MECHANISM OF ACTION OF MONOKETO-ORGANOMYCIN, CYSTAURI-MYCIN AND THEIR PERFORMIC ACID-OXIDIZED MODIFICATIONS

II. INHIBITION OF MITOCHONDRIAL ATPase ACTIVITY IN *ASPERGILLUS NIDULANS* AND IDENTIFICATION OF THE PERFORMIC ACID-OXIDIZED MODIFICATIONS-BINDING PROTEIN

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Performic acid-oxidized modifications of monoketo-organomycin and cystaurimycin[1] were found to possess identical mechanisms of action. Both inhibit mitochondrial ATPase activity in *Aspergillus nidulans*. The corresponding binding-protein was isolated from whole mitochondria and purified ATPase complex using neutral chloroform - methanol mixture. Gel filtration and amino acid analyses gave an estimated molecular weight of 8,000~8,500 dalton and was identified as the smallest subunit of the ATPase complex. This protein is highly hydrophobic (30% polarity) and highly rich in alanine, leucine and glycine. Tyrosine and alanine represent the N- and C-terminal amino acids respectively. Binding studies revealed that neither the N- nor the C- terminal residues of the above two enzyme inhibitors was involved in the binding or inhibition processes. Nonspecific binding to other mitochondrial component(s) was, however, observed with oxidized monoketo-organomycin. This nonspecific binding could be abolished by using the dansylated derivative of the latter compound. The dicyclohexylcarbodiimide (DCCD)-binding protein showed characteristics very similar to what appeared to be the same and one protein capable of binding the performic acid-oxidized compounds. Oligomycin and DCCD had no significant inhibitory effect on binding of the ATPase inhibitors investigated.

This mechanism of action differs from that exhibited by the parent nonoxidized compounds, monoketo-organomycin and cystaurimycin. The latter pair were found to inhibit *in vitro* protein synthesis[2] but not ATPase activity.

Performic acid-oxidized modifications of monoketo-organomycin (ox-MKOM) and cystaurimycin (ox-CYST) are potent growth inhibitors in several bacteria[1,2]. While monoketo-organomycin (MKOM)[3] and cystaurimycin (CYST)[1] could inhibit *in vitro* protein synthesis in isolated *Escherichia coli* ribosomes, their performic acid-oxidized modifications failed[2]. It was then suggested that the *in vivo* growth inhibitory action of the oxidized compounds might be due to their interference with one or more of the other metabolic pathways in intact cells[2]. Screening studies indicated, however, that ox-MKOM and ox-CYST have particular inhibitory actions on mitochondrial ATPase activity in *Aspergillus nidulans* sensitive to oligomycin and DCCD.

Oligomycin[4,5] and DCCD[6] have been found to inhibit the mitochondrial ATPase activity through a similar mode of action and act on the membrane-integrated part of the enzyme[7]. The irreversible mode of action allowed the identification of a DCCD-binding protein on the level of mitochondria[8] and of purified oligomycin-sensitive ATPase complex[9]. The binding protein was identified as a proteolipid due to its solubility in neutral chloroform-methanol (CM) mixtures[8].

The present communication aims to pinpoint the mechanism and site of action of ox-MKOM

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and ox-CYST. Oligomycin and DCCD were used as control inhibitors. Inhibition of mitochondrial ATPase activity by these compounds was investigated and, except for oligomycin, the corresponding binding proteins were isolated from whole mitochondria and purified ATPase complex in *A. nidulans*, characterized and compared.

Materials and Methods

MKOM[3], CYST and their performic acid-oxidized modifications were prepared as described[1]. The [14]C- labelled forms of these compounds were obtained by supplementing the MKOM-producing culture (*Streptomyces* AS- 400)[3] with the appropriate amount of a mixture of the 18 common ^{14}C-labelled amino acids (52 mCi/matom carbon). Oligomycin was obtained from Sigma Chemical Co. and cold DCCD from Nutritional Biochemical Corp. [14]C- Urea and labelled amino acids were from The Radiochemical Centre, Amersham. [14]C- DCCD was prepared as described[10]. Purity and radio-chemical purity were tested by thin-layer chromatography on aluminum oxide (neutral, type E, Merck) using benzene as eluant. [14]C- DCCD prepared showed only one spot when chromatographed solely or in combination with authentic cold DCCD. The specific radioactivity was 21,000 cpm/nmol DCCD.

