Purification and Properties of Two Soluble Coupling Factors of Oxidative Phosphorylation from *Alcaligenes faecalis**

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ABSTRACT: The affinity of coupling factors of oxidative phosphorylation for membrane fragments in the *Alcaligenes faecalis* system decreased 10- to 15-fold when the ionic strength of the medium was lowered from 0.10 to 0.00. Essentially all of the heat-labile coupling factor (HLF) and about 80% of the heat-stable coupling factor (HSF) were eluted with one wash at low ionic strength. The factors were resolved on DEAE-Sephadex columns. Further purification of the HLF by sucrose density gradient centrifugation gave a protein with an *s* value of approximately 13 that was homogeneous as assayed by polyacrylamide gel electrophoresis at pH 9.5. Electron microscopy of negatively stained preparations showed molecules about 90 Å in diameter that were indistinguishable from the 90 Å coupling factors of the mitochondrial and chloroplast systems. The HLF had latent, Mg^{2+}-dependent ATPase activity with a molecular activity of 300–500 moles/min per mole of HLF. Its molecular activity in oxidative phosphorylation was 15,000 moles/min per mole of HLF. The HSF, purified 800×, did not appear to be a nucleic acid as has been previously reported and is now believed to be a heat-stable protein. The heat-stability property was pH dependent, however, with all activity being lost with boiling for one minute at mildly acidic pH. The behavior of the HSF on DEAE-Sephadex columns suggested it could exist in more than one form. Attempts at further purification of the HSF have been hampered by a loss of activity that was dependent on HSF concentration and which is believed to be due to an irreversible self-aggregation and precipitation. The previously characterized polynucleotide factor may have functioned by stabilizing components of the phosphorylating system (the HLF in particular) and though it stimulated oxidative phosphorylation was probably not a coupling factor.

Phosphorylating particles of *Alcaligenes faecalis* prepared by centrifugation from cell-free crude extracts in 0.1 M KCl and 0.25 M sucrose have been resolved into particulate and supernatant fractions by centrifugation in the absence of KCl (Adolfsen and Moudrianakis, 1971b). The former fraction consisted of membrane fragments containing the electron-transport apparatus, and the latter contained the heat-labile and heat-stable coupling factors (HLF and HSF[1]) originally reported by Pinchot in 1953. A reconstitution assay was developed in which there was an *absolute* dependence of the reappearance of phosphorylating activity on the addition of the supernatant fraction to the particulate fraction. In the experiments presented in this paper, the purification of the two coupling factors was followed with this assay, and reconstitution was found to have absolute dependencies on both coupling factors. The HLF was found to be analogous to the 90-Å coupling factor of the mitochondrial system (Kagawa and Racker, 1966) and of the chloroplast system (Howell and Moudrianakis, 1967), and it is probably similar to the 13 S factor isolated from *Micrococcus Lysodecticus* (Ishikawa, 1970). Evidence was obtained to indicate that the HSF was not a nucleic acid as has been previously reported (Pinchot, 1957; Shibko and Pinchot, 1961a,b) but was instead a heat-stable protein. An analogy may also exist between this system and the one described in *Mycobacterium phlei*, in which the presence of at least two coupling factors of oxidative phosphorylation has been proposed (Higashi *et al.*, 1970).

Materials and Methods

Analytical DEAE-Sephadex Resolution of Soluble Coupling Factors. Phosphorylating particles were prepared and stored at −20° in small aliquots, and coupling factors were washed off in 0.25 M sucrose in the absence of KCl as previously reported (Adolfsen and Moudrianakis, 1971a,b). The coupling factor fraction from 25 mg of particles was brought to a final concentration of 0.1 M KCl and 0.01 M Tricine (pH 8.0) by the successive addition of 1.0 M KCl and 0.1 M Tricine (pH 8.0). DEAE-Sephadex A-25, swollen in 0.1 M KCl + 0.01 M Tricine (pH 8.0), was packed in a Sephadex K9 column (bed volume approximately 8 ml). The sample, containing about 1 mg of protein, was applied and eluted with a linear salt gradient consisting of 20 ml each of 0.1 and 1.0 M KCl in 0.1 M Tricine (pH 8.0) + 0.25 M sucrose, utilizing a Buchler Instruments Plexiglas apparatus and vibrator designed for the preparation of sucrose gradients. The flow rate was 20–25 ml/hr, and 1.2- or 1.5-ml fractions were collected with an LKB fraction collector. The elution was monitored at 230 nm to amplify peaks in the protein portion of the profile; the ratio of absorbancies at 230/280 nm for proteins was about seven.

