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Photoaffinity labelling of the β-methoxyacrylate binding site in bovine heart mitochondrial cytochrome bc_1 complex

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A carbene-generating ^{14}C-labelled β-methoxyacrylate derivative ((*E*)-methyl-3-methoxy-2-[4-(3-trifluoromethyl-3-diazirinyl)benzoyloxyphenyl]propenoate, uniformly labelled with ^{14}C in the benzene ring of the benzoyl group) has been used to locate the proteins involved in binding this class of inhibitors to bovine heart mitochondrial ubihydroquinone:cytochrome *c* oxidoreductase. The β-methoxyacrylate photoaffinity label was shown to be a competent inhibitor of electron transport through the protein complex. Under illumination through a narrow bandpass filter, allowing specific photolysis of the diazirine group, the compound bound to cytochrome *b* and weakly to an 8 kDa polypeptide. Apart from some binding to a cytochrome *b* aggregate, other proteins were left unlabelled. The binding could be prevented in the presence of excess amounts of unlabelled β-methoxyacrylate, myxothiazol or stigmatellin but not by antimycin A or HQNO. At high concentrations DBMIB partially competed for the binding site. The binding site for this class of inhibitors is therefore the 'o-site'. Our results indicate that this site is comprised of residues from cytochrome *b* and possibly the 8 kDa polypeptide and that the site may be close to the Reiske iron-sulphur protein.

Introduction

Respiratory electron transport through the mitochondrial ubihydroquinone-cytochrome *c* oxidoreductase (EC 1.10.2.2, cytochrome bc_1 complex, complex III) can be inhibited at three kinetically distinct sites [1]. The susceptible reactions are: (i) reduction of the iron-sulphur centre, (ii) reduction of cytochrome *b* (at the 'o-centre') and (iii) re-oxidation of cytochrome *b* (at the 'i-centre'). The precise location of these sites within the complex and in relation to the ubiquinol/ubiquinone binding sites is a matter of current controversy. In particular, there are a number of inhibitors of cytochrome *b* reduction which may or may not compete at the ubiquinol oxidation site (o-centre). Myxothiazol, mucidin (strobilurin A) and stigmatellin are active at this site. They cause red shifts in the cytochrome *b* 566-nm absorption band, indicating that at least part of the inhibitor binding site resides on that protein [2,3]. Moreover, yeast strains resistant to these compounds have mutations in the DNA coding for cytochrome *b* [4–7]. The consequent amino acid alterations occur in two distinct regions of the protein. On the basis of a current model derived from secondary structure prediction these two separate regions may fold together on one side of the mitochondrial membrane to form a discrete site [7,8].

However, kinetic experiments have indicated that methoxyacrylate analogues of the above compounds interact non-competitively with quinolic electron donors [9]. Additionally, myxothiazol has been shown to have no effect on the binding of a photoaffinity-labelled ubiquinone [10]. The data suggest that the inhibitor and ubiquinone molecules bind at separate sites. The sites must be spatially close; however, since it is the o-centre related *b*-566 spectrum that is specifically shifted by this class of inhibitors.

It seems likely that the conformation and activity of mammalian cytochrome *b* is dependent on a number of non-prosthetic proteins [11,12]. Indeed, controlled disruption of the bc_1 complex [11] and cross-linking studies [13] have indicated that cytochrome *b* and a 13.4 kDa protein are closely associated. An 8 kDa protein has also been shown to co-fractionate with cytochrome *b* [14]. A structural prediction suggests that either of

Abbreviations: DBMIB, 2,5-dibromomethylisopropyl-*p*-benzoquinone; HQNO, 2-heptyl-4-hydroxy-quinoline *N*-oxide; MOA, β-methoxyacrylate; SDS, sodium dodecyl sulphate; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol.

