Biochimica et Biophysica Acta, 509 (1978) 410—418

BBA 78025

IMMUNOLOGICAL PROPERTIES OF MEMBRANE-BOUND ADENOSINE TRIPHOSPHATASE

IMMUNOLOGICAL IDENTIFICATION OF RUTAMYCIN-SENSITIVE $F_0 \cdot F_1$ATPase FROM *MICROCOCCUS LUTEUS* ATCC 4698 ESTABLISHED BY CROSSED IMMUNOELECTROPHORESIS

MANFRED SCHMITT *, KLAUS RITTINGHAUS, PETER SCHEURICH, UDO SCHWULERA and KLAUS DOSE

Institut für Biochemie, Joh. Gutenberg-Universität, Joh. Joachim Becher-Weg 30, D-6500 Mainz (G.F.R.)

(Received October 14th, 1977)

Summary

(1) $F_0 \cdot F_1$ATPase (EC 3.6.1.3) from *Micrococcus luteus* ATCC 4698 was solubilized from plasma membranes by the non-ionic detergent Triton X-100 in the presence of 0.05 M $MgCl_2$.

(2) The antibiotics rutamycin, Dio-9, quercetin, oligomycin, botrycidin, efrapeptin, leucinostatin, valinomycin, and venturicidin as well as *N,N'*-dicyclohexylcarbodiimide and dinitrophenol are potent inhibitors of $F_0 \cdot F_1$ATPase activity.

(3) $F_0 \cdot F_1$ATPase activity is completely inhibited by anti-F_1ATPase antibodies. The inhibition is non-competitive.

(4) Crossed immunoelectrophoresis reveals a reaction of immunological identity of $F_0 \cdot F_1$ATPase and F_1ATPase indicating that both enzymes have in common antigenic sites.

Introduction

Four antigens, not expressed on the outer surface of the plasma membrane, have been detected by crossed immunoelectrophoresis with membrane antiserum in fractions from membranes of *Micrococcus luteus* ATCC 4698 solubilized with the non-ionic detergent Triton X-100 [1]. One of these, ATPase (EC 3.6.1.3), has been localized by ferritin labeling only on the inner

* To whom requests should be addressed at the present address: Institut für Medizinische Mikrobiologie, Joh. Gutenberg-Universität, Augustus-Platz, D-6500 Mainz, G.F.R.

face of the membrane [2]. This ATPase is composed of two structurally distinct entities; these are referred to as F_0ATPase and F_1ATPase [3]. The F_1ATPase from *M. luteus* ATCC 4698 can be solubilized from plasma membranes by osmotic shock treatment [4,5] and purified to homogeneity by several techniques [4—8]. The F_0 moiety of the $F_0 \cdot F_1$ATPase complex is apparently buried in the hydrophobic interior of the membrane [3].

The primary objective of this study was to solubilize the $F_0 \cdot F_1$ATPase complex from plasma membranes of *M. luteus* ATCC 4698 by the detergent Triton X-100 in intact form, to identify it by immunological techniques (crossed immunoelectrophoresis) and to characterize this $F_0 \cdot F_1$ATPase complex by various inhibitors such as antibiotics, *N,N'*-dicyclohexylcarbodiimide, dinitrophenol, and organic tin derivatives.

Moreover, crossed immunoelectrophoresis was applied to reveal the antigenic relationship of $F_0 \cdot F_1$ATPase and F_1ATPase.

Experimental

Materials. Highly purified agarose was from Merck, Darmstadt; complete Freund's adjuvant from Difco, Detroit. Acrylamide, recrystallized four times, was purchased from Roth, Karslruhe. Tris(hydroxymethyl)aminomethane was from BioMol, Ilversheim, and *N,N'*-dicyclohexylcarbodiimide from Sigma, Munich. Na_3-ATP, ferritin, and aldolase were obtained from Boehringer, Mannheim. Dinitrophenol, catalase, hemoglobin, bovine serum albumin, quercetin, and diisopropylfluorophosphate were from Serva, Heidelberg. Efrapeptin, leucinostatin, and rutamycin were a gift from W. Keller-Schierlein, Zürich. Venturicidin was from Chambrian Chemicals, Croydon, and Dio-9 was a gift from Gist-Brocades, Delft. Triethyltin chloride was purchased from Merck, Darmstadt, and tributyltin chloride from Riedel-de Haën, Seelze-Hannover. Sepharose CL-6B was from Pharmacia, Freiburg. All other chemicals were of analytical grade, purchased from Merck, Darmstadt.