DEAE-Sephadex Purification of Coupling Factors on a Preparative Scale. Approximately 1.5 g of phosphorylating

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[1] Abbreviations used are: HLF, heat-labile factor; HSF, heat-stable factor; native particles, phosphorylating particles prepared by centrifugation of membrane fragments from cell-free crude extracts in KCl–sucrose; stripped particles, native particles washed once in sucrose in the absence of KCl to remove coupling factors; reconstituted particles, stripped particles incubated with coupling factors under conditions favoring reconstitution of oxidative phosphorylation; KCl–sucrose, 0.1 M KCl + 0.25 M sucrose; KCl–sucrose–MES, KCl–sucrose + 0.01 M MES buffer at pH 6.8; Tris–sucrose, 0.12 M Tris buffer at pH 6.6 + 0.25 M sucrose; MES, 2(*N*-morpholino)ethanesulfonic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine.

particles (360 ml of a particle suspension at 4 mg/ml) were sedimented at 78,000*g* (30,000 rpm in Spinco No. 30 rotor) for 1 hr and resuspended with a glass tissue homogenizer in 360 ml of 0.25 M sucrose (no KCl). The membrane fragments were then resedimented at 78,000*g* for 2 hr, and the coupling factor supernatant fractions were carefully decanted and pooled. The membrane fragments (stripped particles) were resuspended in 250 ml of KCl–sucrose and then adjusted to 4 mg/ml by the addition of more KCl–sucrose. Small aliquots were stored at −20° for use in reconstitution assays. The coupling factor fraction was concentrated down to about 40 ml in an Amicon Diaflo ultrafiltration cell, using an XM-50 membrane. Very small membrane fragments were partially removed by centrifugation at 10^5g (40,000 rpm in Spinco No. 40 rotor) for 1 hr, and 1 M Tris buffer at pH 6.6 was added to a final concentration of 0.12 M.

The entire sample (about 50 ml, containing about 50 mg of protein) was applied to a DEAE-Sephadex column 2.5 × 27 cm (approximately 150-ml bed volume), which had been previously equilibrated with 0.12 M Tris at pH 6.6 + 0.25 M sucrose (Tris–sucrose). The column was then washed with 100 ml of Tris–sucrose and eluted with a gradient of 200 ml of Tris–sucrose and 200 ml of Tris–sucrose + 0.5 M KCl. The flow rate was about 50 ml/hr and 10-ml fractions were collected and monitored at 280 nm. Conditions for running these preparative columns were slightly different from those for the earlier analytical columns in order to control pH artifacts. The starting condition of 0.12 M Tris (pH 6.6) was selected because this combination of pH and ionic strength gave elution profiles most similar to those originally obtained with small columns.

The fractions containing the HSF were pooled and stored at −20°. Those containing the HLF were pooled, and saturated ammonium sulfate (pH 6.8–7.0) was added to 50% saturation. After 15 min at 0° the precipitate, containing the HLF, was collected by centrifugation and dissolved in 2% sucrose + 0.01 M MES buffer (pH 6.8). The sample was clarified by centrifugation and concentrated to about 1 ml in a Schleicher & Schuell collodion bag apparatus with 2% sucrose + 0.01 M MES buffer (pH 6.8) in the outer chamber. Aliquots of 0.3–0.4 ml were then applied to each of three 4.6-ml sucrose gradients (5–20% in 0.01 M MES buffer (pH 6.8) + 0.25 mM ADP) with a cushion of 0.3 ml of 40% sucrose. The gradients were centrifuged for 12 hr at 35 K in an SW39 or SW50 rotor, and 20 drop fractions were collected. The HLF recovered from the gradient was homogeneous as assayed by polyacrylamide gel electrophoresis at pH 9.5 and was labile by about 5%/week when stored at −20°.

The purification of coupling factors could not be followed quantitatively by recovery of units of activity due to the previously discussed lack of proportionality of reconstituted oxidative phosphorylation to coupling factor concentration (Adolfsen and Moudrianakis, 1971b), which prevented the definition of any type of simple unit of coupling factor activity. The yield of HLF was 7–8 mg (5 mg/g of phosphorylating particles) and this was estimated as 70–80% of the HLF in native particles. Significant losses during purification only occurred in the ammonium sulfate precipitation step and by some possible dissociation into subunits due to high Tris and KCl concentrations. The yield of HSF was about 15 mg of protein (10 mg/g of native particles), which was estimated as about 80% of the HSF in the particles (see Results). The purification was about 80× relative to native particles or 800× relative to the cell-free crude extract.

Reconstitution of Oxidative Phosphorylation. HLF and HSF were mixed at 0°, and a solution of KCl and MES buffer at pH 6.8 was added to bring the volume to 0.15 ml with a final KCl concentration of 0.1 M and a final MES buffer concentration of 0.01 M. Then 0.05 ml of stripped particles (200 μg of membrane protein) was added, and the system was incubated at 30° for 15 min. The concentrations of the HLF and HSF approximated those found in the crude coupling factor fraction when possible. Computed on the basis of protein yields from native particles, these concentrations were 15–20 and 30–40 μg per ml for the electrophoretically pure HLF and the partially purified HSF, respectively. The second stage of the assay involved measurement of the initial velocity of phosphorylation. The reaction was begun by the addition of 0.8 ml of reaction mixture containing DPNH, ADP, [^{32}P]P_i, $MgCl_2$, hexokinase, glucose, and sucrose, was stopped 1 min later by the addition of 1 ml of 10% trichloroacetic acid, and esterified phosphate was determined (see Adolfsen and Moudrianakis, 1971a, for details).

