Pfaff, E., Klingenberg, M., and Heldt, H. W. (1965), *Biochim. Biophys. Acta 104*, 312.
Randerath, K. (1967), *Anal. Biochem. 21*, 480.
Schnaitman, C., and Greenawalt, J. W. (1968), *J. Cell Biol. 38*, 158.
Slater, E. C. (1966), *Comp. Biochem. 14*, 327.
Tener, G. M. (1961), *J. Amer. Chem. Soc. 83*, 149.
Tyler, D. D. (1970), *Biochem. J. 116*, 30P.
Vasington, F. D. (1963), *J. Biol. Chem. 238*, 1841.
Walters, E., and Cooper, C. (1965), *Anal. Biochem. 10*, 370.

Identification of the Dicyclohexylcarbodiimide-Binding Protein in the Oligomycin-Sensitive Adenosine Triphosphatase from Bovine Heart Mitochondria†

Feico S. Stekhoven,* Richard F. Waitkus, and Herman Th. B. van Moerkerk

ABSTRACT: Dicyclohexylcarbodiimide in concentrations not exceeding those necessary for maximal inhibition of ATPase activity binds specifically to a protein in an oligomycin- and carbodiimide-sensitive ATPase complex (OS-ATPase) isolated from bovine heart mitochondria. This protein, designated the carbodiimide-binding protein, has a molecular weight of 13,000–14,000. At concentrations exceeding the maximum inhibition level, the carbodiimide becomes bound also to other proteins, like F_1, present in the ATPase complex. Crude carbodiimide-binding protein can be extracted from OS-ATPase with chloroform–methanol (2:1, v/v) as a water-insoluble proteolipid.

One of the characteristics of the mitochondrial Mg^{2+}-dependent ATPase is its inhibition by oligomycin or dicyclohexylcarbodiimide (Lardy *et al.*, 1958, 1965; Huijing and Slater, 1961; Beechey *et al.*, 1967; Beyer *et al.*, 1967), both inhibitors having a similar mode of action (Beechey *et al.*, 1966, 1967; Racker and Horstman, 1967; Bulos and Racker, 1968a,b; Roberton *et al.*, 1968).

A soluble Mg^{2+}-dependent ATPase, F_1,[1] has been isolated from beef heart mitochondria as well as from yeast mitochondria (Pullman *et al.*, 1960; Schatz *et al.*, 1967; Tzagoloff, 1969a) but these preparations are not inhibited by oligomycin or the carbodiimide (Racker *et al.*, 1961; Racker, 1962, 1963; Bulos and Racker, 1968a; Schatz *et al.*, 1967; Tzagoloff, 1969b), except by high concentrations of the latter inhibitor (Penefsky, 1967) exceeding those necessary for blocking particulate ATPase activity (Beechey *et al.*, 1967; Beyer *et al.*, 1967; Bulos and Racker, 1968a). In addition submitochondrial particles have been isolated, depleted in F_1, and containing only residual ATPase activity. Addition of F_1 to these particles restored particle-bound ATPase activity which is then oligomycin sensitive (Racker *et al.*, 1961; Racker, 1962, 1963; Kagawa and Racker, 1966a). Subsequently it was shown by studies with radioactive rutamycin, an oligomycin analog (Thompson *et al.*, 1961; Lardy *et al.*, 1965) that the F_1-deficient particles are the site of action of the inhibitor (Kagawa and Racker, 1966b).

Later more depleted submitochondrial particulate preparations have been isolated which require different soluble protein factors like oligomycin-sensitivity-conferring protein (OSCP) (MacLennan and Tzagoloff, 1968; Tzagoloff, 1970), also called Fc or Fc_1 (Bulos and Racker, 1968a,b; Knowles *et al.*, 1971), and Fc_2 (Knowles *et al.*, 1971) to confer oligomycin or the carbodiimide sensitivity to F_1-ATPase. Yet neither one of these factors appears to bear the site of oligomycin or the carbodiimide inhibition, as this was found to be located in the particles depleted of the complementary factors used in the assay. It was furthermore found that these particles lose their ability to confer oligomycin or the carbodiimide sensitivity to F_1 after heat or trypsin treatment, indicating that they contain still another protein necessary for reconstitution of the oligomycin-sensitive ATPase (Bulos and Racker, 1968a,b; Knowles *et al.*, 1971).

It is the aim of this article to pinpoint the site of action of the carbodiimide by use of the radioactive chemical as suggested by Bulos and Racker (1968a). This approach is feasible, since in contrast to oligomycin (Kagawa and Racker, 1966b; Bulos and Racker, 1968a) the carbodiimide is irreversibly bound to the particulate preparations as shown by the fact that inhibition of ATPase cannot be released by washing with phospholipids (Holloway *et al.*, 1966; Bulos and Racker, 1968a). Since we felt that the heat- and trypsin-sensitive component in the depleted particles mentioned above could be the carbodiimide-binding protein, we have concentrated our

† From the Department of Biochemistry, University of Nijmegen, Medical School, Geert Grooteplein Noord 21, Nijmegen, The Netherlands. *Received October 25, 1971.* This investigation was supported in part by the Netherlands Organization for Basic Research (Z. W. O.).

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: F_1, coupling factor F_1 (ATPase) (Pullman *et al.*, 1960); OSCP, oligomycin-sensitivity-conferring protein (MacLennan and Tzagoloff, 1968); Fc (Fc_1), rutamycin- (or dicyclohexylcarbodiimide-) sensitivity factor (Bulos and Racker, 1968a,b; Knowles *et al.*, 1971); Fc_2, factor conferring the dicyclohexylcarbodiimide sensitivity to F_1 in presence of Fc_1 and TUA-STA particles (Knowles *et al.*, 1971); OS-ATPase, oligomycin-sensitive ATPase complex isolated from bovine heart mitochondria by the procedure of Tzagoloff *et al.* (1968a); TUA particles, trypsin–urea-treated submitochondrial particles which were sonicated in the presence of ammonia (Bulos and Racker, 1968a); TUA-CF_o, cholate-treated TUA particles (Bulos and Racker, 1968b); CF_o, trypsin–urea–cholate-treated submitochondrial particles (Kagawa and Racker, 1966b); TUA-STA, silicotungstate-treated TUA particles (Knowles and Guillory, 1970; Knowles *et al.*, 1971).

