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Epitope of OSCP oligomycin sensitivity conferring protein exposed at the surface of the mitochondrial ATPase-ATPsynthase complex

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Summary — Immunological studies were designed to study the structure of the oligomycin sensitivity conferring protein (OSCP) integrated in the mitochondrial ATPase-ATPsynthase complex. The monoclonal antibody $2B_1B_1$ used in this study could bind as well to purified or membrane bound OSCP as shown previously by Protein A-gold immunocytochemistry and by competitive immunotitration. In this paper, it is shown that $2B_1B_1$ can also immunoprecipitate the F_0F_1 complex from a Triton X-100 extract. This means that not only, $2B_1B_1$ binds to the surface of OSCP but also that the binding of $2B_1B_1$ did not destroy the interactions between F_0 and F_1 and further demonstrates the external location of the $2B_1B_1$ binding site in the ATPase-ATPsynthase complex. This antigenic site was located on the N-terminal sequence of OSCP, between residues 1 and 72, as demonstrated after chemical cleavage of OSCP with formic acid, hydroxylamine and partial cleavage with cyanogen bromide. The proximity of Tyr and Arg to the epitope was suggested by the lack of $2B_1B_1$ binding to iodinated OSCP and by the susceptibility of this binding to trypsin or to endoproteinase Arg-C treatments of OSCP, respectively. A more precise location of the epitope has been attempted by using the method of synthesis of overlapping octapeptides on solid support. It was found that 2 groups of octapeptides could bind $2B_1B_1$. The first group contained in common the sequence Pro_7-Pro_8-Val_9-Gln_{10}-Ile_{11}-Tyr_{12}- and the second group of peptides contained the sequence Arg_{62}-Ser_{63}-Val_{64}-Lys_{65}. Another monoclonal antibody, AF_4H_7, which competes with $2B_1B_1$, also recognized the first group of peptides. The possible involvement of these 2 fragments in the epitope localized at the surface of OSCP is discussed. In addition, secondary structure theoretical analysis predicts that these 2 domains should be in a β-strand configuration.

ATPase-ATPsynthase / oligomycin sensitivity conferring protein / monoclonal antibody / protein structure / peptide synthesis / membrane protein / pig heart mitochondria

Introduction

The mitochondrial ATPase-ATPsynthase complex is made of a hydrophilic part F_1, bearing the nucleotide and phosphate binding sites related to ATP hydrolysis or synthesis, and of a hydrophobic membrane part F_0 involved in proton translocation. F_1 contains 5 different subunits arranged in a stoichiometry $\alpha_3\beta_3\gamma\delta\epsilon$ [1]. The structure and subunit composition of the mitochondrial F_0 is still a matter of debate [2]. The oligomycin sensitivity conferring protein (OSCP) described as a link connecting F_1 with F_0 is required to render the ATPase activity of F_1 sensitive to oligomycin [3]. Two copies of OSCP per mole of F_1 are present in the

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mitochondrial ATPase-ATPsynthase complex [4]. OSCP appears to function as a "screw" maintaining F_1 in a conformation competent for ATP synthesis [5]. The aim of this work was to study the structure of OSCP integrated in the ATPase-ATPsynthase complex with the help of monoclonal antibodies in order to better understand its functions.

The structural and topographical features of OSCP have been probed by crosslinking studies which have shown that OSCP is close to the α and β subunits of F_1 [6, 7] and to a protein of 24 kDa of F_0 [7]. In addition, OSCP could not be labeled with lipid soluble reagents, which suggested that it was not in contact with the lipid core of the membrane [7]. More recently, by modifying the Cys_{118} with fluorescent analogs, Duszinski *et al.* [8] have proposed that this cysteine which becomes exposed to the aqueous medium upon binding of F_1, was shielded again after formation of an F_0-F_1-OSCP complex. However, models describing the tertiary structure of this subunit as well as that of the ATPase-ATPsynthase complex have not yet been obtained since crystallographic approaches have been unsuccessful. In this paper we have used a monoclonal antibody which probes a very accessible domain of OSCP at the surface of the ATPase-ATPsynthase complex integrated into the mitochondrial membrane. We show that this domain is located on the N-terminal side of the OSCP sequence, the epitope probably containing amino acid residues between 7 and 12, that could be juxtaposed to amino acids 62 to 65 in the structure of the protein.

Materials and methods

Materials

Specific chemicals were purchased from the following sources: IODO-GEN, Pierce; *N*-hydroxysuccinimide-biotin, Sigma; alkaline phosphatase- or horseradish peroxidase-conjugated antimouse IgG sheep antibody, Biosys; Streptavidin-agarose, BRL; dimethylformamide 99.9%, Aldrich; cyanogen bromide purum, Fluka; Immobilon membranes, IPVH, 0.45 μm, Millipore; nitrocellulose membranes, pH 7.1, 0.1 μm, Schleicher and Schuëll; peptide synthesis kit, Cambridge Research Biochemicals. All other chemicals were of the highest grade available.

Methods

Biological preparations
Previously described procedures were used to obtain pig heart mitochondrial F_1-ATPase [10], OSCP [11] and monoclonal antibodies directed against OSCP: $2B_1B_1$ and AF_4H_7 [12]. These antibodies were purified by affinity chromatography on protein A-Sepharose according to the procedure of Ey *et al.* [13]. Protein concentration was estimated by the method of Lowry *et al.* [14]. ATPase activity and oligomycin sensitivity were measured as previously described [15].

Immunoprecipitation of the F_0F_1 complex
A mitochondrial suspension containing 16 mg of protein was sedimented at 9,000 *g* for 2.5 min and washed with 2 ml of buffer containing 4 mM EDTA and 10 mM HEPES-KOH, pH 7.4. After a second centrifugation at 9 000 *g* for 2.5 min the pellet was homogenized and incubated for 20 min at 0°C in 1 ml of 10 mM HEPES-KOH, 150 mM NaCl, 1% Triton X-100, 0.2 mM PMSF to obtain the mitochondrial lysate. Insoluble proteins were eliminated by centrifugation at 100,000 *g* for 20 min. The clear lysate was kept at −80°C. The purified antibody $2B_1B_1$ was biotinylated according to Updyke and Nicolson [16]. Verification was made that the biotinylated antibody was adsorbed to Streptavidine-agarose and that the biotinylation did not modify the affinity of the antibody for OSCP. Indeed, the binding curve of the biotinylated antibody to OSCP (tested by ELISA at various antibody concentrations) was identical to that observed with the unmodified antibody. The biotinylation of the antibody increased the M_r of the heavy and light chains of $2B_1B_1$ as shown by their migration in SDS-PAGE. The mitochondrial lysate (0.7 mg) was incubated overnight at 4°C with 50 μg of biotinylated antibody (theoretical molar ratio OSCP / antibody of 1, assuming that the mitochondrial membrane contained 10% of ATPase-ATPsynthase complex) in 10 mM HEPES-KOH, 150 mM NaCl, 1% Triton X-100, pH 7.4. Streptavidin-agarose (100 μl of a 10% suspension) was added and mixed for 1 h at 4°C. The pellet obtained after centrifugation for 2.5 min at 9,000 *g* was washed 5 times in 10 mM HEPES-KOH, 150 mM NaCl, 0.1% Triton X-100. The final pellet was treated with 50 μl of 150 mM Tris-HCl, 4% 2-mercaptoethanol, 6% SDS, 6 mM EDTA, 30% glycerol and boiled for 10 min. The solubilized proteins were analyzed by SDS–PAGE.