Preparation of highly purified F_1ATPase. The F_1ATPase was prepared from *M. luteus* ATCC 4698 as described by Risi et al. [5] for *Micrococcus* sp. ATCC 398. All the steps of purification were carried out in the presence of 10^{-4} M diisopropylfluorophosphate.

Preparation of detergent-solubilized $F_0 \cdot F_1$ATPase. Membrane-bound ATPase from *M. luteus* ATCC 4698 was prepared according to Muñoz et al. [4]. 50—70 mg membrane protein was suspended in 0.1 M Tris · HCl (pH 7.5) containing 0.05 M $MgCl_2$, 0.045 M Na_2SO_4 and 20% glycerol. Triton X-100 was added in a detergent/protein weight ratio of 2.6 : 1. The suspension was stirred for 30 min at 0°C and then centrifuged at 200 000 × *g*, 45 min. Mg^{2+} was necessary for the solubilization of $F_0 \cdot F_1$ATPase in intact form. Solubilization by Triton X-100 without Mg^{2+} results in a loss of $F_0 \cdot F_1$ATPase activity linked with an increase in F_1ATPase activity. $F_0 \cdot F_1$ATPase was stored at 4°C in the presence of 10^{-4} M phosphatidylcholine.

Analytical disc electrophoresis. Analytical disc electrophoresis was performed in 5% polyacrylamide gels [5]. Electrophoresis of $F_0 \cdot F_1$ATPase was performed in the presence of 0.1% Triton X-100. ATPase activity was identified in situ according to Owen and Salton [1].

Protein determination. Protein was determined according to Lowry et al. [9] with bovine serum albumin as standard. In the presence of Triton X-100, protein was determined according to Soper and Pedersen [10]. Determination of the protein concentration of F_1ATPase and $F_0 \cdot F_1$ATPase in partially purified preparations was carried out by either the Mancini technique (radial immunodiffusion) [12] or by rocket immunoelectrophoresis [13] with purified anti-F_1ATPase antibodies.

ATPase assay. ATPase activity was determined continuously by measurement of the liberated inorganic phosphate as described by Arnold et al. [14]. Specific activity is given in units per mg protein. One unit is defined as the number of μmol of inorganic phosphate liberated during 1 min at 37°C. Inhibitor studies were done with 2% ethanol present during a 20 min preincubation period at 37°C. Controls received an equivalent amount of ethanol only. Blanks containing ethanol did not show any inhibition.

Immunological techniques. Rabbits were injected intradermally and subcutaneously in multiple sites with highly purified F_1ATPase (0.5 mg of protein/rabbit) emulsified in complete Freund's adjuvant. Injections were repeated three times at weekly intervals. Sera were collected a week after the last injection and stored at −37°C. Anti-F_1ATPase antibodies from F_1ATPase of *M. luteus* ATCC 4698 were prepared by immunoaffinity chromatography with immobilized F_1ATPase (*M. luteus*) as described for the purification of anti-F_1ATPase antibodies of *Micrococcus* sp. ATCC 398 [7,11] and then concentrated to the original serum volume. Radial immunodiffusion was made in 1.5% agarose in barbital buffer ($I = 0.02$) at pH 8.6 [12]. Rocket immunoelectrophoresis was performed in 1.5% agarose in barbital buffer ($I = 0.02$) at pH 8.6 (3 h, 300 V) [13]. Crossed immunoelectrophoresis was carried out in 1.5% agarose in barbital buffer ($I = 0.02$) at pH 8.6. No detergent was added. First dimension electrophoresis was carried out for 90 min (300 V, 15°C), second dimension electrophoresis was run for 18 h (80 V, 15°C).