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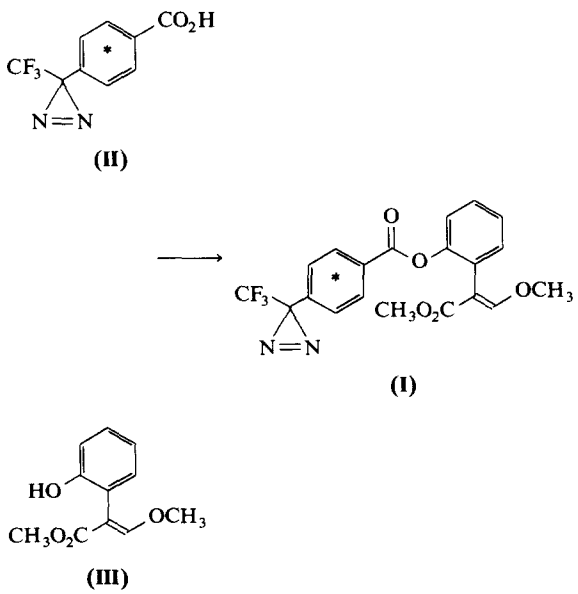
these two proteins may contribute to the 'o'- or 'i'-centres and to proton translocation [11].

The work presented here describes the use of a radiolabelled carbene-generating derivative of a β-methoxyacrylate compound to locate the binding site of this class of inhibitors within the cytochrome bc_1 complex.

Materials and Methods

The β-[^{14}C]methoxyacrylate (I) was prepared by treatment of a solution of the benzoic acid (II) in dichloromethane successively with 1,1′-carbonyl diimidazole and the phenol (III). The benzoic acid (II) was prepared from [U-^{14}C]-4-bromotoluene (Amersham International) by a modification of the route described by Nassal [18]. The phenol (III) was prepared from 2-hydroxyphenyl acetic acid by the method described by Bushell et al. [19]. The ^{14}C labelled ring is indicated with an asterisk. The methoxyacrylate (I) had a radiochemical purity of 96% (17 MBq at a specific activity of 2.59 GBq/mmol). It was stable when stored at −20 °C as a solution in hexane.

The cytochrome bc_1 complex was isolated from fresh bovine hearts according to the method of Hatefi and Rieske [15] and stored at −80 °C in 0.67 M sucrose, 50 mM Tris (pH 8.0) (buffer A). The final protein concentration was assayed by the method of Lowry [16] with the addition of 1% sodium deoxycholate to sample and standard reaction mixtures. Functional unit concentration was determined by reduced-oxidized difference spectrum of cytochrome c_1 at 554 nm ($e = 24.1$ $mM^{-1} \cdot cm^{-1}$). The final concentration was 4.4 nmol cytochrome c_1 per mg protein, which is characteristic of this type of preparation [1]. Electron-transport activity of the complex was measured by addition of ubiquinol 2 at 33 μM final concentration to buffer A containing in addition 0.1% bovine serum albumin, 0.2% horse heart cytochrome *c* and cytochrome bc_1 complex at 25 μg protein per ml. Absorbance changes were measured at 550 nm vs. 557 nm in a Shimadzu UV 3000 dual-wavelength/double-beam spectrophotometer. Correction was made in all subsequent calculations for a slight uncatalyzed reduction of cytochrome *c* (i.e., the rate with no bc_1 complex present).



Scheme I

Illumination of samples was carried out in a Hanau Suntest providing irradiance of 14 W/m^2 at 360 nm, the total radiation intensity between 300 and 630 nm being 830 $W \cdot m^{-1}$. The temperature within the illumination chamber was 25 °C. Photolysis of non-radiolabelled MOA was carried out in methanolic solution in either a sealed quartz dish or in an opaque dish covered with a Spindler and Hoyer UGI filter giving 57% transmission at 360 nm. Progress of photolysis was followed between 200 and 400 nm in a Cary 210 spectrophotometer.

Radiolabelled MOA was diluted in methanol to give a stock concentration of 131 μM. 5 μl aliquots were then added to 1 ml of buffer A containing in addition cytochrome bc_1 complex at 0.295 nM, cytochrome c_1 and with or without other compounds as detailed in figure legends. Samples were then shielded by UG1 filters and illuminated in the Suntest for 10 min at 25 °C. Similar samples were kept in the dark at room temperature. Following incubation, samples were transferred to centrifuge tubes and made up to 50% saturated ammonium sulphate by addition of an equal volume of saturated neutralised solution. Samples were mixed, stood on ice for 10 min then centrifuged at 75 000 × *g* for 30 min. The supernatants were decanted and samples spun again at 10 000 × *g* for 2 min in order to consolidate the pellets and aid removal of any remaining supernatant.

