{-7}$ M, the dissociation constant for the albumin-antimycin complex [18]. However, albumin in the presence of Triton gave a positive result and pentanol-extracted albumin did not enhance antimycin fluorescence like unextracted albumin. Probably pentanol destroys the antimycin-binding site, and this destruction is prevented by Triton.

Antimycin binding to fraction B and fraction C

Fig. 2 shows a gel of the whole enzyme and of fraction B and C obtained after splitting. In agreement with the results of Von Jagow et al. [11] fraction B contains cytochrome *b*. The absorption-difference spectra of fraction B showed that this fraction contains cytochrome *b* and no cytochrome c_1. It also contains subunit 6 (according to the numbering of Marres and Slater [19]) and a little subunit 7 or one of the subunits of band 7. In agreement with the results of Schägger et al. [12] fraction C contains cytochrome c_1, subunits 7 and 8, and also small amounts of the iron-sulphur protein and core proteins. The absorption-difference spectra of fraction C showed that this fraction contains no cytochrome *b*.

Fig. 3 shows the results of the pentanol-extraction method applied to these fractions. The concentration of possible antimycin-binding sites was assumed to be half that of cytochrome *b* in fraction B and the same as that of cytochrome c_1 in fraction C. From the data in this figure it follows that fraction B does not bind antimycin strongly enough to be detected by the pentanol-ex-

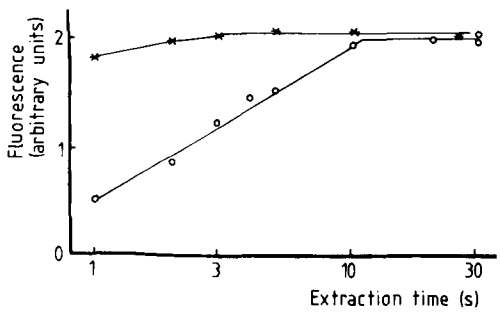

Fig. 1. Binding of antimycin, measured by the pentanol-extraction method. For each point 2 nmol antimycin and 2 nmol binding site were used. ★——★, water; ○——○, cytochrome *c* reductase.

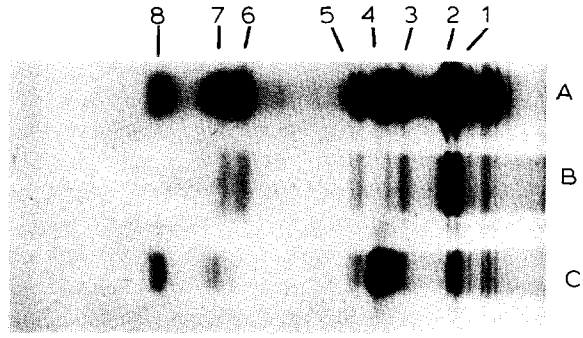

Fig. 2. SDS polyacrylamide slab gel (15%), stained with Coomassie brilliant blue, of (A) intact cytochrome *c* reductase; (B) fraction B and (C) fraction C. The bands of fraction B are somewhat retarded due to the presence of Triton.

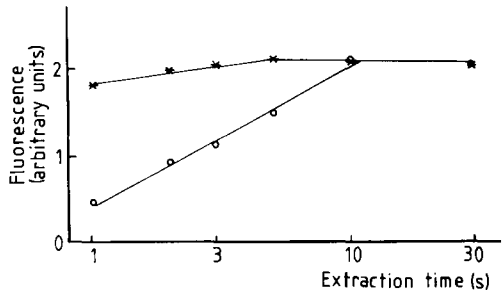

Fig. 3. Binding of antimycin, measured by the pentanol-extraction method. For each point 2 nmol antimycin and 2 nmol possible binding site were used. ★——★, fraction B; ○——○, fraction C.

traction method, and that there is a clear binding in fraction C.

