In contrast, the hydrophobic group attached to the \( \text{E}-\beta\)-methoxyacrylate group is of crucial importance in conferring the site specificity. All active compounds of both \( Q_1 \) and \( Q_i \) sites have two aromatic rings in this group; those with an altered, or only one, ring structure are invariably without effect. This is also true if there are more than three rings, with the possible exception of an extremely weak \( Q_i \) effect on MA 13. It appears that the connection between these two rings is critical for site specificity. An inflexible (MA19) or direct (MA1,2,3) link between them causes them to be \( Q_i \) effectors. A more flexible \(-\text{C} = \text{C}-\) or \(-\text{O}-\)linkage causes them to be \( Q_1 \) effectors. The detailed molecular reason for these effects must await an accurate determination of the three-dimensional structure of this enzyme. Nevertheless, these results already allow us to predict with some confidence the likely \( Q_1 \)-site specificity of other related derivatives of these compounds.

It is already known that MOA-stilbene is a powerful \( Q_1 \)-site effector in the mitochondrial \( bc_1 \) complex [17], in sharp contrast to its weaker \( Q_i \) effect in the thylakoid cytochrome \( b_f \) complex. Some further clarification of this rather unexpected difference might be gained with a study of the \( Q_1 \)-site specificity of the present range of analogous compounds on the cytochrome \( bc_1 \) complex.


Received 27 July 1993

We have the key, can we build a lock? A strategy for the elucidation and molecular modelling of the methoxyacrylate (\( Q_1 \)) binding site of cytochrome \( b \)

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The control of phytopathogens by the natural products myxothiazol, oudemansin and mucidin, known collectively as the methoxyacrylates [1], has provided the impetus for programmes to exploit this series of compounds for agrochemical usage. This can be seen from the number of patents and the range of companies submitting them, together with the announcement of development candidates by BASF [2] and ICI (ZENECA) Agrochemicals [3].

As is often the case much was known about these methoxyacrylate inhibitors. Intrinsic activity studies identified the key features required for inhibitory activity. Molecular modellers and physical chemists at Jealott’s Hill provided data on the conformation of these compounds, which agreed with published X-ray structure of oudemansin [4]. Thus the size and shape of the ‘key’ was known. But what of the ‘lock’ that it fitted? This paper will discuss a biochemical approach to deduce the nature of the lock. It will discuss how molecular genetics, affinity labelling, enzymology, spectroscopy and inhibitor structure–activity studies will be used to assemble a molecular model. What is the reason for wishing to identify the methoxyacrylate-binding site? The answer is that from a precise knowledge of the
chemical environment into which the inhibitor binds, it should be possible to predict the conformation, hydrophobic and electrostatic interactions of inhibitors. This should allow synthetic chemists to focus resources into developing compounds that will be inhibitors at the active site. Using biological data that have already been accumulated also ensures that the compounds have the correct physico-chemical properties to be active in vivo. Such information should enhance the search for new areas of chemistry and the prospects of discovering second- and third-generation compounds.

The mode of action of the natural products mucidin, oudemansin and myxothiazol has been determined as mitochondrial electron transport inhibition - specifically the Q, site of the bc1 complex [1, 5, 6]. Cytochrome b was implicated as the target site both by genetic and biochemical studies. Inhibitor-resistant loci were located on the cytochrome b gene [7]. Difference spectroscopy showed that the addition of myxothiazol or mucidin to dithionite-reduced bc1 complex caused a change in the absorption spectrum of cytochrome b [1]. These data, together with enzymological studies [8], led to the conclusion that the Q, site of the bc1 complex was being blocked. Thus the identity of the lock was given as cytochrome b.

A simple structure of cytochrome b was provided from sequence comparisons and secondary structure predictions [9, 10]. Nine transmembrane a-helices were identified, as well as four invariant histidines, which were proposed as the haem-binding ligands. How would it be possible to build upon these homology and secondary-structure predictions, to order further the transmembrane helices of cytochrome b in the 'two-dimensional membrane', and to identify the methoxyacrylate binding site? A possible way forward lay with molecular genetics. Yeast mutants resistant to inhibitors that act at the bc1 complex had been genetically analysed, and the loci mapped on to the cytochrome b gene. The inhibitors were antimycin A and diuron, which are Q-site inhibitors, and the Q,o-site inhibitors, mucidin and myxothiazol. There were two or three loci on the cytochrome b gene that conferred resistance to each of the bc1 complex inhibitors. Thus it was proposed that when cytochrome b folded into its native form, these separate parts of the protein would come together to form the inhibitor-binding site.

