Biochimica et Biophysica Acta, 548 (1979) 63—71

BBA 47732

OPTIMIZATION OF THE PURIFICATION OF MITOCHONDRIAL F_1-ADENOSINE TRIPHOSPHATASE

FRANCOIS PENIN, CATHERINE GODINOT and DANIELE C. GAUTHERON

Laboratoire de Biologie et Technologie des Membranes du Centre National de la Recherche Scientifique. Université Claude Bernard de Lyon, 43 Bd de 11 Novembre 1918 - 69621 Villeurbanne (France)

(Received March 16th, 1979)

Key words: F_1-ATPase; (Mitochondria, Purification)

Summary

A simple technique of purification of the soluble pig heart mitochondrial F_1-ATPase is described. It consists of removal of extrinsic proteins from mitochondrial membranes before extraction with chloroform and ammonium sulfate fractionation. A high degree of purity, an excellent stability and a good yield are attained after gel filtration through an Ultrogel ACA 34 column equilibrated in the presence of 50% glycerol. The tested properties of the F_1-ATPase prepared by this method are similar to those of the same enzyme extracted by sonication. The enzyme is virtually devoid of tightly bound nucleotides. In addition, some characteristics of the behaviour of the β subunit are shown.

Introduction

Detailed physicochemical studies are only feasible when enzymes or proteins are available in relatively large amounts, in a stable form and with a very good degree of purity. Several methods have been described to purify the mitochondrial F_1-ATPase (EC 3.6.1.3). This enzyme can be extracted from submitochondrial particles either by extensive sonic disruption [1,2], by chloroform treatment [3—6] or else by incubation with diphosphatidylglycerol [7—9]. It must then be further purified by ammonium or protamine sulfate precipitation followed by either ion-exchange [1,2,4,5] or affinity chromatography [10,11] or by gel filtration [6,12]. According to the literature, the purest preparations

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

have been obtained after sonic disruption while higher yields were observed after chloroform extraction. In the present paper, a new method is described combining the advantages of the two previous techniques and which, in a few steps, produces the mitochondrial F_1-ATPase in high yield, with a high degree of purity and excellent stability.

Materials and Methods

Pig heart mitochondria were obtained at 0—4°C as previously described [13]. Submitochondrial particles were prepared according to Senior and Brooks [2]. Protein contents were estimated either with the Lowry procedure [14] or with the Bradford method using Coomassie blue staining [15], taking in both cases defatted bovine serum albumin as the standard. For gel electrophoresis, cylindrical gels (5 × 130 mm) were prepared by mixing 10 g acrylamide, 0.3 g *N,N'*-methylene-bisacrylamide in 0.1 M sodium phosphate buffer containing 0.1% sodium dodecyl sulfate, pH 7.5 (final volume, 100 ml); they were polymerized by adding 0.125 ml of *N,N,N',N'*-tetramethylethylenediamine and 37 mg of ammonium persulfate. Electrophoresis was run at 7 mA/gel for 7 h in 0.1 M sodium phosphate buffer, pH 7.5, containing 0.1% sodium dodecyl sulfate using an Uniscil UE-4 electrophoresis apparatus equipped with a pump permitting the circulation of the buffer between the electrode vessels. Pyronin was used as a tracking dye. After electrophoresis, gels were dipped into the staining solution containing 0.25% Coomassie brilliant blue R, 10% glacial acetic acid, 50% methanol for 4 h at 50°C. Destaining was performed by repeated washings in 25% ethanol/5% methanol/10% acetic acid; this solution was regenerated by passage through activated charcoal. Densitometric traces were obtained with a Vernon PHI 3 gel scanner. For gel filtration, the Ultrogel ACA 34 in 100 mM Tris/sulfate, pH 8.0, supplemented with 5 mM EDTA was first poured into the columns (1.7 × 53 cm or 3.2 × 93 cm) and then equilibrated with the same buffer containing 50% glycerol at a flow rate of 13 and 27 ml/h, respectively, for the small and the large columns. ATPase activity was determined as previously [16,17]; ATP-$^{32}P_i$ exchange activity was measured as described by Conover et al. [18]. The rebinding of F_1-ATPase to F_1-depleted submitochondrial particles was made according to Racker and Horstmann [19] and modified as described earlier [20].

