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A FLUORESCENT DERIVATIVE OF THE OLIGOMYCIN-SENSITIVITY CONFERRING PROTEIN (ACRYLODAN-OSCP). EVIDENCE FOR POLARITY CHANGES IN THE ENVIRONMENT OF CYS_{118} OF OSCP UPON BINDING TO MITOCHONDRIAL F_1

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ABSTRACT. The fluorescent probe, 6-acryloyl-2-dimethylaminonaphtalene (acrylodan) was reacted with the oligomycin-sensitivity conferring protein (OSCP). Acrylodan bound covalently to the single cysteinyl residue of the protein. Acrylodan-OSCP was fully competent in conferring oligomycin sensitivity to the mitochondrial F_o-F_1 ATPase complex. The fluorescence emission peak of acrylodan-OSCP was blue-shifted compared to that of an acrylodan-mercaptoethanol adduct, which means that acrylodan experiences a hydrophobic environment in OSCP. Binding of acrylodan-OSCP to the isolated F_1 was accompanied by a red shift of fluorescence. It was achieved in less than 1 s at 25°C. The titration curve revealed one high affinity OSCP binding site per F_1. Acrylodan-OSCP appears to be an interesting tool for studying the dynamics of structural changes within the mitochondrial ATPase complex. © 1987 Academic Press, Inc.

The oligomycin-sensitivity conferring protein (OSCP) is a small ($M_r \simeq 21\,000$)(1,2) and asymmetrical (axial ratio > 3) protein (3) that interacts with both the catalytic sector F_1 and the proton channel F_o of the mitochondrial ATPase complex. In the presence of OSCP, the ATPase or ATP synthase activity of F_1 is inhibited by oligomycin, an antibiotic which binds to the F_o sector. OSCP contains a single cysteinyl residue, CYS_{118} (4), which can be modified without alteration of the biological efficiency of OSCP in a reconstituted ATPase complex. Through the use of radiolabeled (^{14}C)OSCP obtained by alkylation of CYS_{118} by (^{14}C)NEM (5, 6), and photoactivable OSCP obtained by reaction of CYS_{118} in the dark with the bifunctional reagent azidophenacyl bromide (7), the binding parameters of OSCP with respect to F_1 have been explored. It was found that OSCP binds to the α and β subunits of isolated F_1 (6, 7) and that one mol F_1 binds one mol OSCP with high affinity and two mol OSCP with low affinity (5).

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Abbreviations : OSCP : oligomycin-sensitivity conferring protein ; NEM : N-ethylmaleimide ; Acrylodan : 6-acryloyl-2-dimethylaminonaphtalene.

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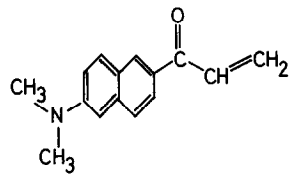

Figure 1. Structure of 6-acryloyl-2-dimethylaminonaphtalene (acrylodan). The vinyl group of acrylodan reacts covalently with the SH group of CYS_{118} of OSCP.

Acrylodan (Figure 1) was recently reported to be a promising reporter for the study of hydrophobic pockets in proteins, due to the strong effect of solvent polarity on its fluorescence spectrum (8). Still more interestingly, acrylodan was found to react selectively and covalently with SH groups in a number of proteins at neutral pH, similarly to aromatic vinyl reagents (9). This latter property proved to be most appropriate for the fluorescent labeling of OSCP as there is only one residue of cysteine in OSCP, CYS_{118}, (4) and the modification of this residue does not alter the biological activity of OSCP (5, 7). The present paper describes the preparation and spectral properties of a fluorescent derivative of OSCP obtained by the condensation of acrylodan with CYS_{118} of OSCP. This new derivative proved to be useful to explore polarity changes in the vicinity of the probe, concomitant with the binding of OSCP to F_1, and to follow binding kinetics.

