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SPONTANEOUS AGGREGATION OF THE MITOCHONDRIAL NATURAL ATPase INHIBITOR IN SALT SOLUTIONS AS DEMONSTRATED BY GEL FILTRATION AND NEUTRON SCATTERING

APPLICATION TO THE CONCOMITANT PURIFICATION OF THE ATPase INHIBITOR AND F_1-ATPase

GÉRARD KLEIN [a], MICHEL SATRE [a], GIUSEPPE ZACCAI [b] and PIERRE V. VIGNAIS [a]

[a] *Laboratoire de Biochimie (INSERM U.191 et CNRS/ERA 903), Département de Recherche Fondamentale, Centre d'Etudes Nucléaires, 85X, 38041 Grenoble Cedex, and* [b] *Institut Laue-Langevin, 156X, 38042 Grenoble Cedex (France)*

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(1) The natural ATPase inhibitor (IF_1) from beef heart mitochondria has a tendency to form aggregates in aqueous solutions. The extent of aggregation and the structure of the aggregates were assessed by gel filtration and small-angle neutron scattering. IF_1 polymerization was found to depend on the salt concentrations, pH of the medium and concentration of IF_1. The higher the salt concentration, the lower the aggregation state. Aggregation of IF_1 was decreased at slightly acidic pH. It increased with the concentration of IF_1 as expected from the law of mass action. (2) Neutron scattering showed the aggregation of IF_1 in 2 M ammonium sulfate solutions. The predominant species is the dimer which has a somewhat elongated shape. (3) The Sephadex G-50 chromatography that is supposed to deprive beef heart submitochondrial particles of loosely bound IF_1 (Racker, E. and Horstman, L.L. (1967) J. Biol. Chem. 242, 2547–2551) was shown to have a limited effectiveness as a trap for IF_1. The reason was that IF_1 released from the particles formed high molecular weight aggregates that were not separated from the membrane vesicles by Sephadex G-50 chromatography. (4) The above observations provide the basis for a simple method of purification of beef heart IF_1 which combines the recovery of the supernatant from submitochondrial particles with the last three steps of the IF_1 preparation described by Horstman and Racker (J. Biol. Chem. (1970) 265, 1336–1344). The particles recovered in the sediment were deprived of IF_1 and could therefore be used for preparation of F_1-ATPase. The advantage of this method is that both IF_1 and F_1-ATPase can be prepared from the same batch of mitochondria.

Introduction

The mitochondrial ATPase inhibitor (IF_1) is a small peptide (M_r 10000) [1], which binds to the β-subunit of the mitochondrial F_1-ATPase [1,2], and thereby controls the catalytic activity of this enzyme [3–5]. It has been suggested that IF_1 has a tendency to aggregate [4,6]. This is consistent with data of cross-linking experiments carried out with chemically radiolabeled IF_1 in which it was shown that [^{14}C]phenylisothiocyanate-modified IF_1, could be cross-linked with 1-ethyl-3-dimethylaminopropylcarbodiimide and *N*-ethoxycarboxyl-2-ethoxy-

Abbreviations: IF_1, beef heart ATPase protein inhibitor; AS particles, submitochondrial particles prepared from beef heart mitochondria by sonication in the presence of ammonium hydroxide at pH 9.0 followed by a Sephadex G-50 treatment; Mops, 4-morpholinepropanesulfonic acid.

1,2-dihydroquinoline to form series of oligomers up to the tetramer [1].

The experiments reported in this paper concern the effect of a number of parameters (salt concentration, pH and concentration of IF_1) on the extent of polymerization of IF_1. For more accurate and convenient evaluation of the polymerization, we used a chemically radiolabeled IF_1. The size and shape of polymerized IF_1 were determined by small-angle neutron scattering. Apart from demonstrating the aggregation of purified IF_1 under different conditions, the present report describes a method which is applicable for preparation of IF_1 and F_1-ATPase from the same batch of mitochondria.