The amino acid changes resulting in resistance to Q, and Q,o-site inhibitors can be determined by sequencing the cytochrome b gene from the various resistant mutants. Thus the sequencing of these mutants should allow the localization of the domains that form the Q, and Q,o sites, and so permit the a-helices to be ordered. The data from DNA sequencing of resistant mutants [11, 12], together with further secondary-structure predictions [13], called into question the nine transmembrane helices model of Widger and Saraste and a new eight transmembrane helices model evolved [14, 15].

The DNA sequencing of inhibitor-resistant cytochrome b genes may identify the regions of cytochrome b that form the binding site, but this information does not determine which amino acids are interacting with the inhibitor. To discover which amino acids interact with specific groups of the inhibitor, an affinity-labelling study is required. This would also confirm that the mutations conferring inhibitor resistance are at the binding site and not having a long-distance effect.

A diazarine photo-affinity label was selected because of the reactivity of the carbene generated upon illumination [16]. The Q, site was believed to be hydrophobic in nature; therefore, an affinity label capable of attacking non-polar bonds was needed. Studies were carried out on model compounds to determine the photostability of potential carrier inhibitors. Thus only the photo-affinity group should be effected upon illumination. Data from the effect of inhibitor structure upon activity, and the feasibility of synthesis, were used in deciding upon the nature of the inhibitor. Thus the photo-affinity-labelled methoxyacrylate IV was synthesized (Figure 1). Biochemical and photo-affinity-binding studies [17] showed that IV inhibited the Q, site of the bc1 complex and bound specifically to cytochrome b of the bc1 complex. To gain an idea of how the methoxyacrylates orientate within the binding site, a second affinity-labelled compound V was prepared. Here the affinity label is connected to a naphthalene-methoxyacrylate. The rationale for the use of this second compound was to put the affinity label closer to the pharmacophore, so that different amino acids would be labelled. It should be possible to deduce from a comparison of the labelling patterns the orientation of these methoxyacrylates within the binding pocket.

After affinity labelling of the bc1 complex with IV, cytochrome b was separated and isolated from the other bc1 complex subunits using SDS/PAGE and then electro-eluted (BioRad 422). The isolated cytochrome b was then digested with Staphylococcus V8 protease, and the resulting peptides separated using SDS/PAGE. A labelled peptide of approximately 17 kDa, which appeared as a single band on high-resolution gradient Phastsystem gels (8–25%)
(Pharmacia), was found. The sequencing of this labelled 17 kDa peptide revealed the sequence TGSNNPTGISSDV, thus implying that the cleavage point is at position 203. The peptides comprising the first 200 residues have not yet been identified. Thus it appears that the diazarine of the affinity-labelled methoxyacrylate inhibitor is close to an amino acid(s) in the carboxyl half of cytochrome b.

Biochemical studies of the \( bc_1 \) complex have identified amino acids that are close to the cytochrome \( b \) haem or inhibitor-binding sites. Characterization of known inhibitor-resistant mutants using kinetics and circular dichroism (c.d.) spectroscopy have shed light on the composition of binding sites [18]. These studies suggest that Phe-129 is not close to haem \( b_{560} \) since, when it is replaced by leucine, no alteration in the c.d.-spectrum is observed. The mutation that results in the change of Ile-147 to Phe does change the c.d.-spectrum, suggesting that Ile-147 is in the region of the \( b_{560} \) haem. By determining the sensitivity of \( bc_1 \) complexes from diverse species, for which the cytochrome \( b \) gene has been sequenced, to various inhibitors it is possible to make deductions about amino acids that may involved in the binding site [19].

The relationship between structure and inhibitory activity of the methoxyacrylates can provide data about the shape of the binding site, and possible electronic interactions between the protein and inhibitor. Examples of how such studies can be used for model building are given below. Inhibitors based on mucidin can be divided into two regions: the 'pharmacophore', which is the methoxyacrylate, and the 'backbone', which represents the rest of the inhibitor.

The importance of the pharmacophore has been discussed previously [20]. Many other variations to the pharmacophore (not mentioned here) result in a loss of activity. Thus it is believed that the methoxyacrylate pharmacophore fits precisely into its binding site. Combining these data with deductions on how the myxothiazol III and mucidin I pharmacophores are accommodated by the same binding site can be used to define the requirements of the binding site. This has led to a proposal that the GQMS or TVIT tetrapeptides of cytochrome \( b \) may bind the ester/amide of these toxophores [21]. It has been revealed that ethyl ketones and methyl amides can replace the ester moiety, to produce active compounds, when the vinyl ether is replaced by an oxime methyl ether as illustrated by pharmacophore II (H. Sauter and F. Rohl, unpublished work).