Results

Isolation and stability of $F_0 \cdot F_1$ATPase

The detergent-solubilized $F_0 \cdot F_1$ATPase from plasma membranes of *M. luteus* ATCC 4698 was homogeneous according to analytical polyacrylamide gel electrophoresis (5% polyacrylamide, 0.1% Triton X-100). It consisted of a single slowly moving band coincident with enzymic activity. A molecular weight of 450 000 ± 30 000 was determined by gel filtration with Sepharose CL-6B. Reference markers were ferritin, catalase, hemoglobin and aldolase. Best solubilization of the $F_0 \cdot F_1$ATPase complex, with respect to ATPase activity and its sensitivity against rutamycin, oligomycin and dicyclohexylcarbodiimide, was achieved at a Triton X-100/membrane protein weight ratio of 2.6 : 1. Furthermore, solubilization of $F_0 \cdot F_1$ATPase by Triton X-100 in the presence of 0.05 M $MgCl_2$ yielded a stable $F_0 \cdot F_1$ATPase complex not contaminated by F_1ATPase (Fig. 2). Higher Triton X-100/membrane protein weight ratios led to the solubilization of a higher amount of membrane proteins. Under these conditions $F_0 \cdot F_1$ATPase was destabilized and F_1ATPase was split off, even in the presence of 0.05 M $MgCl_2$. Optimal $F_0 \cdot F_1$ATPase activity

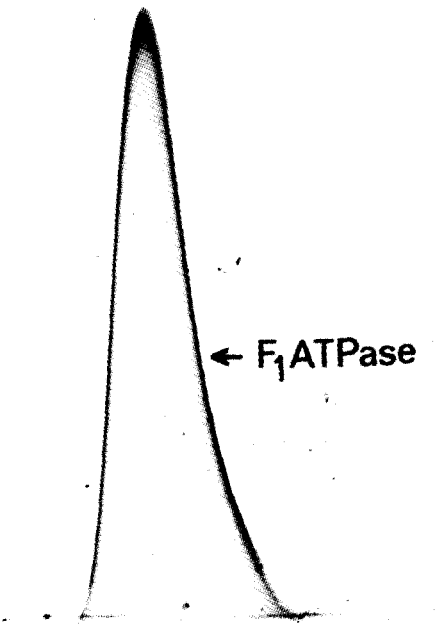

Fig. 1. Crossed immunoelectrophoresis of 4 μg of highly purified F_1ATPase. Concentration of anti-F_1ATPase antibodies: 3% (v/v). First dimension electrophoresis (300 V, 90 min, 15°C): anode to the left; second dimension electrophoresis (80 V, 18 h, 15°C): anode at the top.

was measured at a Mg^{2+}/ATP ratio of 3 : 1 (0.1 M Tris · HCl, pH 7.5, 37°C, 12 μg Triton X-100/mg protein). Specific activity was 0.1 unit. The buffer found to be optimal for stabilizing $F_0 \cdot F_1$ATPase activity contained 0.1 M Tris · HCl (pH 7.5), 20% glycerol, 0.05 M $MgCl_2$, and 10^{-4} M phosphatidylcholine. When stored in this buffer at 4°C (or in liquid N_2) the $F_0 \cdot F_1$ATPase stayed active for several weeks without loosing the F_1ATPase moiety. In the absence of phosphatidylcholine $F_0 \cdot F_1$ATPase was destabilized ($t_{1/2}$ = 2 days) when stored at 4°C or in liquid N_2.

Inhibitor sensitivities of membrane-bound ATPase, $F_0 \cdot F_1$ATPase, and F_1ATPase

A most important criterion for the unimpaired state of the $F_0 \cdot F_1$ATPase is its degree of sensitivity to antibiotics [15,16]. As summarized in Table I the membrane-bound ATPase, the $F_0 \cdot F_1$ATPase, and the F_1ATPase differ in their sensitivity to antibiotics. F_1ATPase is only affected by quercetin, whereas the membrane-bound ATPase and the $F_0 \cdot F_1$ATPase activity is blocked by rutamycin, Dio-9, quercetin, oligomycin, botrycidin, efrapeptin, leucinostatin, valinomycin and venturicidin. *N,N'*-Dicyclohexylcarbodiimide and dinitro-