Covalent modification of OSCP
OSCP was alkylated with *N*-ethylmaleimide according to the procedure of Dupuis *et al.* [6] or iodinated in the presence of IODO-GEN [17] using a molar ratio NaI / OSCP of 10. The slight precipitate which appeared during iodination was removed by centrifugation at 4°C for 2 min in an Eppendorf centrifuge after addition of 0.05% Tween 20 to avoid further precipitation of iodinated OSCP. Unreacted NaI was removed by dialysis against 5 mM HEPES-KOH, containing 0.05% Tween 20.

Amino acid analysis. Protein sequencing
OSCP was hydrolyzed in sealed ampoules in the presence of 6 M tridistilled HCl, 0.05% phenol (v/v) at 110°C for 24, 48 and 72 h. Amino acid analyses were performed on a Kontron automatic amino acid analyser using an AS−70 resin (11 × 0.4 cm column) and a post column ninhydrin detection method.

Automated gas phase sequencing was performed at the Centre de Microanalyse of the CNRS in Solaize on an Applied Biosystem model 470 A apparatus. N-terminal sequence of OSCP was determined after transfer to Immobilon membranes of OSCP separated by SDS gel electrophoresis [18]. The pieces of Immobilon containing OSCP were directly introduced into the gas phase sequencer and the sequencing was performed according to the Applied Biosystem protocol.

Chemical cleavage of OSCP
Formic acid cleavage. Formic acid can cleave preferentially Asp-Pro bonds in proteins. A solution of OSCP (1 mg/ml of 20 mM Tris-H_2SO_4, pH 8.0) was precipitated by addition of 5 vol of cold acetone and kept for 24 h at −20°C. The pellet dissolved in 70% formic acid was incubated overnight at 45°C. The samples were freeze-dried twice after addition of 5 vol of distilled water. The samples were then dissolved in sample electrophoresis buffer and analyzed by SDS−PAGE.

Hydroxylamine cleavage. Hydroxylamine can cleave proteins preferentially at the level of Asn-Gly bonds [19]. Hydroxylamine cleavage was performed on intact OSCP or on the OSCP fragment obtained by formic acid cleavage and separated by SDS−PAGE. The acrylamide bands containing OSCP stained with Coomassie blue were cut out and freeze-dried. The dried gels were incubated for 18 h at 45°C in the presence of 2 M hydroxylamine chlorhydrate, 2 M NaOH, 0.2 M sodium bicarbonate, pH 9.0. The gels were washed 6 times with distilled water to eliminate the salts and incubated for 1 h in 200 mM Tris-HCl, pH 6.0, 15% glycerol (v/v), 2.5% β-mercaptoethanol (v/v), 0.1% SDS. The remaining proteins were then separated by SDS−PAGE in duplicate samples. One sample was stained with Coomassie blue, the other transferred to nitrocellulose for immunological analysis.

Cyanogen bromide cleavage. Cyanogen bromide cleaves Met-X bonds with a high specificity. OSCP precipitated with acetone was solubilized in 70% formic acid. Cyanogen bromide was added at a reagent/protein ratio (w/w) either of 50 for incomplete cleavage or of 500 for complete cleavage. After incubation at room temperature for 15 min to 24 h according to the desired percentage of cleavage, the samples were diluted with 5 vol of distilled water and freeze-dried. Ten μl of 70% (v/v) aqueous glycerol were added to improve peptide recovery. Addition of distilled water and freeze drying were repeated twice. The dried samples were then analyzed by SDS−PAGE and immunodecoration.

Electrophoresis
SDS−PAGE was performed as described by Laemmli [20], using a 15% polyacrylamide separating slab gel (1.5 × 200 × 100 mm) in a Protean II Biorad apparatus. After electrophoresis, the gels could be stained with 0.025% (w/v) Coomassie blue R250 in 1% methanol, (v/v), 25% isopropanol (v/v), 10% acetic acid (v/v) and destained in 10% acetic acid (v/v). In order to test their immunoreactivity, the proteins or peptides separated by SDS−PAGE were transferred to nitrocellulose membranes for 4 h at 200 mA in a Transblot Hoeffer apparatus by the method of Towbin *et al.* [21]. For protein sequencing, the transfer was made in 25 mM CAPS buffer (3[cyclohexyl-1-amino] propane sulfonate) pH 10.4, containing 10% methanol (v/v) for 4 h at 200 mA, according to Matsudaira [18].

Immunodecoration after transfer of proteins to nitrocellulose
The nitrocellulose filters were incubated for 15 min with PBS (phosphatesaline buffer: 10 mM sodium phosphate, 0.9% NaCl (w/v), pH 7.2) containing 1% bovine serum albumin (fraction V, Sigma) and 0.05% Tween 20. The filters were then incubated overnight at 4°C with the antibody (ascitic fluid diluted at 1/1000° in the above buffer). After 6 washes with PBS containing 0.05% Tween 20, the filters were incubated for 1 h at room temperature with an anti-mouse IgG antibody conjugated to either horseradish peroxidase or alkaline phosphatase. The activity of these enzymes was revealed after 3 washes in PBS-Tween and 1 in distilled water using mixtures containing either 0.06% α-naphtol and 0.06% H_2O_2 in 20% aqueous methanol or 0.3 mg/ml nitrobluetetrazolium, 0.15 mg/ml 5-bromo-4-chloro-3-indolylphosphate in 100 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, as substrate for horseradish peroxidase or alkaline phosphatase, respectively.