Materials and Methods

Beef heart mitochondria [7], AS particles [8], F_1-ATPase [9] and IF_1 [10] were prepared according to published methods. The following medium (medium A) was used for the preparation of AS particles: 250 mM KCl, 30 mM Tris-sulfate, 2 mM EDTA and 75 mM sucrose, pH 8.0.

[^{14}C]Phenylisothiocyanate (10.2 mCi/mmol) and [^{14}C]methyl-4-azidobenzoimidate (11 mCi/mmol) were obtained from Radiochemical Center, Amersham, and the Commissariat à l'Energie Atomique, Saclay, France, respectively.

IF_1 was chemically labeled with [^{14}C]phenylisothiocyanate or [^{14}C]methyl-4-azidobenzoimidate as described previously [1,2]. In routine preparations, an average of 1 mol of [^{14}C]phenylisothiocyanate or [^{14}C]methyl-4-azidobenzoimidate was incorporated per mol of IF_1, resulting in a specific radioactivity of $23 \cdot 10^9$ and $15 \cdot 10^9$ dpm/mmol, respectively. It may be recalled that beef heart IF_1 contains 12 lysine residues per mol [11,12], and that its partial labeling by [^{14}C]phenylisothiocyanate or [^{14}C]methyl-4-azidobenzoimidate does not alter its biological activity.

F_1-ATPase activity was assayed as described earlier [9]. ATPase inhibitor activity was calculated from the residual ATPase activity of AS particles after IF_1 binding in a medium containing 250 mM sucrose, 10 mM Mops, 1 mM $MgCl_2$ and 0.5 mM ATP pH 6.5 (medium B).

The protein content of submitochondrial particles was measured by the biuret method [13]. Soluble protein was assayed with the Coomassie blue G-250 binding method [14]. Bovine serum albumin was used as a standard.

SDS-polyacrylamide gel electrophoresis and staining of the gels were carried out as described in a preceding paper [2].

Small-angle neutron scattering experiments were performed at the high-flux reactor of the Institut Laue-Langevin, Grenoble, using the instrument D11 [16]. Samples of IF_1 in H_2O buffer containing 2 M ammonium sulfate, pH 6.5, were placed in 1 mm path length quartz cells. The scattering from the IF_1 sample and from the suspending medium alone were measured with a cell-to-detector distance of 1.84 m and a wavelength of $\lambda = 6$ Å ($\Delta\lambda/\lambda \approx 10\%$), giving a range of scattering vector Q up to 0.12 Å^{-1} (where $Q = 4\pi \sin\theta/\lambda$, θ is half the scattering angle). Two concentrations of IF_1 were examined: 2.5 and 7.2 mg/ml, and the radius of gyration and molecular weight were derived in each case, using the Guinier approximation, as described by Jacrot and Zaccaï [17].

Results and Discussion

Effect of the salt concentration, pH and IF_1 concentration on polymerization of IF_1

Routine filtration assays of IF_1 on Sephadex G-100 consistently showed elution patterns demonstrating the existence of aggregates of IF_1 (Fig. 1A). In such assays, IF_1 in the eluate was monitored by determination of the protein content of the fractions and assay of their inhibitory activity with respect to ATPase of AS particles. For convenience, in subsequent work on aggregation of IF_1, we radiolabeled IF_1; in that case, elution was readily monitored by radioactivity measurement.

As reported in a previous paper [1], IF_1 radiolabeled with [^{14}C]phenylisothiocyanate under appropriate conditions has the same biological and physicochemical properties as unlabeled IF_1. The resulting [^{14}C]phenylisothiocyanate-IF_1 molecule is a suitable material to detect polymerization of IF_1, since its chromatographic behavior on a Sephadex column can be followed easily by radioactivity counting. Fig. 1B shows that the elution pattern of [^{14}C]phenylisothiocyanate-IF_1 chromatographed on a Sephadex G-100 column depends on the salt concentration of the equilibration and elution

