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# Isolation and Characterization of Coupling Factor $F_B$ from Bovine Heart Mitochondria†

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ABSTRACT: An unusually efficient B-type coupling factor with essential sulfhydryl groups has been isolated from bovine heart mitochondria and characterized. This protein, denoted by $F_B$, consists mainly of molecules with approximate molecular weight of 330,000. $F_B$ catalyzes ATP-driven reduction of $NAD^+$ by succinate in the presence of A-particles (submitochondrial particles prepared at pH 9.2 in the presence of ammonia and EDTA) with a specific activity of over 1000 μmol of NADH per min per mg of $F_B$ per 0.5 mg of A-particles at 38° and pH 7.8. After incubation with sodium dodecyl sulfate and 2-mercaptoethanol, $F_B$ showed a single subunit protein band of approximate molecular weight 44,000 in disc gel electrophoresis. These data suggest that the principal molecular species of $F_B$ is made up of eight subunits of equal molecular weight. The kinetic data and amino acid composition of $F_B$ are presented.

At least two mitochondrial coupling factors with essential sulfhydryl groups have been reported in the literature. Factor B of molecular weight 29,200 was first isolated by Lam, Sanadi, and their coworkers (1967, 1969). It catalyzes ATP-driven reduction of $NAD^+$ by succinate in the presence of A-particles with a specific activity of 2–6 μmol of NADH per min per mg of coupling factor per 0.5 mg of A-particles at pH 7.8 and 38°. Racker and coworkers (1970) concluded from their enzymatic and immunological measurements that factor B is probably identical with a coupling factor $F_2$ prepared from bovine heart mitochondria (BHM)[1] by a different procedure (Fessenden et al., 1967). Lam and coworkers (1970) also found another sulfhydryl coupling factor from BHM, factor B′ of approximate molecular weight 45,000, which had a specific activity 20-fold lower than that of factor B and hence was regarded as less pure. The study of these coupling factors is of basic biochemical importance, since they seem to serve as the functional link (Sanadi et al., 1968) between the electron

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[1] Abbreviations used are: BHM, bovine heart mitochondria; HBHM, heavy bovine heart mitochondria; PMB, *p*-mercuribenzoate.

carriers and the terminal enzyme $F_1$ of oxidative phosphorylation.

We wish to report here the isolation of another B-type coupling factor from BHM which for convenience will be denoted by $F_B$. It consists principally of molecular species with approximate molecular weight of 330,000 and catalyzes ATP-driven reduction of $NAD^+$ by succinate in the presence of A-particles with specific activity in the range from 1000 to 2000 μmol of NADH per min per mg of $F_B$ per 0.5 mg of A-particles at pH 7.8 and 38°.

## Materials and Methods

*Materials.* $NAD^+$, $Na_2ATP$, and dithiothreitol were from Sigma Chemical Co. All other chemicals were of reagent grade. Bovine heart mitochondria (BHM) were prepared essentially as described by Löw and Vallin (1963). The fresh BHM preparation, containing 50–60 mg of protein/ml of 0.25 *M* sucrose + 0.01 *M* Tris-chloride buffer at pH 7.4, was mixed with five times its volume of glass-distilled water which had been prechilled at 0°. Ten times its volume of acetone at −30° was subsequently added slowly to the stirred mixture and the precipitate was filtered in the cold through a Büchner funnel. The precipitate collected on filter paper was washed successively with acetone and diethyl ether at −30°, dried in a desiccator, and stored at −70°.

