Electrophoretic Microheterogeneity and Subunit Composition of the 13S Coupling Factors of Oxidative and Photosynthetic Phosphorylation†

Robert Adolfsen,‡ John A. McClung, and Evangelos N. Moudrianakis*

ABSTRACT: Two electrophoretically distinguishable species of the 13S coupling factor of oxidative phosphorylation from *Alcaligenes faecalis* are detectable by standard polyacrylamide gel electrophoresis in the absence of urea, detergents, or any other protein-denaturing reagents. The slower species (type IA) can be converted into the faster species (type IB) by treatment with ATP, and the fast form converts into the slow form when aged at 4°. The enzyme undergoes these conversions both when it is free in solution and when it is membrane bound. The ATP analog adenylyl imidodiphosphate (AMP-PNP) gives the conversion without being hydrolyzed and without causing any apparent change in the mass of the protein, which suggests that the conversion may be a ligand-induced conformational change. Types IA and IB can convert into three other electrophoretically distinguishable species (types IIA, IIB, and III) if the purification procedure involves chromatography on a DEAE-Sephadex column equilibrated in phosphate buffer. These conversions can be prevented if the column is eluted in morpholinoethanesulfonic acid (Mes) buffer and KCl. Type IIA is convertible into type IIB by ATP treatment. Types IA and IB will also convert into types IIA and IIB and finally into type III when aged for extended periods of time at 4°, without a detectable change in mass. Coupling factor activity is lost when type I enzyme converts into type II enzyme, as is the ability of the enzyme to bind to the membrane. However, ATPase activity does not change significantly. The mitochondrial 13S coupling factor shows up to three electrophoretically distinguishable species. The use of phosphate buffer during DEAE-Sephadex chromatography gives conversion of slower species into faster species. ATP treatment does not give interconversions, and aging at 4° gives only a slow dissociation of the enzyme into subunits. The chloroplast 13S coupling factor also shows up to three electrophoretic species. Incubation with ATP does not give interconversions, but a temperature-dependent conversion of the major species into a faster species occurs upon aging. The subunit composition of the three 13S enzymes is very similar by polyacrylamide gel electrophoresis in sodium dodecyl sulfate, the major difference being in the number of classes of small polypeptides.

The molecular apparatus which is responsible for the conservation of energy in the form of ATP is impressively similar in highly diverse living systems. The major coupling factor in mitochondria is a 13S enzyme, about 90 Å in diameter, which exhibits ATPase activity in addition to its coupling factor activity (Pullman et al., 1960; Penefsky and Warner, 1965; Kagawa and Racker, 1966). In spinach chloroplasts the major coupling factor of photophosphorylation is also a 13S ATPase about 90 Å in diameter (Moudrianakis, 1964; Vambutas and Racker, 1965; Howell and Moudrianakis, 1967). In the case of oxidative phosphorylation from the gram-negative bacterium *Alcaligenes faecalis*, the heat-labile factor (HLF[1]) of Pinchot (1953) was recently shown to be a 13S ATPase that is about 90 Å in diameter (Adolfsen and Moudrianakis, 1971c). A 13S coupling factor has also been studied in *Micrococcus lysodeikticus* (Ishikawa, 1966; Muñoz et al., 1969) and *Escherichia coli* (Bragg and Hou, 1972), and a coupling factor having ATPase activity also exists in the gram-positive bacterium *Mycobacterium phlei* (Bogin et al., 1970).

To date, however, no detailed comparative report exists on the behavior of the 13S coupling factors from different systems when electrophoresed under *identical* conditions. The purpose of the present paper is to characterize and compare the electrophoretic properties and subunit composition of the 13S coupling factors from the mitochondrial, chloroplast, and bacterial systems. We previously reported that standard polyacrylamide gels of HLF showed one band, although occasionally two bands of closely similar mobility were evident (Adolfsen and Moudrianakis, 1971c). The studies in the present paper grew out of this preliminary observation, and a total of *five* electrophoretically distinct species of this enzyme have been found to date. Studies on F_1 and CF_1 have revealed as many as three electrophoretically distinguishable species of enzyme in each case. Our ability to visualize all of these different species in polyacrylamide gels depends to a large extent on the conditions employed during electrophoresis. The use of 5% polyacrylamide gels instead of 7.5% polyacrylamide gels gives greater resolution of *large* protein species which have similar mobilities. Another critical factor in achieving this resolution is the amount of protein applied to the gel. Species which have very small differences in mobility tend to fuse

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[1] Abbreviations used are: HLF, heat-labile factor, the 13S coupling factor of oxidative phosphorylation from *Alcaligenes faecalis;* F_1, the 13S coupling factor from beef heart mitochondria; CF_1, the 13S coupling factor from spinach chloroplasts; AMP-PNP, adenylyl imidodiphosphate; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid buffer; tricine, *N*-tris(hydroxymethyl)methylglycine buffer.

into a single band if too much protein is applied; this is particularly true for F_1. Good resolution is usually obtainable when about 5 μg of protein are applied to the gel.

Understanding the microheterogeneity of these enzymes may also be of considerable importance with respect to understanding other properties of coupling factors in general. It is possible, for example, that the affinity of one species of enzyme for ADP and/or ATP differs from that of another species. Aspects of the kinetic interactions of nucleotide with the coupling factor, such as ATP hydrolysis, could also show variations among different molecular forms of the enzyme. Therefore, in order to obtain the maximum degree of understanding of properties, such as enzyme–nucleotide interactions, we believe that it is mandatory that one be aware of precisely what type of microheterogeneity is evidenced by each individual preparation of enzyme that is studied and to know which of the electrophoretic species is used for a particular study.