Activation and Assay of Latent ATPase. Activation was accomplished by the following modifications of the methods described by Farron and Racker (1970): (a) incubation in 20 mM dithiothreitol for 2 hr at room temperature, (b) heating at 65° for 2 min in 25 mM ATP, and (c) adding trypsin, incubating at room temperature for 10 min, and then adding trypsin inhibitor. The ratio in milligrams of protein of HLF–trypsin–inhibitor was 1:3:6. ATPase activity was assayed at 0.5 μg/ml in a 1-ml reaction system containing 5 mM ATP, 5 mM $MgCl_2$, and 10 mM Tricine buffer (pH 8.5). The reaction was terminated after 10 min at 37° by 1 ml of 10% trichloroacetic acid. Inorganic phosphate was assayed by the addition of an equal volume of 0.5 N H_2SO_4 + 0.5% ammonium molybdate + 2.5% $FeSO_4$. Absorbance was read at 660 nm after 10 min at room temperature. The phosphate reagent was prepared immediately before use by mixing equal volumes of 0.5 N H_2SO_4 + 1% ammonium molybdate and 0.5 N H_2SO_4 + 5% $FeSO_4$. The latter solution was slowly oxidized and was prepared fresh weekly.

Electron Microscopy. Samples were negatively stained with 2% phosphotungstic acid at pH 7.0 or 4% ammonium molybdate at pH 7.2 and examined with a Hitachi HU-11B electron microscope operated at 75 kV. An objective aperture of 30 μ diameter was used and liquid nitrogen anticontamination devices were employed both in the specimen chamber and in the diffraction chamber.

Polyacrylamide gel electrophoresis was carried out as described by Davis (1964).

Protein was assayed as previously described (Adolfsen and Moudrianakis, 1971a). A white precipitate developed when a significant amount of KCl was present in the sample. The color developed by the standard, bovine serum albumin, was not affected by the formation of this precipitate, which was removed by centrifugation just prior to reading the absorbancy at 660 nm.

Materials. DEAE-Sephadex A-25 and QAE Sephadex A-25 were obtained from Pharmacia Fine Chemicals, Inc., and Bio-Gel A.5, Tricine and MES buffers were obtained from Calbiochem.

Results and Discussion

DEAE-Sephadex Chromatography of the Crude Coupling Factor Fraction. Four major materials were found in the elution profile of the coupling factor fraction (Figure 1, upper profile). The lower profile in Figure 1 represents

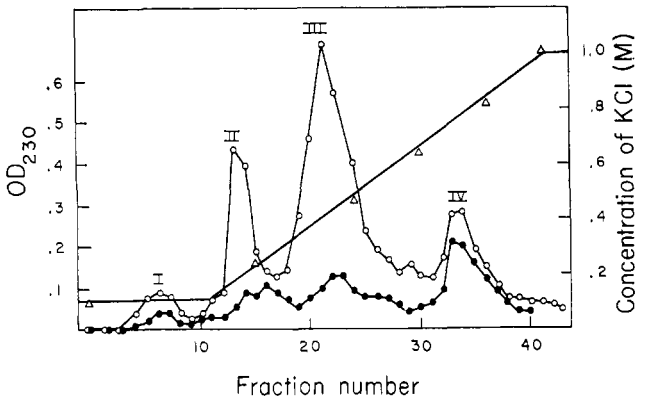

FIGURE 1: DEAE-Sephadex chromatography of the coupling factor fraction. Analytical DEAE columns were run on supernatant fractions obtained by washing native particles in sucrose in the presence or absence of KCl as described in Methods. The fraction volume was 1.2 ml. (○) Supernatant obtained by washing in absence of KCl; (●) supernatant obtained by washing in KCl-sucrose; (Δ) molarity of KCl in the gradient.

the supernatant fraction from particles washed in KCl–sucrose instead of sucrose alone. The difference between the two profiles may be taken as an approximation of the extra material eluted by washing at low ionic strength. Before determining where the coupling factors were, attempts were made to identify what each of the major peaks contained. Peaks I and II had ultraviolet spectra characteristic of proteins. The ratin of OD_{280}/OD_{260} was 1.7 for peak I and 1.6 for peak II.

Spectral examination of peak III showed cytochromes were present. DPNH oxidase activity was also found here. Electron microscopy of negatively stained preparations showed membrane fragments with an average diameter of about 500 Å. Both the oxidase activity and the cytochromes were excluded from a Bio-Gel A.5 column, suggesting that they were both in the small membrane fragments. A comparison of the difference spectra of these membranes and native or stripped particles showed no differences, making it likely that the small membrane fragments contained intact and functional electron-transport systems. The major cytochrome present was c_{552} with smaller amounts of b_{560}; no cytochrome *a* or *o* was detectable.

Peak IV was identified as nucleic acid by its ultraviolet spectrum. Comparison of the upper and lower profiles suggested it was largely contaminating material not completely removed from the particles during their preparation from the cell-free crude extract by centrifugation.