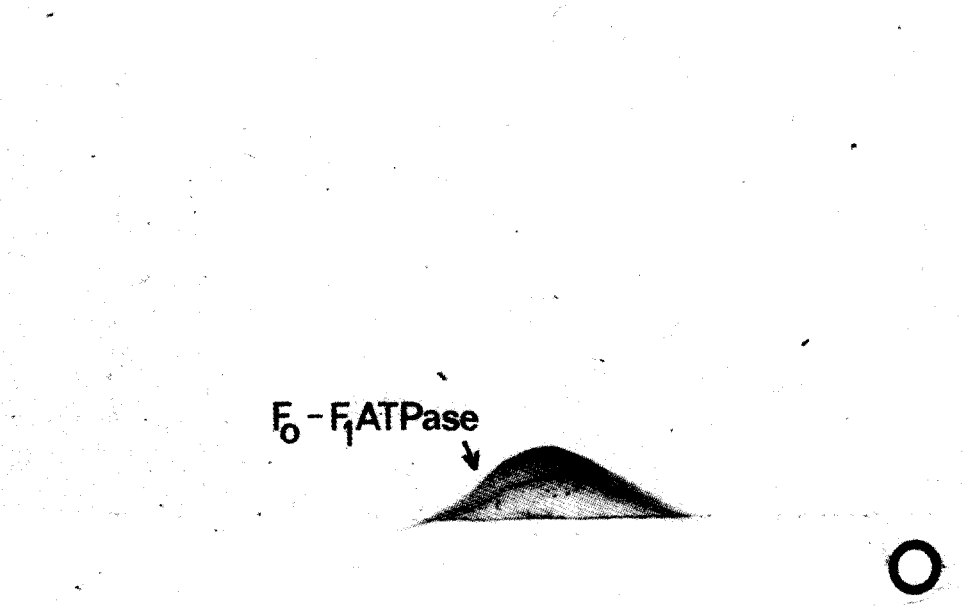

Fig. 2. Crossed immunoelectrophoresis of 2 μg of $F_0 \cdot F_1$ATPase solubilized by Triton X-100. Conditions as indicated in Fig. 1. No Triton X-100 added.

TABLE I

INHIBITION OF ATPase ACTIVITY BY BLOCKING AGENTS

Agent		Inhibition (%) of		
		F_1ATPase *	$F_0 \cdot F_1$ATPase **	Membrane-bound *** ATPase
Rutamycin	($4.6 \cdot 10^{-4}$ M)	—	77	50
Dio-9	(20 μg/ml)	—	49	34
Quercetin	($2 \cdot 10^{-5}$ M)	100	48	11
Oligomycin	($4.6 \cdot 10^{-4}$ M)	—	43	48
Botrycidin	(20 μg/ml)	—	42	53
Efrapeptin	($6 \cdot 10^{-5}$ M)	—	35	14
Leucinostatin	($1.3 \cdot 10^{-4}$ M)	—	28	34
Valinomycin	($5 \cdot 10^{-5}$ M)	—	28	9
Venturicidin	(20 μg/ml)	—	24	49
N,N'-Dicyclohexylcarbodiimide	($4 \cdot 10^{-4}$ M)	8	80	67
Dinitrophenol	($3 \cdot 10^{-4}$ M)	—	49	16
Triethyltin chloride	($2.9 \cdot 10^{-4}$ M)	13	—	62
Tributyltin chloride	($2.9 \cdot 10^{-4}$ M)	24	—	40

* Specific activity: 30 units (0.1 M Tris · HCl (pH 8.0)/0.001 M ATP/0.01 M $CaCl_2$, 37°C).
** Specific activity: 0.1 units (0.1 M Tris · HCl (pH 7.5)/0.001 M ATP/0.003 M $MgCl_2$, 37°C).
*** Specific activity: 0.01 units (0.1 M Tris · HCl (pH 7.5)/0.001 M ATP/0.001 M $MgCl_2$, 37°C).

phenol are potent inhibitors of $F_0 \cdot F_1$ATPase activity and for the ATPase activity in the membrane-bound state (see Table I). In contrast to *N,N'*-dicyclohexylcarbodiimide, dinitrophenol, and the summarized antibiotics, inhibition of the $F_0 \cdot F_1$ATPase activity by the organic tin derivatives triethyltin chloride and tributyltin chloride was not detected.

Immunological studies of $F_0 \cdot F_1$ATPase

The following experiments were carried out with anti-F_1ATPase antibodies purified by immunoaffinity chromatography at immobilized F_1ATPase (F_1ATPase coupled to Sepharose CL-6B) [7,11].