Methods and Materials

Isolation of HLF. The previously described isolation procedure (Adolfsen and Moudrianakis, 1971c), which involved elution of HLF from a DEAE-Sephadex A-25 column in a continuous salt gradient in the presence of Tris buffer, was modified as follows. The 360 ml of crude coupling factor fraction, obtained by washing phosphorylating particles at low ionic strength, was made 0.05 *M* in potassium phosphate (pH 7.0) and applied directly to a DEAE-Sephadex column 4 × 15 cm (bed volume 150 ml), which was equilibrated in 0.05 *M* phosphate (pH 7.0)–0.25 *M* sucrose. After washing with 75 ml of 0.05 *M* phosphate–0.25 *M* sucrose, HLF was eluted stepwise in 0.15 *M* phosphate–0.25 *M* sucrose. Absorbancy of the 5-ml fractions was read at 280 or 230 nm, the peak fractions were pooled, and solid ammonium sulfate was added to a final concentration of 55% of saturation. After at least 15 min of equilibration at 0°, the precipitated enzyme was recovered by centrifugation and dissolved in 3–4 ml of 0.01 *M* Mes (pH 6.8) containing 0.1 m*M* ADP. The enzyme was further concentrated to about 1 ml in a Schleicher and Schuell collodion bag apparatus with 0.01 *M* Mes (pH 6.8) in the outer chamber. (Alternatively, the entire concentration may be done in the collodion bag apparatus.) Sucrose gradient centrifugation was carried out as previously described (Adolfsen and Moudrianakis, 1971c), with 0.01 *M* Mes (pH 6.8) and 0.1 m*M* ADP in the gradients. The enzyme was recovered at a concentration of about 2 mg/ml, with a yield of 6–8 mg from 1.5 g of phosphorylating particles. It was homogeneous as assayed by polyacrylamide gel electrophoresis and showed no apparent decomposition into subunits or aggregation when stored at −20° for as long as a year.

The above procedure yields types IIA, IIB, and III HLF (see text for explanation). To obtain types IA and IB HLF, the 0.05 *M* phosphate buffer was replaced by 0.01 *M* Mes (pH 6.8) containing 0.1 *M* KCl and 0.25 *M* sucrose. HLF was eluted stepwise in 0.01 *M* Mes containing 0.2 *M* KCl and 0.25 *M* sucrose. The remaining details of the procedure are the same as described above.

Isolation of F_1. One gram of beef heart mitochondria was passed through a French pressure cell twice at 6–8 tons pressure in 0.25 *M* sucrose in the *absence* of any salts at a concentration of about 10 mg/ml. Mes buffer was then added to 10 m*M* (pH 6.8) and membranes were removed by centrifugation for 1 hr at 10^5g. The resulting high speed supernatant fraction was concentrated in a Diaflo ultrafiltration cell (operated at 40 psi of nitrogen) down to 10–15 ml, then purified on a DEAE-Sephadex column, concentrated by ultrafiltration, and centrifuged through a sucrose gradient exactly as described above for HLF. The enzyme recovered from the sucrose gradient was *almost* homogeneous when assayed by polyacrylamide gel electrophoresis, and the yield was about 2.5 mg of F_1/g of whole mitochondria.

Both the yield and the purity of the enzyme were increased by using the following modification of the above procedure. After the mitochondria were ruptured in 0.25 *M* sucrose, Mes buffer was added to 10 m*M* (pH 6.8) and KCl was added to 0.1 *M*. Membranes were sedimented by centrifugation for 1 hr at 10^5g. Attempts to purify F_1 from the supernatant fraction showed that the amount of F_1 present was insignificant, indicating that the addition of KCl resulted in its sedimentation with the membranes. The membranes were resuspended in 0.25 *M* sucrose (no salts) and passed through a French pressure cell twice at 6–8 tons pressure to release the enzyme. Less harsh treatments such as simply washing in sucrose alone or in sucrose with 1 m*M* EDTA were ineffective in eluting F_1. Mes buffer (pH 6.8) was added to a final concentration of 10 m*M*, and membranes were removed by centrifugation for 1 hr at 10^5g. Gel electrophoresis of the resulting supernatant fraction showed a large amount of F_1 and only small amounts of contaminating protein species. This supernatant fraction was then concentrated by ultrafiltration, purified on a DEAE-Sephadex column, concentrated further by ultrafiltration, and centrifuged through a sucrose gradient, exactly as described above for HLF. The enzyme was recovered from the sucrose gradient at about 2 mg/ml in 0.01 *M* Mes (pH 6.8) containing 0.1 m*M* ADP and about 15% sucrose (w/v). F_1 was homogeneous as assayed by standard polyacrylamide gel electrophoresis and showed no significant aggregation or dissociation into subunits when stored at −20° for as long as 6 months. The yield of enzyme was about 6 mg/g of whole mitochondria.

Isolation of CF_1. The method was essentially the same as previously described (Howell and Moudrianakis, 1967).

Electrophoresis. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS) was carried out as described by Weber and Osborn (1969), except that the phosphate buffer concentration was 0.05 *M* instead of 0.10 *M* and the gels were run for 2 hr at about 4 mA/gel (50 V). Studies on the mobility of the intact enzyme were carried out essentially as described by Davis (1964), or in a system in which the upper buffer was 0.05 *M* Hepes (pH 7.7) and the lower reservoir buffer was 0.05 *M* imidazole (pH 7.5). In the latter system, Davis's solution A was replaced by a solution containing 12 ml of 1 *M* H_3PO_4, 35 ml of 2 *M* imidazole, 0.2 ml of Temed, and deionized water up to 100 ml (pH 7.7). Solution B was replaced by a solution containing 24 ml of 1 *M* H_3PO_4, 14 ml of 2 *M* imidazole, 0.2 ml of Temed, and deionized water up to 100 ml (pH 5.8). The pH of the gels during electrophoresis was 9.5 in Davis's Tris–glycine system and 8.5 in the Hepes–imidazole system used here. Gels run in the latter system were stacked at about 2 mA/gel (100 V) for about 10 min and were run at about 4 mA/gel (200 V) for about 30 min. The gels, which were about 5 cm long, were polymerized in tubes with an inner diameter of 0.4 cm and a length of about 8 cm. On gels of this size, 5–10 μg of protein gave large, easily visible bands, and as little as 0.1 μg could be visualized as a trace band. Thus, homogeneity of a protein determined by this method means greater than 99% purity. SDS gels of the same size were

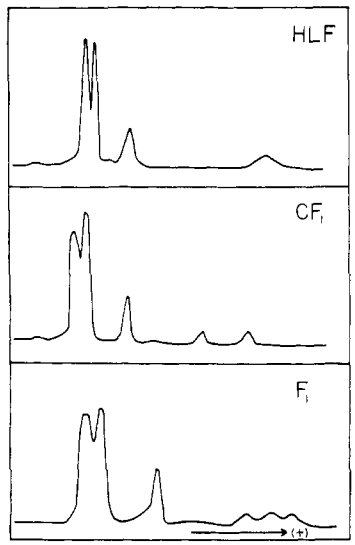

FIGURE 1: SDS gel profiles of HLF, CF_1, and F_1. The gels were scanned at 420 nm using a Gilford linear transport.

also run with 5–10 μg of protein. SDS gels were stained for 4 hr, Hepes–imidazole gels for 2 hr. Staining was performed in 0.05% Coomassie Blue dissolved in 50% methanol–7% acetic acid. Both types of gels were destained by free diffusion in 5% methanol–7% acetic acid and stored in 7% acetic acid (Weber and Osborn, 1969).

