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The insecticide DDT targets the OSCP and subunit D of the *Apis mellifera* ATP synthase

Hassan M. Younis · Ramon Serrano · Reda K. Abdel-Razik · Jan Rydström

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Abstract 1, 1-bis (p-Chlorophenyl) -2, 2, 2-trichloroethane (DDT) has been used for control of malaria mosquitoes and other insect vectors of human diseases since 1945. Its use poses an environmental dilemma and efforts to replace it have been hampered by lack of information about its molecular target. This work identifies the 23 kDa band responsible for the DDT sensitivity in bees, as the OSCP and subunit "d" of the ATP synthase. The OSCP of the bee's ATP synthase contained 207 amino acids compared to 190 in bovine, which is insensitive to DDT, and the identities were only 47%. Subunit "d" of the bees had no counterpart in the bovine. Whether DDT is interacting only with OSCP, only with subunit "d", or with both subunits, remains to be assessed. Identification of the molecular target of DDT will lead the way to new target based insecticides aimed to protect plant, combat malaria and other insect transmitted diseases.

Keywords Mass spectrometry · DDT target · ATP synthase · Subunit "d" · OSCP

H.M.Y: protein isolation, participated in mass spectrometry data analysis, designed research; and wrote the paper. R.S: mass spectrometry data analysis. R.K.A: participated in protein isolation, J. R. and H.M.Y: supervised mass spectrometry in Swegene proteomics core facility.

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H. M. Younis (✉) · R. K. Abdel-Razik
Laboratory of Bioenergetics and Biochemical Toxicology,
Department of Pesticide Chemistry & Technology,
Faculty of Agriculture, Alexandria University,
El-Chatby,
21545 Alexandria, Egypt
e-mail: hassanyounis@gmail.com

R. Serrano
Instituto de Biologia Moleculary de Plants (IBMCP),
Ciudad Politecnica de la Innovacion,
Universidad Politecnica de Valencia-CSIC,
Camino de Vera s/n,
46022 Valencia, Spain

J. Rydström
Jan Rydström, Department of Chemistry, Lundberg Laboratory,
Goteborg University,
Box 462, SE-405 30 Goteborg, Sweden

Introduction

Among a few of the most famous synthetic chemicals employed by man, DDT occupies a unique role unmatched by any other chemical, in particular, through its use in the control of malaria and other insect vectors of human diseases, and later on, to control insect pest species in agriculture (Metcalf 1973; Metcalf et al. 1971; Hayes 1975; Jukes 1977; Roberts et al. 1997). DDT shows wide spectrum effectiveness on most insect species and very low initial toxicity on other animals and man. Because of this, DDT is regarded as one of the few chemicals in history that have had a broad and telling impact on mankind (Metcalf 1973; Hayes 1975).

However, the very properties that have made DDT a successful insecticide, *i, e.* its stability, low water and high lipid solubility, have made it the most insecticide condemned for damaging the environment. Thus, uses of DDT have posed and continue posing an environmental dilemma (Berenbaum 2005). Therefore, while recent reports (UNEP 2007) indicate an increase in the use of DDT, they call for efforts to develop alternatives to eventually reduce and ultimately eliminate its use for disease vector control. Meanwhile, the lack of specific knowledge about DDT's

molecular target makes it difficult for such alternatives to be achieved.

Identification of DDT's molecular target would open the way to develop new insecticides with selective insecticidal properties like DDT but without its negative environmental impact. In the absence of such a molecular target or targets, discoveries of new compounds will remain to be based on high volume, random, screening of corporate data bases of compounds or natural products followed by chemical optimization. The outcomes of such an approach are almost entirely empirical, and most of the resulting compounds would lack selective action and have negative health and/or environmental impacts (Gribble 1994). On the other hand discoveries and design processes would be much improved from knowledge of an isolated target. In such cases combination of its molecular structure determination and computation would emerge as an important, knowledge – based, rational for tailoring and design of target-oriented insecticides.