attention on protein rather than on phospholipid components (Bruni *et al.*, 1971). This choice was supported by the fact that about 75% of the radioactivity from the [^{14}C]carbodiimide-labeled submitochondrial particles could be extracted by chloroform–methanol treatment. Roughly two-thirds of the label was attached to a lipoprotein fraction that could be sedimented by addition of ether to the water-washed chloroform–methanol phase (Cattell *et al.*, 1970, 1971). We chose for our studies an oligomycin-sensitive ATPase complex isolated from beef heart mitochondria according to the procedure of Tzagoloff *et al.* (1968a) with modifications (Tzagoloff *et al.*, 1968b; MacLennan and Tzagoloff, 1968). This preparation contains very little respiratory chain components except flavin, and no other enzymatic activities than ATPase except NADH·ferricyanide reductase and trace amounts of adenylate kinase (Tzagoloff *et al.*, 1968a). It has been shown to contain bound F_1 and OSCP (MacLennan and Tzagoloff, 1968) and apparently contains all the other components involved in conferral of oligomycin or the carbodiimide sensitivity to F_1 including the site of interaction with the inhibitors.

Materials and Methods

Procedures for Mitochondrial Preparations. Beef heart mitochondria were isolated according to Crane *et al.* (1956). The isolation medium (pH 7.4) contained an additional 20 mM EDTA up to the Sharples centrifugation which was performed only once. The resulting mitochondrial cake was homogenized by Potter-Elvehjem homogenization in 0.25 M sucrose–30 mM Tris-sulfate (pH 8) and spun for 25 min at 23,000*g*. The mitochondria were washed once more in the same medium as above and finally suspended in 0.25 M sucrose.

Oligomycin-sensitive ATPase was isolated according to Tzagoloff *et al.* (1968a) with modifications (Tzagoloff *et al.*, 1968b; MacLennan and Tzagoloff, 1968).

The [^{14}C]carbodiimide-labeled OS-ATPase was prepared by incubating a suspension of 10 mg of particle protein/ml of 0.25 M sucrose–10 mM Tris-acetate (pH 7.5) with 1.25 μg of the [^{14}C]carbodiimide/mg of protein (unless otherwise indicated) with shaking at 0° for 4 hr. Particles were spun for 10 min at 12,000*g* and at 4°, and washed two more times by rehomogenization in sucrose–Tris-acetate, and centrifugation as above. Finally they were homogenized in this medium to the original volume.

The carbodiimide-binding protein was extracted from the carbodiimide-labeled OS-ATPase by the method of Cattell *et al.* (1970). The ether sedimented protein was homogenized with a small volume of 10 mM Tris-acetate (pH 7.5).

F_1 was isolated according to Horstman and Racker (1970). All preparations were stored at −70° except F_1 which was stored as indicated (Horstman and Racker, 1970).

Preparation and Source of Inhibitors. Oligomycin was obtained from Sigma Chemical Co., unlabeled dicyclohexylcarbodiimide from Nutritional Biochemical Corp. The carbodiimide was vacuum distilled before use. [^{14}C]Urea which was the starting material for the synthesis of the [^{14}C]carbodiimide was purchased from The Radiochemical Centre (Amersham). [^{14}C]Dicyclohexylurea was prepared from it *via* the method of Amiard *et al.* (1958) on a 0.5-mmole scale (0.5 mCi/0.5 mmole of urea), the procedure being carried out in a sealed tube containing 1 ml of cyclohexylamine. Heating at 160° was for 5 hr. The yield of [^{14}C]dicyclohexylurea after desiccation was 66 mg (58.8%) (melting point 227°). The [^{14}C]-carbodiimide was prepared from it according to Walther (1963) by reaction with $POCl_3$ (30 μl) in 1 ml of dry pyridine in a sealed tube at 80–90° for 2 hr. Final purification was by destillation in a microdestillation apparatus. Solvent (petroleum ether, bp 40–60°) was first removed from the apparatus with a stream of dry nitrogen. Then high vacuum destillation was carried out, first at 40° to remove pyridine, and subsequently at 150° in order to evaporate the carbodiimide. The purified product was dissolved again in petroleum ether, the solvent removed under vacuum in a desiccator, and the final product dried over P_2O_5 under vacuum. The yield of the carbodiimide was 28.5 mg (46.9%) (mp 34–35°). Specific radioactivity was 1 mCi/mmole. Purity and radiochemical purity were tested by thin-layer chromatography on aluminum oxide G (neutral, type E, Merck) using benzene as eluent. Reference (unlabeled dicyclohexylcarbodiimide) and [^{14}C]-dicyclohexylcarbodiimide showed the same R_F of 0.71. No other spots could be seen for either reference or the [^{14}C]-carbodiimide after color development in I_2 vapor. All radioactivity was located at the [^{14}C]carbodiimide spot as tested with a Berthold Dünnschicht-Scanner II. The [^{14}C]carbodiimide was stored at −20° as a solution in petroleum ether.

Assays and Analytical Methods. ATPase assay was performed according to Pullman *et al.* (1960) in the presence of an ATP-regenerating system. OS-ATPase (0.15–0.4 mg of protein) was preincubated for 5 min at 30° in 0.25 M sucrose–10 mM Tris-acetate (pH 7.5) in a total volume of 0.76 ml. The reaction was started by addition of the assay medium components contained in a volume of 0.24 ml. ATP and $MgSO_4$ were both 5 μmoles. Phosphate liberation was corrected for a blank in which OS-ATPase was added after addition of trichloroacetic acid (zero time).

Specific activity of OS-ATPase was determined using particle concentrations varying between 0 and 0.4 mg of particulate protein per ml. A plot of micromoles of inorganic phosphate liberated per minute against milligram of particle protein gave a straight line from the slope of which specific activity (micromoles of P_i liberated per milligram of protein per minute) was calculated.