(a) Inactivation of $F_0 \cdot F_1$ATPase by anti-F_1ATPase antibodies. 1 mg of $F_0 \cdot F_1$ATPase (specific activity, 0.1 unit) was completely inactivated in solution within 9 min by 16 μg of anti-F_1ATPase antibodies (37°C). The inactivation is non-competitive. It is worthwhile mentioning that 1 mg of F_1ATPase (specific activity 30 units) was completely inactivated by 1.2 mg of anti-F_1ATPase (Schmitt, M., unpublished results). Enzymic activity was not maintained by preincubation of the $F_0 \cdot F_1$ATPase · anti-F_1ATPase antibodies complex in the presence of ATP.

(b) Charge shift electrophoresis and crossed immunoelectrophoresis of F_1ATPase and $F_0 \cdot F_1$ATPase. F_1ATPase and $F_0 \cdot F_1$ATPase were subjected to agarose gel electrophoresis in the presence of the non-ionic detergent Triton

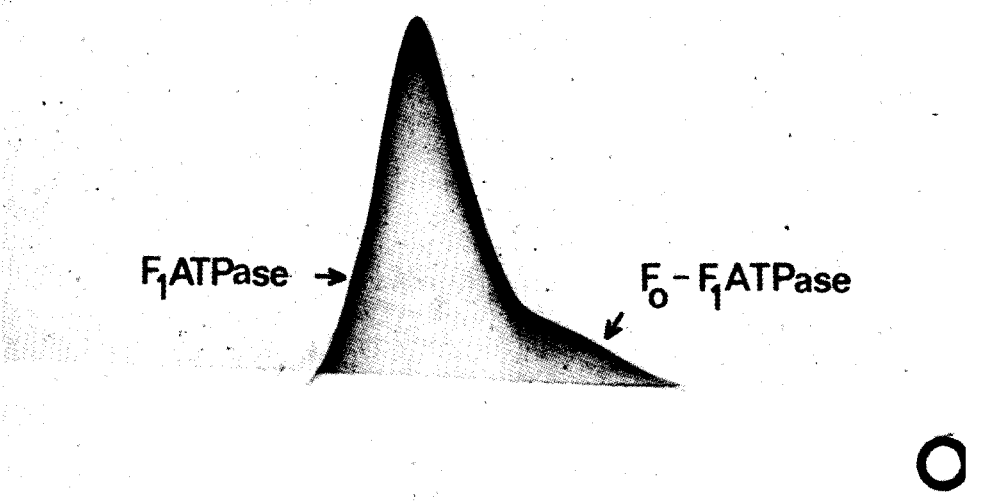

Fig. 3. Crossed immunoelectrophoresis of 2 μg of highly purified F_1ATPase and 2 μg of $F_0 \cdot F_1$ATPase. Conditions as indicated in Fig. 1. No Triton X-100 added.

X-100 according to Helenius and Simons [17]. In comparison to detergent-free electrophoresis the electrophoretic mobility of F_1ATPase and $F_0 \cdot F_1$ATPase remained unchanged. The water-soluble F_1ATPase, as well as the detergent-solubilized $F_0 \cdot F_1$ATPase, yielded single bands in each electrophoretical system; however, the two ATPase preparations differ slightly in their electrophoretic mobility (see Figs. 1 and 2). We could not apply charge shift electrophoresis to distinguish between a hydrophilic and/or amphiphilic character of F_1ATPase and $F_0 \cdot F_1$ATPase because both ATPase preparations are oligomeric enzyme complexes which undergo dissociation into subunits in the presence of the ionic detergent deoxycholate and cetyltrimethylammonium bromide, even in excess of Triton X-100.

$F_0 \cdot F_1$ATPase and F_1ATPase, respectively, formed single precipitin peaks with the anti-F_1ATPase antibodies in detergent-free crossed immunoelectrophoresis. We made use of the different electrophoretic mobilities of F_1ATPase and $F_0 \cdot F_1$ATPase, respectively, to distinguish between the antigenic determinants of both ATPase preparations. The mixture of F_1ATPase and the $F_0 \cdot F_1$ATPase led to a single immunoprecipitate in crossed immunoelectrophoresis (Fig. 3). The fusion of the precipitin lines of F_1ATPase and $F_0 \cdot F_1$ATPase to one single precipitin peak indicates that both enzyme preparations have in common antigenic sites and that the non-ionic detergent Triton X-100 does not denaturate the F_1 moiety of the ATPase complex.