In this context, identification of the mitochondrial ATP synthase as the molecular target through which DDT mediates its insecticidal action (Younis et al. 1978, 2002) would be of great importance. Mitochondrial ATP synthase is the central enzyme in energy conservation in mitochondria (Hong and Pedersen 2008). It uses a proton motive force generated across the membrane by electron flow to drive the synthesis of ATP from ADP and inorganic phosphate (Mitchell 1966). The relationship between the inhibition of this enzyme activity and the mechanism of ion-permeability changes and neurological effects caused by DDT poisoning has been previously described (Younis et al. 2002).

Previous analysis of various preparations of the ATP synthase provided substantial evidence suggesting that the inhibition of its enzyme activity by DDT was dependent on the presence of a specific protein band with an apparent molecular mass of 23 kDa that existed in the preparations from *Apis mellifera* (DDT- susceptible) but was absent from the preparations of the enzyme from DDT-insensitive sources. When this protein band was removed from the DDT-sensitive enzyme, it became insensitive, and when it was resolved from the enzyme it showed specific affinity for DDT and conferred DDT sensitivity upon its addition to the DDT insensitive enzyme (Younis et al. 2002).

The evidence that emerged from these experiments supported the conclusion that, in bees, the 23 kDa protein of the ATP synthase is the DDT target protein; however, its identification by sequencing remained unknown.

The work described here provides evidence using 2D-gels and mass spectrometry peptide analysis that this protein band is a mixture of subunit "d" and oligomycin sensitivity conferral protein (OSCP) of the *Apis mellifera* ATP synthase, and that no obvious counterpart of this protein mixture is present in the DDT-insensitive bovine enzyme.

Materials and methods

Materials

DDT was a standard analytical sample supplied by the U.S. A. Environmental Protection Agency. Oligomycin, phosphoenolpyruvate (PEP), pyruvate kinase (PK) and ATP were purchased from Sigma, St Louis, MO, U.S.A. Soya bean phospholipids (asolectin) was purchased from Associated Concentrates, New York, NY, U.S.A. Honey bees of the species *Apis mellifera* were obtained either from a stock culture (apiary) of the Agriculture Experimental Station at Alexandria University, Alexandria, Egypt, or from the department of Entomology, Uppsala University, Sweden.

Isolation of mitochondria

Mitochondria from bovine heart (Smith 1967) and from thorax flight muscles of bees (Nedergaard and Cannon 1979) were obtained as described. Pellets of mitochondria were suspended in a solution of 20 mM Tris/SO_4 (pH 7.4), 1 mM EDTA, 10 mM succinate, 0.25 M sucrose, 2 mM ATP, 10 mM 2-mercaptoethanol and 10% glycerol, frozen in liquid nitrogen, and kept at −70 °C until used.

Isolation of ATP synthase

Mitochondrial ATP synthases of bees and bovine were solubilized from membranes by sodium cholate (1.5%) in the presence of 0.2 M ammonium sulfate, followed by purification with ammonium sulfate fractionation essentially as described in (Serrano et al. 1976) (method 1). This method yielded an ATP synthase preparation that showed the previously described 23 kDa band (Younis et al. 2002).

The enzyme was also prepared using (method 2), in which the five oxidative phosphorylation complexes were solubilized from mitochondrial membranes by a solution containing 6-aminocaproic acid, digitonin, and n-dodecyl *B*- D- maltoside, followed by separation on gels in a native form (Colorless native gels, CN- PAGE) (Mayer et al. 2007). The band containing ATP synthase, complex V, was excised from several preparative gels followed by linear gradient analytical SDS-PAGE to separate the enzyme subunits. As shown below, preparations of ATP synthase from bees and bovine using this method showed an obvious difference between the two preparations: a protein band in the range of 20 kDa identified as subunit "d" is present in the preparation of the enzyme from bees, but is absent from that of bovine.

Determination of protein and reconstitution with phospholipids

Protein concentration was determined by a modified Lowry method (Lowry et al. 1951) using kits purchased from Pierce co. and BSA for calibration. ATPase was reconstituted with asolectin (95% purified phosphatides), at a ratio of 4.5: 1 of phospholipids to protein before determination of its activity using the cholate dilution method (Racker et al. 1975).