*Coupling Factor $F_B$.* To isolate $F_B$, all preparative operations were carried at 0–4°. For convenience, certain pH measurements were made at room temperature. Inasmuch as the pH of certain Tris buffers are known to vary significantly with temperature, the temperature at which each pH value was determined is given in parentheses in the following procedure. Twenty grams of the above acetone powder was stirred with 200 ml of 100 m*M* Tris-sulfate buffer + 0.2 m*M* dithiothreitol at pH (22°) 8.8 for 10 min. The mixture was then homogenized in a 200-ml Elvehjem-Potter homogenizer by four or five passes of the Teflon plunger. After the mixture was stirred for 10 min longer, its pH (2°) was adjusted from 9.2 to 8.0 with 0.5 *N* $H_2SO_4$ and the mixture was centrifuged for 45 min at 100,000*g*. The supernatant containing $F_B$ was siphoned off. The sediment was resuspended in the 100 m*M* Tris-sulfate buffer + 0.2 m*M* dithiothreitol at pH (22°) 8.8, homogenized, extracted, and centrifuged as before. The two batches of supernatant were combined, made 30% saturation in $(NH_4)_2SO_4$ (0.175 g/ml), adjusted to pH (2°) 8.0, and stirred for 10 min. The precipitate was centrifuged down and discarded. The supernatant was made about 60% saturation in $(NH_4)_2SO_4$ (additional 0.162 mg/ml), stirred for 60 min, and centrifuged. The cream-colored precipitate (fraction I) was dissolved in 8–10 ml of 10 m*M* Tris-sulfate buffer + 0.2 m*M* dithiothreitol at pH (22°) 8.0. The resulting yellow solution was passed through a 2.5 × 45 cm Bio-Gel P-2 column which had been preequilibrated with 10 m*M* Tris-sulfate + 0.2 m*M* dithiothreitol at pH (22°) 8.0 and eluted with the same buffer. The eluted protein fraction was applied to a 2.5 × 20 cm Whatman DE 52 (microgranular form) column which had been preequilibrated with 10 m*M* Tris-sulfate + 0.2 m*M* dithiothreitol at pH (22°) 8.0. The loaded DE 52 column was first eluted with 50 m*M* Tris-sulfate + 0.2 m*M* dithiothreitol at pH (22°) 8.0 (fraction II), followed by elution with 100 m*M* Tris-sulfate + 0.2 m*M* dithiothreitol at pH (22°) 8.0 (fraction III), and finally with 300 m*M* Tris-sulfate + 0.2 m*M* dithiothreitol at pH (22°) 8.0 (fraction IV). Fraction II was found to be quite rich in $F_B$ activity. The protein in fraction II was precipitated by making the mixture about 60% saturation in $(NH_4)_2SO_4$ (0.39 g/ml). The precipitate was redissolved in 50 m*M* Tris-sulfate + 0.2 m*M* dithiothreitol at pH (22°) 8.0 and applied to a 2.5 × 20 cm Bio-Gel P-2 column which had been preequilibrated with a 10 m*M* Tris-sulfate buffer at pH (22°) 8.0 but without dithiothreitol. The loaded column was eluted with the same dithiothreitol-free buffer. The dithiothreitol-free eluate was left standing in air at 0° for 2 hr, whereupon white protein precipitate appeared. After centrifugation for 15 min at 25,000*g*, the white precipitate was gently rinsed with the eluting buffer and then redissolved in 100 m*M* Tris-sulfate + 0.2 m*M* dithiothreitol at pH (22°) 8.0 (fraction IIA). Fraction IIA was again desalted with 2.5 × 10 cm P-2 column which had been preequilibrated with 2.5 m*M* Tris-sulfate dithiothreitol-free buffer of pH (22°) 8.0 and eluted with the same buffer, again left standing exposed to air for 2 hr at 0° and centrifuged. The white precipitate was washed with the same buffer and then redissolved in 100 m*M* Tris-sulfate + 0.2 m*M* dithiothreitol at pH (22°) 8.0 (fraction IIB). Fraction IIB contains the pure coupling factor $F_B$. It can be stored at −70° for 1 week without appreciable loss of biochemical activity. The yield is 5–10 mg.