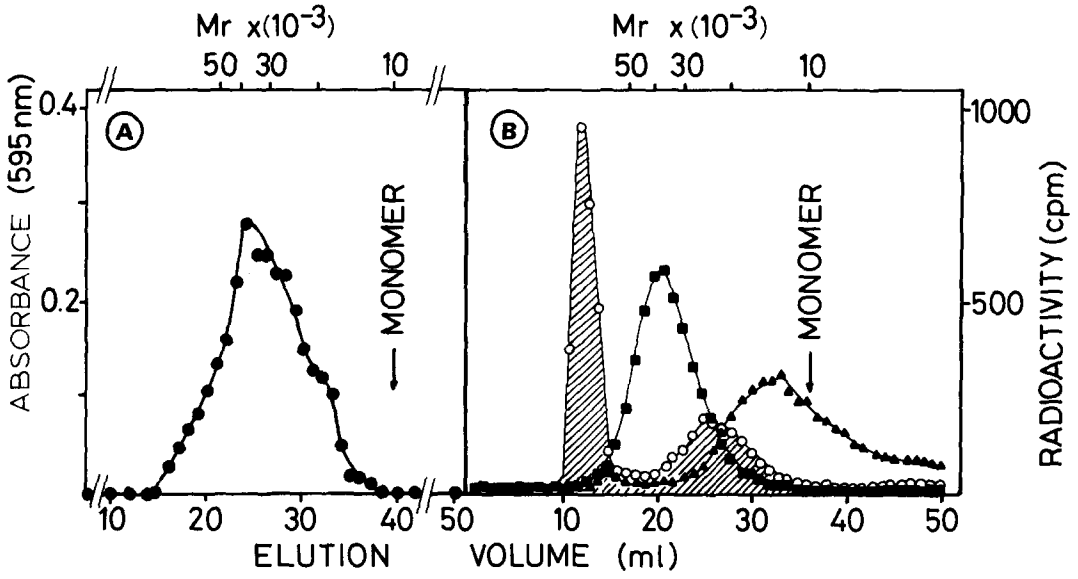


Fig. 1. Elution pattern of unlabeled and ^{14}C-radiolabeled beef heart mitochondria IF_1 on a Sephadex column, suggesting aggregation of IF_1. (A) The Sephadex G-100 column was equilibrated with 2 M ammonium sulfate, pH 8.0. Native IF_1 (0.75 mg/ml) was placed on top of the column and eluted with the equilibration buffer. The column was calibrated with dextran blue, bovine serum albumin, ovalbumin and cytochrome *c* for determination of molecular weight. The protein content was assessed by Coomassie blue and determination of the absorbance at 595 nm. (B) A Sephadex G-100 column was equilibrated with the indicated buffers: (▲——▲) 2 M ammonium sulfate, pH 8.0; (■——■) 250 mM KCl, 75 mM sucrose, 30 mM Tris-H_2SO_4, 2 mM EDTA, pH 8.0; (○——○) 0.1 M ammonium sulfate, pH 8.0. [^{14}C]Phenylisothiocyanate-IF_1 (0.1 mg/ml) was placed on top of the column and eluted with the equilibration buffer. 1 ml fractions were collected and their radioactivities counted.

medium. For convenience, [^{14}C]phenylisothiocyanate-IF_1 was solubilized in the same medium as that used for chromatography. When [^{14}C]phenylisothiocyanate-IF_1 was solubilized in 0.1 M ammonium sulfate, pH 8, it was eluted in the void volume, indicating the formation of large aggregates. On the other hand, when the medium was 2 M ammonium sulfate, pH 8, the radioactivity appeared in a broad band of M_r 20000 with a shoulder at M_r 10000, corresponding to the dimer and monomer forms of IF_1, respectively. Finally, in medium A (250 mM KCl, 75 mM sucrose, 30 mM Tris, 2 mM EDTA pH 8.0, similar to that used for preparation of AS particles), [^{14}C]phenylisothiocyanate-IF_1 was eluted in a broad band whose maximum corresponded to an M_r of 40000, i.e., to a tetrameric form of IF_1. Clearly, the apparent molecular weight of IF_1 depended on the concentration of ammonium sulfate; the higher the salt concentration, the lower the degree of aggregation of IF_1.