The nucleotides tightly bound to F_1 were estimated as follows: the enzyme (3—5 mg protein) kept as a suspension in 4 mM ATP, 1 mM EDTA, 200 mM potassium phosphate, 60% saturated ammonium sulfate, pH 7.5, was sedimented in an Eppendorf bench top centrifuge at room temperature. The pellets were dissolved with 0.5 ml of Tris buffer made of 20 mM Tris/sulfate, 2 mM EDTA, pH 7.8; saturated ammonium sulfate containing 5 mM EDTA (0.75 ml) was added and the samples centrifuged again. This step was repeated four more times and the final pellet dissolved in 0.2 ml Tris buffer; these washings intended to eliminate the nucleotides that are not tightly bound, did not change the specific activity of the enzyme. Trichloroacetic acid was added up to a concentration of 8%. After centrifugation the trichloroacetic acid of supernatants was extracted five times with an equal volume of ether. Residual ether was finally eliminated by bubbling nitrogen. The enzyme preparations

obtained after gel filtration in Ultrogel ACA 34 and kept at −70°C in the presence of 50% glycerol were precipitated by adding 1.5 vols. of saturated ammonium sulfate; the pellet was dissolved in 0.2 ml of Tris buffer and treated as previously with trichloroacetic acid.

ADP and AMP were measured in coupled enzyme assays using the absorbance of NADH as described by Jaworek et al. [21]. ATP was determined by photon counting using the firefly luciferase assay in a L.I.P.E. microphotometer: 100 μl of LUMIT reagent (made by mixing 5 ml LUMAC buffer with 1 vial LUMIT HS) were added to the cuvette containing 150 μl of LUMAC buffer and 50-μl samples containing 20—200 pmol ATP *. Internal standards run simultaneously to check that no ATP was lost during the preparation were always satisfactory when the nucleotide determination was performed the same day as the extraction and the samples kept in ice.

Results and Discussion

Purification procedures

If F_1 was extracted directly from mitochondria or submitochondrial particles by a chloroform treatment as described by Beechey et al. [3], subsequent ion-exchange chromatography or gel filtration could not remove some contaminating proteins. Therefore, a technique was devised to remove these contaminants before the chloroform extraction. The whole procedure is summarized in Table I and includes seven steps.

Removal of extrinsic proteins from mitochondria. Steps 1—4 (Table I). Batches of 50 ml of mitochondrial suspension containing 20 mg of protein/ml of sucrose/Tris buffer: 0.25 M sucrose, 10 mM Tris-HCl at pH 7.6 (step 1) were sonicated for 6 min at 70 W (Branson sonifier B-12) in a refrigerated chamber to maintain the temperature between 0 and 8°C. The suspension was first centrifuged at 20 000 × *g* for 10 min and the supernatant recentrifuged at 100 000 × *g* for 2 h. The pellets were suspended in sucrose/Tris buffer at 30 mg protein/ml (step 2). ATP was added up to a concentration of 4 mM and the pH adjusted to 9.2 with 1 N NH_4OH and allowed to stand overnight at room temperature. The pH was brought back to 8.0 with 1 N HCl (step 3). Batches of 50 ml were sonicated for 5 min at 70 W in a refrigerated chamber to maintain the temperature between 20 and 25°C. After centrifugation (300 000 × *g* for 1 h), the pellets were suspended in sucrose/Tris buffer containing 2 mM EDTA at a concentration of 30 mg protein/ml (step 4).

Chloroform extraction. Step 5 (Table I). Analytical grade chloroform (0.5 vol.) was added to the previous suspension and the two phases were mixed vigorously for 15 s as described by Beechey et al. [3]. The two phases were separated by centrifugation at 600 × *g* for 10 min. The upper, aqueous phase was further centrifuged at 100 000 × *g* for 1 h. The pale yellow supernatant

* The LUMAC buffer contains 0.025 M Hepes (pH 7.75), magnesium, and chelating agents, and LUMIT HS is made of a mixture of luciferin and luciferase purified to obtain high sensitivity and low blanks in ATP determination. Both LUMAC and LUMIT HS are registered trade marks and can be obtained from Lumac B.V., Baanstraat 81, 6372 AD Schaesberg, The Netherlands.