Measurement of the ATPase activity and inhibition

ATPase activity was measured using an ATP-regenerating system, by coupling the reaction to the pyruvate kinase and lactate dehydrogenase system and measuring NADH oxidation at 340 nm (Pullman et al. 1960). The assay mixture contained 50 mM Tris-acetate buffer (pH 7.4), 1 mM $MgCl_2$, 2 mM ATP, 2 mM phosphoenolpyruvate, 0.2 mM NADH, 15 μg/ml pyruvate kinase and 5 μg/ml lactate dehydrogenase. Before the assay, the mixture was incubated at 30 °C for 30 min. and the ATPase protein was preincubated with phospholipids (asolectin) for 3 h at 5 °C. Incubation with the inhibitors, oligomycin and DDT, was performed at 5 °C for 30 min. All samples were brought to room temperature before the assay. The spectrophotometer was connected to a water bath at constant temperature (30 °C) and the absorption was measured at 340 nm for 60 s.

SDS/PAGE of the ATP synthase and separation of subunits

Samples of the purified ATP synthase were prepared for gel electrophoresis in order to separate the individual subunits of the enzyme. The sample buffer contained, SDS, 2-mercaptoethanol, Tris and glycerol- purchased from Biorad. The running buffer used contained Tris (pH 8), SDS and Tricine. Protein and standards were loaded on a commercially made Tris/HCl polyacrylamide gel (Biorad) and a parallel in-house made polyacrylamide (12%, stack 3.9%), and gradient gels (10–18%; 0.7 mm thick) in the presence of 1% SDS. The electrophoresis was run at constant voltage (Laemmli 1970).

Preparation of proteins for mass spectrometry

The protein spots were visualized by Commassie Blue and the protein band of interest, the 23 kDa band, was cut out from the gel and digested with trypsin (Shevchenko et al. 1996). The desalted peptide mixture was then separated using desorption –ionization of the peptide molecules using electrospray ionization, ESI-MS (Fenn et al. 1989). Mass analyses were performed in an ESI-TRAP instrument at the Swegene proteomics core facility, Gothenburg University, Sweden. The search parameters were set to: Fragment mass tolerance (MS/MS accuracy) 0.5 Da, fixed propionamide modification of cysteine and variable modification of oxidized methionine, mass values; mono isotopic, maximum missed cleavage 1. Mass measurements and fragmentation analysis were carried out and the results were used to search against known protein sequences in the databases (Steen and Mann 2004).

Results

Binding of DDT to the ATP synthase of bees and inhibition of its ATPase activity

The ATPase activity of the enzyme preparations from *Apis mellifera,* used in the present study was inhibited up to 97% by 1.5 μM DDT, using an ATP-regenerating system assay, whereas, inhibition by 1 μg/ml of oligomycin under the same conditions amounted to 60%. Reaction conditions were as described under materials and methods.

The above degree of inactivation of the enzyme reaction by DDT was time, concentration and temperature dependent, negatively correlated with temperature, which are criteria of forming chemical bonds. This was confirmed by measuring direct binding of DDT to the ATPase on CN-PAGE, loaded with fixed amounts of the respiratory protein complexes as monitored by scanning at 237 nm to detect bound DDT, where it was found to bind exclusively to the ATP synthase, complex V (results not shown).

Mass spectrometry identification of the 23 kDa protein

The 23 kDa protein band (Fig. 1a) separated on SDS-PAGE as described in method 1, was excised and in-gel digested by trypsin. The resulting peptide masses were analyzed using electrospray ionization (ESI) technique and searched against known protein sequences databases. As seen from the attached peptide summary reports and the histograms in Fig. 1b and c, showing score distribution of the best matching proteins, the 23 kDa band contained a mixture of two proteins, which seem to run together in the electrophoresis, identified from the protein database as the OSCP subunit of the *A. mellifera* ATP synthase, and the subunit "d" of the insect enzyme (Table 1, Fig. 1).

Attempts to separate the two proteins yielded a fraction of OSCP free of subunit "d", referred to as band 2 (Table 1). However, we could not get subunit "d" free of OSCP at this stage.