The extent of IF_1 polymerization was also assessed by using [^{14}C]methylazidobenzoimidate-IF_1, another radiolabeled derivative of IF_1 which, moreover, is photoactivable. Upon photoirradiation, [^{14}C]methylazidobenzoimidate-IF_1 yielded by cross-linking stable oligomers whose molecular weights could be determined by SDS-polyacrylamide gel electrophoresis. Fig. 2 shows that after photoirradiation for 5 min in a medium composed of 0.25 M sucrose, 1 mM $MgCl_2$, 0.5 mM ATP and 10 mM Mops, final pH 6.5, [^{14}C]methylazidobenzoimidate-IF_1 aggregates to give essentially dimers and trimers. Higher aggregates, if any, were present in amounts too small to be visualized by staining on polyacrylamide gels.

A number of other assays were run to test the effect of the pH and IF_1 concentration on the aggregation process. Polymerization of IF_1 was minimal at pH 6.5–7.0, which corresponded to the optimal pH for the binding of IF_1 to F_1-ATPase. Under the same conditions that gave dimers of IF_1 at pH 6.5 on Sephadex gel, trimers were isolated at pH 9.0. Polymerization of IF_1 also depended on

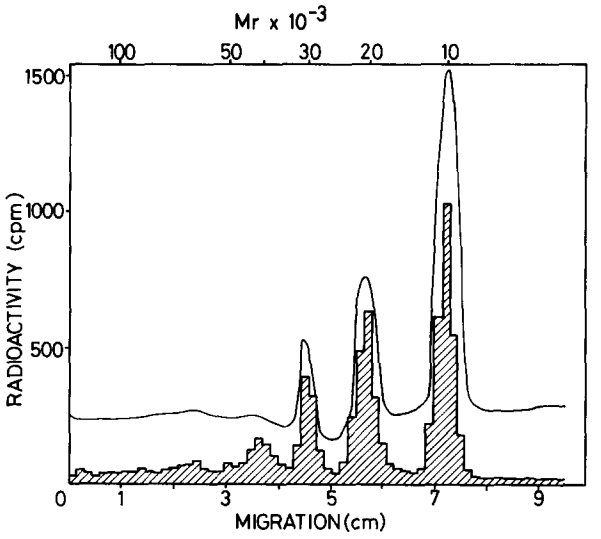


Fig. 2. Photocross-linking of [^{14}C]methylazidobenzoimidate-IF$_1$ as visualized by SDS-polyacrylamide gel electrophoresis. [^{14}C]Methylazidobenzoimidate-IF$_1$ (20 μg) in 0.25 mM sucrose, 1 mM $MgCl_2$, 0.5 mM ATP, 10 mM Mops, pH 6.5, was irradiated for 5 min with a mineral light UVS 11 placed at a distance of 5 cm from the protein sample, and thereafter subjected to SDS-polyacrylamide gel electrophoresis (see Materials and Methods). In the figure, the absorbance of the stained protein bands, and the radioactivity profile (hatched) are superimposed.

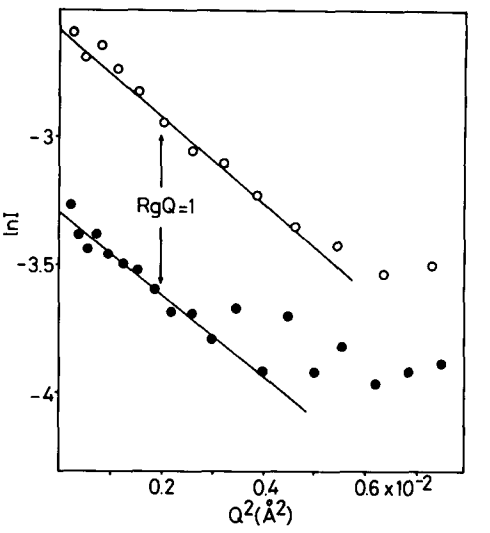


Fig. 3. Guinier plot of the scattering curve of IF$_1$ in H_2O. A straight line was fitted over the range of Q^2 from 0.0003 to 0.005 Å^{-2} to give an R_G value of 22–23 Å and an M_r of about 20000. IF$_1$ was in solution in 2 M ammonium sulfate, pH 6.5, at a concentration of 7.25 mg/ml (O——O) or 2.48 mg/ml (●——●).