Antimycin titration of fraction C

If fraction C binds antimycin more strongly than albumin does, addition of the former should prevent the fluorescence enhancement of antimycin found when it binds to albumin. A problem was that fraction C was isolated in a buffer containing Triton X-100, and we found that Triton already at low concentrations prevents the fluorescence enhancement of antimycin by addition of albumin. Therefore a fraction C had to be obtained without Triton. After the elution of fraction B from the column the hydroxyapatite was washed with buffer (ii) [11] without Triton and a Triton-free fraction C was obtained with 0.4 M sodium phosphate (pH 7.2). Polyacrylamide gels of Triton-free fraction C contained the same bands as the fraction with Triton. When the Triton-free fraction C was tested with the pentanol-extraction method, it showed binding, but less clearly than in Fig. 3. This suggests that the binding is strengthened by the presence of Triton. Fig. 4 shows that increasing amounts of fraction C inhibit the binding of antimycin to the albumin. This means that antimycin binds to fraction C, in agreement with the results of the pentanol-extraction method. The possibility that the effect on the fluorescence is due to quenching of the fluorescence by fraction C is ruled out by the observation that when Triton-free fraction C in a concentration of 4 μM was added to a solution of 15 μM albumin and 4 μM antimycin, the total fluorescence decrease was only about 35%. When 4 μM

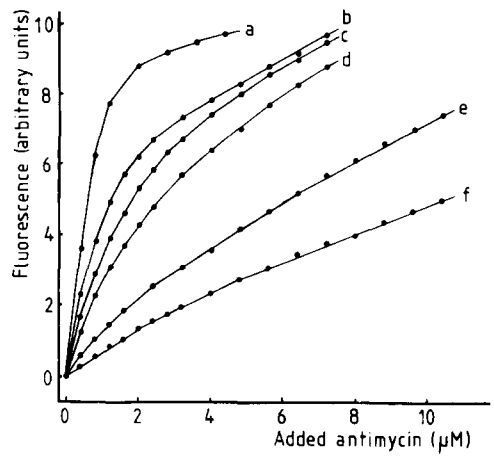

Fig. 4. Binding of antimycin to 1 μM serum albumin and various amounts of Triton-free fraction C: curve a, 0 μM; curve b, 0.5 μM; curve c, 1 μM; curve d, 2 μM; curve e, 4 μM; curve f, 6 μM cytochrome c_1

Triton-free fraction C was added to a solution of 4 μM albumin and 10 μM antimycin (so that the albumin was saturated with antimycin), the fluorescence decreased only from 53 to 46 (arbitrary units). After a few minutes the fluorescence was 41; these results indicate that fraction C can withdraw antimycin from the albumin.

Binding of antimycin to the split products of fraction C

Polyacrylamide gels of fractions D and E (for nomenclature, see Materials and Methods) showed

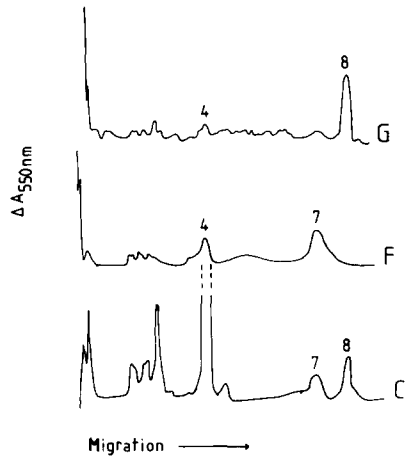

Fig. 5. SDS polyacrylamide slab gel (15%), stained with Coomassie brilliant blue and scanned at 550 nm of (C) fraction C; (F) fraction F; (G) fraction G.

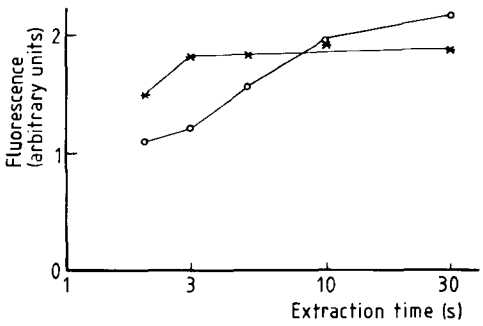

Fig. 6. Binding of antimycin, measured by the pentanol-extraction method. For each point 2 nmol antimycin and 2 nmol possible binding site were used. ★——★, fraction G; ○——○, fraction F.