Inhibitor studies were done with inhibitor present during the 5-min preincubation period at 30°. In case of the carbodiimide inhibition a 4-hr preincubation of OS-ATPase with the carbodiimide at 0° with shaking preceded the 5-min preincubation at 30° because of the slow inhibition kinetics even at 30° (Beechey *et al.*, 1967). Inhibition became maximal during this 4-hr preincubation at 0°. The longer periods (18–24 hr) used by Beechey *et al.* (1967) are unnecessary and lead moreover to a decline in ATPase activity for non-dicyclohexylcarbodiimide-treated OS-ATPase. Oligomycin and the carbodiimide were added from a solution in ethanol in quantities up to 2 and 1.25 μg of inhibitor per mg of protein, respectively. Controls were run containing no inhibitor but an equivalent amount of ethanol (maximally 50 μl).

Protein was determined according to Jacobs *et al.* (1956) or Lowry *et al.* (1951) depending upon whether it was particulate or soluble. The carbodiimide binding protein extracted from OS-ATPase by means of chloroform–methanol (Cattell *et al.*, 1970) was determined according to Hess and Lewin (1965; method C). In each case bovine serum albumin was used as a standard.

Polyacrylamide gel electrophoresis was carried out with the phenol–urea–acetic acid procedure of Takayama *et al.* (1964) or the sodium dodecyl sulfate method of Weber and Osborn (1969). In the latter procedure the incubation medium contained 8 M urea and dialysis was omitted. Excess solvent was removed from proteins in suspension by centrifugation at 4°

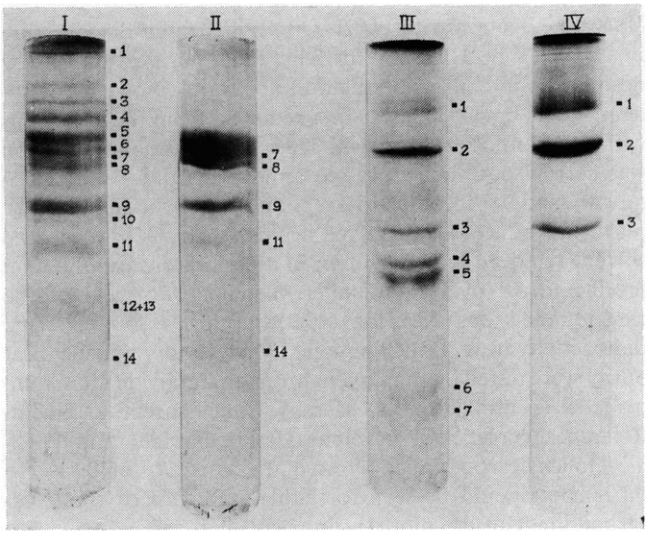

FIGURE 1: Electrophoretic patterns of OS-ATPase and F_1. Gels I and II were simultaneously run according to the sodium dodecyl sulfate method, gels III and IV according to the phenol–urea–acetic acid method as outlined under Materials and Methods. Gels I and III contain OS-ATPase (0.2 mg), gels II and IV F_1 (0.1 mg). Individual protein bands are numbered from top to bottom in reference to corresponding ones in OS-ATPase.

for 10–20 min at 7500*g* prior to their solution in the depolymerizing media. Amounts of proteins used for electrophoresis are reported in the Results section. Electrophoresis time was 2.5 hr in the Takayama method and 6.5–7.75 hr in the method of Weber and Osborn using gels of 7 mm diameter and 60–65 mm length. The gels were stained with a 0.5% solution of Amido Black 10B (Merck) in methanol–acetic acid–water (50:7:43, v/v/v) in the Takayama method for 0.5 hr, followed by electrophoretic destaining against 2% acetic acid with the current at a right angle to the gel axis. Staining with Coomassie Brilliant Blue R250 (ICI) in the sodium dodecyl sulfate method (Weber and Osborn, 1969) lasted 3.5–4 hr and destaining occurred by simple diffusion against the destaining solution which was replaced every day. Full destaining took about 5 days.

Determination of ^{14}C radioactivity was done by means of a liquid scintillation counter computerized for disintegrations per minute (Tri-Carb, Model 3380, Packard Instrument Co., Inc.). Samples were taken to dryness in their counting vials under infrared light, then extracted for a few days at room temperature with 1 ml of Hyamine hydroxide 10-X (Packard Instrument Co., Inc.). The extract was mixed with 10 ml of Insta-Gel emulsifier (Packard Instrument Co., Inc.) and counted. Location of the [^{14}C]carbodiimide-labeled protein after sodium dodecyl sulfate electrophoresis of the [^{14}C]-carbodiimide-labeled OS-ATPase or protein extracted from it by chloroform–methanol was done as follows. The unstained gel was cut into 1-mm slices starting from the top with a gel guillotine (Maurer, 1968), the gel being contained in a Perspex block with a longitudinal hole bored in it. Each slice was weighed and after ten slices the decrease in gel length measured so as to determine the thickness of each slice. Slices were dissolved in counting vials in 2 ml of 30% analytical grade hydrogen peroxide overnight in an oven at 60°. Radioactivity was then counted as described above. Distance of midpoint of each slice from the top of the gel was corrected for compression of the gel in the Perspex block during cutting (usually 3%) and also for gel stretching due to the staining and destaining procedure of a reference gel (about 10%). The count in disintegrations per minute of each slice was plotted against the distance and compared to the protein pattern of the reference gel as shown in the Results section.

The molecular weight of the carbodiimide-binding protein was determined by sodium dodecyl sulfate electrophoresis. Standard proteins of known molecular weight used for calibration (commercial sources in parentheses) were: α-chymotrypsin, type I, from bovine pancreas (Sigma Chemical Co.), β-lactoglobulin, B grade (Calbiochem), trypsin, type I, from bovine pancreas (Sigma Chemical Co.), aldolase from rabbit muscle (Boehringer Mannheim GmbH), and catalase from bovine liver (Boehringer Mannheim GmbH), each running in a separate gel. Molecular weights of these proteins were taken as reported by Weber and Osborn (1969). Electrophoretic mobilities with reference to bromophenol blue as tracking dye were determined as indicated by the same authors.