The data base search of the amino acid sequence of OSCP (*Apis mellifera)* showed its content of 207 amino acids. Likewise, the amino acid sequence of subunit d showed 174 amino acids. The underlined amino acids are those obtained experimentally from MS/MS analysis of the

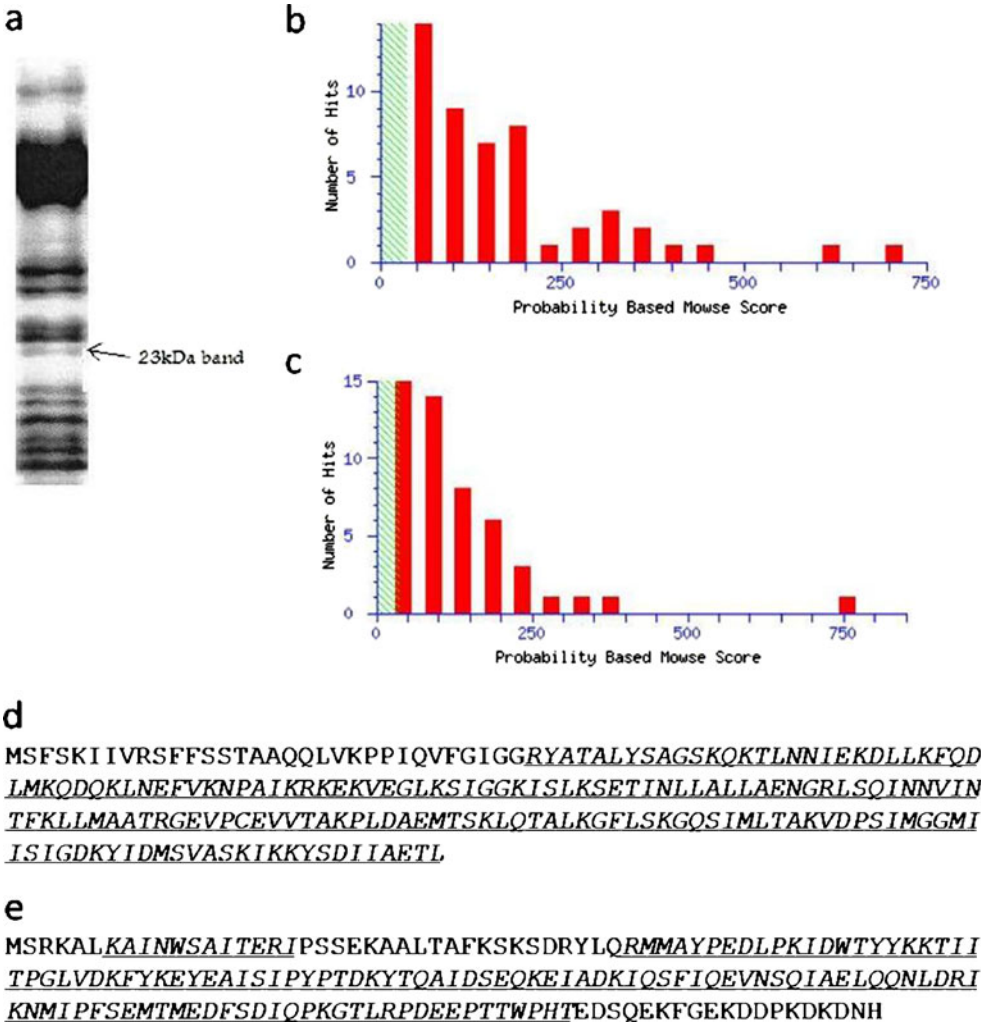

Fig. 1 Mass spectrometry identification of the 23 kDa protein. **a** SDS- PAGE of the ATP synthase prepared as described under method#1, showing the 23 kDa band. **b** Score histogram of Mascot search results illustrates that the OSCP (Score: 706; Mass: 22691 Da) and subunit d (Score: 621; Mass: 20323 Da) are the best matching protein score of the 23 kDa band. **c** The same as in (b), for protein score distribution of fraction of the 23 kDa band containing OSCP (Score: 757; Mass: 22691). **d** The data base search of the amino acid sequence of OSCP (*Apis mellifera*). NCBI Reference Sequence: XP _3927602*;* Locus XP_392760; 207 amino acids; Version XP_392760.2 GI: 66504360. **e** The data base search of the amino acid sequence of subunit d (*Apis mellifera*) NCBI-GI: 48098315, Reference Sequence: XP_394036.1; 174 amino acids. Preparation of proteins for mass spectrometry was carried out as described under materials and methods

respective proteins (Fig. 1d and e). Sequence coverage was 85% for the OSCP and 75% for subunit "d".