the concentration of the enzyme, as could be predicted from the law of mass action. At a concentration of 75 μg/ml in 2 M ammonium sulfate adjusted to pH 6.5, IF$_1$ was eluted as a broad peak corresponding to the apparent molecular weight of a dimer (M_r 20000). At a concentration 10-times higher, besides the dimer, a species with an apparent M_r of 40000, corresponding to a tetramer form, was clearly detected. At a concentration of 1 mg/ml, the tetramer form was predominant.

Solutions of IF$_1$ in 2 M ammonium sulfate were examined by neutron scattering under the conditions described in Materials and Methods. The Guinier plots for the two concentrations used (2.5 and 7.2 mg/ml) are shown in Fig. 3. In a polydisperse solution, the values of the forward scattered intensity and the radius of gyration obtained from the plots are weighted averages for the different types of particles present, with larger particles dominating [18]. When the composition of the solution is known, a molecular weight value can be derived from the forward scattered intensity [17]. The values found were $M_r = 25000 \pm 3000$ and radius of gyration $R_G = 22 \pm 1$ Å at the lower concentration, and $M_r = 19000 \pm 3000$ and $R_G = 23 \pm 1$ Å, at the higher concentration. In this range, therefore, there is no concentration effect. The protein appears to be predominantly in its dimeric form. A globular protein of molecular weight 20000 would have a radius of gyration of 16 Å. The measured value shows the particle to be extended in shape, e.g., an ellipsoid with axial ratios 3 : 1 : 1.

There is an apparent contradiction between the gel filtration and neutron scattering results. Gel filtration of IF$_1$ at the concentration of 1 mg/ml shows a mixture of apparent tetramer and dimer, whereas neutron scattering of IF$_1$ at higher concentrations (2.5 and 7.2 mg/ml) shows essentially a dimer. The molecular weight obtained by neutron scattering is on an absolute scale [17]. This is independent of the hydrodynamic behavior of the protein, in contrast to the gel filtration method. By gel filtration, the apparent molecular weight is determined by calibrating against other particles. These were all globular molecules, which is not the

case for IF_1 as indicated by the high R_G value found in the neutron scattering experiments. An elongated particle is expected to elute at a higher apparent molecular weight [19]. It is a plausible hypothesis, therefore, that the IF_1 aggregates are dimers, the apparent molecular weight found by gel filtration being an overestimate due to the elongated form of the particles.

Yamada et al. [20] have recently reported a monomer-dimer interconversion of beef heart IF_1, depending on the presence of Ca^{2+}. At a critical Ca^{2+} concentration (10^{-6} M), an inactive monomer (M_r 6500) predominates, but, as Ca^{2+} concentration is either increased or decreased, the active dimer (M_r 13000) is formed. In our hands, and with beef heart IF_1 prepared according to the method of Horstman and Racker [10], such a Ca^{2+}-dependent monomer-dimer interconversion could not be demonstrated. IF_1 has an M_r, determined by polyacrylamide gel electrophoresis, of 10000, either in the migration system of Weber and Osborn [21] or in the system of Swank and Munkres [22], with and without Ca^{2+}. Further, the amino acid composition [11,12] shows that the minimal molecular weight of IF_1 is close to 10000. Finally, there is no evidence, based on amino acid sequence [23], that the determined M_r of 10000 corresponds to a duplication of an elementary unit of M_r 5000 in beef heart IF_1.