Identification of the HLF and HSF in DEAE-Sephadex Elution Profiles. Preliminary indications that both the HLF and the HSF were present in the crude coupling factor fraction and that boiling preferentially inactivated the HLF were previously reported (Adolfsen and Moudrianakis, 1971b). Boiled crude coupling factor fraction was therefore capable of serving as a source of HSF. To determine the position of elution of the HLF, stripped particles were incubated with aliquots of the boiled material and each of the fractions from a DEAE-Sephadex gradient under reconstitution conditions and then tested for their ability to catalyze oxidative phosphorylation. In Figure 2A the absorbancy profile of this experiment is shown for reference, along with a profile of protein concentration. Peak II was the major component in the coupling factor fraction. Figure 2B shows

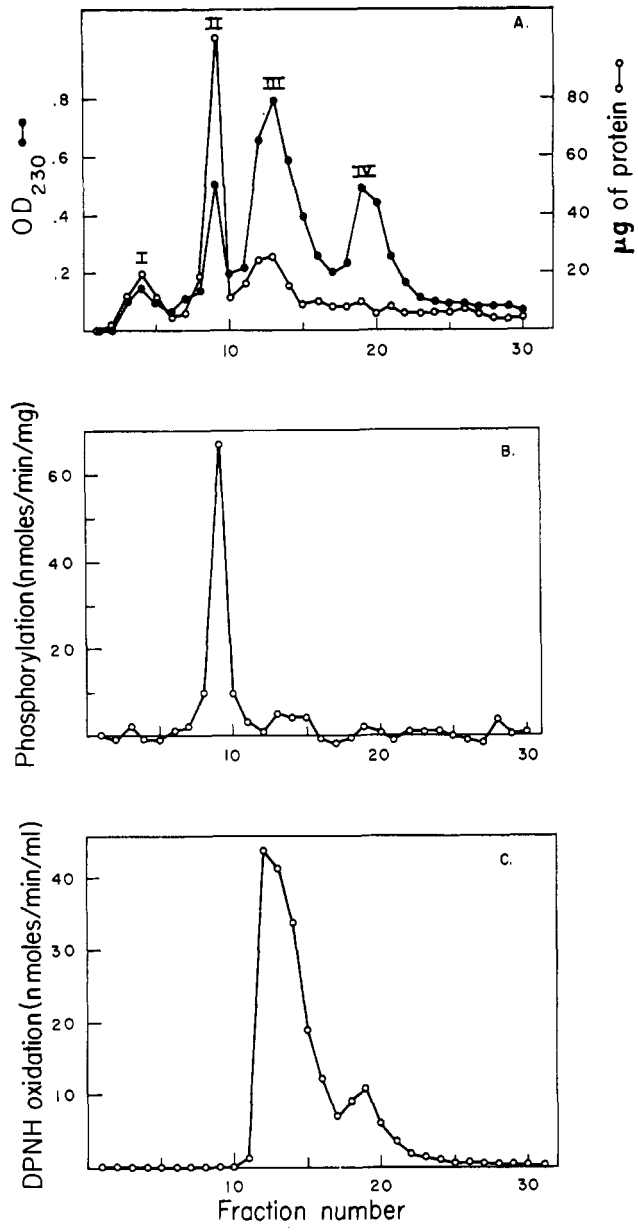

FIGURE 2: Identification of the HLF in DEAE-Sephadex elution profile. The coupling factor fraction was prepared and the KCl gradient was run as described in Methods, collecting 1.5-ml fractions. Part A shows the absorbancy and protein concentration profiles. In part B reconstitution was carried out by incubating 0.15 ml of each fraction with 0.20 ml of boiled coupling factor fraction and 0.05 ml of stripped particles in 0.1 M KCl at 30° for 15 min. The slight variation above and below the zero base line was the result of subtracting the small amount of $[^{32}P]P_i$ not extracted as a phosphomolybdate complex by isobutyl alcohol–benzene and ether. In part C DPNH oxidase activity was measured by determining the rate of decrease of absorbancy at 340 nm in a Cary 14 recording spectrophotometer. The system contained 0.80 ml of each fraction, 0.10 ml of 0.1 M Tricine buffer at pH 7.0 and 0.10 ml of DPNH in 0.01 M Tricine at pH 8.0. The reaction was begun by the addition of DPNH, which had an absorbancy of about 1.6 at the start of the reaction.

that peak II was the HLF. Figure 2C shows complete separation of the HLF and DPNH oxidase activity was achieved. The absolute requirement of reconstitution for the HLF (Figure 2B) should be noted. The assay does not involve stimulation of a residual level of activity in the particles.