Results

OS-ATPase and Its Inhibition by Oligomycin or the Carbodiimide. OS-ATPase split ATP with a specific activity ranging between 1 and 2 μmoles·min^{-1}·mg of protein^{-1} at pH 7.5 and 30°. Maximal inhibition by oligomycin or the carbodiimide, amounting to 83 and 96%, was obtained at concentrations in the incubation medium of 1.6–2.0 μg (4–5 nmoles) of oligomycin/mg of protein, and 1–1.25 μg (5–6 nmoles) of the carbodiimide/mg protein, respectively. Inhibitor blanks containing ethanol in equivalent amounts did not show any inhibition. These data compare fairly well to those of Huijing and Slater (1961), Lee and Ernster (1965), and Bulos and Racker (1968a) with different submitochondrial particles, all derived from heart (equine or bovine). Groot (1970) and Beechey *et al.* (1967), on the other hand, obtained maximal inhibitions for bovine heart submitochondrial particles at concentrations of oligomycin and the carbodiimide which were a factor 4 and 3 lower, respectively. The inhibition curve for commercial nonradioactive carbodiimide (not shown) coincided with that for the [^{14}C]carbodiimide prepared in our laboratory.

Comparison of Electrophoretic Methods. Figure 1 compares the results of electrophoresis of OS-ATPase and F_1 according to the sodium dodecyl sulfate method of Weber and Osborn (1969) (gel I and II) and phenol–urea–acetic acid method of Takayama *et al.* (1964) (gel III and IV). Presence of bound F_1 in OS-ATPase is clearly shown by both procedures. The bands of F_1 (7–9, 11, and 14 in gel II; 1–3 in gel IV) match corresponding bands in OS-ATPase (gel I and III, respectively). The Takayama method gives protein patterns essentially as reported by Tzagoloff *et al.* (1968a) except for a few weak bands of low molecular weight near the bottom of the gel containing OS-ATPase (bands 6 and 7 in gel III of Figure 1). Although the number of predominant bands of F_1 in the two methods of electrophoresis (gel II and IV of Figure 1) is the same, the difference in depolymerizing action between these methods becomes apparent for OS-ATPase (gel I and III of Figure 1): 12–14 bands show up in the sodium dodecyl sulfate method (gel I), 6–8 in the phenol–urea–acetic acid method (gel III). The stronger depolymerizing action of sodium dodecyl sulfate has been documented by Senior and Brooks (1970). Because of its higher resolving power, we have chosen the sodium dodecyl sulfate electrophoresis for routine protein analysis.

Identification of the Carbodiimide-Binding Protein in OS-ATPase. The carbodiimide-binding protein component in

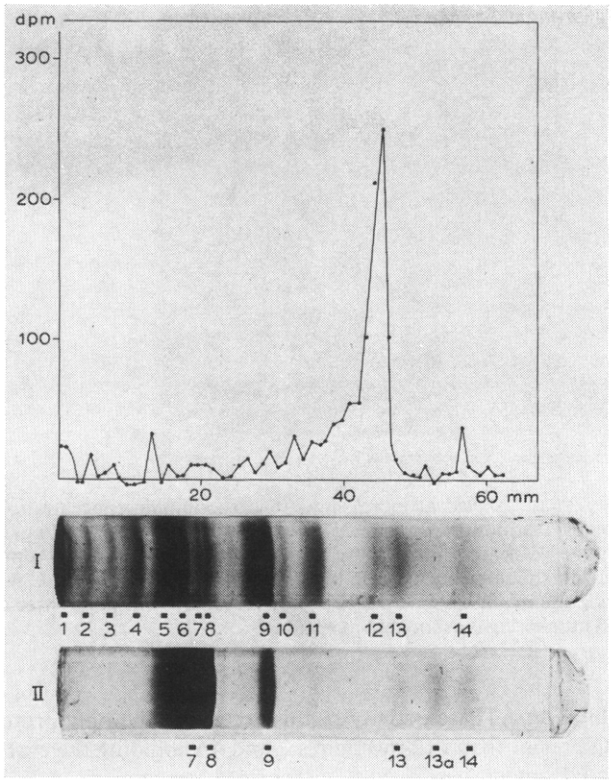

FIGURE 2: Comparison of electrophoretic and radioactive pattern of the [^{14}C]carbodiimide-labeled OS-ATPase. OS-ATPase was [^{14}C]carbodiimide labeled at a concentration of the inhibitor in the incubation medium of 1.25 μg/mg of OS-ATPase. Radioactivity bound to OS-ATPase was 5.23×10^3 dpm·mg of protein^{-1}. Gel I shows the [^{14}C]carbodiimide-labeled OS-ATPase (0.4 mg), gel II is a simultaneous run of F_1 (0.1 mg). The radioactive protein band (12) in OS-ATPase has an electrophoretic mobility of 0.765 relative to tracking dye travel (sodium dodecyl sulfate method). Protein band indication by numbers is as for gels I and II in Figure 1. The present F_1 preparation lacks band 11 seen in gel II of Figure 1 but shows additional bands, 13 and 13a, the latter of which does not occur in OS-ATPase.

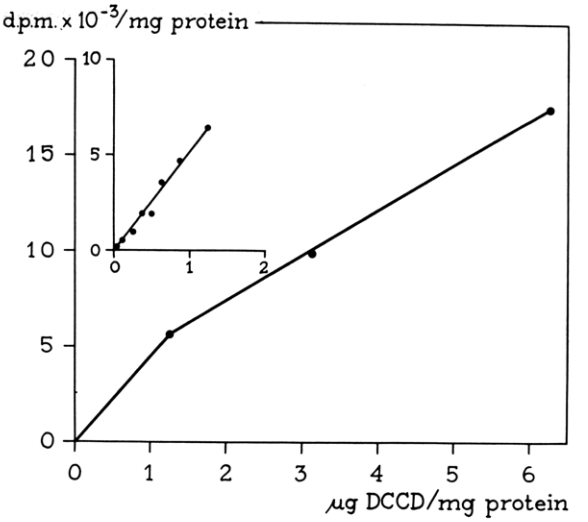

FIGURE 3: Binding course of the carbodiimide to OS-ATPase. OS-ATPase was labeled with the [^{14}C]carbodiimide at concentrations of the inhibitor in the incubation medium, expressed on a protein base, as indicated on the abscissa. The ordinate indicates binding of the carbodiimide expressed as radioactive dpm $\times$ 10^{-3}/mg of protein. The insert shows the result of an analogous experiment with more experimental points covering the carbodiimide concentrations up to 1.25 μg/mg of protein. It has been included in order to show that binding of the carbodiimide is linear below this lowest carbodiimide concentration in the major graph.