Discussion

The preparation of the $F_0 \cdot F_1$ATPase complex

ATPase complexes may be removed and solubilized from the membranes of mitochondria [3], bacteria [18], and chloroplasts [19,20] by a variety of treatments. In contrast to mitochondrial F_1ATPases, the release of F_1 proteins from both bacteria and chloroplast membranes can be achieved by osmotic shock treatment at low ionic strength [4,5] or by washing with EDTA-containing buffers [22]. All the various methods to solubilize complete ATPase complexes ($F_0 \cdot F_1$ATPase) from plasma membranes basically involve the preparation of detergent-solubilized membrane preparations [22,23,24]. Our investigations have been directed towards the solubilization of the $F_0 \cdot F_1$ATPase in intact form and its characterization by antibiotic inhibitors and crossed immunoelectrophoresis. We found a Tris buffer (pH 7.5) containing 0.05 M $MgCl_2$ and Triton X-100 in a detergent/membrane protein weight ratio of 2.6 : 1 to be optimal for solubilizing the $F_0 \cdot F_1$ATPase complex. In such a complexed form the $F_0 \cdot F_1$ATPase is stabilized and no F_1ATPase becomes released. Solubilization by Triton X-100/membrane protein weight ratios greater than 2.6 and/or $MgCl_2$ concentrations less than 0.05 M resulted in an insufficient release of $F_0 \cdot F_1$ATPase from the plasma membrane. As Mg^{2+} is required for the binding of the F_1 factor to the F_0 moiety it seems that part of the force holding the water-soluble factor to the detergent-soluble factor is coulombic.

The effects of various inhibitors

Lloyd and Edwards [15] as well as Lardy et al. [16] have previously shown that the various inhibitors of the mitochondrial ATPase may be classified into

those inhibitors which interact with the $F_0 \cdot F_1$ATPase by binding to the F_0 factor and those which react with the F_1ATPase itself. Our experiments with antibiotics that inhibit mitochondrial F_1ATPase and $F_0 \cdot F_1$ATPase complexes (Table I) showed that the F_1ATPase from *M. luteus* ATCC 4698 is affected by quercetin, but not by efrapeptin. Lardy et al. [16] have suggested that efrapeptin binds at, or close to, an ATP binding site of mitochondrial F_1ATPase. Our data indicate, however, that the binding site for efrapeptin is not located at the F_1 factor of the *Micrococcus* ATPases. The other antibiotics which we have tested inactivate both the membrane-bound and the detergent-soluble ATPase activity. In contrast to the mitochondrial ATPase [25,26] organic tin derivatives have no effect on the *Micrococcus* $F_0 \cdot F_1$ATPase. *N,N'*-Dicyclohexylcarbodiimide is known to be a potent inhibitor of both the bacterial and mitochondrial ATPases [15,27—31]. This applies also to the membrane-bound and the $F_0 \cdot F_1$ATPase activity of *M. luteus* ATCC 4698.

Immunological identification

In order to achieve solubilization of the $F_0 \cdot F_1$ATPase complex the non-ionic detergent Triton X-100 has been used. Solubilization with ionic detergents failed to detach an enzymatic active form from the plasma membranes. The capacity of crossed immunoelectrophoresis has so far seldom been exploited in the study of microbial membrane proteins [1,32]. Owen and Salton [1] have prepared a Triton X-100-soluble protein fraction from the plasma membrane of *M. luteus* ATCC 4698. They demonstrated by crossed immunoelectrophoresis that the detergent-soluble ATPase complex showed lines of partial identity reflecting structurally identic antigenic sites of ATPase components. In our study we demonstrate that in crossed immunoelectrophoresis with anti-F_1ATPase antibodies the two different ATPase preparations, such as F_1ATPase and $F_0 \cdot F_1$ATPase, respectively, from a coalescing precipitate with an identical precipitin line. This result unambiguously indicates that both the F_1 protein of the $F_0 \cdot F_1$ATPase complex and the soluble F_1ATPase interact in an identical, or almost identical, way with anti-F_1ATPase antibodies.