that these fractions had about the same subunit composition as fraction C. However, fractions F and G each contained mostly one of the small subunits. The concentrations of these subunits were determined by scanning the gels (Fig. 5) and comparing the areas of the peaks with the areas of the corresponding peaks in the gels of fraction C, in which the concentration of all subunits was set to be equal to that of cytochrome c_1, as is also that of the antimycin-binding site. Fig. 6 shows the results of the pentanol-extraction method applied to these fractions. The data show clearly that there is binding in fraction F and none in fraction G, from which it can be concluded that the antimycin-binding site is located in fraction F. A scan of a gel of fraction C at 280 nm after repeated extraction with ether to remove all Triton, and before staining with Coomassie brilliant blue, showed a clear absorption at that wavelength at the position of band 7. Since the hinge protein has hardly any absorption at 280 nm [12], this means that band 7 is derived at least partly from a protein other than the hinge protein. Fig. 7 shows a gel of fraction F at a much higher concentration than in Fig. 5. In this gel another band is visible beteen band 7 and the loation of band 8.

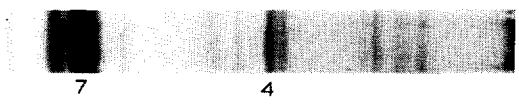

Fig. 7. SDS polyacrylamide slab gel (15%), stained with Coomassie brilliant blue, of fraction F.

TABLE I

AMINO ACID COMPOSITION EXPRESSED IN MOL% OF THE TOTAL AMOUNTS OF AMINO ACIDS WITHOUT CYSTEINE AND TRYPTOPHAN

n.d., not determined.

Amino acid	Fraction *F*	Hinge protein	Non-heme-containing peptide
Asx	9.1	9.6	9.4
Thr	4.9	5.5	5.9
Ser	7.9	5.5	4.5
Glx	19.1	28.8	21.7
Pro	4.4	2.7	3.9
Gly	7.5	1.4	3.6
Ala	6.6	4.1	7.0
Cys	n.d.	(6.8)	(3.0)
Val	5.1	6.8	6.2
Met	1.6	–	0.5
Ile	1.5	–	2.4
Leu	11.1	13.7	12.2
Tyr	1.9	–	2.2
Phe	2.9	2.7	4.1
Lys	5.9	6.8	6.7
His	3.5	4.1	3.3
Arg	7.1	8.2	6.5
Trp	n.d.	–	(1.1)

Amino acid composition of fraction F

In Table I the amino acid composition of fraction F is given. For comparison the composition of the hinge protein is also given [12], and that of the so-called 'non-heme-containing peptide of cytochrome c_1' given in Ref. 20.

Discussion

Although we have used the absorption coefficient for cytochrome c_1 and antimycin given in Materials and Methods, in later experiments Tervoort et al. [21] found a little higher absorption coefficient: 19.2 $mM^{-1} \cdot cm^{-1}$ for the peak of cytochrome c_1 at 552.4 nm, reduced minus oxidized, instead of 17.1 $mM^{-1} \cdot cm^{-1}$ for the peak at 553 nm (and hence 20.1 $mM^{-1} \cdot cm^{-1}$ for the peak-trough difference) used in our laboratory [13]. However, at the same time it was found (Berden, J.A. and De Vries, S., unpublished results) that also the absorption coefficient of antimycin is about 10% higher than 4.8 $mM^{-1} \cdot cm^{-1}$

commonly used [17], so that in most cases the errors compensate each other.

The pentanol-extraction method is based on the reversible binding of antimycin to the binding site and the fact that antimycin is very soluble in organic solvents. The specificity of the method is demonstrated by the fact that there is no positive result when cytochrome *c* oxidase is tested. Attempts to make the method suitable for quantitative measurements of antimycin-binding sites have failed. If the method is carried out with concentrations of antimycin-binding sites less than that of the antimycin, or the binding of antimycin is much weaker than in the intact enzyme, curves are obtained intermediate between those in case of binding and non-binding, shown in Fig. 1. In the case of relatively weak binding the results do not change upon raising the amount of antimycin-binding sites. This is the case with fraction F.