Materials. [^{3}H]AMP-PNP and unlabeled AMP-PNP were obtained from ICN Pharmaceuticals, Inc. [^{3}H]ATP was obtained from New England Nuclear. [γ^{32}P]ATP was prepared as described by Weiss et al. (1968). Other nucleotides and buffers were obtained from Sigma Chemical Company.

Results and Discussion

Subunit Composition of the 13S Coupling Factors of Oxidative and Photosynthetic Phosphorylation. Profiles of gels of the three 13S enzymes electrophoresed in sodium dodecyl sulfate (SDS) are shown in Figure 1, and the molecular weights of the polypeptide subunits are listed in Table I. The apparent similarities and dissimilarities in the sizes of the polypeptides among the different enzymes were verified by *coelectrophoresis* of pairs of enzymes in the *same* gel. In these studies the two major subunits of HLF migrated exactly with the two major subunits of F_1. The small subunit of HLF migrated with the fifth subunit of F_1. When CF_1 was coelectrophoresed with either HLF or F_1, a stack of three bands was observed. Subunit 1 of CF_1 was significantly larger than subunit 1 of HLF or F_1 and subunit 2 of CF_1 was the same size as subunit 1 of HLF or F_1. The third subunit of CF_1 was the same size as the third subunit of HLF, both of which were significantly larger than the third subunit of F_1. The fourth subunit of CF_1 was larger than the fourth of F_1, but the fifth subunit of CF_1 was about the same size as the fourth of F_1. It is interesting that HLF resembles F_1 more closely with respect to the size of the first two subunits, while it resembles CF_1 more closely with respect to the size of the third subunit.

The subunit composition of F_1 has been studied in a number of different laboratories (Brooks and Senior, 1971; Caterall and Pedersen, 1971; Tzagoloff and Meagher, 1971; Knowles and Penefsky, 1972; Van de Stadt et al., 1972). Our values for the molecular weights of the subunits are in good agreement with most of these data. Occasionally only one subunit in the vicinity of 55,000 is reported instead of two (Senior and Brooks, 1970). Subsequently, Senior and Brooks (1971) showed that resolution of subunits 1 and 2 was achieved on 10% polyacrylamide gels but not on 5% gels. However, other factors may also be important, since Van de Stadt et al. (1972) found only one band while using 12% polyacrylamide gels.

Table I: Molecular Weights of Subunits of HLF, CF_1, and F_1 [a].

Subunit	HLF	CF_1	F_1
1	59,000	62,000	59,000
2	54,000	59,000	54,000
3	43,000	43,000	35,000
4	12,000	21,000	14,000
5		14,000	12,000
6			10,000

[a] Standard curves for the determination of subunit molecular weights were constructed using the following proteins: bovine serum albumin (molecular weight 68,000), ovalbumin (43,000), chymotrypsin (25,700), myoglobin (17,200), and cytochrome *c* (11,700). The subunit molecular weights reported here are average values in which a deviation of about ±5% is equivalent to ±2 standard deviations.

A band in the region between subunits 2 and 3 has been observed in some preparations of HLF but not in others. A band between subunits 2 and 3 of yeast mitochondrial F_1 has also been reported (Tzagoloff and Meagher, 1971). It is conceivable that this band represents a contaminant that is present in some preparations of HLF and not in others, but the band was not removed by centrifuging the enzyme through a second sucrose gradient. Also, when a preparation of CF_1 was aged for about 3 weeks at 4° in 10 m*M* Tris (pH 8.0), a number of new bands began to appear. These bands arose at the expense of the major subunits, which stained much less intensely. Splitting of the major subunit bands into two or three bands of closely similar mobility also occurred. Similar splitting of the major subunit bands has also been seen in some preparations of HLF and F_1 after storage for several weeks at −20° or after repeated freezing and thawing. Thus, the extra band that is present in some preparations of HLF may represent partial polypeptide fragmentation rather than contamination.

The number of small subunits reported in preparations of F_1 has varied from 0 to 3. The possibility that some of the small subunits may be lost during the isolation procedure was raised by Brooks and Senior (1971) to explain differences in the amount of ATPase inhibitor subunit found in F_1 prepared by different methods. The *consistent presence of three small subunits* in our preparations of F_1 may be attributable to the avoidance of such harsh treatments as heating at 65°.

Microheterogeneity of HLF. Electrophoresis of the homogeneous enzyme in 7.5% polyacrylamide gels at pH 9.5 sometimes showed two bands migrating closely together instead of one band (Adolfsen and Moudrianakis, 1971c). The extra band cannot be ascribed to contamination by another molecule of similar size and electrophoretic mobility because SDS gels showed the same pattern of four subunit bands regardless of whether one or two bands were seen in standard polyacrylamide gels of the intact enzyme. Further indication that the two bands are different electrophoretic species of the same enzyme was obtained in an experiment in which samples of HLF were incubated for 1 hr at 37° with 1 m*M* ATP prior to electrophoresis. The untreated

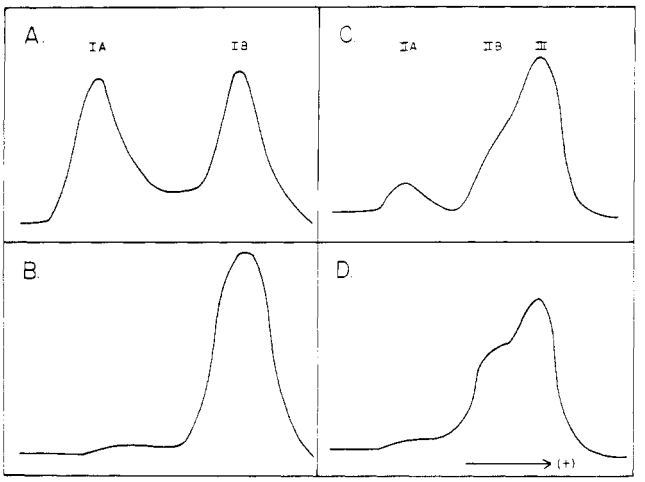

FIGURE 2: ATP-induced conversion of the slow form into the fast form. (A) The crude coupling factor fraction was incubated for 30 min at 37° in the absence of ATP, and 0.1 ml was electrophoresed in a 5% polyacrylamide gel in the Hepes–imidazole buffer system (see Methods and Materials for details); after staining and destaining, the section of the gel containing the slow and fast forms was scanned at 420 nm using a Gilford linear transport. (B) The crude coupling factor fraction was incubated for 30 min in 1 m*M* ATP, and 0.1 ml was electrophoresed. (C) A sample of purified HLF was incubated for 30 min at 37° in the absence of ATP, and about 5 μg was electrophoresed. (D) A sample of purified HLF was incubated for 30 min at 37° in 1 m*M* ATP, and about 5 μg was electrophoresed.

control enzyme showed two bands, while the enzyme incubated with ATP showed only the faster of the two bands. The results were the same using either of the two electrophoretic systems described under Methods and Materials. The conversion was observed most clearly with very small amounts of enzyme (5 μg) in 5% polyacrylamide gels run in the Hepes–imidazole buffer system, so subsequent studies were carried out using this system.