Resolving protein contents of the 23 kDa band

In an attempt to resolve the protein contents of the 23 kDa band, we used the colorless native gel (CN-PAGE) method in the presence of digitonin to purify and isolate the enzyme subunits (method 2). This method enabled isolation of the enzyme in a native form. Parallel blue native (BN) gels were carried out as well (Mayer et al. 2007).

The ATP synthase band (complex V) (Fig. 2a) was cut out from the gel and analyzed in 2D Tricine- SDS- PAGE. A parallel preparation of the enzyme from bovine heart was carried out side by side with that from bees (Fig. 2b).

The 23 kDa protein belongs to the group of the intermediate molecular mass subunits of the enzyme ranging from 25 to 18 kDa, that includes, subunits "a", "b", OSCP and "d" described before for the enzyme from bovine heart (Rees et al. 2009). As shown here, the present 2-D BN-SDS-PAGE system was able to separate these proteins from bees as well as those from a bovine source (Fig. 2b). Protein bands of the enzyme from both sources were cut from the gel and subjected for mass spectrometry

Table 1 Mass spectrometry identification of the 23 kDa protein purified from *Apis mellifera* mitochondrial ATP synthase. The enzyme was purified by method 1, described under the section of Materials and Methods. The 23 kDa protein band of the enzyme separated on SDS/PAG (10–18%, 0.7 mm thick "Fig. 1") was cut, digested by trypsin and the desalted peptide mixture was separated using ESI-MS as described

Definition	Accession no.	Relative molecular mass (Da)	Score	Queries matched	No. Amino acids
Band 1					
OSCP	gi|66504360	22691	706	27	207
Sub.d	gi|48098315	20323	621	21	174
Band 2[a]					
OSCP	gi|66504360	22691	757	41	207