*A-particles.* Heavy bovine heart mitochondria (HBHM) with 200–300 mg of protein and prepared as previously described (Löw and Vallin, 1963; Chen et al., 1975) were suspended at 2° in 20 ml of 2 m*M* Tris-HCl buffer containing 0.05 *M* sucrose and 0.6 m*M* EDTA at pH (22°) 7.8. The mixture was adjusted to pH (2°) 9.2 with 0.5 *M* $NH_4OH$ and sonicated by means of a Branson W-350 sonifier for two 2.5-min periods, separated by a 1–2-min cooling period, with an average power output of 80 W and 15% duty cycle pulses. During the sonication the temperature of the ice-chilled, magnetically stirred sample rose from 2 to 6°. After the debris was separated by centrifugation at 18,000*g* for 10 min, the supernatant was centrifuged at 100,000*g* for 45 min. The resulting pellet was resuspended in 10 ml of 0.25 *M* sucrose + 10 m*M* Tris-sulfate at pH (22°) 7.5. The suspension was again centrifuged at 100,000*g* for 45 min and the final pellet was resuspended in a smaller volume of the same buffer to give a final suspension containing about 10 mg of protein/ml. Yield of A-particles, about 20%; specific activity, 10–20 nmol of NADH per min per 0.5 mg of protein.

*Assay of $F_B$ Activity.* For each assay a 50-μl sample of A-particles containing 0.5 mg of protein was incubated with 2.5–10 μl of pH (22°) 8.0 $F_B$ solution for 1 min at 38° and then mixed with 3 ml of assay mixture at 38° of the following composition: Tris-sulfate, 50 m*M*, pH (22°) 7.8; $MgCl_2$, 3.3 m*M*; succinate, 6.7 m*M*; bovine serum albumin, 2 mg/ml; ATP, 2 m*M*; $NAD^+$, 1 m*M*; KCN (added last), 3.3 m*M*. The rate of ATP-driven reduction of $NAD^+$ by succinate was measured by monitoring the linear increase in absorbance at 340 nm with time by means of a Gilford spectrophotometer. The specific activity of $F_B$ (expressed in μmol of NADH per min per mg of $F_B$ per 0.5 mg of A-particles at pH 7.8 and 38°) is calculated from the observed enhancement of the rate of NADH formation due to the added $F_B$. Protein concentrations were determined by the biuret method.

*Molecular Weight.* Molecular weight determinations were performed by gel filtration using Sephadex G-200 purchased from Pharmacia Fine Chemicals. The gel column used had a 2.2 cm × 70 cm bed volume and was operated at

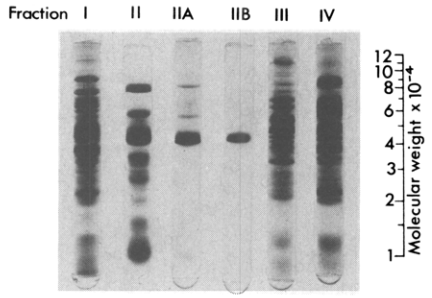


FIGURE 1: Sodium dodecyl sulfate gel electrophoresis of $F_B$-containing protein fraction from bovine heart mitochondria at various stages of purification. I, II, IIA, IIB, III, and IV refer to the corresponding protein fractions described in the Materials and Methods section. The logarithmic molecular weight scale on the right was determined by the best linear semilogarithmic plot determined by the dansylated standard proteins. Fraction IIA was obtained from fraction II by oxidative precipitation. Fraction IIB, which contains pure $F_B$, was obtained from fraction IIA by oxidative precipitation again.