The ATP-induced conversion of the slow species into the fast species was observed both in preparations of the crude enzyme—the supernatant fraction obtained by washing phosphorylating particles at low ionic strength (Adolfsen and Moudrianakis, 1971b)—and in the purified, homogeneous enzyme recovered from the sucrose gradient. The densitometric tracings of gels shown in Figure 2A and B show the conversion in the case of the crude or unpurified enzyme. Incubation of this enzyme with 1 m*M* P_i or AMP had no effect at all, while a partial conversion of the slow species into the fast species was observed when the enzyme was incubated with 1 m*M* ADP. Since the preparations of crude enzyme contain a small amount of adenylate kinase, it was considered possible that ATP produced by adenylate kinase was responsible for the apparent effect of ADP. When the crude enzyme was incubated with 1 m*M* ADP for 30 min at 37° in the presence of hexokinase (1 EU/ml), glucose (10 m*M*), and $MgCl_2$ (5 m*M*), there was no conversion at all. Since the conversion was completely inhibited by having hexokinase present to prevent any buildup of ATP by adenylate kinase, it appears that the nucleotide-induced conversion is entirely specific for the triphosphate.

In studies on the purified enzyme, the faster species was often observed to split into two bands. Great variation in the relative amounts of these three species has been observed from one preparation of enzyme to the next. Some preparations contained about equal amounts of the slow and fast species with barely a trace of the third, fastest species, while others (such as the one shown in Figure 2C) showed only small amounts of the slow and fast species with a predominance of the third species. The gel tracing in Figure 2D shows that the ATP-induced conversion of the slow species into the fast species did not involve the third species. Better resolution of the second and third species was obtainable by applying less protein to the gels—as little as 2 μg. Similar dilutions of the crude enzyme preparation failed to separate the fast species into two distinct species. Thus, the third species that is present in preparations of the purified enzyme may arise during the purification procedure from one or both of the other two species.

The effects of various conditions on the ATP-induced conversion of the slow species into the fast species in preparations of crude enzyme are as follows. The conversion was often seen with 0.1 m*M* ATP, but sometimes it was only partial; a concentration of 1 m*M* ATP was routinely used to assure complete conversion. The conversion was seen when the pH was anywhere between 5.5 and 9.0; higher or lower values were not tested. It was temperature-independent, occurring anywhere from 0 to 50°. It was also quite rapid, going to completion as fast as the enzyme could be applied to the gel and its separation from nucleotide effected by the electric field; this length of time was estimated as less than 10 min, the time required for the focusing of the tracking dye and its migration to the end of the stacking gel. Divalent cations (1 m*M* $MgCl_2$ or 1 m*M* $CaCl_2$) or EDTA (1 m*M*) had no effect on the mobility of the enzyme or the amount of enzyme in the slow and fast bands, nor did they interfere with the ATP-induced conversion. P_i, AMP, and ADP (1 m*M*) did not interfere with the conversion, while 1 m*M* PP_i caused a slight inhibition.

Conversion of the Fast Species into the Slow Species by Aging. When the crude coupling factor fraction was stored at 4° in unbuffered 0.25 *M* sucrose, a time-dependent conversion of the fast species into the slow species was observed (Figure 3). Without any aging almost all of the HLF appeared as the fast band, while after 6 days more than half of it was detected in the slow band. On the seventh day a significant change in the mobility of both the slow and fast bands occurred. In addition, the distribution of mass appeared to shift in favor of the fast species. Further aging resulted in the complete disappearance of the slow species, a slight further increase in the mobility of the remaining material, and finally a sharpening up of the band when no further changes in mobility occurred. The identity of the bands as fast or slow species was established in a separate set of gels, in which the samples were treated with 1 m*M* ATP to eliminate the slow species (data not shown). In another aging experiment in which the enzyme was stored in 10 m*M* Mes buffer (pH 6.8), the conversion of the fast species into the slow species stopped after 1 or 2 days with approximately equal amounts of each species present, and incubation with 1 m*M* ATP for 10 min at 37° gave only a partial conversion of the slow species back into the fast species. This apparent interference by Mes buffer was largely overcome by including 1 m*M* $MgCl_2$ in the incubation mixture. When the enzyme was aged in 10 m*M* Tris (pH 8.0), the conversion of the fast species into the slow species went almost to completion, and subsequent incubation with ATP gave complete conversion back into the fast species. The peculiar change in mobility which occurred on the seventh day in the previous experiment (Figure 3) occurred on the seventh day in the system aged at pH 6.8 but did not occur in the system aged at pH 8.0.

Interconversions of the Slow and Fast Species of the

Membrane-Bound Coupling Factor. To determine whether the slow species could convert into the fast species while it was membrane-bound, 2-ml samples of phosphorylating particles in 0.1 *M* KCl–0.25 *M* sucrose–0.01 *M* Mes (pH 6.8) were incubated for 5 min at room temperature either with no additions or with 1 m*M* P_i or AMP or ADP or ATP. The particles were then washed once in sucrose alone to elute the coupling factors (Adolfsen and Moudrianakis, 1971b). Electrophoresis of the resulting crude coupling factor fractions showed that preincubation of the membranes with P_i or AMP gave no changes in the amounts of slow and fast species present, while ADP gave a partial conversion of the slow species into the fast species and ATP gave a complete conversion. The partial effect of ADP was attributed to the contamination by adenylate kinase, as described above. The ATP-induced conversion was also observed when the membranes that had been exposed to ATP were washed once in KCl–sucrose–Mes *before* coupling factors were washed off the membranes at low ionic strength. This eliminated the possibility that the ATP-induced conversion could have occurred *after* the dissociation of the coupling factor as a result of incomplete removal of ATP from the membrane pellet.