Critical examination of conditions of depletion of submitochondrial particles in bound IF_1. Fate of IF_1 during the preparation of AS particles

The A submitochondrial particles, as prepared in the method of Racker and Horstman [8], still contain bound IF_1. To remove IF_1 from the A particles, it was proposed to pass these particles through a Sephadex G-50 column equilibrated with 2 mM EDTA, 75 mM sucrose, 250 mM KCl and 30 mM Tris-sulfate, pH 8 (medium A) [8]. The IF_1-depleted particles are called AS particles. The conclusion that IF_1 had been removed during Sephadex chromatography was based on the increased ATPase activity of AS particles. However, as previously shown in Fig. 1, IF_1 in solution in the same medium as that used for the above Sephadex chromatography aggregates mostly in a tetrameric form; this finding casts some doubt on the ef-

TABLE I

REACTIVATION OF THE ATPase ACTIVITY OF AS PARTICLES INHIBITED BY [^{14}C]PHENYLISOTHIOCYANATE-IF_1

AS particles (2.28 mg protein) (step 1) were incubated for 15 min at 25°C with 11 μg of [^{14}C]phenylisothiocyanate-IF_1 in 550 μl medium B (cf. Materials and Methods) (step 2). The particles were sedimented by centrifugation for 15 min at 25000 rpm in a Sorvall centrifuge SS1. The sediment was resuspended in medium A (cf., Materials and Methods), and after 1 h at 25°C, the suspension was filtered on a column of Sephadex G-50 equilibrated in medium A. The fraction that corresponded to the void volume contained the major part of the ATPase activity and the ^{14}C radioactivity (step 3). It was centrifuged for 20 min at 25000 rpm in a Sorvall centrifuge SS1. The sediment, resuspended in 250 mM sucrose and 10 mM Mops, pH 6.5, was assayed for ATPase and radioactivity (step 4).

Step	Fractions	ATPase specific activity (μmol P_i formed/min per mg)	Inhibition of ATPase activity (%)	Amount of [^{14}C]phenylisothiocyanate-IF_1 (pmol/mg)
1	AS particles	4.25	0	-
2	AS particles with bound [^{14}C]phenylisothiocyanate-IF_1 (medium B)	0.55	87	272
3.	Particles from step 2, resuspended in medium A and filtered on Sephadex G-50	3.80	10	200
4.	Particles from step 3, sedimented by centrifugation	3.80	10	16