Growth Conditions and Isolation of Mitochondria

The oligomycin-sensitive *A. nidulans* was grown in liquid defined medium[11] for 18 hours at 37°C with shaking (200 rev./minute). Mycelia were harvested by filtration, homogenized[12] and mitochondria were separated[13]. Phenylmethylsulfonyl fluoride, 5 mM, was included in the isolation buffer.

Purification of ATPase Complex

The method of MARAHIEL *et al.*[14] was followed for purified ATPase complex preparation. ATPase activity was assayed at pH 6.5 as described[15].

Labelling and Isolation of DCCD-, ox-MKOM- and ox-CYST-binding Proteins

On assaying ATPase activity on the level of mitochondria and of purified ATPase complex, the dansylated forms[1], *i.e.* with blocked NH₂ groups, of [14]C- labelled ox-MKOM and ox-CYST (dans-[14]C- ox-MKOM and dans-[14]C- ox-CYST respectively) were found also inhibitory to the enzyme activity and were thus included in the binding and inhibitory studies along with their corresponding nondansylated forms.

For inhibitory and binding studies, whole mitochondria or submitochondrial particles[14] were suspended in 10 mM Tris-acetate, pH 7.5, and incubated at 0°C for 4 hours with DCCD and for 6 hours in case of oxidized compounds and their dansylated derivatives. DCCD was added as methanolic solution. The other compounds were dissolved in the incubation buffer and added. Controls lacking inhibitors were always set up. In all cases, including the controls, methanol was added to 1%. After incubation, removal of unbound materials was conducted as recommended[16] followed by lyophilization of mitochondria. Controls were treated similarly.

The inhibitors-binding proteins were isolated by incubating the lyophilized mitochondria with dry neutral CM (2:1, v/v) for 30 minutes at room temperature with stirring and centrifuged. Extraction of mitochondria was repeated twice. The various combined extracts were separately flash evaporated to near dryness, 30°C, cooled to 0°C, 4 volumes of pre-cooled diethyl ether added and incubated over night at −20°C. The precipitates were again dissolved in CM and insoluble material was removed. Precipitation was repeated three times.

Proteins were determined by the method of LOWRY *et al.*[17] using crystalline bovine serum albumin as a standard. Protein was hydrolyzed in 6 N HCl (containing one drop of 1% phenol) at 110°C for different times and up to 96 hours. Amino acids were analyzed in an automatic Biotronic LC 600 E-analyzer. End group analyses were made as described before[1].

[14]C- Radioactivities were measured in a Packard Tri-Carb liquid scintillationspectrometer. Samples of proteins and the 1-mm gel slices were dissolved in 0.1 M Tris-acetate buffer containing 1% (w/v)

sodium dodecylsulfate, pH 8, at 50°C for 12 hours, scintillation solution added and radioactivity assayed.

Results and Discussion

Previous studies on the very closely related ox-MKOM and ox-CYST showed that they possess a similar, though not identified, mode of action[2]. The only chemical difference between these two compounds is the presence of an additional tripeptide, glycine-phenylalanine-glutamic, at the N- terminal region in ox-MKOM with subsequent determination of glycine as the N- terminal amino acid. Cysteic acid, the N- terminal residue in ox-CYST, is thus masked by this tripeptide in ox-MKOM[1,2]. That ox-MKOM is of relatively low growth inhibitory effect compared with ox-CYST[1,2] was attributed to a possible activity-masking role played by this terminal tripeptide on the biologically active ox-CYST-moiety in ox-MKOM molecule. Thus the two above [14]C- labelled compounds were dansylated[1] to block the NH_2 groups in their N- terminal amino acids and which are the only free NH_2 in their molecules[1]. In this way it would be possible to investigate the role played by these groups in their inhibitory action. In addition, it would provide, through fluorescence and/or radioactivity, a convenient way for identification of the corresponding binding proteins. Coincidence or noncoincidence of fluorescence and/or radioactivity with a given protein peak would provide a reliable way to investigate these possibilities.