Membranes aged for 1 day at 4° showed no apparent increase in the amount of slow species present in the crude coupling factor fraction that was prepared from them. However, after 1 week at 4° nearly half of the enzyme had converted into the slow form. Thus, the interconversion induced by aging also occurs when the enzyme is membrane-bound, although at a slower rate than when it is free in solution.

One final question of importance with respect to the membrane-bound coupling factor is what species exist while the enzyme is synthesizing ATP. Phosphorylating particles were incubated at 37° for 15 sec with 2 m*M* DPNH, 5 m*M* P_i, 0.25 m*M* ADP, and 8 m*M* $MgCl_2$, conditions that were optimal for oxidative phosphorylation (Adolfsen and Moudrianakis, 1971a). The membranes were chilled and coupling factors were washed off at low ionic strength. Electrophoresis showed that the untreated control system contained the usual amount of the slow species, while the experimental system contained HLF exclusively as the fast species. In another experiment, it was found that this conversion was prevented by the addition of hexokinase to the reaction mixture. These results suggest that this conversion may be attributable to *free* ATP which was produced by oxidative phosphorylation and not to events occurring *during* ATP synthesis.

Coelectrophoresis of Purified and Unpurified HLF. The observation that aging the crude coupling factor fraction for a week at 4° resulted in an increase in the electrophoretic mobilities of the slow and fast species raised the question as to what the mobilities of the species in the preparations of purified enzyme were with respect to the species in the crude coupling factor fraction. The results of the coelectrophoresis study shown in Figure 4 indicate that *none* of the three species present in the preparations of purified enzyme have the same mobility as *either* of the two species in the crude coupling factor fraction. In short, *five* electrophoretically distinguishable species of HLF exist. In another experiment, the purified enzyme was coelectrophoresed with the crude coupling factor fraction that was aged for 10 days at 4°. The results showed that the enzyme which had undergone its maximal increase in mobility migrated exactly with the third and fastest species of HLF that was present in the preparation of purified enzyme.

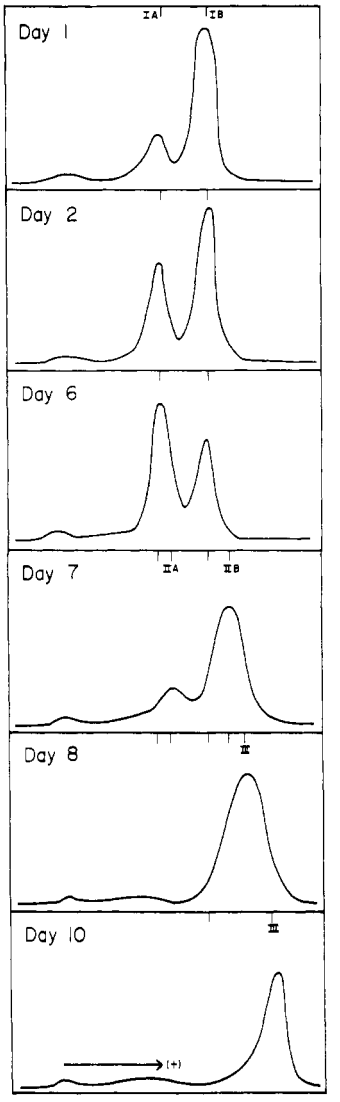

FIGURE 3: Effect of aging on microheterogeneity of HLF in the crude coupling factor fraction. Samples of crude coupling factor (equal amounts of protein) were aged for the indicated length of time at 4° in unbuffered 0.25 *M* sucrose and then were electrophoresed. The section of the gel containing HLF was scanned. The tracings are aligned with respect to the small amount of a contaminant on the left side of each panel. The weights of the paper for the combined areas under the peaks are (arbitrary units): 48.5, 50.0, 51.5, 49.5, 51.2, and 37.0 for days 1, 2, 6, 7, 8, and 10, respectively. The decrease of about 30% in the size of the HLF peak that occurred between the eighth and tenth day is attributable to lability of the enzyme (dissociation into subunits). This lability did *not* occur when the experiment was carried out in the presence of 1 m*M* $MgCl_2$, which stabilizes the enzyme.

The following nomenclature is proposed in order to refer to these five species of enzyme without confusion. The two species in the crude coupling factor fraction will be referred to as type IA (crude slow form) and type IB (crude fast form). The first two species in preparations of the purified enzyme will be referred to as type IIA (purified slow species) and type IIB (purified fast species). The third and fastest species in preparations of purified enzyme will be referred to as type III. The mobilities of these species are listed in Table II. Types IA and IB are particularly close in mobility and may be distinguished best by determining whether or not treatment with ATP results in a change in mobility. The fastest species was placed in a separate category because it seems to be an end product in the changes

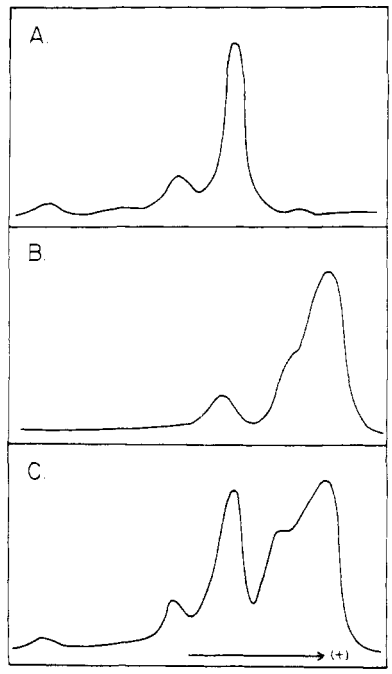

FIGURE 4: Coelectrophoresis of purified HLF with HLF in the crude coupling factor fraction. (A) Crude fraction; (B) purified HLF; (C) coelectrophoresis of crude enzyme and purified enzyme.

undergone by the enzyme during aging (Figure 3); type I ages into type II and then into type III. Also, when purified HLF, obtained as a mixture of types IIA, IIB, and III, was aged for 3 days at 4° in 10 m*M* Mes buffer (pH 6.8) at 0.1 mg/ml, it converted completely into type III enzyme.