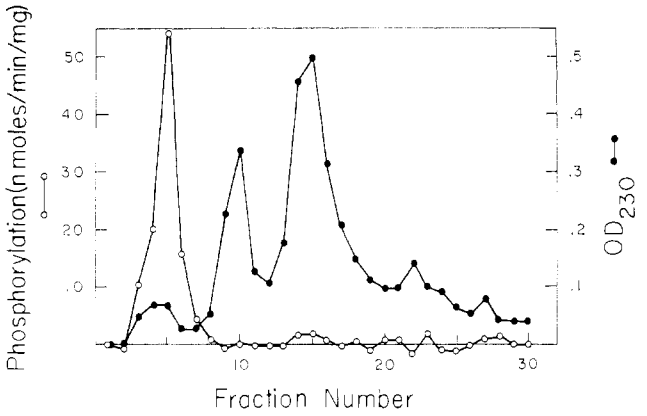

FIGURE 3: Identification of the HSF in DEAE-Sephadex elution profile. The coupling factor fraction was prepared and run on an analytical DEAE-Sephadex column as described in Methods, with one difference: The particles were washed once in KCl–sucrose before the coupling factors were washed off in sucrose alone. Reconstitution was accomplished by incubating 0.20 ml of each fraction with 0.15 of HLF purified through DEAE Sephadex and 0.05 ml of stripped particles in KCl-sucrose at 30° for 15 min.

TABLE I: Distribution of the HSF in Membranes and Supernatant Fractions of Washes of Native Phosphorylating Particles in the Presence and Absence of KCl.[a]

Boiled Supernatant from	Sp Act. of Phosphorylation
I. Native Particles[b]	82
II. Particles washed once in KCl–sucrose[c]	54
Sup 1, KCl–sucrose	16
III. Particles washed three times in sucrose alone[d]	20
Sup 1, sucrose alone	161
Sup 2, sucrose alone	39
Sup 3, sucrose alone	8

[a] HSF was prepared by boiling the various membrane and supernatant fractions for 5 min and then removing denatured protein by centrifugation. HSF activity in reconstitution was assayed as described in Methods. [b] Particles were boiled at a concentration of 4 mg/ml. [c] Particles were washed once in KCl–sucrose at a concentration of 4 mg/ml and both the supernatant and membrane fraction were boiled and tested for HSF activity. [d] Particles were washed three times in sucrose alone at a concentration of 4 mg/ml. All three supernatant fractions and the final membrane fraction were boiled and tested for HSF activity.

The location of the HSF in the elution profile was determined in a similar experiment. This time native particles were washed one extra time in KCl–sucrose before the coupling factors were washed off in sucrose alone. The purpose of the extra wash was to remove as much contaminating nucleic acid as possible, to facilitate the identification of the HSF if it were a polynucleotide as previously claimed (Pinchot, 1957; Shibko and Pinchot, 1961b). The absorbancy profile of Figure 3 shows that most of peak IV was eliminated. However, the activity profile shows that the HSF was in peak I instead of in the peak IV region. While this did not exclude the possibility that the HSF contained nucleic acid, it certainly argued against its being exclusively polynucleotide. The nature of this factor will be dealt with in more detail below.

Interaction of Coupling Factors and Membrane Fragments. After the resolution of the coupling factors on DEAE-Sephadex it was possible to estimate their individual affinities for membrane fragments at low ionic strength. The specific activity of reconstituted oxidative phosphorylation in a system containing membrane fragments, HSF, and supernatant fraction obtained by washing membrane fragments a second time at low ionic strength was 3 nmoles/min per mg of particle protein. The binding constant of the HLF was therefore very close to zero in the absence of KCl.

Table I shows the distribution of the HSF between particulate and aqueous phases of the system at high and low ionic strength. Some HSF was eluted from the particles by washing in the presence of KCl, but a much larger amount was eluted by washing in its absence. Three washes in sucrose alone were necessary to elute all of the HSF that could be eluted. About 80% was eluted in the first wash in sucrose alone, giving a binding constant of 0.2 at low ionic strength. By comparison to the previously determined binding constant of 30 in the presence of KCl (Adolfsen and Moudrianakis, 1971b) the reduction in affinity obtained by decreasing the ionic strength of the medium was about 15-fold. Another estimate of about 10-fold was obtained by comparing reconstituted phosphorylation in the two "Sup 1" fractions in Table I. It seems permissible to extend these estimates to the HLF since a reduction in its affinity (previously estimated as 10 in the presence of KCl) by this amount would give a number not experimentally distinguishable from zero.

It may be wondered how the reconstitution assay could have an absolute requirement for added HSF when only about 80% of it was eluted by one wash in sucrose in the absence of KCl. This may be understood in view of the previously reported sigmoid shape of the saturation curve of stripped particles with crude coupling factor fraction (Adolfsen and Moudrianakis, 1971b), which dictated that very low concentrations of coupling factors would not be detectable. This lack of proportionality may also have introduced some degree of error in our attempts to determine binding constants.

Characterization of the HLF. Lability was a major problem in studies on the HLF. The data in Table II show that lability during storage at −20° was lower in all systems in which sucrose was present. Lability in the absence of sucrose was increased by KCl, ATP, and MgATP and was decreased by ADP and MgADP. The same pattern was observed in the presence of sucrose except that ATP stabilized instead of destabilized the enzyme. The data also show that nucleotides were capable of increasing the level of reconstituted activity and that this phenomenon was modified by sucrose.