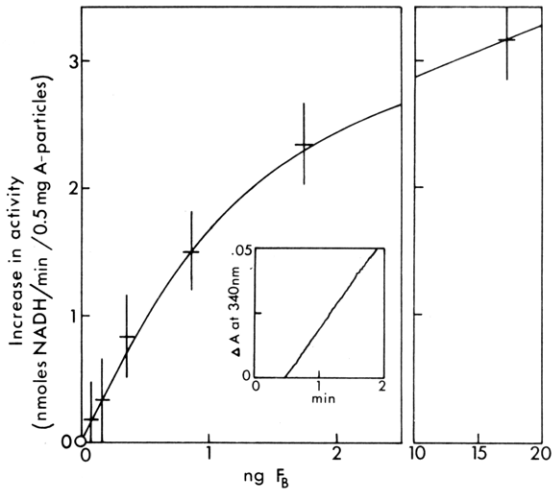


FIGURE 2: Enhancement of the activity of A-particles by $F_B$. According to this curve, the specific activity of $F_B$ is between 1000 and 2000 $\mu$mol of NADH per min per 0.5 mg of A-particles at pH 7.8 and 38°. The activity of A-particles without added $F_B$ was 16.8 nmol of NADH per min per 0.5 mg of A-particle protein. The increase in the activity of A-particles with more than 35 ng of added $F_B$ remains constant at 3.7 nmol of NADH per min per 0.5 mg of A-particles, corresponding to a stimulation ratio of 1.22. A typical measurement is given in the insert which shows the linear increase of absorbance at 340 nm. In each series of such measurements, $F_B$ was diluted with the same buffer containing dithiothreitol and added to the assay mixture containing bovine serum albumin.

5° with a flow rate of about 12 ml/hr. Marker proteins of the following molecular weights were supplied by Sigma Chemical Co.: thyroglobulin (Type I), 669,000; pyruvate kinase (Type II), 237,000; aldolase (Grade I, from rabbit muscle), 155,800; bovine serum albumin, 67,000; ovalbumin, 45,000; myoglobin (Type II, from sperm whale), 17,800. The value 155,800 used for aldolase is that calculated from its recently published amino acid sequence (Lai, 1975). In both calibration of the column and studies on $F_B$, the column buffer was 0.05 *M* Tris-HCl (pH 8.0) containing 1 m*M* dithiothreitol. Protein elution was either monitored at 280 nm using a Gilson M-UV-RP monitor or, at low protein concentrations, by collecting fractions and measuring intrinsic protein fluorescence with a Hitachi MPF-2A spectrofluorometer.

As a further check on column determinations in the $F_B$ molecular weight range, measurements were made on bovine liver glutamate dehydrogenase (GDH). For determinations on GDH, the column buffer was 0.05 *M* phosphate (pH 7.8) containing 0.1 m*M* GTP and 0.1 m*M* NADH. In this medium, GDH exists as a hexamer of apparent molecular weight 310,000 as measured by light scattering (Eisenberg and Tomkins, 1968). Chromatography on our G-200 column, based on the elution behavior of marker proteins in 0.05 *M* phosphate buffer at pH 7.8, also yielded a molecular weight of 310,000.

Sodium dodecyl sulfate gel electrophoresis and amino acid analysis of the purified $F_B$ were carried out as described by Chen et al. (1975) in a separate report.

## Results and Discussion

*Purity and Activity of $F_B$.* The sodium dodecyl sulfate gel electrophoresis patterns of the protein fractions containing $F_B$ at different stages of purification are shown in Figure 1. The total protein as well as the specific activity of each fraction in a typical preparation is listed in Table I. Apparently oxidative precipitation, i.e., precipitation due to the formation of intermolecular disulfide linkages upon exposure to air is the most effective purification step. Fraction IIB, obtained from fraction IIA by a second oxidative precipitation, is electrophoretically homogeneous and considered to be pure $F_B$. The subunit molecular weight of $F_B$ determined from the linear semilogarithmic plot calibrated with dansylated standard proteins is 44,000 ± 1000.