[a] Band 2 is the fraction separated from band 1

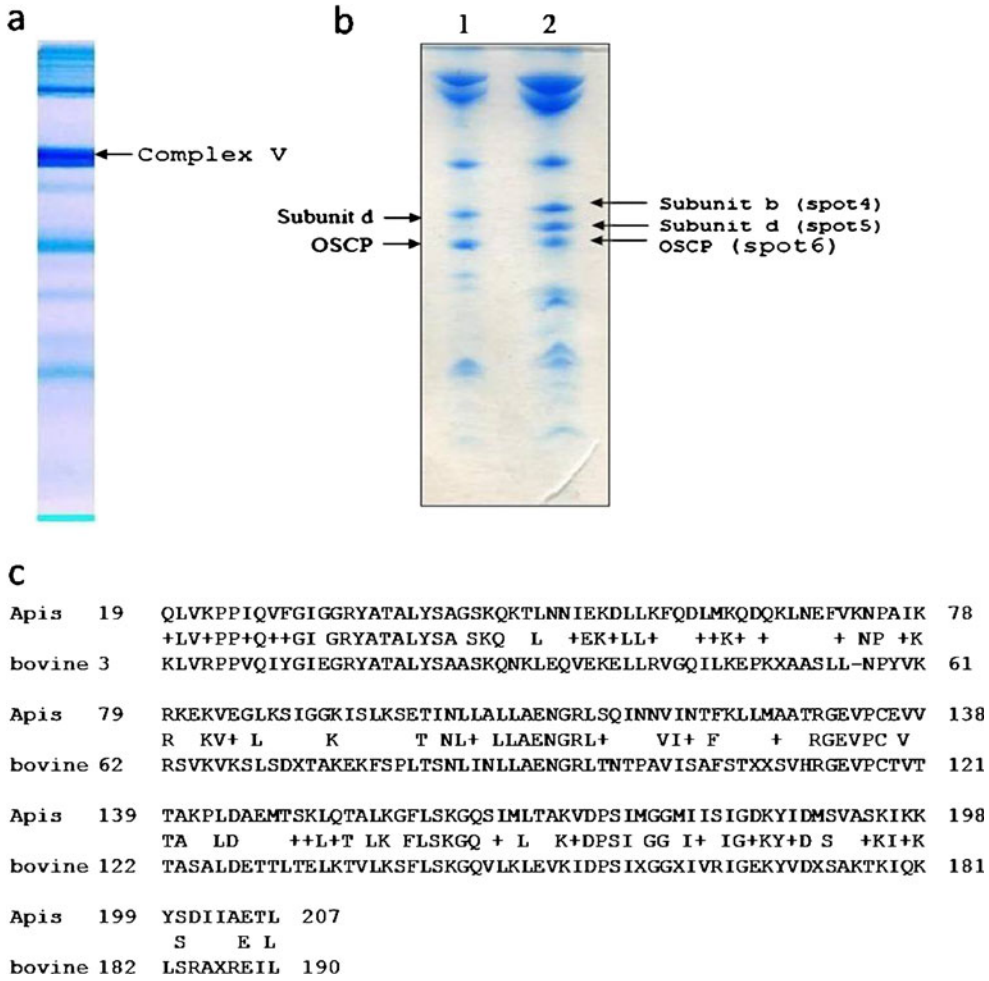

Fig. 2 Resolving protein contents of the 23 kDa band. **a** ATP synthase from bee's thoracic flight muscles and bovine heart mitochondria extracted by digitonin (2 g/g of protein) and resolved by 1- D BNE. **b** The spot corresponding to the ATP synthase was cut from the 1-D BN gel and resolved by 2 D Tricine –SDS-PAGE using ***a*** linear gradient of acrylamide from 10% to 18% in the presence of 0.1% SDS (Laemmli 1970). Lane 1 is the enzyme from bovine heart and Lane 2 is the enzyme from bees. **c** Alignment of the sequences of the two OSCPs, i.e., that of Apis *(*XP 392760.2), which contains 207 amino acids and that of bovine, *Boss Taurus,* (PDB; 2wsss; Gi; 268612216) which contains 190 amino acids. PSI-BLAST- Position Scoring Matrices (PSSM) Blast 2sequences. Plot of gi|66504360|ref|XP_392760.2|vs gi|26812216|pdp|2WSS|S

proteomic analysis as described. The results are summarized in Table 2.

Spots numbers 5 and 6 (*A. mellifera)* (Table 2), were those of proteins found in the 23 kDa band described under method 1. The present gel system has resolved these proteins, the OSCP and chain "d", components of the 23 kDa band. Their respective de novo amino acid sequences, Fig. 1d & e were the same, whether the proteins were prepared by method 1 or method 2. The relative molecular mass for the OSCP and subunit d of the *Apis mellifera* enzyme differed from those reported for these subunits of the bovine enzyme (Rees et al. 2009). Figure 2c

Table 2 Mass spectrometry identification and sequence coverage of protein subunits of ATP synthase from Apis and bovine heart mitochondria separated by two dimensional gels as described under method # 2. The band containing the ATP synthase, separated in a native form on CN-PAGE, from the two sources, Apis and bovine (Fig. 2), was cut from gels and analyzed on linear gradient analytical SDS PAGE to separate the enzyme subunits (Fig. 2). The enzyme from bees differed from that of bovine in the protein spot no 5, identified as subunit "d" that was absent in bovine. Protein spots were cut out from gels, digested by trypsin, desalted, separated, searched in the data bases and identified as described under methods

Spot no	Accession no	Description	Relative mol. mass	Score	No of assigned peptides	Sequence coverage
Apisc mellifera						
4	gi| 66529463	Fo ATP synthase Subunit B chain Mitochondria	27763	708	8	38.6%
5	gi| 48098315	Fo ATP synthase Subunit d Mitochondria	20323	787	17	75%
6	gi| 66504360	ATP synthase "O" Subunit Mitochondria (OSCP)	22691	911	16	85%
Boss taurus						
4	gi|110591029	Chain D sub- Complex of The stator	24653	912	15	——
5	gi|74268299	ATP synthase "O" subunit (OSCP)	23305	703	13	86%