MATERIALS AND METHODS

OSCP was purified from beef heart mitochondria as described in (2) and stored in liquid nitrogen. Beef heart mitochondrial F_1 was prepared according to (10). OSCP activity prior and after labeling was determined by its ability to confer oligomycin sensitivity to F_1 as previously described (5). 6-Acryloyl-2-dimethylaminonaphtalene (acrylodan) was purchased from Molecular Probes (4849 Pitchford Avenue Eugene, OR 97402 U.S.A.) and was used as an acetonitrile solution prepared freshly before experiments. Unreacted acrylodan was separated from OSCP by filtration-centrifugation (5) through short Sephadex G50 columns (11). Absolute fluorescence spectra were recorded on a SLM 8000 fluorometer. Kinetic and binding assays were carried out with a high sensitivity fluorometer (Biologic Co, ZIRST, 38240 Meylan, France) with the excitation light set at 400 nm and the emission light passed through a K55 Baltzer filter (maximal transmission at 552 nm, half-maximal transmission at 525 and 568 nm). Both fluorometers were equipped with thermostated cuvette holders and stirring devices. All fluorescence measurements were made at 25°C. Protein was determined by the method of Bradford (12). Bovine serum albumin was used as a standard.

RESULTS

Preparation of acrylodan-OSCP and demonstration of the cysteine specificity in the addition reaction

Acrylodan reacts with thiol compounds to give covalent and highly fluorescent derivatives (8). Reaction with thiol groups in proteins occurs

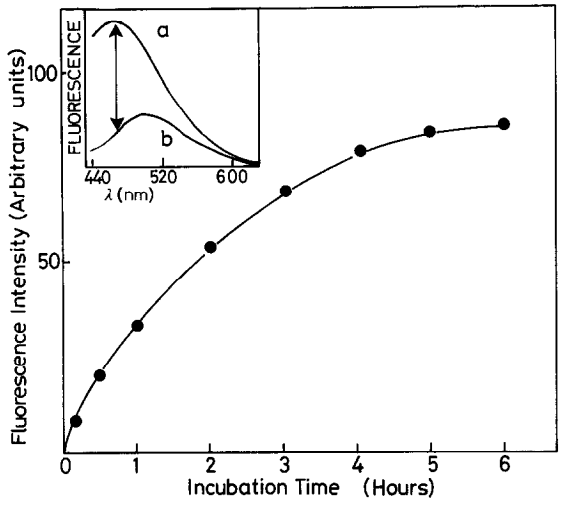

Figure 2. Fluorescence changes resulting from the addition of acrylodan to native OSCP and NEM-pretreated OSCP. NEM-OSCP was prepared and purified as described in (5). One nanomol OSCP or one nanomol NEM-OSCP was let to react with 5 nanomol acrylodan in 2.5 ml of 100 mM KCl, 20 mM Hepes pH 7.0 and 0.005% Tween 20 for 6 h at 25°C. The emission fluorescence spectra of the reaction products obtained with OSCP (a) and NEM-OSCP (b) were recorded (insert). The difference in emitted light between the two spectra at 470 nm (corresponding to the peak of acrylodan-OSCP) was plotted as a function of the time of incubation with acrolydan. Temperature 25°C.

specifically at neutral pH (9). Upon addition of acrylodan to OSCP at pH 7.4, a fluorescent adduct accumulated with time. The reaction was virtually terminated after 4 to 5 hrs at room temperature or 10 hrs at 4°C.

In routine preparations, the reaction was carried out at a molar ratio of 5 mol acrylodan to 1 mol OSCP in 20 mM Tris sulfate pH 7.4 at 4°C for 12 hours. Unreacted acrylodan was removed by Sephadex chromatography, using the centrifugation-filtration technique (11, 5). The yield of acrylodan-OSCP was routinely of the order of 70% with respect to OSCP.