Inhibition of ATPase activity in whole mitochondria by oligomycin, DCCD, dans- and nondans-forms of the other inhibitors is shown in Fig. 1. ATPase activity was halfmaximally inhibited at (μg/mg protein): 0.18 oligomycin, 0.5~1 DCCD, 2.5~5 ox-MKOM and 1.75~2.5 ox-CYST. The same values were measured when either nondans- or dans-[14]C- ox-CYST was used. This indicates that the presence of free NH_2 group in cysteic acid, the N-terminal residue, is not essential for inhibiting ATPase activity by ox-CYST. With dans-ox-MKOM the results were, however, almost identical with those obtained with either ox-CYST or its dans-form. This shows that blocking of the free NH_2 group in the N-terminal amino acid, glycine, in ox-MKOM resulted in rendering this compound more inhibitory to ATPase activity. At this stage, it was thought that the free NH_2 group in ox-MKOM molecule might be responsible for some sort of steric hindrance and/or for being able, under the experimental conditions adopted, to react nonspecifically with other protein(s) or component(s) in mitochondria or in ATPase complex itself and which is not involved in ATPase inhibition (see later) and hence the high amounts required to exert the same degree of inhibition exhibited by its dans-form (Fig. 1).

It is worth pointing out that the same results and values were recorded with the cold preparations of the compounds investigated. In addition, dansyl chloride—the dansylating agent— was of no inhibitory action on ATPase activity when used under the same conditions.

Fig. 1. Inhibition of mitochondrial ATPase by oligomycin, DCCD, ox-MKOM and ox-CYST.
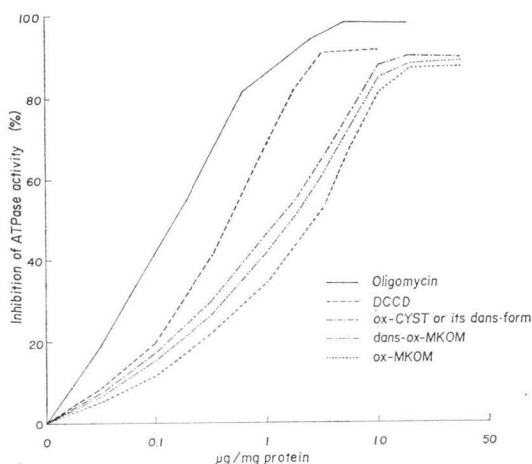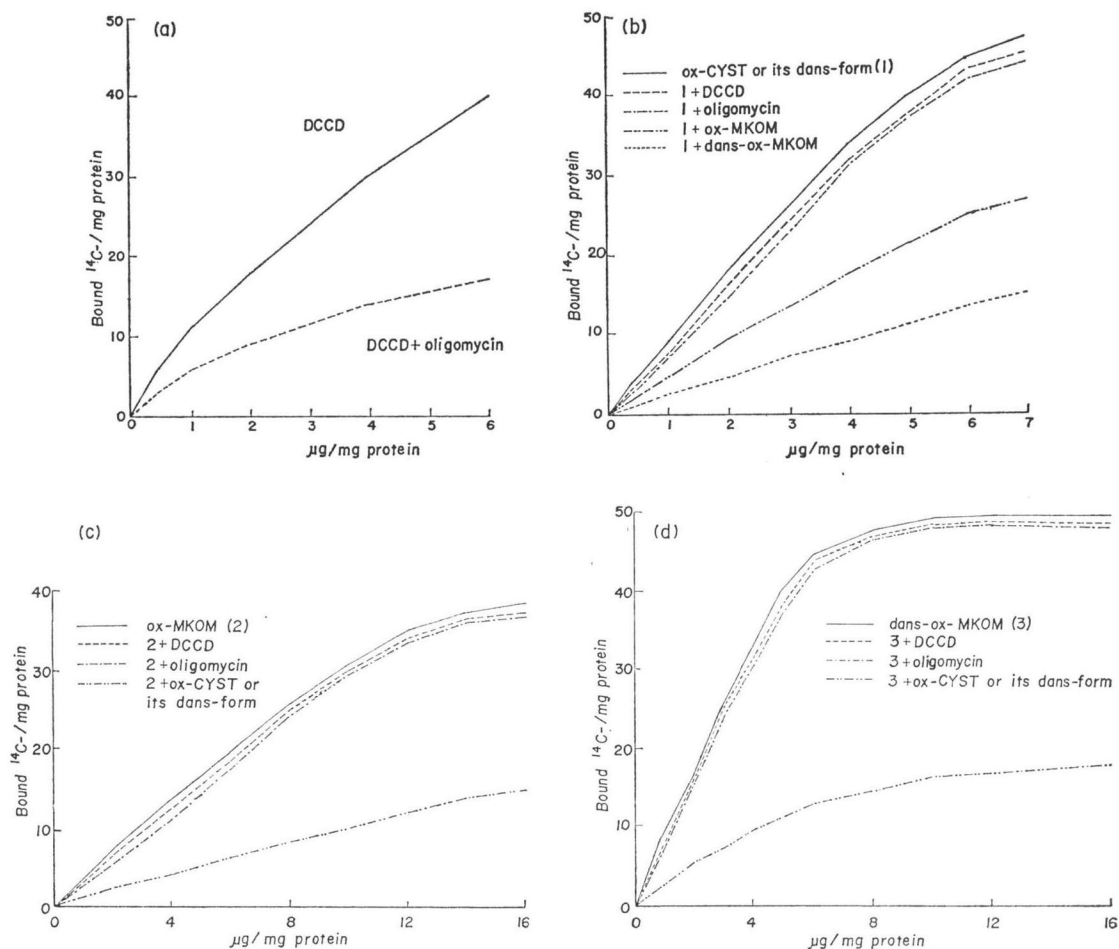
Fig. 2.   Binding of DCCD, ox-MKOM and ox-CYST to ATPase complex in *Aspergillus nidulans*.