The backbone not only contributes lipophilicity to carry the pharmacophore into the \( Q_{b} \)-binding site, it also has steric requirements for optimal activity. By determining the activity of inhibitors with restricted conformational freedom, the shape of the binding cavity can be deduced. The further probing of this cavity with more flexible
Table I

Inhibition of lamb heart mitochondrial respiration

*IC*₅₀ is the dose required to inhibit the rate of oxygen consumption of lamb heart mitochondria by 50%.

<table>
<thead>
<tr>
<th>Compound</th>
<th><em>IC</em>₅₀ (µM)</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>VI</td>
<td></td>
<td>![Structure VI]</td>
</tr>
<tr>
<td>o-Stilbene</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>m-Stilbene</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>p-Stilbene</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>Z-Stilbene isomer</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>VII</td>
<td></td>
<td>![Structure VII]</td>
</tr>
<tr>
<td>Acetylene</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>VIII</td>
<td></td>
<td>![Structure VIII]</td>
</tr>
<tr>
<td>Dibenzoalane</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>IX</td>
<td></td>
<td>![Structure IX]</td>
</tr>
<tr>
<td>m-Terminal phenoxy</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td>p-Terminal phenoxy</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>X</td>
<td></td>
<td>![Structure X]</td>
</tr>
<tr>
<td>m-Terminal phenethoxy</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>p-Terminal phenethoxy</td>
<td>0.9</td>
<td></td>
</tr>
</tbody>
</table>

compounds allows the limits of the binding site to be explored. The rigid backbones of the acetylene VII and dibenzoalane VIII, confer good activity and a known conformation (see Table I for activity). The stilbene VI can also fit into this shape, but its *m*- and *p*-isomers show an increasing loss of inhibitory activity (30-fold and 700-fold, respectively) indicating that there is a 'ceiling' to the binding site. The *Z*-isomer of VI (with respect to the stilbene) is one hundred times less active than its *E*-isomer. Here the terminal phenyl of the *Z*-isomer is protruding into either the ceiling or the side of the binding site. The binding pocket can now be probed with the more flexible ether-linked compounds such as IX and X. The *m*-substitution of the terminal phenyl in compounds IX and X confers greater inhibition than the *p*-substituted isomers. The more flexible three-ring phenyl ether structures can adopt conformations similar to those of VI, VII and VIII. This is achieved by the central phenyl being orthogonal to the acrylate phenyl with the terminal phenyl in the same plane as the acrylate phenyl. The steric volume of the binding-site inhibitors may have to adopt conformations that are not of the lowest energy or distort the binding site; both of these effects will contribute to a decreased inhibitory activity. The methoxyacrylate structure–activity data have provided a tool that can be used to
validate putative binding-site models, such as tight steric requirements for the pharmacophore and hydrogen-bonding interactions, correct volume and shape to accommodate the various backbones, and possible hydrogen bonds to the aromatic rings of the backbone.

Molecular models of cytochrome b can be constructed from spacefilling or molecular graphics based on the above studies. The transmembrane helices can be ordered by forming a four α-helix bundle around the two haems [22]. The other helices can be positioned based on the results of molecular genetic studies [11, 12, 23] and data from homology comparisons can be used to further define the cytochrome b structure (M. Degli Esposti et al., unpublished work). The modelling of the binding site is more difficult since, from the mutation studies, it appears to be formed by the extra membrane loops. The mitochondria prepared from yeast mutants with Gly→Arg, Asn→Tyr and Leu→Ser were all resistant to a phenyl methoxyacrylate. An interpretation of these observations is that the three mutation sites are close to, or affecting, pharmacophore binding, but nothing is known about the amino acids that bind the backbone. The affinity-labelling study was undertaken to provide this data and further progress towards building the methoxyacrylate binding site will be dependent on obtaining such information.

What is naturally bound in the methoxyacrylate site? Is it ubiquinol? Are these inhibitors binding in an analogous manner to that seen with herbicides that compete for the Q<sub>i</sub> site of photosystem II? The affinity-labelling study showed that one of the smaller subunits was labelled above background levels [17]. Does part of this subunit occupy the Q<sub>i</sub> site and act as the controlling factor for ubiquinol oxidation? If so, there should be a domain in either the iron-sulphur protein, or cytochrome c<sub>b</sub>, of bacterial bc systems, that is analogous to this subunit.

Miss G. Cooper, Mrs B. J. Jager and Dr R. M. Mansfield, ICI (ZENECA) Agrochemicals are thanked for assistance with biochemical studies; Dr M. Hutchings (ICI, Blackley), Mr E. Savins, and Mr K. Beaumont ICI (ZENECA) Agrochemicals, for synthesis of affinity-labelled compounds; Dr A.-M. Colson-Corbisier, Louvain la Neuve and Dr M. Degli Esposti, Bologna for collaborative research; and Dr M. Davison, ICI (ZENECA) Pharmaceuticals for peptide sequencing.