For electrophoresis, pellets were resuspended by vortexing for 30 s in 30 μl of a solution containing 10 mM Tris (pH 8.0), 1 mM ethylenediaminetetraacetic acid, 2.5% SDS, 0.01% bromophenol blue, 5% β-mercaptoethanol and 12% glycerol. Samples were incubated at room temperature for 10 min before electrophoresis. Proteins were stacked at 30 V for 3 h in a 1.5 mm thick 4.5% acrylamide zone with 3% crosslinking. The resolving gel consisted of 16% acrylamide with 2% crosslinking in which the samples were separated at 100 V overnight at 6 °C. The buffer in both gel zones was 0.112 M sodium acetate, 0.112 M Tris (pH 6.4). The cathode buffer was 0.1 M Tris, 0.1 M tricine, 0.1% SDS (pH 8.0). The anode buffer was 0.2 M Tris (pH 8.9). The method of Schagger and Von Jagow [17] proved to be successful at resolving lower-molecular-weight proteins. However, using this method we could not obtain satisfactory separation of cytochromes *b* and c_1 for

later interpretation of fluorographs. We therefore adopted the method given above. Following electrophoresis gels were either (i) blotted (LKB Novablot) on to nitrocellulose (pore size, 0.2 μm) followed by protein visualization with amido black and then sprayed with Amplify (Amersham International) or (ii) fixed, stained and destained according to Ref. 17 then soaked for 10 min in amplify prior to drying under vacuum. Blots or dried gels underwent fluorography with Hyperfilm-MP (Amersham International) backed by a GRI Rapid-X intensification screen (Genetic Research Instrumentation Ltd.) at $-80\,^{\circ}C$ for 3 weeks.

Similar gels were run using preparative well combs to resolve the equivalent of 12 individual samples. Following staining and destaining, protein bands were excised, freeze-dried and combusted (Packard Tri-carb sample oxidizer giving 91% combustion efficiency) prior to scintillation counting.

Results

The spectra in Fig. 1 show the progression of photolysis of the 'cold' photoaffinity labelled β-methoxyacrylate when illuminated with or without a UG1 filter. It was envisaged that the filter would be necessary in later experiments to prevent photodamage to the cytochrome complex. The diazirine group has a peak at 348 nm with shoulders at 330–340 and 360–370 nm [18]; these features could clearly be seen in the spectra shown here. Photolysis without the filter (Fig. 1a) led to a loss of these bands and concurrent appearance then disappearance of a band absorbing at 300 nm. The latter effect was due to formation then photolysis of a diazo moiety from the original diazirine. In the presence of the filter (Fig. 1b), photolysis of the diazirine group was relatively unaffected (full decay in about 6 min). However, the diazo group decayed only slowly, photolysis being complete within 40 min. This was not envisaged as a potential problem in binding studies since only about 20% of the diazirine groups take this route to the active carbene [18].

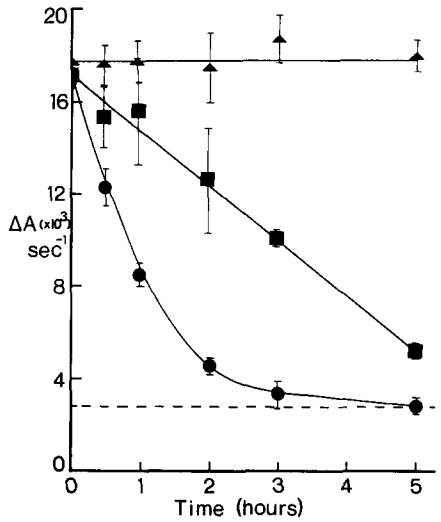


Fig. 2. The effect of illumination on the ubiquinol/cytochrome *c* oxidoreductase activity of isolated cytochrome bc_1 complex. Samples were prepared in 'buffer A' with 0.1% bovine serum albumin and cytochrome complex at 0.2 μM cytochrome c_1 and placed in a Hanau Suntest in quartz dishes with (■———■) or without (●———●) shielding by the UG1 filter. A control sample (▲———▲) was kept in the dark at room temperature. Samples were taken at the given intervals and horse heart cytochrome *c* added to 2 mg per ml. The reaction was started by addition of 33 μM ubiquinol 2 and absorbance changes followed at 550 nm (with 557 nm reference beam). The dashed line represents the rate of uncatalysed cytochrome *c* reduction in the absence of cytochrome bc_1 complex. Error bars indicate the standard deviation from three separate experiments.