Effects of oligomycin and DCCD on binding of the other inhibitors were also investigated. Whole mitochondria were incubated separately with different amounts of various [14]C- labelled compounds in the presence and absence of either cold DCCD or oligomycin. After binding and removal of unbound material[16], the purified ATPase complex was isolated[14]. Results are shown in Fig. 2.   Binding of ^{14}C-DCCD was highly reduced in the presence of oligomycin (Fig. 2a) suggesting that the two inhibitors are bound to the same inhibitory site. Similar results were recorded in *Neurospora crassa*[16].   At 2 µg of DCCD optimal binding was recorded and where no nonspecific binding[9] was observed. At 5 µg ox-CYST or its dans-form (Fig. 2b) and dans-ox-MKOM (Fig. 2d) nearly equal amounts of these inhibitors were bound per mg ATPase complex protein.   This amount is almost twice that bound of ox-MKOM (Fig. 2c) under the same conditions.   Nearly half amount of ox-MKOM added, in the form of [14]C- radioactivity, was detected in the insoluble material left after solubilization of the sub-mitochondrial particles in the presence of Triton X-100[14] and could not be separated from the insoluble material or identified.   In contrast, this insoluble material was found devoid of radioactivity or fluorescence-radioactivity combination, depending on the compound used, with subsequent detection

of the markers exclusively in the ATPase complex isolated.

DCCD and oligomycin were of no significant effect on binding of the tested compounds to the ATPase complex (Fig. 2b~2d). The presence of cold ox-CYST or its dans-form reduced markedly the binding of ^{14}C-ox-MKOM as well as its dans-form to ATPase complex. The inverse relationship is true. This indicates, and apart from the nonspecific binding of ox-MKOM to other mitochondrial component(s) other than ATPase complex, that ox-MKOM, ox-CYST and their dans-forms would have bound to the same inhibitory site and which appears to be different from that of DCCD and oligomycin.

In the binding studies the amounts used of the various compounds were the lowest ones capable of exerting maximal inhibition of ATPase activity in order to avoid any nonspecific binding especially in case of DCCD[9].

CM extraction of the binding proteins appeared to be very specific. The amount of radioactivity recovered from either whole mitochondria or purified ATPase complex labelled with either ox-CYST, dans-ox-CYST or dans-ox-MKOM was about 95~98% of that added. With ox-MKOM only 50% of the original counts in whole mitochondria was recovered in CM extract. Almost the same amount of radioactivity was recovered from purified ATPase complex, about 97% recovery.