Isolation of Type I HLF. Studies were undertaken to determine where in the purification procedure the conversion of type I enzyme into types II and III enzyme occurred, so that conditions could be obtained under which this conversion did *not* occur. It should be noted that the conversions occurring in the isolation procedure cannot be ascribed simply to aging, because the isolation procedure takes no more than 2 days. The isolation procedure used up to this time involved applying the crude coupling factor fraction to a DEAE-Sephadex column equilibrated in 0.05 *M* phosphate buffer (pH 7.0), eluting the enzyme in 0.15 *M* phosphate, concentrating HLF by ultrafiltration, and running a preparative sucrose gradient (see Methods and Materials for complete details). It was found that the enzyme was type I when it was applied to the sucrose gradient and types II–III when it was recovered from the gradient. However, the simple passage of the enzyme through the sucrose gradient did *not* cause the conversion. This was determined by concentrating the entire crude coupling factor fraction down to 1 ml by ultrafiltration methods and purifying it through two successive sucrose gradients; the DEAE-Sephadex chromatography step was omitted in this experiment. The nearly pure enzyme was recovered from the second sucrose gradient as a mixture of types IA and IB. This result implicated the earlier step, DEAE-Sephadex chromatography, as somehow priming the coupling factor to undergo the conversion in the subsequent sucrose gradient step. It was then found that changing the buffer in the DEAE-Sephadex column from 0.05 *M* phosphate (pH 7.0) to 0.01 *M* Mes (pH 6.8) containing 0.1 *M* KCl eliminated the problem of conversion in the subsequent sucrose gradient. Thus, exposure of the enzyme to *phosphate* buffer during DEAE-Sephadex chromatography may tend either to accelerate those events

Table II: Electrophoretic Mobility of the Various Species of HLF.

Species	R_f	Species	R_f
IA	0.52	IIB	0.60
IB	0.56	III	0.63
IIA	0.54		

which occur during aging for extended periods of time at 4°, or, alternatively, to set in motion those events that ultimately lead to an increase in the mobility of the enzyme. Factors other than phosphate buffer may be involved, however, since occasionally a preparation of enzyme isolated using phosphate buffer in the chromatography step was type I and a preparation isolated in Mes buffer and KCl was a mixture of types II and III. This happened with a frequency of not more than 10%. However, it emphasizes the importance of performing electrophoresis on each individual preparation of enzyme to specifically determine what kind of microheterogeneity it shows.

Mechanism of Interconversion of Types IA and IB HLF. It is important to know whether types IA and IB HLF are alternate conformational states of the enzyme or are the results of a reversible interconversion involving dissociation and reassociation of a subunit(s) or cofactor(s). If the interconversions were due to a simple association–dissociation equilibrium that was controlled by ATP, then the conversion which involves *association* of the subunit or cofactor should *not* be observed if the type IA–IB enzyme mixture was completely free of dissociated subunits or cofactors. To achieve complete removal of these hypothetical small substances, HLF was purified through two successive sucrose gradients; the recovered enzyme contained approximately equal amounts of the two species. It was found that treatment with ATP gave a complete conversion of type IA into type IB, and that aging at 4° gave a nearly complete conversion of type IB into type IA. The fact that the conversion could still proceed in *both* directions ruled out the simple association–dissociation model proposed above. The data do not exclude the rather unlikely possibility that something can dissociate from one form of the enzyme without giving a change in electrophoretic mobility and then associate with the other form and result in a change in mobility.

To obtain further evidence that subunit dissociation is not involved in the interconversion of types IA and IB HLF, separate studies of the polypeptide composition of the two species were undertaken. HLF was electrophoresed in the Hepes–imidazole system, the bands corresponding to types IA and IB HLF were eluted in 0.1% SDS and 1% 2-mercaptoethanol, and the recovered material was electrophoresed in SDS polyacrylamide gels. Both types IA and IB HLF showed *all four classes* of polypeptide subunits.

Another important question is whether the ATP-dependent conversion requires the hydrolysis of ATP or simply the association of the ligand with the enzyme. These two possibilities were distinguishable by using an analog of ATP which is not subject to hydrolysis by most ATP-utilizing enzymes, such as AMP-PNP (Yount et al., 1971). To establish first whether HLF could hydrolyze AMP-PNP, 100 μg of enzyme was incubated for 5 hr at 37° in 0.15 ml of incubation mixture consisting of 0.1 m*M* [^{3}]AMP-PNP, 1 m*M* $MgCl_2$, and 30 m*M* Tris (pH 8.0). The reaction mixture was analyzed on a Dowex 1-Cl^- column as previously described (Adolfsen and Moudrianakis, 1972), using the following modified elution schedule: AMP was eluted in five

2-ml fractions of 0.01 *N* HCl, ADP-NH_2 (the product of hydrolysis of AMP-PNP) was eluted in six 2-ml fractions of 0.01 *N* HCl–0.01 *M* KCl, and AMP-PNP was eluted in six 2-ml fractions of 0.01 *N* HCl–0.1 *M* KCl. No increase in the amount of AMP or ADP-NH_2 was detectable over the low level present in the reagent radionucleotide that was incubated for 5 hr under the same conditions but without enzyme. It was estimated that the lower limit of detection of hydrolysis was 1–2%, which was equivalent to *less than one turnover* in 5 hr at 37°. In experiments in which the enzyme was incubated with [^{3}H]ATP, it was found that *all* of the ATP was hydrolyzed in less than 1 hr. In studies on the effect of AMP-PNP on microheterogeneity, it was found that incubation of the enzyme with 0.1 m*M* AMP-PNP for 5 min at 0° resulted in a partial conversion of type IA into IB, whereas the conversion was almost complete when the incubation was carried out in the presence of 1 m*M* $MgCl_2$ in addition to the AMP-PNP. The rapidity of the conversion at 0°, along with the absence of any detectable hydrolysis of AMP-PNP after 5 hr at 37°, leads to the conclusion that the conversion of type IA into IB is the result of a ligand-induced conformational change.

The above conclusion was strengthened by negative results in experiments designed to detect formation of a phosphoprotein. There were less than 10^{-4} mol of acid-precipitable ^{32}P/mol of HLF after incubation with 0.1 m*M* [γ-^{32}P]ATP for an hour at 37°. When a similarly treated enzyme was denatured in 8 *M* urea at pH 7.0 or 9.0 and passed through a Sephadex G-50 M column equilibrated in the same solvent, no radioactivity was excluded with the polypeptide chains. These data suggest that formation of a stable phosphoprotein does not occur upon incubation with ATP in vitro and is not involved in the conversion of type IA into type IB HLF, but they do not rule out the possibility of transient phosphoprotein formation during ATP hydrolysis.