Competitive ELISA
The ability of native or modified OSCP to bind the antibody $2B_1B_1$ was tested by measuring the amount of antibody remaining free in solution and able to bind to OSCP coated to polystyrene plates after incubation with increasing concentrations of native or modified OSCP. The wells of 96-well polystyrene NUNC plates were coated with OSCP at a concentration of 1 mg in 100 ml of PBS, as described previously [22]. The antibody $2B_1B_1$ (ascitic fluid diluted at 1/8000° in PBS-BSA) was incubated for 3 h at 4°C in Eppendorf tubes with OSCP (chemically modified or not) or with a synthetic peptide analogous to an OSCP fragment at increasing concentrations. The antigen-antibody mixture (50 μl) was then incubated for 1 h (except otherwise indicated) in the wells of

the polystyrene plates containing coated OSCP to titrate the amount of antibody remaining free in the incubation mixture. This amount was then estimated by ELISA using horseradish peroxydase conjugated anti-mouse IgG, as described previously [22].

The competition between the antibodies $2B_1B_1$ and AF_4H_7 for their binding to OSCP was made by using purified antibodies iodinated with [^{125}I]NaI in the presence of IODO-GEN, as described above for OSCP. The binding of the iodinated antibody to OSCP was tested by RIA [22] at a fixed concentration of iodinated antibody (corresponding to 50% of its maximal binding) in the presence of increasing concentrations of the other unlabeled antibody.

Synthesis of peptides homologous to the sequence of beef heart OSCP

The synthesis of a peptide containing the region of OSCP including the sequence Ala_{26}-Glu_{38} of beef heart OSCP, which is the most antigenic in the sequence as predicted by the method of Parker *et al.* [23] was performed by using a semi-manual LKB peptide synthetizer and the chemistry of the Fmoc-amino acids [24]. The immunoreactivity of this peptide was tested by competitive ELISA after purification of the peptide by reverse-phase HPLC on a C18 column with 50 Å pore diameter. The peptide was eluted with a linear gradient of 0.04% trifluoroacetic acid in acetonitrile (v/v) and of 0.1% trifluoroacetic acid.

A family of all possible overlapping octapeptides spanning the regions Phe_1-Lys_{30} and Arg_{41}-Met_{72} covalently bound to separate polyacrylic/polyethylene pins bearing β-alanine-hexamethylene diamine as a spacer was synthetized using the Fmoc amino acids and following the protocol given by Cambridge Biochemicals Research. Each octapeptide was acetylated before removal of the protecting groups. The pins saturated with PBS containing 0.05% Tween, 1% egg albumin and 1% bovine serum albumin were incubated overnight at 4°C with the antibody $2B_1B_1$ (ascitic fluid diluted at 1/1000° in PBS-Tween containing 1% bovine serum albumin). After 4 washes in PBS-Tween, the pins were incubated for 1 h with horseradish peroxydase conjugated anti-mouse IgG diluted at 1/1000° in PBS-BSA. The pins were washed again 3 times in PBS-Tween and once in distilled water. The peroxidase activity was revealed by incubating the pins in a 96-well microtitration plate containing in each well 175 µl of substrate buffer (80 mM citrate - phosphate buffer, pH 4.5, 2.5 mM ABTS, 0.18 mM H_2O_2). The optical density was read at 410 nm in a Dynatech microtitration plate reader.

Antigenic potential and secondary structure predictions

The antigenic potential along OSCP sequence was studied using the ANTHEPROT program [25] which includes several methods: the surface probability of Emini *et al.* [26], the hydrophilicity profile determined from the inverse scale of hydrophobicity according to Kyte and Doolittle [27], the structural dissimilarity scale relative to Gly of Padlan [28], and Antigon which combines methods using theoretical [29] and experimental [23] hydrophilicity parameters with that of prediction of polypeptide chain flexibility [30]. The boundaries of domains were estimated according to Vonderviszt and Simon [31].

Results

Immunoprecipitation of the F_0F_1 complex with the anti-OSCP monoclonal antibody $2B_1B_1$

The monoclonal anti-OSCP antibody, $2B_1B_1$ immunoprecipitates the F_0F_1 complex (Fig. 1-lane 2) from a mitochondrial Triton extract (Fig. 1, lane 1). Indeed, in addition to OSCP, subunits of F_1 such as α, β or γ can be easily visualized in the immunoprecipitate as well as subunits known to belong to F_0 such as the 24 or the 20 kDa proteins. Additional bands present in the immunoprecipitates can be attributed to the heavy and light chains of the $2B_1B_1$ antibody and to a protein of 17 kDa which dissociates from streptavidin during the treatment of the beads with the electrophoresis sample buffer. This 17 kDa protein is therefore a degradation product of streptavidin and not a protein associated with the F_0F_1 complex. The simultaneous

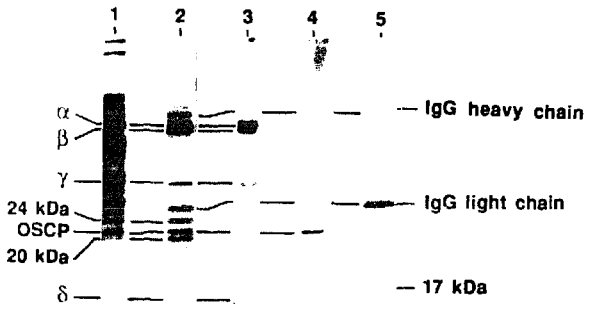

Fig. 1. Immunoprecipitation of F_0F_1 with the monoclonal antibody $2B_1B_1$. Experimental conditions as described in *Materials and methods*. *Lane* 1: mitochondrial Triton extract, *lane* 2: immunoprecipitate from mitochondrial Triton extract, *lane* 3: F_1, *lane* 4: OSCP, *lane* 5: Streptavidin-agarose incubated with the antibody in the presence of Triton X-100 but in the absence of mitochondria.

immunoprecipitation of F_0 and F_1 subunits with OSCP proves not only that OSCP has an external location in the F_0F_1 complex but also that the binding of the antibody does not disrupt the interactions between F_0 and F_1 subunits.

Comparison between the amino acid composition of pig and beef heart OSCP

The antidoby $2B_1B_1$ is able to recognize beef heart OSCP as well as pig heart OSCP [12]. The amino acid sequence of beef heart OSCP has been established by Ovchinnikov *et al.* [32]. In order to determine whether it was reasonable to use the beef heart OSCP sequence in the localization of the $2B_1B_1$ epitope, we have compared the amino acid composition of pig heart OSCP to that of beef heart OSCP. Table I shows that the differences observed between amino acid analysis of pig and beef heart OSCP are of the same order of magnitude as the differences

Table I. Comparison between amino acid analysis of pig and beef heart OSCP.