The inhibitor-binding proteins were dissolved in suitable buffer and equal amounts were subjected to gel electrophoresis[18] along with purified ATPase complex. Results are shown in Figs. 3 and 4. As clearly seen, only a single protein capable of binding all the inhibitors investigated including DCCD was detected (Fig. 3). This applies not only for ATPase complex but also for whole mitochondria (Fig. 4a~4d). The bound ^{14}C-inhibitors migrated with the smallest ATPase subunit of about 8,000 dalton. With ^{14}C-DCCD, the radioactivity did not exactly coincide with the protein peak. Based on similar observation in *N. crassa*, it was suggested that the electrophoretic and chromatographic behaviour of DCCD-binding protein might have been changed by the bound inhibitor[16]. Except for DCCD,

Fig. 3. Gel electrophoresis[14] of ATPase complex showing ox-CYST-binding protein.
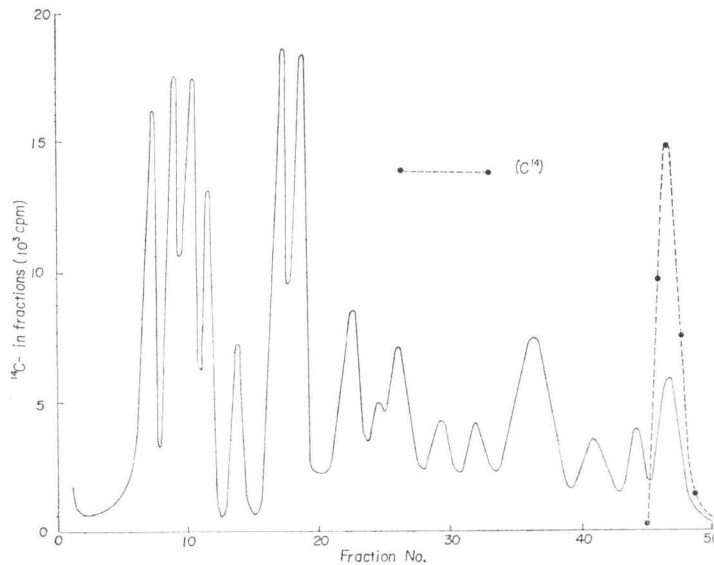Similar results were obtained with DCCD, dans-ox-CYST, ox-MKOM and its dans-form.

Fig. 4. Gel electrophoresis[19] of protein (isolated from either whole mitochondria or purified ATPase complex) capable of binding:
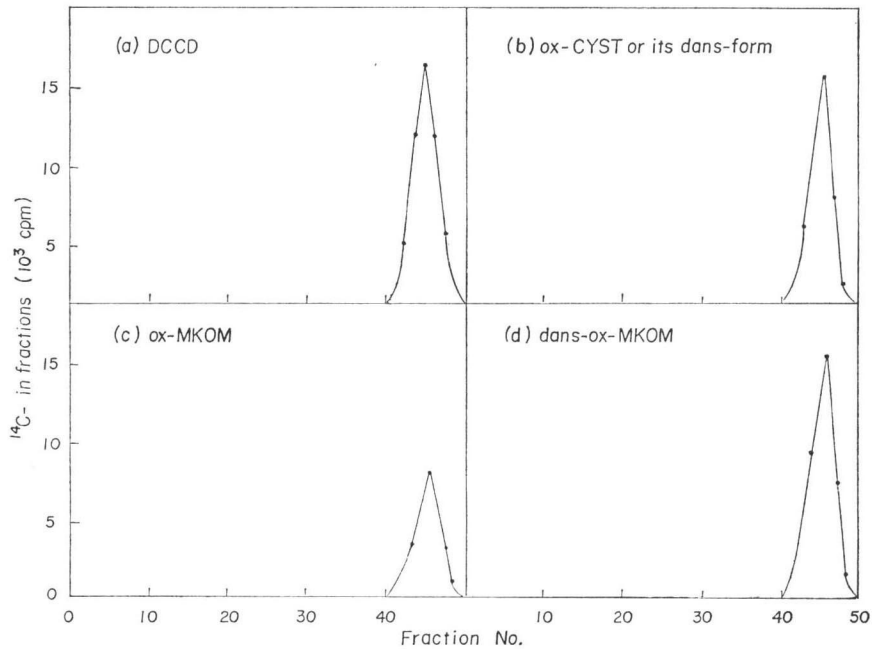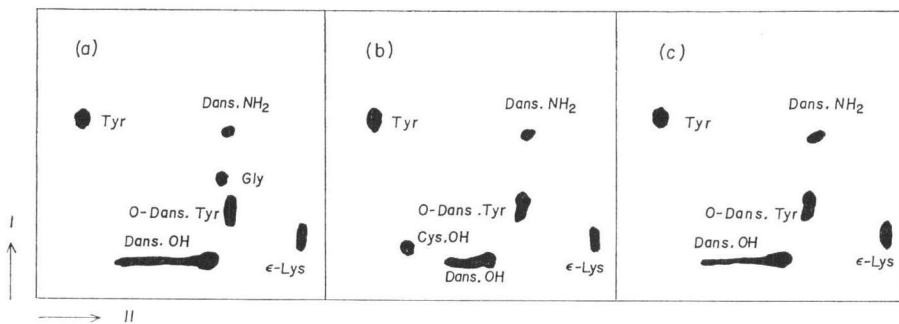