shows alignment of the sequences of the two OSCPs, i.e., that of Apis (XP 392760.2), which contains 207 amino acids and that of bovine, *Boss Taurus,* (PDB; 2wsss; Gi; 268612216) which contains 190 amino acids. It showed differences in 17 amino acids. Positions marked (+) indicate semi-conserved amino acids (62%). Identity is 47%. Moreover, the first 19 amino acids of *Apis* OSCP are not present in bovine OSCP (Fig. 2c). This part of the bee's OSCP may contain the DDT binding site. In addition, the bovine enzyme had no corresponding structure of the *Apis* enzyme "d" subunit (Fig. 2b). Hence, DDT may not just bind to the OSCP but to subunit "d" as well. The overall DDT binding site then may contain subunit "d" and OSCP.

Discussion

The discovery and use of DDT during and after the World War II marked the beginning of the era of synthetic organic insecticides (Lauger et al. 1944). DDT was critical in eradication of malaria worldwide and still in use as a major component of its control today (UNEP 2007). However, its continued use in the environment ever since has evoked contentious arguments (Metcalf 1972; Jukes 1977; Roberts et al. 1997) and efforts to replace it have been hindered by the lack of knowledge about its molecular target. The present study is the first to identify such a target that provides new clues for specific insecticidal design.

Identification of the DDT-target described in the present work used tandem mass spectrometry (MS/MS) data from the 23 kDa protein band of the ATP synthase associated with the inhibition of the enzyme activity by DDT. Sequence *a*nalysis of this protein band showed its composition to be a mixture of the enzyme subunits "d" and OSCP. Both subunits are components of the peripheral stalk (the stator) of the ATP synthase. The peripheral stalk attaches the F_1 domain to the membrane domain via the OSCP (Rees et al. 2009). Such structure plays a crucial role as a stator in coupling the mechanical rotation to chemical synthesis of ATP. Interaction of DDT with one or more of these components of the peripheral stalk would impair its function in coupling the energy stored in the electrochemical gradient of protons (the trans-membrane proton motive force "pmf") to ATP synthesis. An integral structure of the stator is required for such an energy transfer processes. Interaction of DDT with OSCP and/or subunit "d" would interrupt such a structure, freeze the mechanical rotation of the enzyme and block synthesis of ATP.

The differences existing between the two subunits, OSCP and subunit "d", in bees and th bovine enzyme, explained in the results section, allow DDT to interact selectively with the enzyme in bees and inhibit its activity. Particularly, the first 19 amino acids of *Apis* OSCP, which are not present in bovine OSCP, may contribute to DDT binding.

On the other hand, the ATP synthase subunit "d" of the bee's enzyme had no obvious counterpart in the bovine ATPase. Its amino acid sequences and molecular weight (Figs. 1 and 2) are different from that of the enzyme from bovine heart (Rees et al. 2009) and rat liver (Higuti et al. 1993). In both sources, rat and bovine, it is composed of 160 amino acid residues compared to 174 in bees. Moreover, the sequence of the rat subunit "d" is highly homologous with that of subunit "d" of bovine heart while both have no homology with subunit "d" of the bee's enzyme. Yet, the selective presence of this subunit in the insect enzyme, as a component of the 23 kDa band, is suggestive of it playing a role in the binding and selective action of DDT. Interaction of DDT with this selective protein subunit of the insect enzyme may constitute a crucial role in blocking of the enzyme activity.

Nevertheless, the DDT pocket "niche" may include a structure provided by the two protein subunits, subunit "d" and OSCP. The two proteins, as shown above, are rich in hydrophobic amino acids which comprise about 30% of subunit "d" and 50% of the OSCP that could facilitate their association with the hydrophobic structure of DDT (Bowman et al. 1960). However, it will be necessary to learn more about the nature of DDT's interaction with the two subunits, whether it is interacting only with OSCP, only with subunit d, or with both OSCP and subunit d. Reconstitutions of DDT sensitivity with just the OSCP or just subunit "d" would be necessary steps towards this goal. On the other hand, an X-ray structure of a sub-complex of the enzyme containing these two proteins in the presence of DDT would provide a clear explanation of the way DDT interacts with these proteins and how it resides in the enzyme. Such experiments which can provide structural information necessary to understand the way DDT interacts with the enzyme are still challenges for the future.

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