Amino acid	Pig heart	Beef heart			
	mol / 100 mol[a]	mol / mol[b] OSCP	amino acid analysis[c]	amino acid analysis[d]	sequence[e]
Asp+Asn	5.13±0.17	10	10	9	10
Thr	5.41±0.02	10	12	11	13
Ser	9.9 ±0.04	19	18	13	16
Glu±Gln	11.22±0.17	21	19	24	19
Pro	3.48±0.08	7	8	9	8
Gly	5.00±0.13	9.5	8	12	9
Ala	8.83±0.32	17	17	18	16
Val	8.76±0.47	17	16	18	16
Cys		1[f]	1	1	1
Met	3.70±0.02	7	7	6	8
Ile	6.30±0.22	12	11	11	11
Leu	9.73±0.03	18.5	23	24	23
Tyr	2.39±0.17	5	4–5	4	5
Phe	1.77±0.17	3	4–5	4	4
Lys	12.16±0.47	23	20	17	21
His	1.75±0.05	3	1	1	1
Arg	3.89±0.04	7	9	9	9

[a]Mean ± SEM (N = 3).
[b]Values were calculed on the basis of 190 amino acids in the sequence corresponding to an M_r value of 21 000. Ser and Thr were extrapolated to zero time of hydrolysis of OSCP. Ala, Val, Ile, Leu and Lys were measured after 72 h of hydrolysis.
[c,d]According to Dupuis *et al.* [41][c] and Senior [42][d] on the basis of 190 amino acids.
[e]According to Ovchinnikov *et al.* [32].
[f]Measured by chemical modification with [^{14}C]*N*-ethylmaleimide [4].

between the beef heart OSCP amino acid analysis and its composition deduced from the sequence. For further control, the N-terminal sequence of pig heart OSCP was also compared to that of beef heart OSCP. No difference could be observed for the first 18 amino acids that have been sequenced.

Effects of chemical modifications of OSCP on antibody binding

In a first approach to identify the domain of OSCP recognized by the antibody and exposed at the surface of the F_0F_1 complex, the effects of the modification of specific amino acids of OSCP on antibody binding was tested. Ovchinnikov *et al.*, [32] have shown that the amino acid sequence of beef heart OSCP contains a single cysteinyl residue, Cys_{118}. This cysteine can be alkylated by *N*-ethylmaleimide [6]. The labeling of pig heart OSCP by the same procedure with [^{14}C]*N*-ethylmaleimide similarly revealed the presence of one cysteine in OSCP, not involved in oligomycin sensitivity of the ATPase activity of the reconstituted F_0F_1 complex. The binding of either $2B_1B_1$ or AF_4H_7 to OSCP covalently modified with 0.9 mol of [^{14}C]*N*-ehylmaleimide per mole [33] was identical to the binding of these antibodies to unmodified OSCP. Therefore, the alkylation of the cysteine, which is very likely at the same position as Cys_{118} of beef heart OSCP, has no effect on the binding of the antibody to OSCP. On the contrary, Figure 2 shows that $2B_1B_1$ could not bind any more when easily accessible tyrosines of OSCP were iodinated. Under the conditions used, an average of 3 tyrosines were iodinated per mole of OSCP. These results suggest that at least 1 tyrosine is present in the $2B_1B_1$ binding site or close to it while the cysteine of OSCP must be remote from this site. The same results were obtained with AF_4H_7.

Recognition by $2B_1B_1$ and AF_4H_7 of OSCP and of its framents obtained by cleavage with formic acid, hydroxylamine or cyanogen bromide

When the binding of iodinated $2B_1B_1$ to OSCP was measured by RIA in the presence of increasing concentrations of unlabeled AF_4H_7, the radioactivity was progressively decreased to zero. Conversely, the presence of $2B_1B_1$ completely inhibited the binding of iodinated AF_4H_7. Therefore, although these antibodies had been obtained from 2 different fusions, they recognized the same or at least overlapping epitopes.

Treatment of OSCP with trypsin at a molar ratio trypsin/OSCP of 1/1000 for 1 min to 1 h produced a large number of peptides. However only a band very close to that of OSCP could react with $2B_1B_1$. A similar result was obtained with endoproteinase Arg-C. These results suggest that an Arg-X bond, very susceptible to the trypsin or endoproteinase Arg-C attack, is close to the epitope of $2B_1B_1$. Therefore, these types of enzymatic cleavage could not be further used to isolate a small fragment of OSCP recognized by $2B_1B_1$.

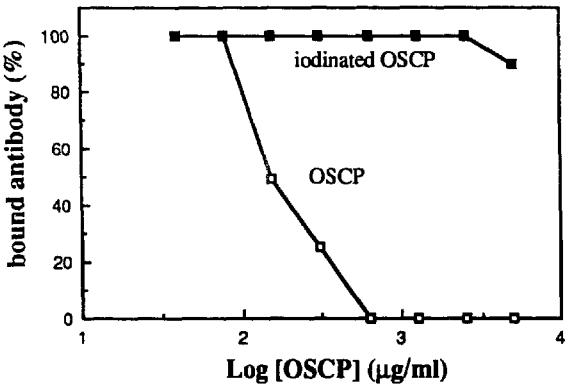

Fig. 2. Effect of OSCP iodination on its binding to the monoclonal antibody $2B_1B_1$. The monoclonal antibody $2B_1B_1$ purified on Protein A-Sepharose was incubated for 3 h at a concentration of 0.5 µg/ ml of buffer containing 20 mM Tris–H_2SO_4, pH 8.0, 100 mM NaCl, 0.02% Tween 20 with increasing amounts of OSCP (□) or iodinated OSCP (■). Aliquots of 50 µl were then transferred to the wells of microtitration plates coated with 1 µg of OSCP. After a 2 h-incubation at 0–4°C, the amount of $2B_1B_1$ bound to the wells was titrated by ELISA using horseradish peroxidase conjugated anti-mouse IgG.

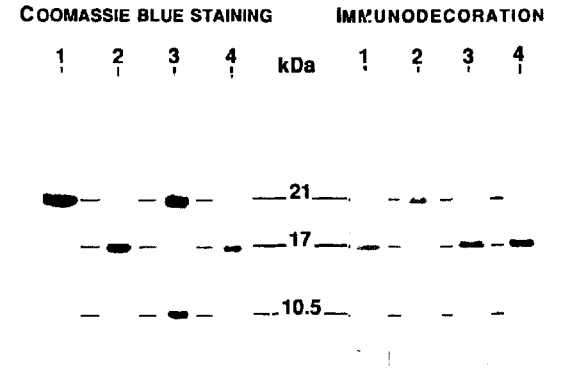

Fig. 3. Recognition by $2B_1B_1$ of fragments of OSCP obtained by cleavage with formic acid and hydroxylamine. Experimental conditions as described in *Materials and methods*. *Lanes* 1: purified OSCP; *lanes* 2: fragment obtained by cleavage with 70% formic acid, *lanes* 3: fragments obtained by cleavage with hydroxylamine, *lanes* 4: fragments obtained by hydroxylamine cleavage of the 17 kDa peptide obtained by formic acid cleavage of OSCP.