Acknowledgements

The authors would like to thank Professor Dr. H.U. Wolf for very useful suggestions in the initial phase of this research and Professor Dr. M. Dierich for helpful discussion and for revising the manuscript. We are indebted to Miss C. Brand for editing the manuscript. This work was supported in part by Deutsche Forschungsgemeinschaft, grant Do 192/1. This work is part of the thesis of M. Schmitt, K. Rittinghaus and P. Scheurich for a Dr. rer. nat. degree at the Johannes-Gutenberg-Universität, Mainz.

References

1 Owen, P. and Salton, M.R.J. (1975) Proc. Natl. Acad. Sci. U.S. 72, 3711—3715
2 Oppenheimer, J.D. and Salton, M.R.J. (1973) Biochim. Biophys. Acta 298, 297—322
3 Pedersen, P.L. (1975) J. Bioenerg. 6, 243—275
4 Muñoz, E., Salton, M.R.J., Ng, M.H. and Schor, M.T. (1969) Eur. J. Biochem. 7, 490—501
5 Risi, S., Höckel, M., Hulla, F.W. and Dose, K. (1977) Eur. J. Biochem. 81, 103—109
6 Hulla, F.W., Höckel, M., Risi, S. and Dose, K. (1976) Eur. J. Biochem. 67, 469—476

7 Schmitt, M., Risi, S. and Dose, K. (1977) 11th FEBS Meeting, Copenhagen, A 4-13 717
8 Höckel, M., Hulla, F.W., Risi, S. and Dose, K. (1976) Biochim. Biophys. Acta 429, 1020—1028
9 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
10 Soper, J.W. and Pedersen, P.L. (1976) Biochemistry 15, 2682—2690
11 Schmitt, M., Risi, S., Müller, H.W. and Dose, K. (1977) Hoppe Seyler's Z. Physiol. Chem. 358, 301—302
12 Mancini, G., Carbonara, A.O. and Heremans, J.F. (1965) Immunochemistry 2, 235—254
13 Laurell, C.B. (1966) Anal. Biochem. 15, 45—52
14 Arnold, A., Wolf, H.U., Ackermann, B.P. and Bader, H. (1976) Anal. Biochem. 71, 209—213
15 Lloyd, D. and Edwards, S.W. (1977) Biochem. J. 162, 581—590
16 Lardy, H., Reed, P. and Lin, C.C. (1975) Fed. Proc. Fed. Am. Soc. Exp. Biol. 34, 1707—1710
17 Helenius, A. and Simons, K. (1977) Proc. Natl. Acad. Sci. U.S. 74, 529—532
18 West, I.C. (1974) Biochem. Soc. Spec. Publ. 4, 27—38
19 Leech, R.M. (1974) Biochem. Soc. Spec. Publ. 4, 19—25
20 Penefsky, H.S. (1974) in The Enzymes (Boyer, P.D., ed.), 3rd edn., Vol. 10, pp. 375—394, Academic Press, New York
21 Beechey, R.B. (1974) Biochem. Soc. Spec. Publ. 4, 41—62
22 Roisin, M.P. and Kepes, A. (1972) Biochim. Biophys. Acta 275, 333—346
23 Tzagoloff, A. and Meagher, P. (1971) J. Biol. Chem. 246, 7328—7336
24 Swanljung, P. and Ernster, L. (1971) in Energy Transduction in Respiration and Photosynthesis (Quagliariello, E., Papa, S. and Rossi, C.S., eds.), pp. 839—840, Adriatica Editrice, Bari
25 Griffiths, D.E., Houghton, R.L., Lancashire, W.E. and Meadows, P.A. (1975) Eur. J. Biochem. 51, 393—402
26 Griffiths, D.E., Cain, K. and Hyams, R.L. (1977) Biochem. Soc. Trans. 5, 205—207
27 Clarke, D.J. and Morris, J.G. (1976) Biochem. J. 154, 725—729
28 Altendorf, K. (1977) FEBS Lett. 73, 271—275
29 Fillingame, R.H. (1976) J. Biol. Chem. 251, 6630—6637
30 Hare, J.F. (1975) Biochem. Biophys. Res. Commun. 66, 1329—1337
31 Harold, F.M., Baarda, J.R., Baron, C. and Abrams, A. (1969) J. Biol. Chem. 244, 2261—2268
32 Johansson, K. and Hjerten, S. (1974) J. Mol. Biol. 86, 341—352