Fig. 5. N-Terminal analyses of the isolated proteins binding: (a) ox-MKOM or its dans-form, (b) ox-CYST or its dans-form and (c) DCCD or the protein isolated from the controls. All the dans-amino acids shown were further identified by mixing with their corresponding authentic samples.

exact coincidence of [14]C- label and/or fluorescence with the protein peaks was always recorded in case of the other inhibitors used. Homogeneity of the isolated proteins was confirmed by using different gel systems[18,19] and by column chromatography analyses in 80% formic acid on P-30 Biogel. A single band on gels and one peak in P-30 was always obtained.

N-Terminal analyses[1] of the various isolated inhibitors-binding proteins along with that isolated from controls are shown in Fig. 5. In all cases tyrosine was the only N- terminal amino acid detected. O-Dans-tyrosine and ε-dans-lysine (ε-Lys) detected together with tyrosine (Fig. 5a ~ 5c) would have been derived from the internal tyrosine and lysine residues respectively in the proteins. These amino acids are lacking in the inhibitors molecules.[1,2] Glycine (Fig. 5a) and cysteic acid (Fig. 5b) would have

been produced from the N-terminal residues in ox-MKOM and ox-CYST respectively[1]. Proteins binding the dansylated forms of the latter inhibitors gave similar results. Hydrazinolysis[1] revealed alanine as the C-terminal amino acid of the various proteins analyzed. Except for the DCCD-binding protein and that isolated from controls, taurine, the C-terminal amino acid of the inhibitors used, was the only other amino component detected with alanine.

The above results show that all of the inhibitors used in the binding experiments seem to bind specifically to what appeared to be the one and same protein. The latter was identified by gel electrophoresis to be the smallest subunit of ATPase complex. The results show also that neither the N- nor the C-terminal residues of ox-MKOM or ox-CYST was involved in the binding process. These results were expected in case of the two above inhibitors in light of their strong competition for binding to the same inhibitory site but were rather unexpected in case of DCCD whose presence had no significant effect on the binding of the other compounds. This would denote that the isolated inhibitor-binding protein might possibly contain at least two different inhibitory sites and that binding of a given ATPase inhibitor to only one of them appears to be sufficient to block the enzyme activity. At present, however, the possibility that several closely related polypeptides may be present in the proteolipid and that one or more of them could bind DCCD while the other(s) could bind ox-MKOM and ox-CYST could not be ruled out.

In a recent study on DCCD-binding protein in *N. crassa* and yeast, it was reported[20] that the proteolipid isolated is present in the ATPase complex as oligomer, probably as hexamer, and that the modification of one of the probably six sites of the subunit oligomer is sufficient to block the enzymatic activity of the complex.

The results obtained with ox-MKOM are of especial interest. It was with this compound alone that nonspecific binding to unidentified mitochondrial component(s) not involved in ATPase inhibition was observed. This behaviour of the inhibitor was mainly due to the presence of the free NH_2 groups in the N-terminal residue in the additional tripeptide present in its molecule (see-above). This fits in logically in the light of the fact that blocking of these groups by dansylation abolished this nonspecific binding

Table 1. Amino acid composition of DCCD-, ox-MKOM- and ox-CYST-binding proteins in *Aspergillus nidulans*