The cleavage of OSCP with formic acid produced a fragment of 17 kDa that could be revealed by immunodecoration (Fig. 3, lanes 2). The sequence of beef heart OSCP containing an Asp-Pro bond in position 155-156, this 17 kDa fragment should correspond to the first 155 amino acids of the sequence.

Treatment of OSCP with hydroxylamine also cleaved OSCP. A fragment of 10.5 kDa recognized by the antibody was obtained (Fig. 3, lanes 3). Beef heart OSCP contains an Asn-Gly bond in position 92-93. It is therefore likely that the 10.5 kDa peptide contains the 2 parts of OSCP cleaved approximately in the middle of the sequence. When the fragment of 17 kDa obtained by formic acid treatment was cleaved by hydroxylamine, an identical band of 10.5 kDa recognized by $2B_1B_1$ was obtained (Fig. 3, lanes 4). From these experiments, it could be concluded that the epitope recognized by the antibody was located within the first 92 amino acids.

The complete cleavage of OSCP by cyanogen bromide did not produce any peptide that could be detected by immunodecoration (not shown). However, incomplete cleavage of OSCP by cyanogen bromide produced several peptides recognized by $2B_1B_1$ (Fig. 4, lanes 2 and 3). Their apparent molecular weights were of about 17 kDa and 12 kDa. In addition, a peptide of about 8 kDa gave a faint but reproducible staining by immunodecoration (not clearly visible on the actual figure). On the contrary a 15 kDa band stained with Coomassie blue could not be detected by immunodecoration. The N-terminal sequence of the 17 kDa and of the 15 kDa peptides could be performed after transfer to an Immobilon membrane. The N-terminal sequence of the 17 kDa peptide corresponded to the N-terminal sequence of OSCP. Taking into account the location of the methionines on beef heart OSCP, a peptide of 17 kDa should correspond to a fragment spanning the sequence 1–159 or 1–163. The peptide of 15 kDa had an N-terminal sequence beginning by Ala-Ala-Ser-Leu-Leu(?)-Asn. This sequence corresponds to a fragment beginning at Ala_{52} on the beef heart OSCP sequence. Taking into account its size, this 15 kDa peptide should correspond to a fragment of OSCP spanning the sequence 52–190 or 52–186. The lack of recognition of this peptide by $2B_1B_1$ indicated that the cleavage at the level of Met_{51} destroyed the epitope suggesting either that the epitope was located at the level of the first 52 amino acids or else that the epitope was destroyed by cleavage of the Met_{51}-Ala_{52} bond. The latter hypothesis was based on the fact that no peptide that would correspond to the fragment Phe_1-Met_{51}, that is a peptide of about 6 kDa was found to be reactive with the antibody by immunodecoration. On the contrary, a peptide of 8 kDa that could correspond to the fragment Phe_1-Met_{72} was slightly immunoreactive. All the peptides that were found to be immunoreactive with the antibody $2B_1B_1$ was also recognized by AF_4H_7.

Prediction of secondary structure and antigenic domains of OSCP

Figure 5 shows that the fragments of the OSCP sequence that are predicted as accessible to the surface and located between amino acids 25 and 85 (Fig. 5, *SURPRO*) also correspond to hydrophylic regions (Fig. 5, *KYDOO*) and to regions predicted as antigenic according to the method

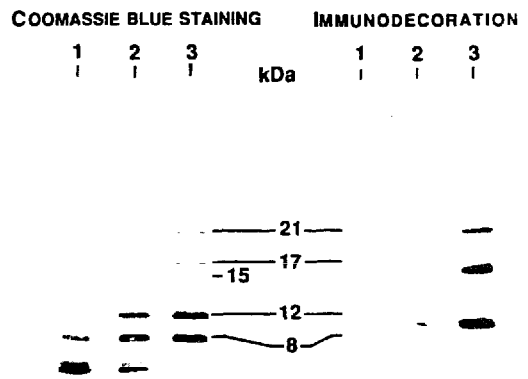

Fig. 4. Cleavage of OSCP with cyanogen bromide. OSCP (0.2 mg) dialyzed against 0.1 M ammonium bicarbonate, pH 8.0 containing 0.02% 2-mercaptoethanol was freeze-dried in a Speed-Vac concentrator, dissolved in 1 ml distilled water, divided into 3 aliquots and freeze-dried again. 150 μl of cyanogen bromide (10 mg/ml of 75% formic acid) were added to each sample. Distilled water (1 ml) was added to each sample after incubation either at room temperature for 15 min (sample 3) or 1 h (sample 2) or at 37°C for 24 h (sample 1). The samples were freeze-dried. Addition of 1 ml of water and freeze-drying were repeated twice. The samples were dissolved in 100 μl of electrophoresis sample buffer and analyzed by SDS–PAGE gel electrophoresis in a 15–25% gradient gel. Two lanes were run for each sample: one was stained with Coomassie blue, the other was transferrred to nitrocellulose. After transfer, the nitrocellulose blot was saturated with bovine serum albumin and incubated successively with $2B_1B_1$ (ascitic fluid diluted at 1/5000e with PBS-BSA) and with alkaline-phosphatase-conjugated anti-mouse IgG as described in *Materials and methods*. The presence of immunoreactive peptides was revealed in the presence of α-naphtol and H_2O_2.

of Parker *et al.* [23] (Fig. 5, *ANTIGON*). The dissimilarity scale relative to Gly of Padlan predicts somewhat different antigenic regions. However, several segments are also predicted as antigenic between amino acids 30 and 90 (Fig. 5, *DISSIMILARITY*).

The prediction of the secondary structure in the 1–90 region indicates a high α-helix content between amino acids 20 and 56. Although the boundaries of the predicted helices are rather broad (depending on the method of prediction) there are 3 helices which are jointly predicted by the double prediction and the homology method. It is important to note that, with these methods, the expected prediction success of these copredicted helices is at the 70% level [34]. Two smaller α-helices are predicted between amino acids 66–78 and 85–91. Two β-strands are also generally found around amino acids 7–14 and 62–66.