DEAE-Sephadex columns have been run in the presence of 0.25 M sucrose due to its stabilizing effect on the HLF. Columns run without sucrose sometimes gave an HLF that was nonfunctional in reconstitution assays. The coupling factor after the sucrose gradient step lost about 50% of its activity in one week when stored at −20°. When the gradient contained 0.25 mM ADP, the lability decreased to about 5%/week. This further stabilization of the enzyme by ADP was found to be optimal at 0.25 mM.

TABLE II: Partial Stabilization of HLF Activity in Reconstitution.[a]

Additions	Sp Act. of Phosphorylation at Time = 0		% Act. Lost after 4 Days	
	−Sucrose	+Sucrose	−Sucrose	+Sucrose
None	61	65	48	37
0.1 M KCl	61	60	82	57
1 mM ATP	81	57	93	26
1 mM MgATP	56	48	98	83
0.25 mM ADP	82	70	43	36
0.25 mM MgADP	81	71	33	31

[a] One aliquot of HLF purified through a preparative DEAE-Sephadex column was passed through a Bio-Gel A.5 column in 0.01 M Tricine buffer (pH 7.0) and another, in buffer + 0.25 M sucrose. The excluded fractions were pooled and split into six aliquots, to which the additions noted in the table were made. Reconstitution was performed as described in Methods, except that the HSF contained 0.2 M KCl. The aliquots were assayed before and after storage for 4 days at −20°. All numbers reported are averages of duplicate systems. The specific activities of phosphorylation reported are for the first day, before storage at −20° for 4 days.

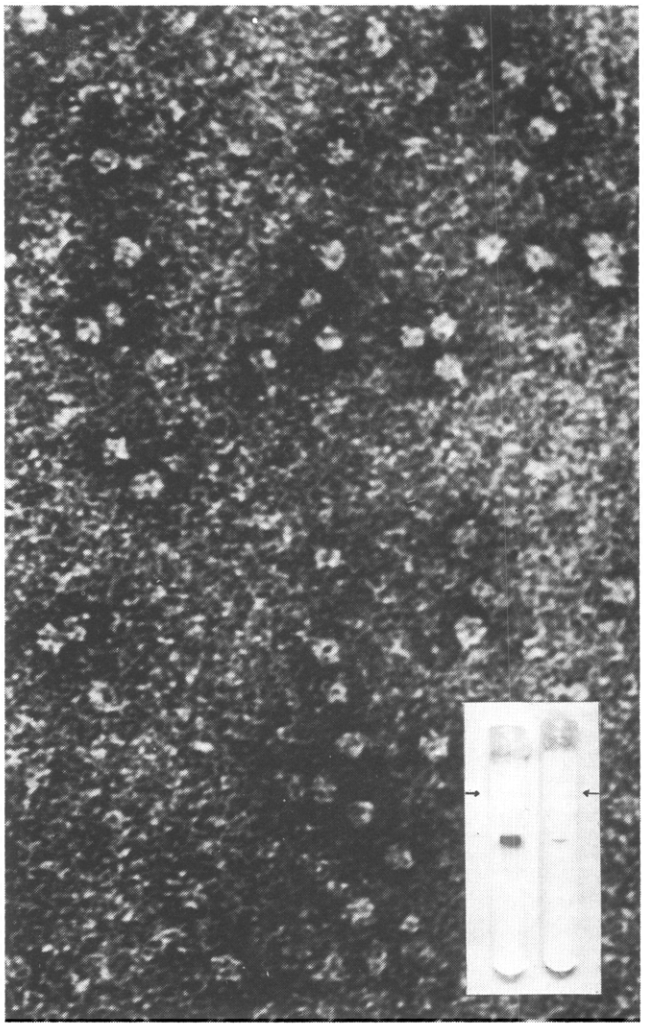

FIGURE 4: Negative staining of the HLF with 2% phosphotungstic acid. The HLF was purified through an analytical DEAE-Sephadex column and electron microscopy of a negatively stained preparation was carried out as described in Methods. Magnification was 550,000×. The inset shows the sucrose gradient purified HLF electrophorecsed with a sample of 20 μg (right) and 200 μg (left); arrows indicate origins.

The sedimentation coefficient of the HLF in the sucrose gradient was approximately 13 S. Electron microscopy of negatively stained preparations (Figure 4) showed that it was approximately 90 Å in diameter and indistinguishable from the 90-Å coupling factor of the mitochondrial system (Kagawa and Racker, 1966) and the chloroplast system (Howell and Moudrianakis, 1967). The inset in Figure 4 shows the HLF electrophoresed at low and high protein concentration. Its mobility was identical to that of the chloroplast factor.

The molecular activity of the HLF functioning in oxidative phosphorylation could be estimated from the following facts: (1) the yield of HLF from native particles was 5 mg/g; (2) its molecular weight was approximately 3×10^5, from its *s* value of 13 and by comparison with the chloroplast factor; (3) the specific activity of oxidative phosphorylation in native particles was 250 nmoles/min per mg (typical preparation). The value arrived at for the molecular activity was 15,000 moles of ATP synthesized per min per mole of HLF.