As previously reported, NEM is able to alkylate the single cysteinyl residue of OSCP, CYS_{118} ; alkylation is specific of CYS_{118} even when NEM is added in excess (5). As shown in Fig.2, the covalent binding of NEM and acrylodan to OSCP was mutually exclusive. A sample of native OSCP and one of OSCP previously alkylated by NEM (NEM-OSCP) were incubated with a 5 fold molar excess of acrylodan at 25°C for various periods of time. After removing the unreacted acrylodan by Sephadex filtration, the emission fluorescence spectra were recorded. The spectra illustrated in the insert of Fig.2 clearly indicate that alkylation of OSCP by NEM prevents binding of acrylodan. The red-shifted fluorescence spectrum observed in the case of NEM-OSCP might be due to some unspecific binding of acrylodan which is approximated to 20% of the total binding. The difference at 470 nm between the emission spectra of native OSCP and NEM-OSCP reacted with acrylodan was

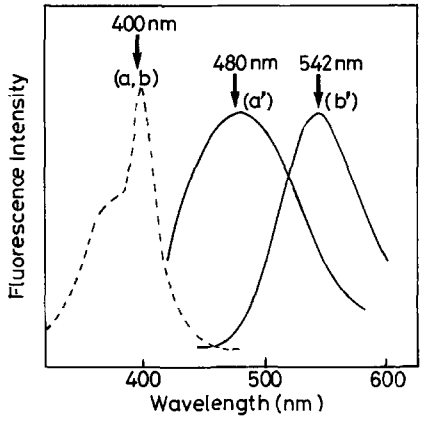

Figure 3. Fluorescence spectra of acrylodan-OSCP and acrylodan-mercaptoethanol. The acrylodan adducts were prepared by reacting a 5 molar excess of acrylodan with OSCP and mercaptoethanol respectively for 12h at 4°C at pH 7.4, as described in Results. The excitation spectra of acrylodan-OSCP and acrylodan-mercaptoethanol are superimposable (a, b). The emission spectra of acrylodan-OSCP (a') and acrylodan-mercaptoethanol (b') were recorded using excitation light at 400 nm. Temperature 25°C.

found to slowly increase with time up to a plateau that was attained after 4-5 hours at 25°C. In summary, the present results show that acrylodan, in accordance with its thiol reactivity, binds to CYS_{118} in OSCP. Other experiments carried out with a reconstituted F_1-F_o complex, similar to those reported with NEM-OSCP (5), indicated that acrylodan-OSCP, like NEM-OSCP, was as active as unmodified OSCP in conferring oligomycin-sentivity to membrane-bound F_1. In other words, acrylodan-OSCP mimicks native OSCP, as does NEM-OSCP.

Fluorescent properties of the acrylodan-OSCP adduct

The emission spectra of protein-acrylodan adducts depend on the nature, hydrophilic or hydrophobic, of the environment of the fluorescent probe ; with increasing hydrophobicity of the environment, the emission spectrum is more and more blue-shifted and conversely, when the protein-acrylodan adduct is treated with a denaturating agent, the emission spectrum is red-shifted, which reflects exposure of the fluorophore to the solvent (8).

The fluorescence spectra of the acrylodan-mercaptoethanol and acrylodan-OSCP adducts are shown in Fig.3. Whereas the excitation spectra of the two adducts were superimposable with the same peak at 400 nm, their emission spectra were markedly different. The emission peak of acrylodan-OSCP (480 nm) was blue-shifted compared to that of acrylodan-mercaptoethanol (542 nm), suggesting that the OSCP-bound acrylodan is in a hydrophobic environment.