Recognition by $2B_1B_1$ and by AF_4H_7 of synthetic peptides identical to peptides present in the beef heart OSCP sequence

In a first approach, a peptide containing the sequence of beef heart OSCP from Ala_{26} to

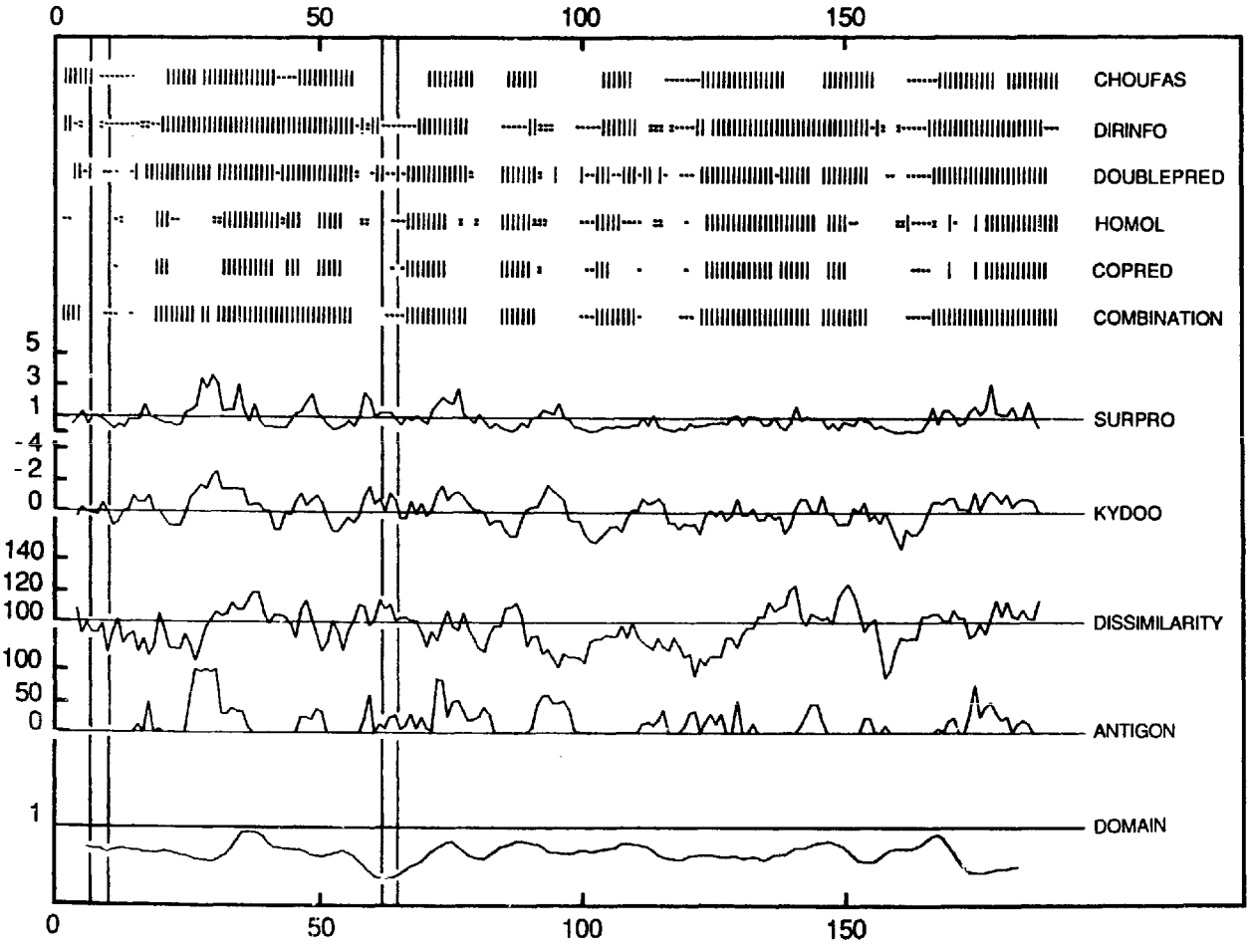

Fig. 5. Prediction of the secondary structure and of the immunogenic potential of bovine OSCP. The predictions have been obtained by using the ANTHEPROT computer program package. The different graphs correspond to the following methods: CHOUFAS [37]; DIRINFO [38]; DOUBLEPRED [39]; HOMOL [40]; COPRED [25]; COMBINATION [25]; SURPRO [26]; KYDOO [27]; DISSIMILARITY (using a window of 7 amino acids) [28]; ANTIGON (combined method including hydrophilicity and flexibility parameters using a window of 7 amino acids) [23]; and DOMAIN using a window of 11 amino acids [31]. The α-helices are indicated by vertical bars, the β-strands by dashes and the turns by semi-columns. The remaining aperiodic regions are not represented.

Glu_{38} was synthesized and purified. This peptide corresponds to a region of OSCP considered to have a high antigenic potential by all tested prediction methods (Fig. 5). This peptide was also located inside the smallest OSCP fragment (amino acids 1 to 72, *see above*) that could be recognized by $2B_1B_1$. After synthesis, this peptide was tested for its immunoreactivity towards $2B_1B_1$ by 2 methods. Firstly, 10 μg of the peptide were spotted onto an Immobilon membrane in a Dot-Blot apparatus (BioRad) and tested by immunodecoration. No binding of the antibody could be detected. Secondly, the peptide was used in a competitive ELISA assay, under conditions similar to those described in Figure 2. In this case, the antibody was preincubated with the peptide or with OSCP at a concentration giving a half-maximum response for OSCP at a concentration of 0.1 nM. When the peptide was preincubated with the antibody no competition occurred, *i.e.* no decrease in the ELISA response was observed even for peptide concentrations as high as 1 mM (data not shown), that is a concentration 10^7 higher than that of OSCP.

A family of overlapping octapeptides spanning 2 regions of OSCP from amino acids 1 to 30 and 41 to 72 has been synthesized and kept covalently bound to the extremety of plastic pins, according to the method of Geysen [35]. The pins were tested for their immunoreactivity towards the antibodies $2B_1B_1$ and AF_4H_7. Figure 6 shows that a first group of octapeptides close to the N-terminal end of OSCP were able to significantly bind the antibodies. It mainly contained 3 consecutive octapeptides spanning the region from Val_5 to Ile_{14}. The sequence of amino acids common to these peptides is: Pro_7-Pro_8-Val_9-Gln_{10}-Ile_{11}-Tyr_{12}. A second group of 5 octapeptides able to bind $2B_1B_1$ but not AF_4H_7 was located between the Pro_{58} and the Leu_{69}. The sequence of amino acids common to these 5 octapeptides is: Arg_{62}-Ser_{63}-Val_{64}-Lys_{65}. Similar profiles were obtained in 3 different experiments. All positive octapeptides in the first group contain a Tyr in their sequence. In the second group, a Tyr is present in the first 2 octapeptides. Pins bearing the immunoreactive octapeptides were iodinated [17]. The immunoreactivity was selectively suppressed for the pins containing a Tyr in their sequence. When the pins bearing the OSCP octapeptides were tested using either unspecific mouse IgG or an anti-β subunit monoclonal antibody, no significant binding could be demonstrated, which indicated the specificity of the $2B_1B_1$ or AF_4H_7 binding.