OS-ATPase was identified by labeling the ATPase complex with the [^{14}C]carbodiimide followed by separation of its protein components *via* gel electrophoresis and analysis of the gel for radioactivity. The resulting radioactivity pattern is compared to the protein pattern of a reference gel as shown in Figure 2. OS-ATPase (gel I in Figure 2) was first incubated with the [^{14}C]carbodiimide at a concentration that maximally inhibited ATPase activity (1.25 μg of the carbodiimide/mg of protein, *cf.* Results section). Under these conditions there is but one sharp radioactive peak coinciding with a low molecular weight protein (band 12 in gel I) with a mobility of 0.76. Electrophoresis of F_1 (gel II in Figure 2) along with the [^{14}C]carbodiimide-labeled OS-ATPase indicates that F_1 does not contain the carbodiimide-binding protein band 12 occurring in OS-ATPase, as could be expected from the results of Racker and associates (Racker *et al.*, 1961; Racker, 1962, 1963; Kagawa and Racker, 1966b; Bulos and Racker, 1968a). Non-dicyclohexylcarbodiimide-labeled OS-ATPase showed the same protein pattern as its dicyclohexylcarbodiimide-labeled counterpart (*cf.* gel I of Figure 1) with the same electrophoretic mobilities.

Analysis of the Carbodiimide-Binding Specificity. In order to gain more insight into the specificity of the carbodiimide binding, OS-ATPase was incubated with different concentrations of the radioactive inhibitor, at and above the maximum inhibition level of Figure 2. The results of this experiment are shown in Figure 3. Up to 1.25 μg of the carbodiimide/mg of protein in the incubation medium gave a straight line for the carbodiimide binding expressed as dpm·10^{-3}·mg of protein^{-1} (see insert). At that particular point a break occurs in the graph, followed by a binding course of lower affinity with a slope of 50% of that before the break. This indicates that other components as the one labeled in Figure 2 started to bind the carbodiimide at concentrations above the break point in Figure 3. The gel electrophoretic pattern shown in Figure 4 indicates that these other components are, in part at least, proteins.

Figure 4 shows the protein patterns of OS-ATPase incubated with different concentrations of the [^{14}C]carbodiimide along with the radioactivity patterns. At a concentration of 1.25 μg of the [^{14}C]carbodiimide/mg of protein in the incubation medium, corresponding with the break point in Figure 3, again only one protein with a mobility of 0.76 (band 12) had become labeled (Figure 4A). At higher concentrations of the carbodiimide (2.5 and 5 times that used in Figure 4A) other proteins, including F_1 (band 7–9, and 11, *cf.* gel I and II of Figure 1 and 2), bound the inhibitor in increasing amounts (Figure 4B,C). This is in agreement with results of Penefsky (1967) who found F_1-ATPase to be inhibited 97.5% after treatment of the enzyme with 7.63 μg of the carbodiimide/mg of protein according to our calculation. The 0.76 mobility protein (band 12) exceeds, however, all other labeled proteins by hundreds of disintegrations per minute (Figure 4B,C). These results are also in agreement with those of Holloway *et al.* (1966), suggesting specific and nonspecific binding sites for the carbodiimide in bovine heart submitochondrial particles (CFo) which were depleted in F_1, lipids, and respiratory chain components (Kagawa and Racker, 1966b).

From the above data it can be concluded that specificity of the carbodiimide to protein binding in OS-ATPase is restricted to concentrations of the inhibitor up to those just maximally

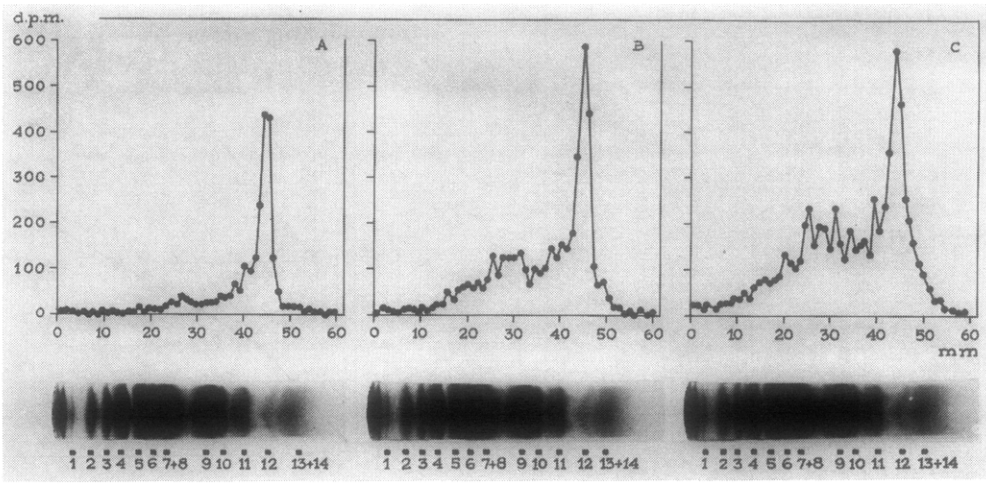

FIGURE 4: Comparison of electrophoretic and radioactive patterns of OS-ATPase labeled at increasing [^{14}C]carbodiimide concentrations. OS-ATPase was labeled with the [^{14}C]carbodiimide at a concentration in the incubation medium of 1.25 μg·mg of protein^{-1} (A), 3.12 μg·mg of protein^{-1} (B), and 6.25 μg·mg of protein^{-1} (C). Specific radioactivities of the [^{14}C]carbodiimide-labeled OS-ATPase were 5.6 $\times$ 10^3 dpm·mg of protein^{-1} (A), 9.82 $\times$ 10^3 dpm·mg of protein^{-1} (B), and 17.49 $\times$ 10^3 dpm·mg of protein^{-1} (C). Gels were loaded in A, B, and C with 0.8 mg of protein. Electrophoretic mobility of the protein band (12) carrying the major radioactive peak was 0.755–0.764 in these three experiments (simultaneous runs, sodium dodecyl sulfate method). Protein band numbering is as for gel I in Figure 1.