Use of the filter to protect the biological sample was necessary, as can be seen in Fig. 2 in which electron-transport activity of the bc_1 complex following pro-

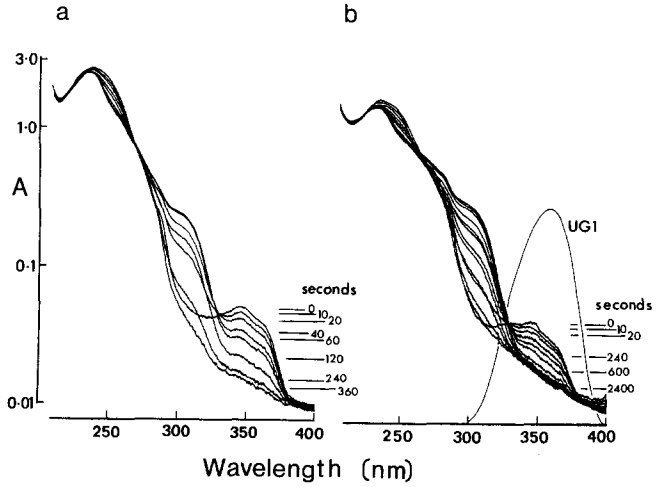


Fig. 1. Progression of photolysis of non-radiolabelled photoaffinity β-methoxyacrylate in the absence (a) and presence (b) of protection by a UG1 glass filter. Compound was added to methanol giving a 16.3 μM solution in a quartz dish with a grease sealed lid. Illumination of samples was carried out in a Hanau Suntest apparatus and samples taken for assay at the specified times.

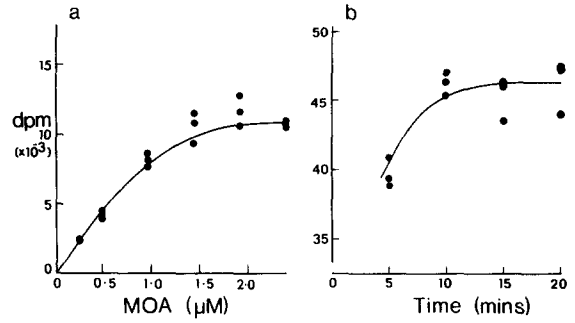


Fig. 3. The effect of (a) dose and (b) time of incubation on binding of radiolabelled photoaffinity β-methoxyacrylate to cytochrome bc_1 complex. Samples in (a) were illuminated for 10 min, the β-methoxyacrylate dose level in (b) was 0.6 μM. Other conditions were as in Materials and Methods. Samples were recovered by precipitation twice with 50% ammonium sulphate prior to scintillation counting.

longed illumination is presented. After 10 min the unprotected sample had lost over 10% activity whereas the filter-protected sample had lost only 1% activity. As noted above, a 6–10 min illumination was required for photolysis of the β-methoxyacrylate. In order to be sure that the photoaffinity label was binding to active centres we used the UG1 filter in all studies.

The concentration of photoaffinity labelled β-methoxyacrylate required to inhibit 50% of ubiquinol-cytochrome c_1 oxidoreductase activity was found to be 1.37 moles per mol cytochrome c_1. This ratio of inhibitor to sample was adopted in later experiments in an attempt to obtain adequate labelling of the MOA binding site yet with minimised non-specific attachment. In a dose-response trial using radiolabelled MOA, this level of inhibitor was found to give about 70% of the saturated level of binding to the cytochrome bc_1 complex as a whole unit (Fig. 3a). Similarly, illumination for 10 min proved to be the exposure time beyond which no further binding occurred (Fig. 3b).