TABLE I

PURIFICATION OF PIG HEART MITOCHONDRIAL F_1-ATPase

Step	Proteins (mg)	ATPase activity	
		Total (units) *	Specific (units · mg^{-1})
1. Mitochondria	6400	9 850	1.54
2. First sonication	3660	19 400	4.3
3. Alkaline treatment	3660	27 400	7.5
4. Second sonication	3000	21 500	7.1
5. Chloroform extraction	142	8 500	60
6. Ammonium sulfate precipitation	90	7 950	89
7. Gel filtration	67	7 800	117

* Units: μmol ATP hydrolyzed/min.

was adjusted to pH 8.0 and supplemented with 4 mM ATP (final concentration).

Ammonium sulfate precipitation. Step 6 (Table I). A saturated $(NH_4)_2SO_4$ solution containing 5 mM EDTA was added dropwise with continuous stirring until a 37% saturation; the pH was maintained at 8.0 with 1 N KOH. The precipitate was removed by centrifugation (10 000 × *g* for 15 min).

The supernatant fraction was brought to 60% saturation with solid ammonium sulfate and the pH adjusted to 8.0 with 1 N KOH. The mixture was allowed to stand for 10 min at room temperature and kept overnight at 0—4°C. The precipitate was collected by centrifugation and could be either suspended in a medium containing 60% $(NH_4)_2SO_4$, 4 mM ATP, 1 mM EDTA, 40 mM Tris/sulfate, pH 8.0 at 0—4°C or dissolved in a buffer made of 100 mM Tris/sulfate, 5 mM EDTA, 50% glycerol, pH 8.0 (glycerol buffer). In the latter medium, the enzyme was stable at any temperature ranging from —70°C to room temperature. At the end of this step, the enzyme was almost pure as shown by its specific activity (Table I) and dodecyl sulfate gel electrophoresis (Fig. 1, upper trace). Ion-exchange chromatography could not improve enzyme purity.

Gel filtration. Step 7 (Table I). A highly purified enzyme (Fig. 1, trace I*) could be obtained by gel filtration: the enzyme was dissolved in glycerol buffer at a concentration of 25 mg protein/ml. After removal of insoluble material by 5 min centrifugation in an Eppendorf bench-top centrifuge, the enzyme was applied on top of an Ultrogel ACA 34 column (3.2 × 93 cm) equilibrated with the glycerol buffer at room temperature. The ATPase activity eluted in a single symmetrical peak superimposed with the protein peak I* (Fig. 2). The specific activity of the enzyme was constant all along the peak and the total activity introduced was recovered as shown in Table I. The eluted enzyme was concentrated with an Amicon ultrafiltration cell equipped with a PM 10 membrane. Some inactive material eluted in later fractions (peak II*) corresponding partly to contaminants and partly to depolymerized F_1-ATPase; free subunit β seems to be the major peptide present (Fig. 1, trace II*).

The technique described here includes some steps besides the chloroform extraction that are slightly more time consuming than the techniques described