The HLF had no ATPase activity unless subjected to one of the three treatments described in Methods. The specific activities of Mg^{2+}ATPase activated by trypsin, heat treatment, and dithiothreitol were 0.9, 1.2, and 1.8 μmoles per min per mg, respectively, corresponding to molecular activities of 270, 360, and 540 moles per min per mole HLF. Ca^{2+} gave rates of hydrolysis about 70% as high as Mg^{2+}.

There was considerable suggestive evidence for the existence of more than one form of the HLF. Sucrose gradient activity profiles were not symmetrical and consistently showed a shoulder of activity sedimenting just slightly slower than the main peak of activity. The peak in the corresponding protein profiles was skewed just slightly to the left *or* to the right of the main peak in the activity profiles, suggesting different amounts of the two forms may be present in different preparations. In addition, polyacrylamide gels occassionally showed two bands migrating very close together. The second band could have been a different functional or nonfunctional form of the HLF. A completely nonfunctional HLF obtained from a DEAE-Sephadex column run in the absence of sucrose showed only one band. A functional and nonfunctional form of the analogous chloroplast latent ATPase is also known (Bennun and Racker, 1969). In preparations of the HLF in which a significant amount of activity had been lost, a number of trace bands were also observed. These were probably degradation products of the HLF, either subunits or subunit clusters. Further investigations along these lines are in progress.

Characterization of the HSF. The activity profile in Figure 4 showed that the HSF eluted in peak I, suggesting it might not be a polynucleotide as has been previously claimed. In view of this discrepancy it was considered advisable to ascertain whether the factor in peak I was really the HSF and not an entirely different coupling factor. A study of the

stability of the peak I factor from an analytical DEAE-Sephadex column (solvent 0.1 M KCl + 0.01 M Tricine (pH 8.0) + 0.25 M sucrose) showed a 15% loss of activity after 5 min at 100° and a 75% loss in half an hour. By contrast, the HLF lost 100% of its activity after only 30 sec at 100°. Since only a negligible amount of activity was lost by boiling the peak I factor for a length of time sufficient to denature most proteins (1 or 2 min), it was considered permissible to continue to refer to it as the HSF.

The heat-stability property was found to be pH dependent. When HSF from a preparative DEAE-Sephadex column (solvent 0.12 M Tris (pH 6.6) + 0.25 M sucrose) was heated for 1 min it lost 100% of its activity. The reason for the loss was the high-temperature coefficient of Tris buffer. The pH of Tris–sucrose was measured as 4.5 at 100° with an Instrumentation Laboratory No. 14093 combination electrode designed to read pH from 0 to 130°. When the pH of the HSF solution was adjusted to 9 prior to boiling and then readjusted to the original pH for the reconstitution assay, no significant loss of activity was observed.

HSF activity profiles from preparative DEAE columns showed that the major portion of the HSF did not bind to the column at all while about 10% bound weakly and eluted as a shoulder. This suggested the HSF could exist in more than one form. A complete separation of the two forms was achieved when pooled HSF fractions from analytical DEAE-Sephadex column runs were rechromatographed on QAE-Sephadex in 0.1 M KCl + 0.01 M Tricine (pH 8.5) + 0.25 M sucrose. The bulk of the active material did not bind but a smaller amount did and was eluted in a gradient from 0.1 to 0.5 M KCl. The two modes of behavior could be explained as different conformational states or as due to self-aggregation.

Efforts directed at further purification have thus far been unsuccessful due to one peculiar property. While the HSF prepared from analytical columns was completely stable for 3 months at −20°, it quickly lost activity when it was concentrated. Concentration by ultrafiltration in a collodion bag apparatus resulted in a complete loss of activity. Concentration by ammonium sulfate precipitation gave only 10–20% recovery of activity. Lyophilization also gave about 20% recovery of activity. This loss may have been the result of an irreversible aggregation and precipitation that was facilitated by higher concentrations of the HSF. Suggestive of this was the slight turbidity that developed in the material concentrated by ultrafiltration. In addition, recovery of protein applied to Bio-Gel P columns was only about 30%, suggesting that precipitation had occurred on the column.

All of the information available on the HSF at this point made the postulate that it was a heat-stable protein much moret enable than identitfying it as a nucleic acid. One such heat-stable protein coupling factor has already been reported in the *Micrococcus lysodeikticus* system (Yamashita and Ishikawa, 1965; Ishikawa, 1970). Polyacrylamide gel electrophoresis showed two major, one minor and two trace bands in peak I.