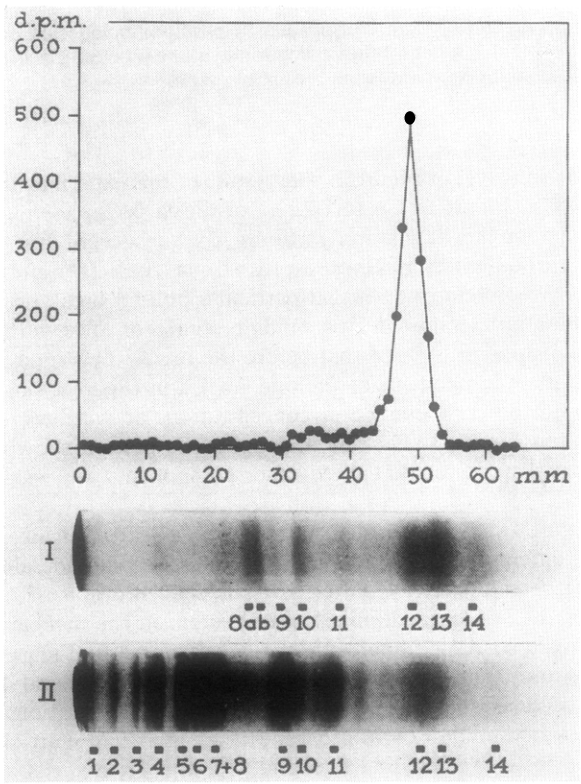

FIGURE 5: Comparison of electrophoretic and radioactivity pattern of the carbodiimide-binding protein isolated from the [^{14}C]carbodiimide-labeled OS-ATPase by chloroform–methanol (2:1, v/v) extraction. OS-ATPase was labeled with the [^{14}C]carbodiimide at a concentration of 1.25 μg·mg of protein^{-1} in the incubation medium and protein extracted from it as described at Materials and Methods. Specific radioactivity of the [^{14}C]carbodiimide-labeled OS-ATPase was 6.96 $\times$ 10^3 dpm·mg of protein^{-1} and that of the extracted protein 4.46 $\times$ 10^4 dpm·mg protein^{-1}. Gel I shows the extracted protein (0.114 mg), gel II for comparison the [^{14}C]carbodiimide-labeled OS-ATPase (0.3 mg) before extraction. Electrophoretic mobility of radioactive protein (band 12) in gel I was 0.760, the corresponding protein band in gel II had an electrophoretic mobility of 0.774 (simultaneous runs, sodium dodecyl sulfate method). Band numbering is as for gel I in Figure 1. Bands 8a and 8b in gel I show up only faintly in the starting material OS-ATPase (gel II).

inhibiting ATPase activity of this complex. The only protein component (band 12 in Figures 2 and 4A) binding the carbodiimide under these conditions has an electrophoretic mobility of 0.762 $\pm$ 0.0025 (range 0.744–0.781, 19 gels in 10 different experiments). This protein will be designated as "the carbodiimide-binding protein."

Extraction of the Carbodiimide-Binding Protein in Chloroform–Methanol. Cattell *et al.* (1970, 1971) have reported that treatment of the [^{14}C]carbodiimide-labeled submitochondrial particles with chloroform–methanol (2:1, v/v) dissolves 73–78% of the radioactivity, of which roughly two-thirds resided in a protein fraction that sedimented out of the water-washed chloroform–methanol phase after addition of ether. This method was applied to OS-ATPase after incubation with a maximally inhibiting level of the [^{14}C]carbodiimide (1.25 μg·mg of protein^{-1}). Figure 5 (gel I compared to radioactivity pattern) shows that the carbodiimide-binding protein could be extracted by this procedure, although it is still in an impure form showing seven protein bands (8a and -b, 9–11, 13, and 14) besides the radioactive one (band 12). Further purification may be feasible *via* chromatography over Sephadex LH-20 (Cattell *et al.*, 1970, 1971). The radioactive band (mobility 0.76) virtually coincides with the carbodiimide-binding protein band in the [^{14}C]carbodiimide-labeled OS-ATPase (band 12 in gel II of Figure 5, mobility 0.77). This means that chloroform–methanol extraction and subsequent ether treatment as used in the isolation procedure do not affect the electrophoretic mobility of the carbodiimide-binding protein, which will allow an unambiguous molecular weight determination.

Molecular Weight of the Carbodiimide-Binding Protein. The molecular weight of the carbodiimide-binding protein was determined from its electrophoretic mobility exactly as described by Weber and Osborn (1969), using calibration proteins in the molecular weight range from 11,000 to 60,000 as indicated in the Materials and Methods section. The amounts of protein used per gel (20 μg of α-chymotrypsin, 10 μg of β-lactoglobulin, 20 μg of trypsin, 7 μg of aldolase, and 7 μg of catalase) gave sharp bands, allowing accurate determination of electrophoretic mobilities in the range from 0.21 to 0.90. A linear relationship between log molecular weight and mobility was obtained as calibration graph.

Preparations of the carbodiimide-binding protein were: OS-ATPase (0.3 mg), the [^{14}C]carbodiimide-labeled OS-ATPase (0.3 mg, 1.25 μg of the carbodiimide/mg of protein in incubation medium), and protein extracted by chloroform–methanol from the latter (0.114 mg). The carbodiimide-binding protein bands (designated 12 in Figures 2, 4, and 5) in these three preparations showed electrophoretic mobilities ranging from 0.76 to 0.78. By interpolation a molecular weight of 13,000–14,000 was determined from the calibration graph in two different experiments in which calibration proteins and the three dicyclohexylcarbodiimide-binding protein containing preparations were simultaneously run.