The possibility that sulfhydryl groups or disulfide interchanges might be involved in the conversion was also investigated. It has been established that the interconversion of two species of bovine mercaptalbumin occurs as a consequence of disulfide interchanges which are accelerated at alkaline pH (Foster et al., 1965; Nikkel and Foster, 1971). This interconvertibility was completely inhibited by alkylating a sulfhydryl group and was restored by adding a reducing agent. The rate of conversion of type IB HLF into IA HLF by aging at 4° was significantly faster at pH 8.0 than at pH 6.8, but neither 1% 2-mercaptoethanol nor 1 m*M* *N*-ethylmaleimide had an observable effect on the conversion. These results exclude the possibility that the conversion involves sulfhydryl groups that are readily accessible to these reagents, but they do not rule out the possibility that the conversion involves inaccessible groups.

Mechanism of Conversion of Type I HLF into Types II and III HLF. SDS polyacrylamide gels of type I and type II–III HLF preparations both showed four classes of polypeptide subunits. Also, when a sucrose gradient purified preparation of type II–III HLF was aged into type III and then purified through another sucrose gradient to remove any dissociated subunits, all four classes of polypeptides were still found by SDS gel electrophoresis. Thus, these conversions also do not appear to involve any changes in the mass of the protein. However, the above observations do not exclude the possibility that a change in subunit stoichiometry may occur. For example, the possibility that there might be two subunit number 4 polypeptides in type I HLF and only one in type II or III HLF is not determinable at the present time. This question cannot be answered until the problem of subunit stoichiometry of coupling factors in general is solved. The latter is presently under consideration in this laboratory.

One way an enzyme could change its mass without changing the *number* of polypeptides in it is by partial proteolysis, which could involve removal of segments of some of the polypeptides. Changes in the molecular weights of the subunits of phosphorylase kinase have been detected by SDS gel electrophoresis during the time course of activation of this enzyme by trypsin (Hayakawa et al., 1973). Coelectrophoresis of type I HLF with type II–III HLF in the same SDS gel showed that there were *no changes* in the size of any of the four polypeptide subunits. At this point mention must be made of the problem of partial polypeptide fragmentation that may occur in some aged preparations of enzyme (see above). There was *no correlation* between the presence or absence of the band between subunits 2 and 3 and the microheterogeneity of the preparation; this band has been seen in both type I and type II–III enzyme preparations.

Another possibility is that something other than a polypeptide may be lost from the enzyme during the conversion of type I into types II and III. For example, the microheterogeneity which has been observed for bovine superoxide dismutase is related to the metal content of the enzyme (Rotilio et al., 1972). Three new species of enzyme with electrophoretic mobility greater than the original enzyme were observed in starch gels when Cu^{2+} and/or Zn^{2+} were removed from the enzyme by treatment with chelating agents. Kinetic studies on the ATPase activity of HLF raised the possibility that this enzyme might contain tightly bound metal ions (Adolfsen and Moudrianakis, 1973). When HLF was incubated with 1 m*M* EDTA at 4°, a time-dependent decrease in the intensity of the bands corresponding to types IA and IB HLF was observed; the bands were almost completely gone after 4 days. Types II and/or III did not appear as type I disappeared. The effect of EDTA may be to increase the lability of the enzyme by promoting dissociation into subunits. If bound metals are present and are slowly removed by EDTA, these metals may play a role in the quaternary structure of the enzyme.

Correlation of ATPase Activity and Coupling Factor Activity with Microheterogeneity. The previously reported kinetic studies on the ATPase activity of HLF (Adolfsen and Moudrianakis, 1973) were performed entirely on type II–III HLF. In a reaction mixture containing 10^{-4} *M* ATP, hydrolytic activity was enhanced by both $MgCl_2$ and KCl, with the monovalent cations activating the enzyme more strongly than the divalent cations. The specific activity of the enzyme in the presence of both ionic activators was usually about 300 nmol per min per mg. The ATPase activity of type I HLF was also activated by both monovalent and divalent cations and to about the same extent as type II–III HLF. The only difference known thus far is that the specific activity of the type I enzyme is slightly lower, about 150 nmol per min per mg. Thus, it is not possible at the present to make any substantial correlation between ATPase activity and microheterogeneity.

ATPase activity may also be demonstrated in situ in an electrophoresed gel of HLF simply by incubating the gel in 50 m*M* Ca-ATP and 10 m*M* Tris (pH 8.0). A white precipitate, calcium phosphate, marks the positions of both type IA and IB HLF. Of course, it is questionable whether it is

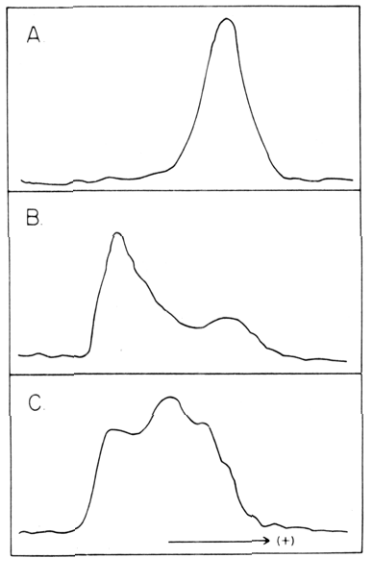

FIGURE 5: Microheterogeneity of F_1. (A) F_1 purified using phosphate buffer in the DEAE-Sephadex column chromatography step; (B and C) F_1 purified using a solution of KCl and Mes buffer in the DEAE-Sephadex step.

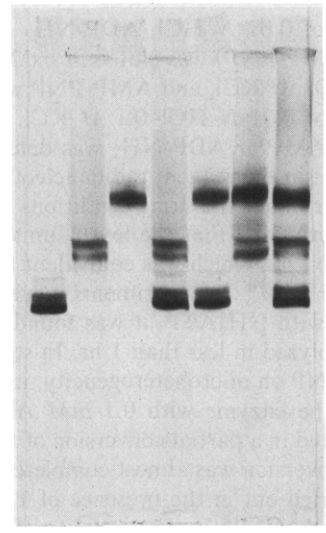

FIGURE 6: Comparison of the electrophoretic mobilities of HLF, CF_1, and F_1. From left to right the gels are: (1) HLF, showing a trace of type IIA, with about equal amounts of types IIB and III comprising the bulk of the preparation; (2) CF_1, showing a trace of its slowest species, with a major amount of its middle species and a significant amount of its fastest species; (3) F_1, showing the fastest species, with a slight trace of a slower species; (4) HLF and CF_1 coelectrophoresed; (5) HLF and F_1 coelectrophoresed; (6) CF_1 and F_1 coelectrophoresed; (7) all three enzymes coelectrophoresed.

possible to speak of ATPase activity of type IA HLF, since it converts into type IB as soon as it is exposed to ATP.