We were attempting to use the photoaffinity-labelled MOA to characterise the binding site of this general class of inhibitors. It was therefore necessary to demonstrate that the photoaffinity label blocked electron transfer at the appropriate location and perturbed the cytochrome *b* spectrum in a predictable fashion. Addition of succinate to mitochondria led to reduction of cytochromes *a*, *b* and c_1 and appearance of the appropriate α-bands (Fig. 4a, reduction–oxidation). Following addition of the photoaffinity-labelled compound, only cytochrome *b* remained reduced by succinate. In common with other MOA's therefore the compound inhibits electron flow between cytochromes *b* and c_1. The MOA's are known to bind at the 'o-centre' of the cytochrome bc_1 complex causing a red shift in the *b*-566 spectrum [9]. On the other hand, antimycin binds

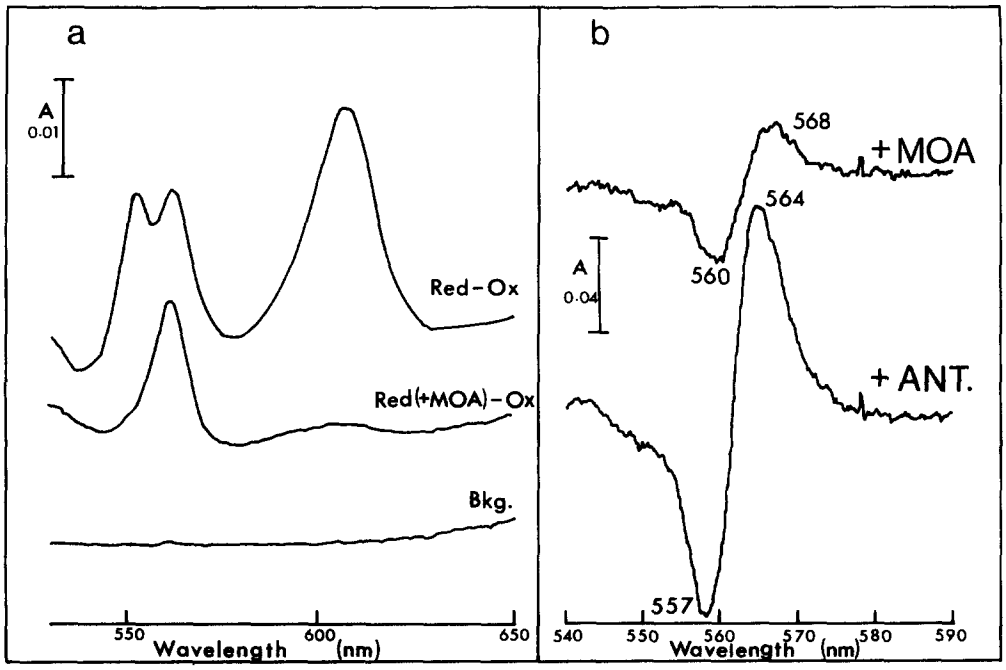


Fig. 4. The effect of affinity labelled β-methoxyacrylate on (a) reduction of mitochondrial cytochromes and (b) the spectrum of cytochrome *b*. In (a) bovine heart mitochondria (16 mg protein per ml) were added to a solution containing 0.25 M sucrose, 50 mM Tris-HCl (pH 8.0) which was split between reference and sample cuvettes and the background (Bkg.) spectrum recorded. Sodium succinate (4 mM) was then added to the sample cuvette in the presence (middle trace) or absence (upper trace) of 16 μM β-methoxyacrylate. In (b) sample and reference tandem cuvettes were prepared, bovine heart mitochondria were added to give 16 mg protein per ml and reduced with excess sodium dithionite. Inhibitor (10 μM antimycin) was added to sample mitochondria and reference buffer whilst an equivalent volume of ethanol was added to reference mitochondria and sample buffer (lower trace). The band shift so obtained was reversed by addition of the same amount of antimycin to reference mitochondria, the above procedure was then repeated using 40 μM β-methoxyacrylate instead of antimycin (upper trace).