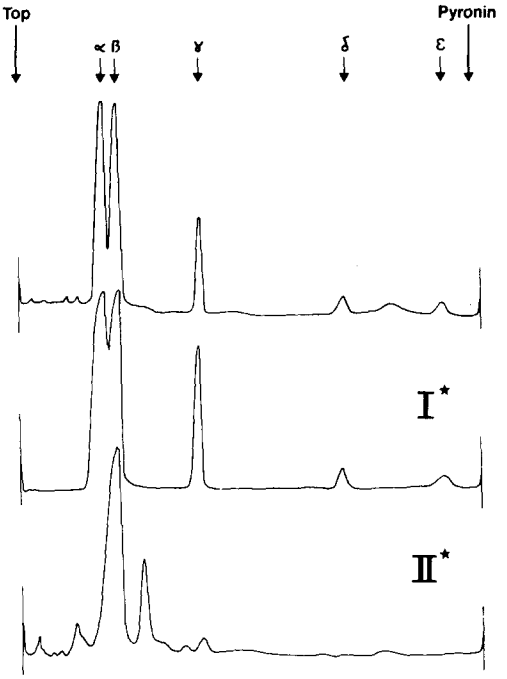

Fig. 1. Densitometric traces obtained after polyacrylamide gel electrophoresis of mitochondrial F_1 extracted by chloroform. Upper trace: before gel filtration, 26 μg protein, middle trace I*: after gel filtration, fraction I* of Fig. 2, 41 μg protein; lower trace II*: after gel filtration, fraction, II* of Fig. 2, 20 μg protein.

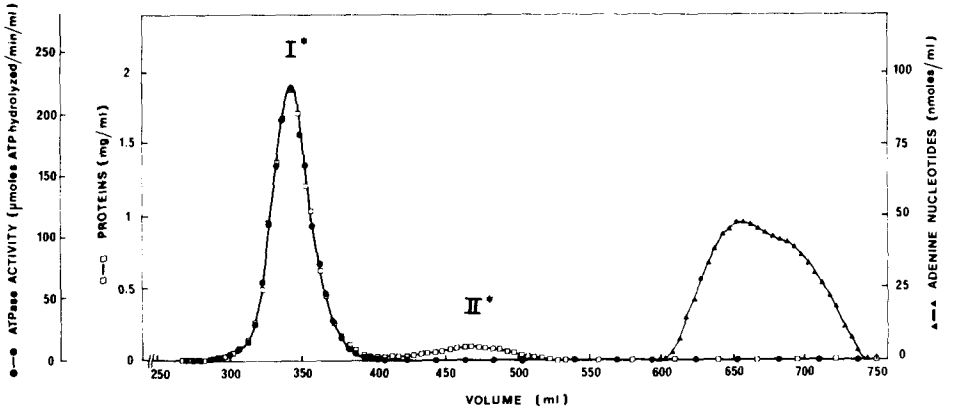

Fig. 2. Gel filtration on Ultrogel ACA 34 of mitochondrial F_1 extracted by chloroform. The column (3.2 X 93 cm) was eluted with 100 mM Tris/sulfate, 5 mM EDTA, 50% glycerol, pH 8.0, at room temperature at a flow rate of 20 ml/h maintained by hydrostatic pressure. Mitochondrial F_1 (90 mg protein) was applied on top of the column at a concentration of 25 mg protein/ml. ATPase activity was measured using a spectrophotometric assay [16]. Proteins were estimated according to Bradford [15]. Adenine nucleotides were determined by the ratio between absorbance at 259 and 280 nm.

by other authors [3,5,6] but they are, however, necessary. The alkaline treatment (step 3, Table I) insures a more complete elimination of the proteic inhibitor [22] since this step increases the total activity; the subsequent sonication (step 4, Table I) removes contaminants that are otherwise extracted during the chloroform treatment and cannot be completely eliminated either by ion-exchange chromatography or by gel filtration. The fractionation by ammonium sulfate precipitation (step 6, Table I) made after the chloroform extraction permits an evasion of the ion-exchange chromatography which increases the content in β subunit (see below). The presence of depolymerized F_1 subunits after such a step has indeed been noticed in several preparations [2,4,23].

β Subunit. To separate extra amounts of the β subunit often present in F_1-ATPase preparation, a gel filtration on a column of Ultrogel ACA 34 can be used in the final step of purification. F_1-ATPase was first extraction by sonication and then purified by ammonium sulfate precipitation and DEAE-Sephadex chromatography according to Di Pietro et al. [24]; finally, the gel filtration was applied. One can see in Fig. 3 that peak II is present in a larger amount than it was when F_1 was extracted with chloroform. The densitometric traces obtained after dodecyl sulfate gel electrophoresis of this fraction indicate that this peak II contains almost exclusively the β subunit (Fig. 4, trace II). The trace of the enzyme obtained just after ion-exchange chromatography on DEAE-Sephadex A-50 shows that the β subunit was apparently present in larger amounts than the α subunit (Fig. 4, upper trace). In contrast after gel filtration on Ultrogel ACA 34, both peaks α and β were equivalent (Fig. 4, trace I).