Amino acid	mol/100 mol		
	DCCD-binding protein[+]	ox-MKOM- & its dans-form -binding protein	ox-CYST- & its dans-form -binding protein
Aspartic	7.28	7.16	7.31
Arginine	3.14	3.11	3.16
Histidine	0.65	0.00	0.00
Threonine	1.20	1.22	1.22
Serine	8.64	8.60	8.63
Glutamic acid	6.72	6.75	6.75
Proline	5.63	5.63	5.65
Glycine	14.38	14.50	14.30
Alanine	14.08	14.12	14.15
Valine	6.16	6.20	6.23
Methionine	4.74	4.70	4.80
Isoleucine	6.13	6.14	6.16
Leucine	9.74	9.76	9.80
Tyrosine	3.95	3.95	3.97
Phenylalanine	5.20	5.17	5.23
Lysine	2.13	2.10	2.15
Tryptophan	n.d.	n.d.	n.d.
Cystine	n.d.	n.d.	n.d.
Total	99.77	99.11	99.51

n.d.=Not determined, +=part of this data has been published[22].

behaviour. The latter would thus stand for the obvious reduction in the apparent inhibitory efficiency of ox-MKOM compared with the very closely related ox-CYST and dans-ox-MKOM. Nonspecific binding appears thus to lead to a shortage in the free amount available of ox-MKOM to be bound to the proper inhibitory site in the proper protein in the ATPase complex and hence the low inhibitory effect. Thus the presence of the additional tripeptide, glycine-phenylalanine-glutamic, with its NH_2 free group in glycine, would accordingly be responsible for what could be denoted as an "activity pseudo-masking effect". Similar conclusion was previously derived from growth-inhibitory studies[2].

The inhibitor-binding process was found, as in the case of DCCD, to be a time-dependant process (results are not given) indicating formation of a chemical bond. In all cases, the isolated inhibitor-protein complex could not be resolved back to their individual components with any one of 8 M urea-, sodium dodecyl sulfate-containing buffers, and extensive washing with phospholipids[21] followed by column and electrophoretic chromatography.

Amino acid analyses of DCCD- and other inhibitors-binding proteins are given in Table 1. The molecular weight of these proteins is estimated as 8,500 dalton and the polarity[21] as $29 \sim 30 \%$. These proteins are characterized by being highly hydrophobic and by their high content of alanine, leucine and glycine and low content of histidine and threonine.

At present the mechanism and site of binding of ox-MKOM and ox-CYST or their dans-forms are still unknown. Sequencing of such proteins is now in progress to determine the site of binding of the inhibitors used. It has been, however, suggested[20] that in yeast and in *N. crassa* DCCD-binding proteins it is the glutamic acid residues which are modified by this inhibitor and the possible mechanism of such binding was discussed.

References

1) IMAM, G. M. & H. KÜNTZEL: Further studies on ASN-136 and monoketo-organomycin. Cystaurimycin, a broad spectrum substance produced by partial enzymic digestion of monoketo-organomycin. J. Antibiotics 30: 314~320, 1977

2) IMAM, G. M. & H. KÜNTZEL: Mechanism of action of monoketo-organomycin, cystaurimycin and their performic acid-oxidized modifications. I. Effects on bacterial growth and ribosomal peptidyl transferase activity. J. Antibiotics 32: 935~942, 1979

3) IMAM, G. M.: Monoketo-organomycin, a new antibiotic. Proc. Egypt. Acad. Sci. 27: 137~144, 1977

4) PULLMAN, M. E. & G. SCHATZ: Mitochondrial oxidations and energy coupling. *In* Annual Review of Biochemistry. Vol. 36, p. 539, (P. D. BOYER, *ed.*) Annual Review Inc., California, U.S.A. 1967

5) GLEHN, M.; R. NORRESTAM, P. KIERKEGAARD, L. MARON & L. ERNSTER: Three-dimensional structure of oligomycine. FEBS Lett. 20: 267~269, 1972

6) BEECHEY, R. B.; C. T. HOLLOWAY, I. G. KNIGHT & A. M. ROBERTON: Dicyclohexylcarbodiimide-an inhibitor of oxidative phosphorylation. Biochem. Biophys. Res. Commun. 23: 75~80, 1966