at the 'i-centre' of the bc_1 complex causing a spectral red shift of the *b*-562 haem. In order to determine whether the affinity labelled compound bound at the same site as other MOA's, its effect on the reduced cytochrome *b* difference spectrum was examined (Fig. 4b). Initially antimycin A4 (Sigma Chem. Co.) was added at saturation levels to the sample giving a red shift with a characteristic peak at 564 nm and trough at 557 nm. A similar amount was then added to the reference cuvette to cancel out this effect. The affinity labelled MOA was then added and a band shift with peak at 568 nm and trough at 560 nm, indicative of binding at the 'o-centre', was observed [24,25]. The reverse experiment, in which binding sites were first saturated with affinity-labelled MOA, then with antimycin, produced similar band shifts (Results not shown). We therefore conclude that the compound being used here affects electron transport in the same manner and binds at the same site as other β-methoxyacrylate-type inhibitors.

Having established the spectral effects of binding the photoaffinity-labelled MOA and the appropriate exposure and dose conditions, we investigated the proteinaceous binding site. Resolution of cytochromes *b* and c_1 on SDS-polyacrylamide gels has consistently proved problematic. In particular, cytochrome *b* runs anomalously in different gel types and with different pre-treatments [20,21]; this problem was discussed extensively in Ref. 1. We used the tetramethylbenzidine/H_2O_2'haem-staining' technique [22] to identify the location of cytochrome c_1 in a variety of different gel systems. We were unable to satisfactorily resolve cytochrome c_1 from cytochrome *b* using the classical Laemmli method [23] or the Schagger system [17] (see also Fig. 6 in [9]). The method we finally adopted gave us the greatest resolution of cytochrome *b* from other bands. However, cytochrome c_1 had mixed mobility giving diffuse haem-containing bands between 28 and 31 kDa (Fig. 5, band IV). When radioactivity in the stained bands was assayed by scintillation counting a clear pattern emerged. Only three bands showed labelling significantly higher than the background count. These were a band at 66 kDa (labelled 'Agg'), band III (cytochrome *b* at 33 kDa) and band IX (at 8 kDa). Using a variety of pretreatments (warming, boiling, ±SDS, ±β-mercaptoethanol, Results not shown), we concluded that the 66 kDa band was a doublet of cytochrome *b* that appeared upon heating. Cytochrome *b* and this aggregate between them bound about 80% of the total label detected on the gel. All other bands individually bound about 2% of the total label except for the 4% bound to band IX. The incorporation of radiolabelled compound was high; 2.14% of the labelled β-methoxyacrylate added to samples became bound to cytochrome *b* and its aggregate. This equates to 10% of the available sites being occupied. This result appears to

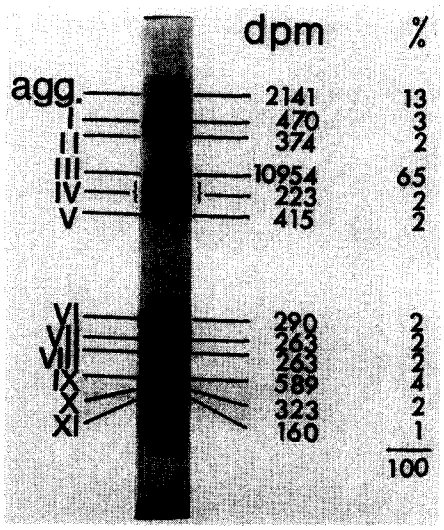


Fig. 5. SDS-polyacrylamide gel electrophoresis of photoaffinity labelled cytochrome bc_1 complex. Following electrophoresis gels were stained and destained then prepared for scintillation counting as described in Materials and Methods. The results, in disintegrations per min, are the average of five separate radiolabelling experiments.

be unequivocal: the β-methoxyacrylate-type inhibitors bind primarily to cytochrome *b* with a minor component on an 8 kDa polypeptide.