When HLF was assayed for coupling factor activity as previously described (Adolfsen and Moudrianakis, 1971c), using boiled crude coupling factor fraction as a source of heat-stable coupling factor, it was found that type II–III HLF failed to reconstitute oxidative phosphorylation. In contrast, heat-labile coupling factor activity was observed for the type I enzyme. One possible mechanism by which coupling activity could be lost is that the enzyme might lose its ability to bind to the membrane. The ability of HLF to interact with membranes was tested by incubating HLF with membranes and heat-stable factor in 0.1 *M* KCl and 0.01 *M* Mes (pH 6.8). These conditions were optimal for the reconstitution of oxidative phosphorylation (Adolfsen and Moudrianakis, 1971b,c). The reconstituted phosphorylating particles were sedimented by centrifugation, and gel electrophoresis was performed on the supernatant fractions. The *absence* of a band corresponding to HLF in these gels would indicate that HLF had bound to the membranes. The gels showed that *none* of the type I enzyme had remained behind in the supernatant fraction, while *all* of the type II enzyme had remained behind. Thus, the loss of coupling factor activity that occurs when type I HLF converts into type II HLF appears to be directly related to the loss of the ability of the enzyme to bind to the membranes.

Microheterogeneity of F_1. Preparations of F_1 isolated using phosphate buffer in the DEAE-Sephadex column usually contained a single electrophoretic species (Figure 5A). When the column was equilibrated in Mes buffer and KCl, the enzyme recovered from the subsequent sucrose gradient usually contained two species, with the slower one being predominant (Figure 5B). Coelectrophoresis of the enzyme in Figure 5A with the enzyme in Figure 5B established that the material in Figure 5A had the same mobility as the faster species in Figure 5B. Thus, the use of phosphate buffer in the DEAE-Sephadex column promoted conversion of the slower species into the faster species. The use of Mes buffer and KCl in the column did not give any conversions, as judged by coelectrophoresis of the F_1 in the crude coupling factor fraction (before anion exchange chromatography) with the enzyme subsequently recovered from the sucrose gradient.

Some preparations of F_1 contained a third electrophoretic species which had a mobility between that of the slow and fast species (Figure 5C). The R_f's of these three species are 0.28, 0.32, and 0.37. None of these species can be contaminants, because SDS gels revealed only those six polypeptides shown in Figure 1C. Resolution of the species of F_1 in gels was more difficult than for HLF because the mobilities of the species of F_1 were lower. Optimal resolution was obtained when small amounts of protein (not more than 5 μg) were electrophoresed in 5% polyacrylamide gels in the Hepes–imidazole buffer system. Application of larger amounts of protein resulted in fusion of the bands into a single, wide band.

ATP treatment did not result in any detectable interconversions of species, nor did aging the enzyme at 0.1 mg/ml in 10 m*M* Mes (pH 6.8) and 0.25 *M* sucrose for several days at 4°. However, after several days of aging in 10 m*M* Tris (pH 8.0) and 0.25 *M* sucrose, a new species appeared which had a mobility greater than that of the fastest species noted above (R_f 0.46). This new species may represent the first stage of dissociation of the enzyme into subunits, since two other bands of very high mobility also appeared (R_f 0.91 and 0.94), which probably were subunits. With further aging an increasing amount of material remained at the interface of the stacking and running gels, which may be aggregated subunits. Our interpretation of these results is consistent with the findings of Penefsky and Warner (1965) and Warshaw et al. (1968).

Microheterogeneity of CF_1. The crude coupling factor fraction obtained from spinach chloroplasts by EDTA extraction showed a single major species of CF_1 (R_f 0.47) and a small amount of a minor species with a slightly greater mobility (R_f 0.50). Occasionally a smaller amount of a

species with a mobility slightly less than that of the major species was also present (R_f 0.44). Purification of CF_1 from this fraction involves precipitation with ammonium sulfate and centrifugation through a sucrose gradient (Howell and Moudrianakis, 1967b). Coelectrophoresis of the purified, homogeneous enzyme with the crude coupling factor fraction showed that no changes in the mobility of the enzyme occurred during this purification procedure. However, when the enzyme was aged overnight at 4° at a concentration of 0.1 mg/ml in 10 m*M* Mes (pH 6.8) and 0.25 *M* sucrose, a partial conversion of the major species into the fastest species was observed. The rate of conversion was greater when the enzyme was aged at room temperature. Incubation of CF_1 with ATP prior to gel electrophoresis has not yet been seen to result in interconversion of species. Microheterogeneity in preparations of homogeneous CF_1 has also been noted by Farron (1970).

Comparison of the Microheterogeneity of HLF, CF_1 and F_1. A comparison of the electrophoretic mobilities of the three 13S enzymes is shown in Figure 6. HLF has the greatest mobility, CF_1 is slower, and F_1 is slowest. In another coelectrophoresis experiment, the fastest species of CF_1 had *almost* the same mobility as the slowest species of HLF (type IA). With respect to other electrophoretic properties, F_1 is similar to HLF in that the use of phosphate buffer in the DEAE-Sephadex column favors the conversion of slow species into faster species. It is dissimilar in that ATP does not appear to be able to induce any species interconversions, and in that aging at 4° results only in a slow degradation of the enzyme into subunits. CF_1 is similar to HLF in that aging at 4° results in conversion of a slow species into a faster species, but it is dissimilar in that ATP does not induce any species interconversions. The apparent failure of ATP to induce species interconversions in the cases of F_1 and CF_1 does *not* mean that ATP cannot interact with these enzymes in a manner similar to the way in which it interacts with HLF. It means only that, if similar interactions do occur, the effects may not be detectable by gel electrophoresis.

It is conceivable that electrophoretic microheterogeneity may be paralleled by functional microheterogeneity, or that different functional states may exist for each of the various electrophoretic species. In view of these possible complexities, it is clearly of great importance in studies involving coupling factors in general to be aware of exactly what forms of HLF or F_1 or CF_1 are used *in each individual experiment.* Failure to be aware of such problems may give rise, for example, to apparent discrepancies in the results obtained by different investigators. Perhaps it would be best to study the species that are present in major proportions immediately after the elution of the enzyme from the membrane, taking this as the *most native species.* If this point of reference is acceptable, then future studies should emphasize the properties of type IB HLF, the slowest species of F_1, and the species of CF_1 with intermediate mobility. At the very least, the nature of the microheterogeneity of the enzyme used in any given series of studies should be clearly specified.

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