The exact specific activity of $F_B$ depends on the quality of A-particles used in the assay. With freshly prepared A-particles, the specific activity of $F_B$ is usually over 1000 $\mu$mol of NADH per min per mg of $F_B$ per 0.5 mg of A-particles at 38° and pH 7.8. $F_B$ can be stored in frozen solution at −70° for a week without significant loss of activity. Figure 2 shows the effect of preincubation with different concentration of $F_B$ on the ATP-driven reduction of $NAD^+$ by succinate catalyzed by A-particles. According to this curve, the specific activity of this particular batch of $F_B$ is between 1000 and 2000 $\mu$mol of NADH per min per mg of $F_B$ per 0.5 mg of A-particles.

In spite of the high specific activity of $F_B$, Figure 2 shows that even at 20 ng of $F_B$ per 0.5 mg of A-particles the rate of ATP-driven reduction of $NAD^+$ by succinate is enhanced only 20%. It seems likely that other soluble factors, which had been leached out during the sonication step, are also needed for full restoration of the coupling of phospho-

Table I: Activity of Protein Fractions Containing $F_B$ at Different Stages of Purification.[a]

| | Protein fraction[b] | | | | | |
|---|---|---|---|---|---|---|
| | I | II | III | IV | IIA | IIB |
| Mg of protein | 550 | 135 | 65 | 149 | 29.2 | 21.9 |
| Specific activity[c] | 10.4 | 15.8 | 2.51 | 2.10 | 406 | 1090 |

[a] 20 g of acetone powder containing 12.2 g of protein was extracted twice at 2° with 100 m*M* Tris-sulfate containing 0.2 m*M* dithiothreitol at pH (22°) 8.8. [b] The isolation of different protein fractions is described in the Materials and Methods section. [c] The specific activities are expressed in $\mu$moles of NADH formed per min per mg of $F_B$ per 0.5 mg of A particles at 38° and pH 7.8. The crude extract of acetone powder made from fresh BHM is from 2 to 4.5 $\mu$mol of NADH per min per mg of protein per 0.5 mg of A particles. This value is considerably higher than the extract of acetone powder made from lyophilized BHM reported by Lam et al. (1967).

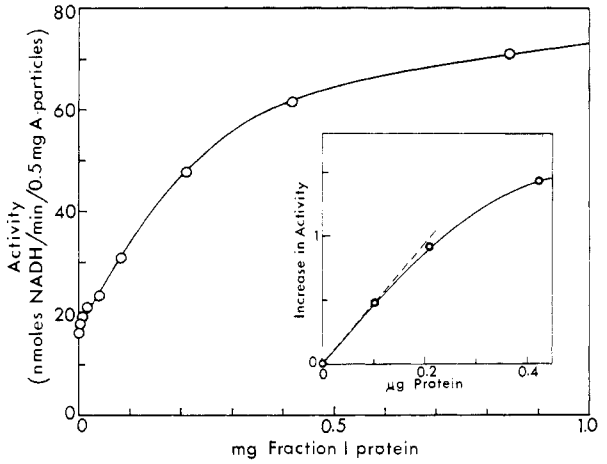


FIGURE 3: Effect of fraction I protein on the activity of A-particles. The activity of A-particles without added $F_B$ was 16.4 nmol of NADH per min per 0.5 mg of A-particle protein.

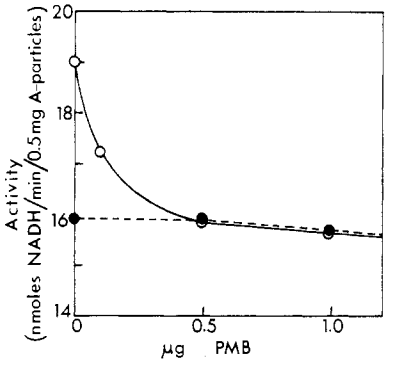


FIGURE 4: Effect of *p*-mercuribenzoate (PMB) on the activities of $F_B$ and A-particles. For measurements with $F_B$ (○), 5, 10, or 20 μl of PMB solution and 1.75 μg of $F_B$ in 5 μl of solution were mixed and kept at 0° for 1 min. The solution was then mixed with 50 μl of A-particles, incubated for 1 min at 38°, subsequently mixed with 3 ml of the assay mixture, and measured as described in the Materials and Methods section. For measurements without $F_B$ (●), each PMB solution was kept by itself at 0° for 1 min, then mixed with 50 μl of A-particles, incubated for 1 min at 38° without added $F_B$, and subsequently assayed in the same way.