In order to locate the binding site more precisely, we carried out photoaffinity labelling subsequent to pre-incubation of samples with various other inhibitors of Complex III activity (see Fig. 6). Following electrophoresis, gels were either electroblotted on to nitrocellulose or dried prior to fluorography. Either technique has some disadvantages. With blotting, the transfer of higher molecular weight proteins was significantly less efficient than smaller polypeptides. This meant that comparison of the intensity of different bands on fluorographs could not be quantitative. On the other hand, when gels were soaked in fluorographic reagent before drying, we found that some polypeptides, especially the smaller ones, became diffuse and in some cases began to elute from the gel. However, with these considerations in mind, the fluorographs from both methods gave comparable results. Track a of Fig. 6 pictorially illustrates the results given in Fig. 5 for binding of the mOA. The major site of binding was cytochrome *b* and its aggregate; a minor site was detected on band IX. There was also a broad area of label running at the bottom of the gel in the position where lipids and SDS electrophorese. The labelling here was partly the result of covalent binding to lipids and detergent present in the sample but also to non-specific adsorption of the chemical to the precipitated material. This may explain the uneven extent of labelling in this position between samples. Track b contains a sample that was incubated with the photoaffinity label in the dark; no binding occurred under these conditions. The samples in tracks c–i were incubated with various inhibitors of mitochondrial elec-

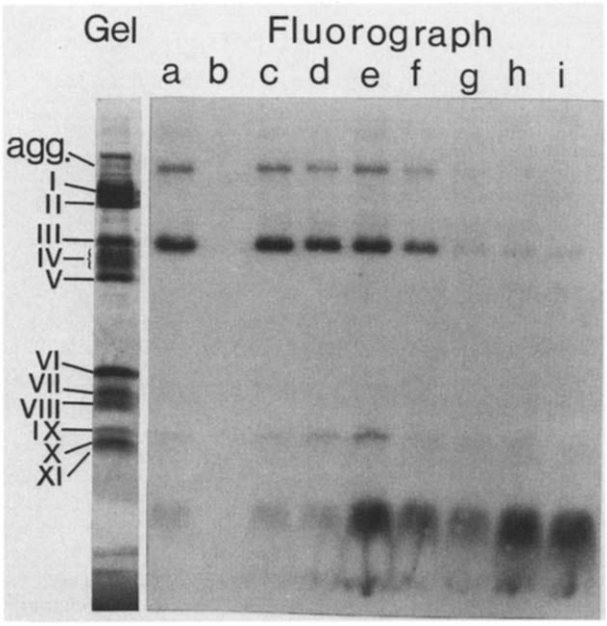


Fig. 6. The effect of various mitochondrial electron transport inhibitors on binding of the radiolabelled photoaffinity MOA to cytochrome bc_1 complex. Samples were pretreated, electrophoresed and subjected to fluorography as described in Materials and Methods. This example is from a dried gel. The various treatments were (a) control; (b) dark, and pre-incubation with (c) 6 μM antimycin A4; (d) 60 μM HQNO; (e) 60 μM DBMIB; (f) 600 μM DBMIB; (g) 6 μM (*E*,*E*)-methyl 3-methoxy-2-(styrylphenyl)propenoate; (h) 6 μM myxothiazol and (i) 6 μM stigmatellin.

tron transport before addition of the photoaffinity label and subsequent illumination. The competing compounds were added at 10 times the rate of the labelled β-methoxyacrylate except for HQNO (track d) at 100 × and DBMIB (tracks e and f) at 100 and 1000 ×. This was in accordance with the inhibitory efficiency of each chemical relative to antimycin A as noted in Ref. 1. It can be seen that inhibitors at the 'i-centre', antimycin (track c) and HQNO (track d), did not prevent binding of the labelled β-methoxyacrylate. Similarly DBMIB at the lower dose (track e) had no effect. However, at a higher rate (track f) it prevented about 50% of labelling. This may suggest that the DBMIB binding site, presumed to be associated with the iron-sulphur protein, is adjacent to the β-methoxyacrylate binding site. The sample in track g was preincubated with a 'cold' β-methoxyacrylate ((*E*,*E*)-methyl 3-methoxy-2-(2-styrylphenyl)propenoate [9,19]) and binding of the radiolabelled compound was effectively inhibited. This indicates that the binding site of the photoaffinity label was identical to other β-methoxyacrylates. It is therefore a valid probe. Tracks h and i were the result of pre-incubation with myxothiazol and stigmatellin respectively. These 'o-centre' inhibitors significantly reduced the binding of radiolabelled compound suggesting that β-methoxyacrylates, myxothiazol and stigmatellin bind at the same site.