Discussion

In a previous study [7], it was shown that the anti-OSCP monoclonal antibody $2B_1B_1$ could bind to inverted submitochondrial particles (ETP). Indeed, protein A-gold particles could be visualized by immunocytochemistry after incubations of ETP bound to electron microscopic grids successively with $2B_1B_1$ and with protein A-gold. In addition, the total amount of OSCP titrated after a previous dissociation of these membranes with urea was equal to the amount of OSCP measured by competitive ELISA using intact inverted membranes in the competition. These experiments indicated that the epitope of OSCP recognized by $2B_1B_1$ was accessible to the inner surface of the mitochondrial membrane and that all OSCP molecules present in mitochondria appeared similarly accessible to the antibody. In this paper, we demonstrate that not only is the epitope recognized by this antibody easily accessible at the surface of the inner mitochondrial membrane but also that the binding of this antibody to OSCP did not destroy the interactions between F_0 and F_1. Indeed, the whole F_0F_1 complex could be immunoprecipitated by using the anti-OSCP monoclonal antibody, $2B_1B_1$. The whole F_0F_1 complex was often immunoprecipitated by using polyclonal antibodies raised against F_1 since the first report of Tzagoloff and Meagher [36]. To our knowledge, it is the first example showing that this complex could be immunoprecipitated by using an anti-OSCP antibody. This demonstrates very clearly that OSCP occupies an external location in the F_0F_1 complex and should not be, as shown in some textbooks, a protein sequestered between F_0 and F_1.

To further understand the structure of OSCP in view of this conclusion, the identification of the epitope recognized by this antibody was necessary. The sequence of the pig heart OSCP was unknown, however that of beef OSCP was and the antibody recognized beef as well as pig heart OSCP. The antigenic site present in pig heart OSCP should, therefore, also be present in beef heart OSCP. We show in this paper that the amino acid analyses of the 2 proteins were very close. The N-terminal sequence of the 2 proteins was identical at least for the first 18 amino acids and predictions of cleavage based on the beef heart sequence were proved correct using the pig heart OSCP. Indeed, the size of the fragments obtained by formic acid, hydroxylamine and cyanogen bromide corresponded to the predictions

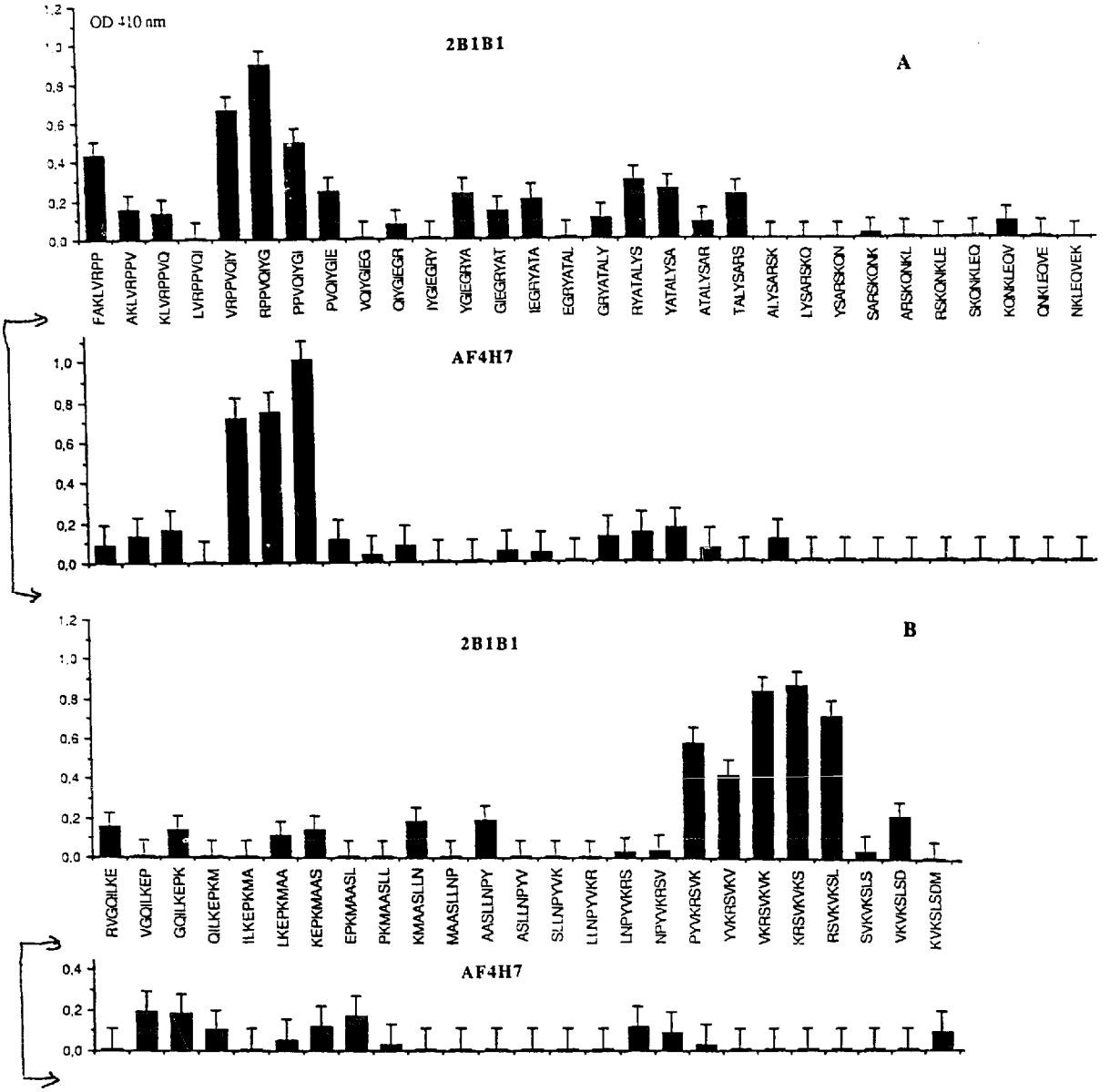

Fig. 6. Recognition by $2B_1B_1$ or by AF_4H_7 of overlapping synthetic octapeptides identical to peptides present in the sequence of beef heart OSCP. Experimental procedures as described in *Materials and methods*. (**A**) Overlapping octapeptides spanning the sequence of OSCP from residues 1 to 30. (**B**) Overlapping octapeptides spanning the sequence of OSCP from residues 41 to 72. A series of octapeptides unrelated to OSCP and synthesized together with the peptides homologous to OSCP were tested for their immunoreactivity to $2B_1B_1$ or to AF_4H_7 together with the OSCP peptides, to serve as controls. The range of optical densities read fore these control assays is indicated as vertical bars. The tests were repeated twice on the same pins after removing the antibodies bound to the pins by treatment with SDS and methanol at 60°C, as described by the manufacturer. The differences in optical densities read for the three series of assays always lay between the range indicated by the vertical bars.

the predictions made with beef heart OSCP. This indicates that the Asn-Gly, the Asp-Pro and the Met-X bonds should be in the same positions in beef and pig OSCP.