rylation to oxidation. Figure 3 shows an activity vs. concentration curve obtained similarly by using fraction I, which contains many other proteins, instead of fraction IIB. The limiting slope gives the specific activity of fraction I as 4.5 μmol of NADH per min per mg of fraction I protein per 0.5 mg of A-particles, but at high protein concentration the rate is enhanced by a factor of 4.4.

Normally in the isolation of enzymes for simple substrates, the total enzymatic activity of the preparation decreases as the purification progresses because of unavoidable loss. However, by multiplying each specific activity in Table I by the number of milligrams of protein in that fraction, we find that fraction IIB (or $F_B$) contains ten times as much total activity as fraction II from which it was prepared. This unusual observation is probably due to the complexation of $F_B$ with another soluble component in fraction II which competes with the binding sites of A-particles for $F_B$. Since each binding site presumably already has this binding component, it is not expected to bind the $F_B$ which has already been complexed. Consequently, some of the $F_B$ activity in fraction II may be undetectable by the present assay method.

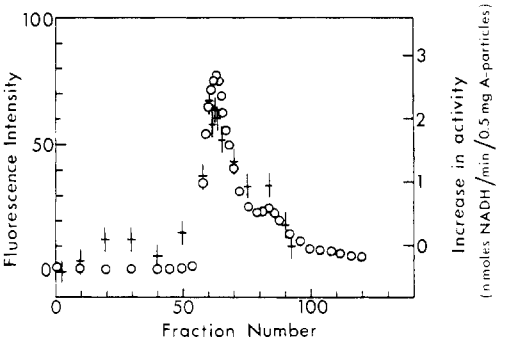


FIGURE 5: Gel filtration chromatography of $F_B$ (fraction IIB) on Sephadex G-200 performed in 50 m*M* Tris-sulfate + 1 m*M* dithiothreitol (pH 8.0). Circles represent the protein elution profile, as detected by intrinsic protein fluorescence (excitation wavelength, 292 nm; emission wavelength, 350 nm). Crosses represent $F_B$ activity profile, assayed by ATP-driven reduction of $NAD^+$ by succinate in the presence of A-particles.

*Effect of Sulfhydryl Reagents.* The effect of *p*-mercuribenzoate (PMB) on the activity of $F_B$ is summarized in Figure 4. The data show that PMB is a much more effective inhibitor for $F_B$ than for A-particles. Since 0.09 μg of PMB in 3 ml of assay mixture inhibited 50% of the activity of $F_B$, the approximate concentration of PMB for 50% inhibition is $(0.09 \times 10^{-6}/357.2)(1000/3) \approx 10^{-7}$ *M*, the expected order of magnitude for the binding constant of PMB by protein sulfhydryl groups. More incisive labeling experiments with 2′-bromo-2,4-dinitroacetanilide (Chen et al., 1975) show that $F_B$ indeed has essential cysteinyl sulfhydryl groups.

*Molecular Weight.* The molecular weight of $F_B$ was estimated by gel filtration with a calibrated Sephadex G-200 column. According to the results of eight measurements similar to the one illustrated in Figure 5, the molecular weight of the principal molecular species of isolated $F_B$ at 5° is 330,000 ± 25,000. There is also a minor component of varying amount of molecular weight near 90,000. Since both components are biologically active and since dodecyl sulfate gel electrophoresis of fraction IIB gives only one subunit protein band of approximate molecular weight 44,000, we conclude that the major and minor components are different oligomeric forms of $F_B$ made of the same protein subunits: a major molecular species made of eight subunits of equal molecular weight and a minor molecular species made of two subunits of the same molecular weight. Whether the octamer and the dimer were present as such in the intact mitochondrial inner membrane or were formed after isolation cannot be deduced from the present experimental results. Indeed the molecular weight of isolated $F_B$ was found to vary with temperature and composition of the medium. For example, at 23° and in 50 m*M* Tris-sulfate, 1 m*M* ATP, 1 m*M* dithiothreitol, and 2 m*M* EDTA at pH 8.0, gel filtration with a calibrated Sepharose 6-B column gave essentially a single protein band of approximate molecular weight 240,000.