7) ROBERTON, A. H.; C. T. HOLLOWAY, I. G. KNIGHT & R. B. BEECHEY: A comparison of the effects of NN-dicyclohexylcarbodi-imide, oligomycine A and aurovertin on energy-linked reactions in mitochondria and submitochondrial particles. Biochem. J. 108: 445~456, 1968

8) CATTEL, K. J.; C. R. LINDOP, I. G. KNIGHT & R. B. BEECHEY: The identification of the site of action of NN- dicyclohexylcarbodi-imide as a proteolipid in mitochondrial membranes. Biochem. J. 125: 169~177, 1971

9) STEKHOVEN, F. S.; R. F. WAITKUS & TH. B. MOERKERK: Identification of the dicyclohexylcarbodiimide-binding protein in the oligomycin-sensitive adenosine triphosphatase from bovine heart mitochondria. Biochemistry 11: 1144~1150, 1972

10) FILLINGAME, R. H.: Identification of dicyclohexylcarbodiimide reactive protein component of adenosine 5-triphosphate energy-transducing system of *Escherichia coli*. J. Bacteriol. 124: 870~883, 1975

11) PENTECORVO, G.; J. A. ROPER, L. M. HEMMONS, K. D. McDONALD & A. W. J. BUFTON: The genetics of *Aspergillus nidulans*. Adv. Genet. 5: 141~238, 1953

12) PIENIAZEK, N. J.; P. P. STEPIEN & A. PASZEWSKI: An *Aspergillus nidulans* mutant lacking cystathionine β-synthase: Identity of *l*-serine sulfhydrylase with cystathionine β-synthase and its distinctness from O-acetyl- *l*-serine sulfhydrylase. Biochim. Biophys. Acta 297: 37~47, 1973

13) JINKS, J. L.: *In* Cytoplasmic Inheritance. in Fungi. p. 325 (W. J. BURDETEE, *ed.*) Halden-Day Inc., San Francisco, 1963

14) MARAHIEL, M. A.; G. M. IMAM, P. NELSON, N. J. PIENIAZEK, P. P. STEPIEN & H. KÜNTZEL: Identification of an intramitochondrially synthesized proteolipid associated with the mitochondrial ATPase complex as the product of a mitochondrial gene determining oligomycin resistance in *Aspergillus nidulans*. Eur. J. Biochem. 76: 345~354, 1977

15) BAGINSKI, E. S.; P. P. FOA & B. ZAK: Determination of phosphate: Study of labile organic phosphate interference. Clin. Chim. Acta 15: 155~158, 1967

16) SEBALD, W.; TH. GRAF & G. WILD: Cytoplasmic synthesis of the dicyclohexylcarbodiimide-binding protein in *Neurospora crassa*. *In* Genetics and Biogenesis of Chloroplasts and Mitochondria. pp. 167~174, (TH. BUCHER *et al*, eds). Amsterdam, The Netherlands, Elsevier, North-Holland Biomedical Press, 1976

17) LOWRY, O. H.; N. J. ROSEBROUGH, A. L. FARR & R. J. RANDALL: Protein measurement with FOLIN phenol reagent. J. Biol. Chem. 193: 265~275, 1951

18) SWANK, R. T. & K. D. MUNKRES: Molecular weight analysis of oligopeptides by electrophoresis in polyacrylamide gel with sodium dodecyl sulfate. Anal. Biochem. 39: 462~477, 1971

19) SEBALD, W.; W. MACHLEIDT & J. OTTO: Products of mitochondrial protein synthesis in *Neurospora crassa*. Determination of equimolar amounts of three products in cytochrome oxidase on basis of amino acid analysis. Eur. J. Biochem. 38: 311~324, 1973

20) SEBALD, W.; TH. GRAF & H. B. LUKINS: The dicyclohexylcarbodiimide-binding protein of mitochondrial ATPase complex from *Neurospora crassa* and *Saccharomyces cerevisiae*. Eur. J. Biochem. 93: 587~599, 1979

21) CAPALDI, R. A. & G. VANDERKOOI: The low polarity of many membrane proteins. Proc. Natl. Acad. Sci., U.S.A. 69: 930~932, 1972

22) TURNER, G.; G. M. IMAM & H. KÜNTZEL: Mitochondrial ATPase complex of *Aspergillus nidulans* and the dicyclohexylcarbodiimide-binding protein. Eur. J. Biochem. 97: 565~571, 1979