The determination of the smallest peptide that could be recognized by $2B_1B_1$ after chemical cleavage of OSCP is summarized in Figure 7. Acid formic cleaved OSCP by giving rise to a 17 kDa peptide able to bind $2B_1B_1$. This 17 kDa peptide could be cleaved by hydroxylamine giving a peptide of 10.5 kDa still able to bind $2B_1B_1$. The smallest peptide obtained by partial cleavage with cyanogen bromide corresponded to the N-terminal fragment of OSCP containing the 72 first amino acids. It could therefore be concluded that the antigenic site recognized by $2B_1B_1$ was located in this N-terminal fragment. The same conclusions were reached with the antibody AF_4H_7. The fact that the binding of $2B_1B_1$ could be demonstrated by immunodecoration neither with the N-terminal fragment Phe_1-Met_{51} nor with the C-terminal fragment

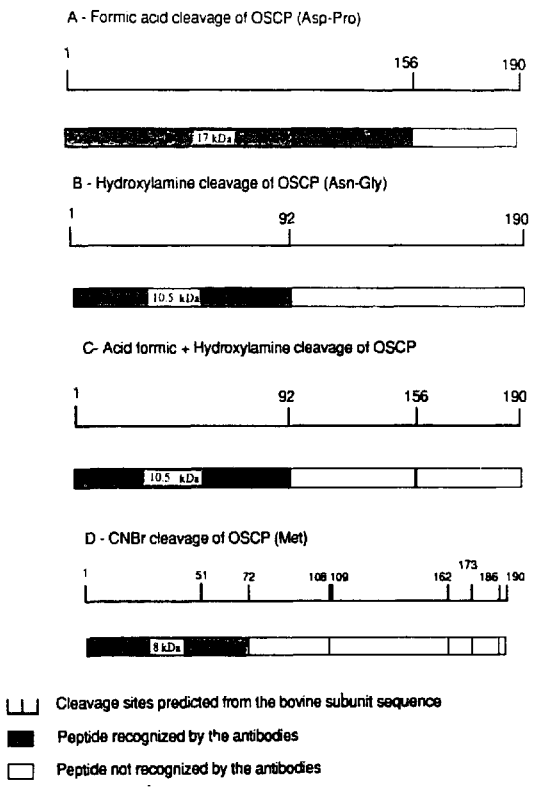

Fig. 7. Summary of the effects of chemical cleavage of OSCP on the binding of the monoclonal antibody $2B_1B_1$ to OSCP.

starting at Ala_{52} (15 kDa) suggested that Met_{51} was important in the structure of the antigenic site either directly, as being a part of the epitope or indirectly, as being a part of the epitope or indirectly, as being important in conferring to OSCP an adequate structure for $2B_1B_1$ binding.

The systematic synthesis of overlapping peptides chosen along the N-terminal sequence of beef heart OSCP has permitted the identification of 2 groups of peptides reacting with $2B_1B_1$. The first, which is located between Pro_7 and Tyr_{12}, also reacted with AF_4H_7. This reactivity towards this same group of peptides explains very well the competition between the 2 antibodies for binding to OSCP. The second group of immunoreactive peptides, located between Arg_{62} and Lys_{65} was specific to $2B_1B_1$. The fact that 2 relatively remote regions were immunoreactive towards $2B_1B_1$ was an unexpected finding for a monoclonal antibody such as $2B_1B_1$. Indeed, previous experiments suggested that the epitope recognized by $2B_1B_1$ should be sequential: the affinity of the antibody to OSCP was the same for OSCP integrated in active membrane-bound ATPase-ATPsynthase complex as for OSCP present after denaturation of the membranes by urea. The most likely explanation for this apparent discrepancy is, that, after denaturation with urea OSCP was able to recover its initial conformation, at least, as far as the domains involved in the antibody binding are concerned.

The identification of these 2 regions is consistent with all the results observed either after covalent modification of OSCP or after enzymatic or chemical cleavage of OSCP. The fragment Pro_7-Tyr_{12} contains a Tyr while the fragment Arg_{62}-Lys_{65} is close to the Tyr_{59}. This can explain why the iodination of OSCP prevented the binding of $2B_1B_1$. The fragment Arg_{62}-Lys_{65} contains an Arg while the fragment Pro_7-Tyr_{12} is close to Arg_6 which is present in the sequence of pig heart OSCP as shown by the N-terminal sequence analysis. The presence of these Arg explains why the immunological response towards $2B_1B_1$ was lost very rapidly after trypsin or endoproteinase Arg-C treatments. The fact that neither the N-terminal fragment Phe_1-Met_{51} nor the C-terminal fragment starting at Ala_{52} bind the antibodies can be related to either a lack of immunoreactivity of the small amount of peptide bound to nitrocellulose or to a difference in the structure of the peptides bound to nitrocellulose.

In conclusion, our working hypothesis on the structure of OSCP is that the fragment close to

the N-terminal end, Pro_7-Tyr_{12} should belong to the epitope recognized by the 2 antibodies. The fragment Arg_{62}-Lys_{65} could also be at least partly involved in the $2B_1B_1$ binding domain. These peptides would therefore be located in close proximity in the structure of OSCP. Since we have shown that this antigenic site is very accessible at the surface of OSCP, we can conclude that the Pro_7-Tyr_{12} and, possibly Arg_{62}-Lys_{65} parts of the OSCP sequence are located at the surface of the ATPase-ATPsynthase complex. It is interesting to examine the secondary structure predicted on these fragments. We have shown that 2 β-strands were predicted by several prediction methods of secondary structure. One would be around amino acids 7–14 and the other between amino acids 62–65, the latter sequence being more hydrophilic. It is noteworthy that these regions exactly match the position of the sequence recognized by the antibody $2B_1B_1$. These 2 β-strands were close to α-helices. One can therefore elaborate a structure of 2 β-strands maintained at the surface of OSCP eventually with the help of adjacent α-helices. The fragment 62–65 of the OSCP sequence is also located at the limit of a domain predicted by the method of Vonderviszt and Simon [31]. This might be of importance in the accessibility of this region. These interpretations remains hypothetical and will await a confirmation by crystallographic X-ray analysis. The information gathered in this immunological study may however be of help in the modelisation of the structure of the protein.

Acknowledgments

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