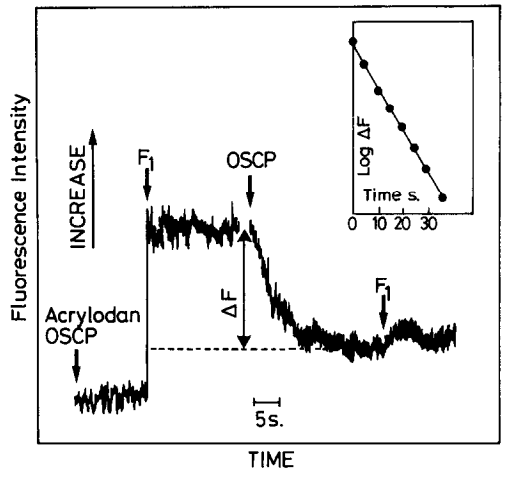

Figure 4. Kinetics of binding of acrylodan-OSCP to beef heart mitochondrial F_1. The fluorescence emission light after passage through a K_{55} filter was recorded, using an excitation light centered at 400 nm and an emission light centered at 558 nm (cf. Materials and Methods). To 2.5 ml of the saline medium (see legend of Figure 2), the following additions were made : 0.2 nanomol acrylodan-OSCP, 0.8 nanomol F_1, 5 nanomol native OSCP, and 0.8 nanomol F_1. Temperature 25°C.

Fluorescent changes reflecting the binding of acrylodan-OSCP to mitochondrial F_1

Upon addition of purified F_1 to acrylodan-OSCP, the fluorescence emission peak at 480 nm was shifted to 495 nm. This red shift indicates changes in the environment of acrylodan, possibly a partial extrusion of acrylodan into the solvent due to conformational changes in the OSCP molecule, in keeping with data reported by Prendergast et al. (8) for a number of other proteins.

Using a band pass centered at 552 nm for the emitted light (cf Materials and Methods), the kinetics of binding of F_1 to acrylodan-OSCP were recorded (Fig.4). The reaction was completed in less than 1 s at 25°C. Acrylodan-OSCP was efficiently, but slowly chased from F_1 upon addition of 25 fold excess of unlabeled OSCP. The residual fluorescence after completion of the chase was not significantly decreased by doubling the amount of unlabeled OSCP. Plots of the fluorescence decrease in a semi-log scale were linear (Insert Fig.4). From the half time of the chase reaction, 7 s, the rate order constant for the dissociation of the OSCP-F_1 complex could be approximated to 0.1 s^{-1}. Based on a value of 0.08 µM for the dissociation constant relative to the high affinity binding of OSCP to F_1 (5), the rate order constant for the binding of OSCP to F_1 at 25°C was found to be about 1.2 $\mu M^{-1}. s^{-1}$. This value fits well with the fact that the binding of 0.8 µM OSCP to an excess of F_1 was completed in less than 1 s (Fig.4).

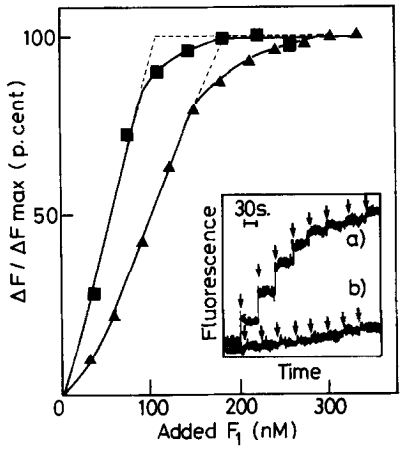

Figure 5. Stoichiometry and specificity of the binding of acrylodan-OSCP to beef heart mitochondrial F_1. The experimental conditions were similar to those of Figure 4. Increasing amounts of F_1 were added to two fixed concentrations of OSCP namely 112 nM (■—) and 186 nM (▲—). The amount of high affinity OSCP binding site(s) in F_1 was approximated from the intercept of the titration curves with the plateau of fluorescence (dotted lines). In the insert, fluorescence changes resulting from addition of acrylodan-OSCP to native F_1 (b) and trypsinized F_1 (b) are compared. Temperature 25°C.

Mild trypsinization of F_1, while not influencing ATPase activity, completely inhibits the specific interaction of F_1 with OSCP (5, 13). Consistent with these results, fluorescence changes are no longer observed when trypsinized F_1, instead of native F_1, is added to acrylodan-OSCP (Fig.5 Insert). This points to the specificity of the interaction between F_1 and acrylodan-OSCP.