*Amino Acid Composition.* The amino acid composition of $F_B$ is summarized in Table II which also includes the amino acid composition of factor B determined by Lam et al. (1969). As expected, the amino acid composition of $F_B$ is different from that of factor B as well as that of any subunits of the coupling factor $F_1$ (Knowles and Penefsky, 1972).

In view of the important role of the B-type coupling fac-

Table II: Amino Acid Composition of the Coupling Factor $F_B$.

| Amino Acid | $F_B$ Mole % | $F_B$ No. of Residues per Subunit of Mol wt 44,000 | Factor B[a] Mole % | Factor B[a] No. of Residues per Molecule of Mol wt 29,200 |
|---|---|---|---|---|
| Cysteine (total)[b] | 1.58 | 6.0 | 1.55[c] | 3.99 |
| Aspartic acid | 11.0 | 41.7 | 9.80 | 25.4 |
| Threonine | 4.55 | 17.3 | 4.90 | 12.7 |
| Serine | 4.82 | 18.3 | 5.98 | 15.2 |
| Glutamic acid | 10.4 | 39.6 | 10.75 | 27.9 |
| Proline | 6.03 | 22.9 | 5.18 | 13.4 |
| Glycine | 7.69 | 29.2 | 9.20 | 23.8 |
| Alanine | 5.66 | 21.5 | 8.60 | 24.3 |
| Valine | 6.63 | 25.2 | 6.57 | 17.0 |
| Methionine | 1.84 | 7 | 1.83 | 4.74 |
| Isoleucine | 5.37 | 20.4 | 5.33 | 13.8 |
| Leucine | 8.71 | 33.1 | 9.58 | 24.8 |
| Tyrosine | 3.08 | 11.7 | 2.61 | 6.74 |
| Phenylalanine | 3.50 | 13.3 | 3.46 | 8.94 |
| Tryptophan | 1.45 | 5.5 | 0.74 | 1.90 |
| Lysine | 7.00 | 26.6 | 6.72 | 17.4 |
| Histidine | 2.40 | 9.1 | 2.53 | 6.55 |
| Arginine | 8.29 | 31.5 | 3.98 | 10.3 |

[a] Values quoted from the work of Lam et al. (1969). [b] Determined as carboxymethylcysteine after incubating $F_B$ with iodoacetate and mercaptoethanol in the presence of 7.5 *M* urea at 40° (Chen et al., 1975). [c] Determined as cysteic acid.

tors in oxidative phosphorylation, it would be of interest to know whether $F_B$ and factor B represent independent endogenous molecular species or whether the biologically less active factor B of molecular weight 29,000 represents a fragment of $F_B$ produced by the isolation process. An inspection of the numbers of amino acid residues in Table II shows that except for alanine each subunit of $F_B$ contains a larger number of residues of each of the other amino acids than factor B. Since the difference in the numbers of alanine residues in factor B and $F_B$ is not much larger than the experimental uncertainties, it would be risky to rule out the latter possibility. Further considerations on this question should be postponed until both proteins are sequenced.

## Acknowledgment

We are indebted to Dr. D. R. Sanadi for whose expert advice on the preparation of their factor B and A-particles, to Mr. D. R. Saunders for amino acid analysis, to Mrs. I. T. Blazy and B. Stone for preparation of mitochondria, and to Mrs. K. A. Connolly for her valuable assistance.

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