The effect of competing inhibitors on binding to cytochrome *b* in the band III position was mirrored by changes in labelling on the aggregated protein in all tracks. This was partly also the case with binding to the 8 kDa protein, band IX. In the instance shown in Fig. 6 the 'o-centre' inhibitors in tracks g, h and i appeared to have competed out the radiolabelled compound. However, the effect was not as marked as that upon cytochrome *b* (band III). Indeed, in other experiments not shown here, the extent of displacement was variable. This may be because the 8 kDa protein is a peripheral part of the 'o-centre' binding site or may be related to the flexible nature of the photoaffinity label.

Discussion

We have prepared a ^{14}C-labelled, carbene-generating derivative of a β-methoxyacrylate compound in order to investigate the binding site within the mitochondrial cytochrome bc_1 complex of this class of inhibitors. It was shown by spectroscopy that the compound interacts with the protein complex in the same way as other members of its class and therefore provides a valid probe for the location of the binding site. Our main finding was that the compound binds almost exclusively to cytochrome *b* with some binding to an 8 kDa polypeptide. There was almost no non-specific binding to other proteins. This result provides structural evidence to support existing kinetic, spectral and molecular biological data regarding sites of inhibition within the bc_1 complex. Inhibitors of mitochondrial electron transport known to bind at the so-called 'o-centre' were able to compete out the photoaffinity label confirming that this centre is primarily located on cytochrome *b*.

The 8 kDa polypeptide may also contribute to this binding site. In this respect it is interesting that this protein has been previously associated with cytochrome *b* following fractionation experiments [14] and structural prediction studies [11]. The relatively light labelling of the 8 kDa polypeptide in relation to cytochrome *b* need not suggest that this protein is only a peripheral part of the binding site. Instead it may be a consequence of the structure of our probe. There are two features of the molecule that should be considered. Firstly, the diazirine group is distal to the methoxyacrylate group. Therefore, the covalent bond formed with the protein upon photolysis will be at a point approx. 0.85 nm from the attachment site of the β-methoxyacrylate. Secondly, the molecule is extremely flexible about the bonds connecting the two benzene rings. One can envisage that when located in the binding site the molecule may exist in a range of configurations in which the diazirine group only infrequently lies adjacent to the 8 kDa protein. Close association of the 8 kDa protein with an active centre is suspected, since it has been shown that this is the DCCD-binding protein

and is therefore involved with proton translocation [14].

We have found that at high relative concentrations DBMIB will partly compete with the β-methoxyacrylate inhibitor. One possible explanation for this effect is that there may be a limited spatial link between the 'o-centre' and the iron-sulphur protein which is the site of inhibition by this compound. However, the photoaffinity β-methoxyacrylate did not label the iron-sulphur protein as might have been expected if that were the case. Alternatively, the photoaffinity labelled MOA may have bound directly to the DBMIB molecules present in such large excess. The methoxyacrylate molecules would not then have been able to enter an otherwise vacant binding site on the protein. We are not able, therefore, to make any firm conclusions about the relative locations of the DBMIB and MOA binding sites.

It has been proposed [9] that the β-methoxyacrylates inhibit non-competitively with ubiquinone so that use of this class of compounds in mutant studies might not give adequate information about the in vivo quinone binding site. We therefore intend to use the radiolabelled photoaffinity β-methoxyacrylate to carry out competition experiments with ubiquinone in order to investigate the extent of overlap of the binding sites. Synthesis of a compound with the photoaffinity group closer to the β-methoxyacrylate binding group will also enable us to determine the proximity of the 8 kDa polypeptide to the 'o-centre' and the orientation of the bound molecule within the site. Additionally, it should now prove possible to sequence labelled band III in an attempt to determine some of the amino-acids present in the β-methoxyacrylate binding site. This will help to further elucidate the three dimensional structure of cytochrome *b*.

In conclusion, these experiments have shown that an affinity labelled β-methoxyacrylate bound specifically to cytochrome *b* with a minor attachment to an 8 kDa protein and that the binding site is part of the so-called 'o-centre'.

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