Discussion

Dicyclohexylcarbodiimide-Binding Protein as Possible Site of the Carbodiimide Inhibition. The present study points to a high specificity of the carbodiimide to protein binding in OS-ATPase up to a level of the inhibitor just causing maximal inhibition. The role of the carbodiimide-binding protein in conferral of oligomycin or the carbodiimide sensitivity to F_1-ATPase has to be established *via* future reconstitution studies using in addition OSCP (MacLennan and Tzagoloff, 1968; Bulos and Racker, 1968a,b), Fc_2 (Knowles and Guillory, 1970; Knowles *et al.*, 1971), phospholipids (Kagawa and Racker, 1966b; Roberton *et al.*, 1966; Bulos and Racker, 1968b), and Mg^{2+} (Bulos and Racker, 1968a) as minimal components necessary for restoration of this inhibitor sensitivity.

Evidence that the site of inhibitory interaction with oligomycin or the carbodiimide has a protein character and is not F_1, OSCP, or Fc_2 can briefly be summarized here. (1) F_1, OSCP, or Fc_2 are not inhibited by oligomycin or the carbodiimide, since after removal of excess inhibitor from these factors they were still fully active in reconstitution of oligomycin- or the carbodiimide-sensitive ATPase activity in the presence of submitochondrial particles depleted of the complementary factors (Racker, 1963; Kagawa and Racker, 1966b; Bulos and Racker, 1968a; Knowles *et al.*, 1971). In line with these data it was shown in the Results section that the carbodiimide-binding protein was absent from F_1. In addition OSCP has a molecular weight of 17,000–18,000 determined by molecular sieving with Sephadex G-100 (MacLennan and Tzagoloff, 1968) or sodium dodecyl sulfate electrophoresis (O. Li and D. R. Sanadi, unpublished data). These values are by 3000–5000 higher than the molecular weight of 13,000–14,000 found for the carbodiimide-binding protein, making it unlikely that the carbodiimide-binding protein is identical with OSCP. (2) Treatment of the complementary factor depleted submitochondrial particles with oligomycin or the carbodiimide (excess inhibitor removed) gives particles which confer inhibition to F_1-ATPase, as such or in the presence of phospholipids, OSCP, OSCP + asolectin, or OSCP + Fc_2 depending on the degree of depletion (Kagawa and Racker, 1966b; Roberton *et al.*, 1966, 1968; Bulos and Racker, 1968a,b; Knowles *et al.*, 1971). (3) Short periods of heat (50–60°) or trypsin treatment destroy oligomycin and the carbodiimide sensitivity in these particles: TUA, TUA-CFo, or TUA-STA, depleted in F_1 + OSCP, F_1 + OSCP + phospholipids, and F_1 + OSCP + Fc_2, respectively (Bulos and Racker, 1968a,b; Knowles *et al.*, 1971). TUA and TUA-CFo still contain Fc_2 but this protein is heat stable (Knowles and Guillory, 1970; Knowles *et al.*, 1971). Loss of oligomycin or the carbodiimide sensitivity in TUA-CFo could not be restored by phospholipids (Bulos and Racker, 1968b).

Thus, it is likely that the site of the carbodiimide inhibition is a protein different from F_1, OSCP, or Fc_2. The 0.76 mobility protein (band 12) in OS-ATPase is a favorite candidate, since it is the only protein labeled by the [^{14}C]carbodiimide at concentrations which do not exceed the maximum inhibition level.

After conclusion of our experiments, Cattell *et al.* (1971) have given additional support for the protein nature of the inhibition site. Chloroform–methanol extractable radioactivity from the [^{14}C]carbodiimide-labeled submitochondrial particles is not attached to phospholipids as checked by thin-layer chromatography. All radioactivity of the crude proteolipid fraction derived from this extract resides in the protein moiety remaining at the origin. In this fraction only one protein with an electrophoretic mobility corresponding to a molecular weight of 10,000 bound the inhibitor. This was the major radioactive protein component in the [^{14}C]carbodiimide-labeled submitochondrial particles. The molecular weight of 10,000 is 3000–4000 lower than the one found by us for the carbodiimide-binding protein. No explanation for this difference in molecular weight can be given at the moment.

Acknowledgments

The authors are indebted to Dr. R. B. Beechey for sending data pertinent to the synthesis of the [^{14}C]carbodiimide as well as for valuable discussions on the study of the carbodiimide-binding protein. We thank Dr. E. Racker for sending us a preprint of the work on Fc_2 and Dr. D. R. Sanadi for providing us with unpublished data concerning OSCP.

References

Amiard, G., Heymes, R., and Velluz, L. (1958), *Chem. Abstr. 52*, 426*f*.

Beechey, R. B., Holloway, C. T., Knight, I. G., and Roberton, A. M. (1966), *Biochem. Biophys. Res. Commun. 23*, 75.

Beechey, R. B., Roberton, A. M., Holloway, C. T., and Knight, I. G. (1967), *Biochemistry 6*, 3867.

Beyer, R. E., Crankshaw, D. L., and Kuner, J. M. (1967), *Biochem. Biophys. Res. Commun. 28*, 758.

Bruni, A., Pitotti, A., Contessa, A. R., and Palatini, P. (1971), *Biochem. Biophys. Res. Commun. 44*, 268.

Bulos, B., and Racker, E. (1968a), *J. Biol. Chem. 243*, 3891.

Bulos, B., and Racker, E. (1968b), *J. Biol. Chem. 243*, 3901.

Cattell, K. J., Knight, I. G., Lindop, C. R., and Beechey, R. B. (1970), *Biochem. J. 117*, 1011.

Cattell, K. J., Lindop, C. R., Knight, I. G., and Beechey, R. B. (1971), *Biochem. J. 125*, 169.

Crane, F. L., Glenn, J. L., and Green, D. E. (1956), *Biochim. Biophys. Acta 22*, 475.

Groot, G. S. P. (1970), Ph.D. Thesis, Mondeel-Offsetdrukkerij, Amsterdam.

Hess, H. H., and Lewin, E. (1965), *J. Neurochem. 12*, 205.

Holloway, C. T., Roberton, A. M., Knight, I. G., and Beechey, R. B. (1966), *Biochem. J. 110*, 79P.

Horstman, L. L., and Racker, E. (1970), *J. Biol. Chem. 245*, 1336.

Huijing, F., and Slater, E. C. (1961), *J. Biochem. (Tokyo) 49*, 493.