Other studies have shown that the β subunit precipitates at the same ammonium sulfate concentration as F_1 and is retained by, and eluted from, the

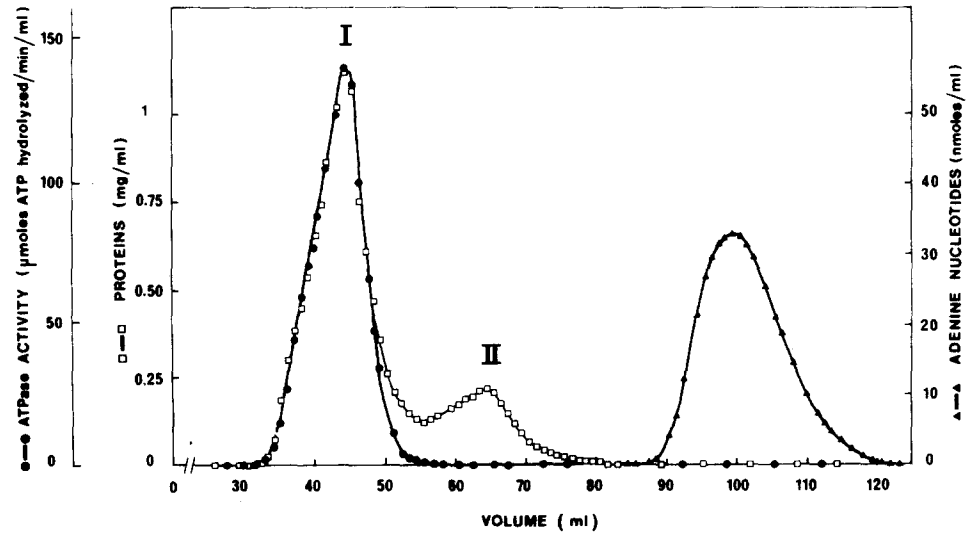

Fig. 3. Gel filtration on Ultrogel ACA 34 of mitochondrial F_1 extracted by sonication. F_1 was prepared according to Di Pietro et al. [24] up to the DEAE-Sephadex chromatography steps. All conditions are the same as in Fig. 2 except that the column was smaller (1.7 × 53 cm), the flow rate slower (6.8 ml/h) and that 20 mg proteins were applied.

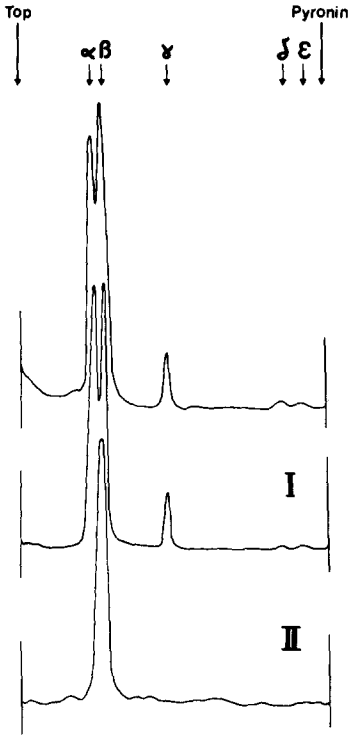

Fig. 4. Densitometric traces obtained after polyacrylamide gel electrophoresis of mitochondrial F_1 extracted by sonication. The enzyme was prepared as previously described [24]. Upper trace: enzyme obtained after DEAE-Sephadex A-50 chromatography, 32 μg protein; middle trace: I after gel filtration on Ultrogel ACA 34 fraction I of Fig. 3, 20 μg protein; lower trace II, fraction II of Fig. 3: 14 μg protein.