The cytochrome *b* in fraction B obtained after splitting of the enzyme by the method of Von Jagow et al. [11] does not bind antimycin. The Triton-free fraction C, on the other hand, bound antimycin about as strongly as albumin, which binds much more weakly than does the intact cytochrome *c* reductase. The relatively weak binding may be the result of a change of a high-affinity site into a low-affinity site due to the splitting procedure. Another interpretation is that during the splitting procedure the high-affinity site disappears and another, low-affinity site can now be detected, but this is unlikely because such a site has not been found in bovine heart cytochrome *c* reductase.

On splitting the complex into a cytochrome *b*- and a cytochrome c_1-containing fraction by the method of Von Jagow et al. [11], subunit 6 (and some subunit 7) is associated with cytochrome *b* and most of subunit 7 with cytochrome c_1. With a different splitting procedure [19], subunit 6 remains associated with cytochrome c_1 and subunit 7 with cytochrome *b*.

Products obtained by splitting according to the procedure of König et al. [10] did not show a normal binding of antimycin. A crude cytochrome c_1-containing fraction (obtained after the first splitting in that procedure) showed only at high concentrations a little binding with the pentanol-extraction method; in polyacrylamide gels of that fraction there was also a weak band 7 visible. The precipitated cytochrome *b*-containing fraction did not show binding at all. Apparently, the binding capacity is lost by that precipitation, due to denaturation.

Schägger et al. [12] identified their fraction F as the hinge protein, a subunit with a very high content of glutamic acid residues, the amino acid sequence of which has been determined by Wakabayashi et al. [20]. This subunit is thought to play a role in the interaction between cytochrome c_1 and cytochrome *c*. The question is whether our antimycin-binding fraction F contains the same protein. Band 7 has probably been derived from more than one subunit. The stoichiometry to cytochrome c_1 is 2 : 1 [19] instead of 1 : 1 in the case of the antimycin-binding site [22]. The hinge protein has only a faint colour after staining with Coomassie brilliant blue [20] in contrast to subunit 7 in Ref. 19 and in Fig. 2. The staining of the protein in fraction F is weaker than of the subunit 7 in the intact cytochrome bc_1 complex (and fraction B contains a band of maybe 'another' subunit 7) but not a band as weak as one might expect if it was pure hinge protein. Band 7 has also a clear absorption at 280 nm although the hinge protein has not, because of the lack of tyrosine and tryptophan. The amino acid composition shows similarities and discrepancies when compared to that of the hinge protein [20]. The amino acid composition of fraction F resembles that of the so-called 'non-heme-containing peptide of cytochrome c_1' given in Ref. 20, especially the lower content of glutamic acid residues than the pure hinge protein. This 'protein' is probably the hinge protein together with one or more other proteins. Hence we conclude that our fraction F consists also of the hinge protein and another subunit. Maybe the weak band below the strong band 7 in Fig. 7 is the hinge protein.

It is known that the hinge protein is very tightly bound to cytochrome c_1 from which it is very difficult to dissociate [20]. The crude cytochrome c_1-containing fractions obtained by the method of Silman et al. [9] and König et al. [10], therefore contain hinge protein. Those fractions contain native cytochrome c_1, and probably also native hinge protein, but they do not bind antimycin. This makes it unlikely that the hinge protein contains

the antimycin-binding site. Therefore, we suggest that the antimycin-binding protein is that polypeptide in band 7 that appears in our fraction F together with the hinge protein, with a molecular mass of about 12000. This would be in disagreement with the results of Das Gupta and Rieske [3], where the antimycin-binding protein is a subunit that in gel electrophoresis migrates with the highest mobility of the subunits (our band 8). Our results agree, however, with the data reported by these authors in 1982 [5] although they propose that band 7 represents the cytochrome c_1-associated polypeptide. From our experiments it seems probable that the subunit in fraction F on its own binds antimycin with about the same strength as does albumin or a little stronger, but that the binding becomes very strong by the interaction with cytochrome *b*, which is prevented if the cytochrome *b* is modified, as for example in Ref. 7, where the mutants have become resistant to antimycin.

The finding that the antimycin-binding protein is probably a supporting link between cytochrome *b* and cytochrome c_1 [22] is in agreement with the fact that subunit 7 is isolated under some conditions together with cytochrome *b* and under other conditions with cytochrome c_1, as described above.

Acknowledgements

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