Jacobs, E. E., Jacob, M., Sanadi, D. R., and Bradley, L. B. (1956), *J. Biol. Chem. 223*, 147.

Kagawa, Y., and Racker, E. (1966a), *J. Biol. Chem. 241*, 2461.

Kagawa, Y., and Racker, E. (1966b), *J. Biol. Chem. 241*, 2467.

Knowles, A. F., and Guillory, R. J. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 895 Abs.
Knowles, A. F., Guillory, R. J., and Racker, E. (1971), *J. Biol. Chem. 246*, 2672.
Lardy, H. A., Johnson, D., and McMurray, W. C. (1958), *Arch. Biochem. Biophys. 78*, 587.
Lardy, H. A., Witonsky, P., and Johnson, D. (1965), *Biochemistry 4*, 552.
Lee, C.-P., and Ernster, L. (1965), *Biochem. Biophys. Res. Commun. 18*, 523.
Lowry, O. H., Rosebrough, N., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
MacLennan, D. H., and Tzagoloff, A. (1968), *Biochemistry 7*, 1603.
Maurer, H. R. (1968), Disk Elektrophorese, Berlin, Walter de Gruyter & Co.
Penefsky, H. S. (1967), *J. Biol. Chem. 242*, 5789.
Pullman, M. E., Penefsky, H. S., Datta, A., and Racker, E. (1960), *J. Biol. Chem. 235*, 3322.
Racker, E. (1962), *Proc. Nat. Acad. Sci. U. S. 48*, 1659.
Racker, E. (1963), *Biochem. Biophys. Res. Commun. 10*, 435.
Racker, E., and Horstman, L. L. (1967), *J. Biol. Chem. 242*, 2547.
Racker, E., Pullman, M. E., Penefsky, H. S., and Silverman, M. (1961), *Proc. 5th Int. Congr. Biochem., Moscow 5*, 303.
Roberton, A. M., Holloway, C. T., Knight, I. G., and Beechey, R. B. (1966), *Biochem. J. 100*, 78P.
Roberton, A. M., Holloway, C. T., Knight, I. G., and Beechey, R. B. (1968), *Biochem. J. 108*, 445.
Schatz, G., Penefsky, H. S., and Racker, E. (1967), *J. Biol. Chem. 242*, 2552.
Senior, A. E., and Brooks, J. C. (1970), *Arch. Biochem. Biophys. 140*, 257.
Takayama, K., MacLennan, D. H., Tzagoloff, A., and Stoner, C. D. (1964), *Arch. Biochem. Biophys. 114*, 223.
Thompson, R. Q., Hoehn, M. M., and Higgins, C. E. (1961), *Antimicrob. Ag. Chemother.*, 474.
Tzagoloff, A. (1969a), *J. Biol. Chem. 244*, 5020.
Tzagoloff, A. (1969b), *J. Biol. Chem. 244*, 5027.
Tzagoloff, A. (1970), *J. Biol. Chem. 245*, 1545.
Tzagoloff, A., Byington, K. H., and MacLennan, D. H. (1968a), *J. Biol. Chem. 243*, 2405.
Tzagoloff, A., MacLennan, D. H., and Byington, K. H. (1968b), *Biochemistry 7*, 1596.
Walther, H. (1963), *Chem. Abstr. 58*, 2382*f*.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.

Effect of Antimycin A and 2-Heptyl-4-hydroxyquinoline *N*-Oxide on the Respiratory Chain of Submitochondrial Particles of Beef Heart†

J. R. Brandon,‡ J. R. Brocklehurst, and C. P. Lee*

ABSTRACT: Comparative studies of the effects of antimycin A and 2-heptyl-4-hydroxyquinoline *N*-oxide (HOQNO) on some of the chemical and physical properties of the respiratory chain of submitochondrial particles from beef heart have been made. (1) Antimycin A and HOQNO exhibit similar inhibition of both forward (NADH and succinate oxidases) and reversed (from ascorbate + PMS → NAD^+ supported by added ATP) electron flow of the respiratory chain. On a molar basis the amount of antimycin A required to give complete inhibition is approximately equal to the amount of cytochrome b_{561}. Antimycin A is 10 times more efficient than HOQNO. (2) Both compounds induce an absorption increase with a peak at 565.5 nm accompanied by a shoulder at 557–558 nm with either NADH- or succinate-reduced, terminally inhibited particles. The spectral changes titrate with antimycin A or HOQNO in the same way as the inhibitory effects, but somewhat less of either compound is required (on a mole to mole basis) to attain the maximal effect. (3) The spectral peak at 566 nm induced by antimycin A consists of two components: reduction of cytochrome b_{566} and a red shift of the reduced form of cytochrome b_{561}. HOQNO induces *only* the reduction of cytochrome b_{566}. (4) The antimycin-A-induced red shift is seen most clearly in a dithionite-reduced preparation where there is no interference from cytochrome b_{566}. (5) ATP induces a partial reduction of cytochrome b_{566} in the succinate–KCN-pretreated preparation. No red shift was observed.

It is now generally accepted that there are two major *b* cytochromes associated with the mitochondrial inner membrane which can be distinguished spectrophotometrically (Chance, 1952, 1958; Chance and Schoener, 1966; Chance *et al.*, 1966; Slater *et al.*, 1970a,b; Sato *et al.*, 1971a,b; Wikström, 1972) and potentiometrically (Dutton *et al.*, 1970, 1971; Wilson and Dutton, 1970; Rieske, 1971). One, cytochrome b_{561}, is characterized by a single α band at 561 nm at room temperature and a midpoint potential of +30 mV, and is fully reducible by succinate or NADH in anaerobiosis.

† From the Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pennsylvania 19104. *Received September 29, 1971.* This investigation has been supported by grants from the U. S. National Science Foundation (NSF GB23253), and the U. S. National Institutes of Health (NIH, GM12202). J. R. Brandon is a recipient of an NIH postdoctoral fellowship (5F-02GM49877). J. R. Brocklehurst is a recipient of a Wellcome Research travel grant. C. P. Lee is an NIH career development awardee, (1-K4-GM38822).

‡ Present address: Department of Chemistry, Ohio State University, Columbus, Ohio 43210.