DEAE-Sephadex A-50 column exactly in the same conditions as the F_1-ATPase. The other dissociated subunits of F_1 are eluted at lower ionic strength. The similarity between the behaviour of the subunit and of the F_1-ATPase explains the higher content in the β subunit found in some preparations. As the F_1-ATPase can be dissociated into its subunits either by cold denaturation and addition of chaotropic agents [25] or by drastic sonication, ammonium sulfate precipitation and ion-exchange chromatography can be used to prepare the β subunit.

This similarity in the behaviour of the β subunit and the F_1-ATPase seems to indicate tht the β subunit has an external position in the overall complex of the soluble F_1-ATPase.

Properties of chloroform-extracted mitochondrial F_1

The F_1-ATPase prepared by chloroform extraction of pig heart mitochondria and purified through an Ultrogel column has a very high specific activity and its properties are very similar to those of the same enzyme prepared according to Di Pietro et al. [24] by a method similar to that of Senior and Brooks [2]: V = 35 ± 3 μmol P_i/min per mg protein in Tris/maleate buffer; K_m = 0.28 ± 0.02 mM for MgATP. Kinetic studies made at variable MgATP concentrations in the presence of ADP indicate a cooperativity between ATP and ADP sites since the

TABLE II

NUCLEOTIDE CONTENT OF F_1 EXTRACTED WITH CHLOROFORM

Gel filtration is carried out in the presence of 50% glycerol on Ultrogel ACA 34 as described in the text.

	mol/mol of F_1 (380 000 daltons)		
	ATP	ADP	AMP
Before gel filtration	0.33 ± 0.03 (4)	1.8 ± 0.2 (6)	<0.1
After gel filtration	0.27 ± 0.04 (12)	0.17 ± 0.18 (4)	<0.1

Hill coefficient for MgATP was equal to 1 in the absence of ADP and 1.6 in the presence of 1 mM ADP; this result indicates that the regulatory properties of the enzyme are well preserved by this purification procedure [16]. The capacity of reconstitution of ATP-$^{32}P_i$ exchange activity with F_1-depleted submitochondrial particles was identical to that obtained with the F_1-ATPase prepared according to Di Pietro et al. [24].

When kept in 50% glycerol the enzyme was quite stable. At −70°C the activity did not change for at least 7 months. At room temperature there was no change for more than a month and at 0—4°C the activity remained constant for 3 months even if diluted at 0.2 mg protein/ml. After removal of glycerol by ammonium sulfate precipitation, for example, the enzyme became cold labile. After purification of the enzyme by gel filtration in the presence of 50% glycerol the ratio between absorbance at 280 nm and 260 nm ranged from 2.15 to 2.25; this indicates, according to Garrett and Penefsky [26] that the enzyme should be lacking in tightly bound nucleotides. A direct determination of these nucleotides tightly bound to F_1 (Table II) showed that the ATP content (about 0.3 mol/mol of F_1) was not significantly modified during the gel filtration. The enzyme contained about 2 mol of ADP tightly bound/mol of F_1 before gel filtration while ADP was barely detectable after passage through the Ultrogel ACA 34 column in the presence of 50% glycerol. No AMP was present.

Finally, the F_1 prepared by chloroform extraction is insensitive to oligomycin and sensitive to aurovertin [27] exactly as the F_1 purified by other methods.

In conclusion, the technique described here brings several improvements when compared to other techniques. It is a simple one since it can be performed in three days, including the preparation of the mitochondria. It gives a higher yield since we obtain about 100 mg F_1-ATPase starting with 10 g of mitochondria while most other methods give about 100 mg F_1-ATPase starting from 10 g of submitochondrial particles [2,5,23]. The purity is very satisfactory as judged by the specific activity and the gel electrophoresis. Besides its efficiency in the final purification of the enzyme, the gel filtration in the presence of 50% glycerol has many advantages: it increases tremendously the stability of the enzyme and it eliminates almost completely the tightly bound nucleotides and therefore the enzyme thus prepared can be directly used to study the binding of externally added nucleotides. All the tested properties of this preparation: kinetic constants, ADP inhibition, capacity to reconstitute ATP-$^{32}P_i$ exchange in depleted submitochondrial particles, subunit composi-

tion, sensitivity to inhibitors are identical to the properties of the enzyme extracted by sonication [2,24].