Based on the fluorescence shift which results from the binding of F_1 to acrylodan-OSCP, titrations of the OSCP binding site(s) of F_1 were carried out at two different concentrations of acrylodan-OSCP, namely 112 nM and 186 nM, (Fig.5). Extrapolation of the initial linear portion of the two titration curves to the plateau corresponding to the maximal fluorescence increase gave values of 105 nM and 180 nM respectively, indicating that one mol acrylodan-OSCP is able to bind with high affinity to one mol isolated F_1.

DISCUSSION

Chemical modifications that do not alter the activity of functional proteins are often used with the aim to study the interactions of the labeled proteins in biological complexes. Along this line, OSCP has been alkylated at its single cysteinyl residue CYS_{118} by (^{14}C)NEM (5) and by a bifunctional photoactivable reagent azido phenacyl bromide (6) without loss of biological activity. The resulting radiolabeled and photolabeled derivatives of OSCP have been used to study interactions of OSCP with the

subunits of mitochondrial F_1. The present paper describes the preparation and the spectral properties of a fluorescent derivative of OSCP, obtained by condensation of acrylodan with CYS_{118}. The wavelength difference between the emission peaks at 542 nm for acrylodan-mercaptoethanol and at 480 nm for acrylodan-OSCP, strongly suggests that acrylodan bound to CYS_{118} in OSCP experiences a hydrophobic region of the protein molecule ; indeed, the sequence data (4) indicate that the amino acid residues in the central sector of OSCP encompassing CYS_{118} are predominantly hydrophobic. Conversely, the fluorescence of acrylodan-OSCP is red-shifted upon addition of mitochondrial F_1, suggesting a change in the conformation of OSCP, resulting in exposure of the fluorophore to the aqueous medium. Titration of the OSCP binding sites of F_1 with acrylodan-OSCP indicated one high affinity acrylodan-OSCP binding site per F_1. This corroborates previous results obtained with radiolabeled OSCP (5). As OSCP interacts with the α and β subunits in isolated F_1 (6, 7), and there are 3 α and 3 β subunits per F_1 (for review 14), this strengthens the view that mitochondrial F_1 exhibits a partial reactivity for OSCP (5).

REFERENCES

(1) Tzagoloff A, Mac Lennan D.H. and Byington K.H. (1968) Biochemistry 7, 1596-1602.
(2) Senior A.E. (1971) J. Bioenerg. 2, 141-150.
(3) Dupuis A, Zaccaï G. and Satre M. (1983) Biochemistry 22, 5951-5956.
(4) Ovchinnichov Y.A, Modyanov N.N., Grinkevich V.A., Aldanova N.A., Trubetskaya O.E., Nazimov I.V., Hundal T. and Ernster L. (1984) FEBS Lett. 166, 19-22.
(5) Dupuis A, Issartel J.P., Lunardi J, Satre M. and Vignais P.V. (1985) Biochemistry 24, 728-733.
(6) Dupuis A, Lunardi J., Issartel J-P. and Vignais P.V. (1985) Biochemistry, 24, 734-739.
(7) Dupuis A. and Vignais P.V. (1985) Biochem. Biophys. Res. Commun. 129, 819-825.
(8) Prendergast F.G., Meyer M, Carlson G.L, Iida S. and Potter J.D (1983) J. Biol. Chem. 258, 7541-7544.
(9) Friedman M, Krull L.H. and Cavins J.F. (1970) J. Biol. Chem. 245, 3868-3871.
(10) Knowles A.F. and Penefsky H.S. (1972) J. Biol Chem. 247, 6617-6623.
(11) Penefsky H.S. (1977) J. Biol. Chem. 252, 2891-2899.
(12) Bradford M.M. (1976) Anal. Biochem. 72, 248-254.
(13) Hundal T, Norling B. and Ernster L. (1983) FEBS Lett. 162, 5-10.
(14) Vignais P.V. and Satre M. (1984) Mol. Cell. Biochem. 60, 33-70.