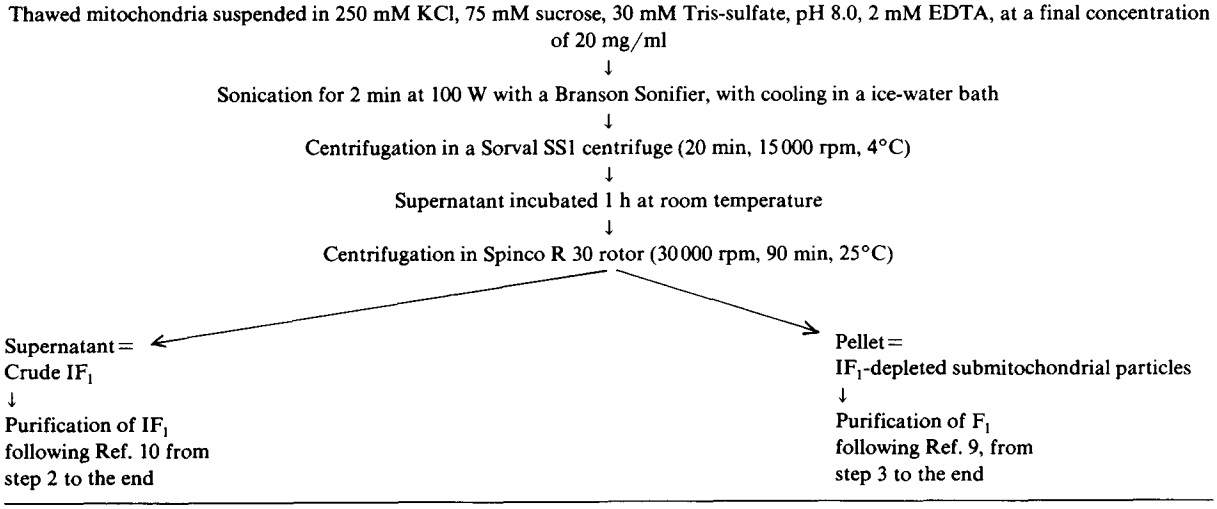
ficiency of the Sephadex G-50 chromatography to separate IF_1 from AS particles. To clarify this point, the following experiment was devised. AS particles were preincubated with [^{14}C]phenylisothiocyanate-IF_1 in medium B, pH 6.5 (see Materials and Methods) to facilitate the interaction between [^{14}C]phenylisothiocyanate-IF_1 and particles. The binding efficiency was assessed by the extent of inhibition of the ATPase activity in particles which reached 87% in the present experiment (table I, step 2). The particles with their bound [^{14}C]phenylisothiocyanate-IF_1 were recovered by centrifugation, resuspended in medium A, and then filtered on a column of Sephadex G-50 equilibrated with medium A (pH 8.0) (Table I, step 3). The void volume contained the AS particles and the ^{14}C radioactivity; at this stage, the AS particles had recovered 90% of their original ATPase activity, probably due to the release of the bound [^{14}C]phenylisothiocyanate-IF_1 into the medium. Following centrifugation for 15 min of the void volume of the chromatography step, the AS particles were recovered in the pellet, and separated from the [^{14}C]phenylisothiocyanate-IF_1 which remained in the supernatant (Table I, step 4). These data make it clear that Sephadex G-50 chromatography is not necessary to remove IF_1 from the A particles. In fact when the AS particles with their bound [^{14}C]phenylisothiocyanate-IF_1 (Table I, step 2) were resuspended and incubated for 1 h in medium A, and then directly collected by centrifugation, avoiding the Sephadex step, their ATPase activity had considerably increased, and the bound ^{14}C radioactivity had dropped to a negligible level. Van de Stadt and Van Dam [24] reported that a high ionic strength activated the ATPase activity of MgATP particles; however, there was no direct indication of a release of IF_1. The ^{14}C-radiolabeled IF_1 used here shows a correlation between the increased ATPase activity of the particles and the release of IF_1.

An improved procedure for the preparation of IF_1, based on the spontaneous aggregation of IF_1

For routine preparation of AS particles, the A particles obtained as described by Racker and Horstman [8] are passed through Sephadex G-50; the pass-through fraction is centrifuged at 100000 $\times g$ for 90 min to collect the AS particles [8]. As demonstrated above, IF_1 was present together with the AS particles in the pass-through fraction; however, after centrifugation, IF_1 remained in the supernatant. When trichloroacetic acid was added to the supernatant at a final concentration of 5%, a precipitate was formed, which consisted of virtually pure IF_1. The only contaminating protein (less

TABLE II

PURIFICATION SCHEME FOR CONCOMITANT PURIFICATION OF F_1 AND IF_1



than 5%) had an M_r of 60000, and was probably serum albumin. These findings were used to set up a quick method of preparation of IF_1 which is summarized briefly here.

Beef heart mitochondria were sonicated in medium A for 2 min (see Table II). The suspension was first centrifuged for 20 min at 15000 rpm in a Sorvall SS1 apparatus to remove unbroken mitochondria. The supernatant containing the submitochondrial particles was left for 1 h at 25°C, and was then centrifuged at 30000 rpm in a Spinco rotor 30 for 90 min at 25°C. The supernatant contained the released IF_1 which could be further purified by the procedure of Horstman and Racker [10], starting from step 2 of this procedure. The pellet consisted of submitochondrial particles depleted of IF_1, similar to AS particles. These particles could be used as such, or for further purification of F_1-ATPase by the procedure of Knowles and Penefsky [9], starting from step 3 of this procedure. As an example, starting from 8.3 g of beef heart mitochondrial protein, it was possible to prepare 60 mg of pure ATPase with a specific activity of 103 μmol ATP hydrolyzed/min per mg and 4.8 mg of pure IF_1. It is clear that an obvious advantage of the new procedure is that the same batch of beef heart mitochondria can be used to prepare IF_1 and F_1-ATPase.

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