Acknowledgements

This work was supported by grants from the Délégation Générale à la Recherche Scientifique et Technique (No. 77-7-0277) and from the Centre National de la Recherche Scientifique (LP 5421). We thank Dr. A. Di Pietro for stimulating discussions during the course of this work. Thanks are also due to Prof. Slater and Dr. Bertina for a generous gift of aurovertin.

References

1 Pullman, M.E., Penefsky, H.S., Datta, A. and Racker, E. (1960) J. Biol. Chem. 235, 3322—3329
2 Senior, A.E. and Brooks, J.C. (1970) Arch. Biochem. Biophys. 140, 257—266
3 Beechey, R.B., Hubbard, S.A., Linnett, P.E., Mitchell, A.D. and Munn, E.A. (1975) Biochem. J. 148, 533—537
4 Barrett, R.H. and Selwyn, M.J. (1976) Biochem. J. 156, 315—322
5 Spitsberg, V.L. and Blair, J.E. (1977) Biochim. Biophys. Acta 460, 136—141.
6 Kopecky, J., Houstek, J. and Drahota, Z. (1977) Mol. Cell. Biochem. 18, 77—80
7 Toson, G., Contessa, A.R. and Bruni, A. (1972) Biochem. Biophys. Res. Commun. 48, 341—347
8 Bruni, A. and Bigon, E. (1974) Biochim. Biophys. Acta 357, 333—343
9 Bruni, A., Pitotti, A., Palatini, P., Dabbeni Scala, F. and Bigon, E. (1979) Biochim. Biophys. Acta 545, 404—414.
10 Hulla, W., Höckel, M., Risi, S. and Dose, K. (1976) Eur. J. Biochem. 67, 469—476
11 Tuena de Gomez-Puyou, M. and Gomez-Puyou, A. (1977) Arch. Biochem. Biophys. 182, 82—86
12 Drahota, Z. and Houstek, J. (1977) Biochim. Biophys. Acta 460, 541—546
13 Gautheron, D.C., Durand, R., Pialoux, N. and Gaudemer, Y. (1964) Bull. Soc. Chim. Biol. 46, 645—660
14 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
15 Bradford, M.M. (1976) Anal. Biochem. 72, 248—251
16 Godinot, C., Di Pietro, A. and Gautheron, D.C. (1975) FEBS Lett. 60, 250—255
17 Godinot, C., Penin, F. and Gautheron, D.C. (1979) Arch. Biochem. Biophys. 192, 225—234
18 Conover, T.E., Prairie, R.L. and Racker, E. (1963) J. Biol. Chem. 238, 2831—2837
19 Racker, E. and Hosrtmann, L.L. (1967) J. Biol. Chem. 242, 2547—2551
20 Blanchy, B., Godinot, C. and Gautheron, D.C. (1978) Biochem. Biophys. Res. Commun. 82, 776—781
21 Jaworek, D., Gruber, W. and Bergmeyer, H.U. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H.U., ed.), 2nd english edn., pp. 2127—2131, Academic Press, New York
22 Pullman, M.E. and Monroy, G.C. (1963) J. Biol. Chem. 238, 3762—3769
23 Knowles, A.F. and Penefsky, H.S. (1972) J. Biol. Chem. 247, 6617—6623
24 Di Pietro, A., Godinot, C., Bouillant, M.L. and Gautheron, D.C. (1973) Biochimie 57, 959—967
25 Penefsky, H.S. and Warner, R.C. (1965) J. Biol. Chem. 240, 4694—4702
26 Garrett, N.E. and Penefsky, H.S. (1975) J. Biol. Chem. 250, 6640—6647
27 Lardy, H. and Lin, C.H.C. (1969) in Inhibitors as Tools in Cell Research (Bücher, Th. and Sies, H., eds.), pp. 279—281, Springer-Verlag, Berlin