A direct attempt was made to determine whether peak I contained any nucleic acid at all. Peak I from a preparative column run was precipitated in 5% $HClO_4$, collected by centrifugation, dissolved in 1 N KOH, and incubated at 40° for 20 hr. If the HSF were a polynucleotide of the RNA type (Pinchot, 1957), it should have been hydrolyzed to 2′–3′-ribonucleotides by this treatment. Protein and any DNA that might have been present were precipitated by the addition of $HClO_4$ to a pH of less than 1. After the precipitate had been removed by centrifugation, the supernatant was neutralized with KOH and $KClO_4$ was spun down. All centrifugation steps were preceded by chilling on ice for at least 10 min. The supernatant was then applied to a Dowex 1-Cl^- column in a Pasteur pipet (bed volume 1 ml). Any 2′–3′-ribonucleotides that may have been present were eluted in 5 ml of 0.01 N HCl. No ribose was detectable in this fraction by the orcinol method performed as described by Schneider (1957). Computations based on the limit of detection indicated the entire preparation, originally containing about 15 mg of protein, contained less than 2 μg of ribose. Assuming a molecular weight for nucleotides as 350 and that of amino acids as 100, there could not have been more than 0.005 nucleotide residue/amino acid residue. Spectral examination of the material from the Dowex column showed it contained a total of about 0.5 unit of absorbancy at 260 nm. From this figure there could not have been more than 0.002 nucleotide residue/amino acid residue. The material absorbing at 260 nm amounted to about 0.1% of the total nucleic acid absorbancy of the crude coupling factor fraction, while the purified nucleic acid factor of Shibko and Pinchot (1961b) amounted to about 5% of the total nucleic acid released from phosphorylating particles by boiling. The extremely low nucleic acid content of peak I contrasted to the amount of nucleic acid in the preparations of Shibko and Pinchot strongly suggested that the HSF studied here and their polynucleotide factor were entirely different entities. Finally, it is quite possible that the small amount of nucleic acid in Peak I was the leading edge of a small nucleic acid peak eluting between the HSF and HLF in preparative column runs. The peak fractions of this material did not contain HSF activity.

With respect to the previously characterized nucleic acid factor, its stimulation of phosphorylating activity by 50–80% (Shibko and Pinchot, 1961b) may not be sufficient to warrant its being taken as a coupling factor. Synthetic nucleic acids can stimulate oxidative phosphorylation, and a stimulation of reconstituted phosphorylation by ADP and ATP was observed in Table II of this paper. A major difference between the particles studied here and those used in earlier investigations was that poor conditions for storage of the cell-free crude extract resulted in a rapid loss of oxidative and phosphorylating activities by about 90% (Adolfsen and Moudrianakis, 1971a). The possibility of atypical or artifactual events occurring in such a system must be given some consideration. If nucleic acids have any role in this system, it may be to stabilize the HLF. Shibko and Pinchot (1961b) reported that their preparations of HLF contained about 5% nucleic acid, and that its removal destroyed the activity of the HLF. The fact that ADP was found to be critical for the stability of the HLF in the present paper suggested it could substitute for polynucleotide.

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References

Adolfsen, R., and Moudrianakis, E. N. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 734 Abs.

Adolfsen, R., and Moudrianakis, E. N. (1971a), *Biochemistry 10*, 434.

Adolfsen, R., and Moudrianakis, E. N. (1971b), *Biochemistry 10*, 440.
Bennun, A., and Racker, E. (1969), *J. Biol. Chem. 234*, 1325.
Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.
Farron, F., and Racker, E. (1970), *Biochemistry 9*, 3829.
Higashi, T., Bogin, E., and Brodie, A. F. (1970), *Arch. Biochem. Biophys. 136*, 331.
Howell, S., and Moudrianakis, E. N. (1967), *Proc. Nat. Acad. Sci. U. S. 58*, 1261.
Ishikawa, I. (1970), *J. Biochem. (Tokyo) 67*, 297.
Kagawa, Y., and Racker, E. (1966), *J. Biol. Chem. 241*, 2475.
Pinchot, G. B. (1953), *J. Biol. Chem. 205*, 65.
Pinchot, G. B. (1957), *J. Biol. Chem. 229*, 1.
Schneider, W. C. (1957), *Methods Enzymol. 3*, 680.
Shibko, S., and Pinchot, G. B. (1961a), *Arch. Biochem. Biophys. 93*, 140.
Shibko, S., and Pinchot, G. B. (1961b), *Arch. Biochem. Biophys. 93*, 257.
Yamashita, S., and Ishikawa, S. (1965), *J. Biochem. (Tokyo), 57*, 232.

Purification and Kinetic Properties of Aconitate Isomerase from *Pseudomonas putida**

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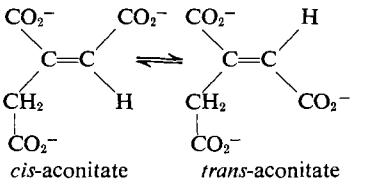
ABSTRACT: Aconitate isomerase isolated from *Pseudomonas putida* grown on *trans*-aconitate has been partially purified 45-fold to a specific activity of 116 IU/mg. The enzyme, which catalyzes the interconversion of *cis*- and *trans*-aconitate, has a maximum rate at pH 8.5 and a molecular weight approximated to be 78,000 ± 10,000 by Sephadex chromatography. In assays initiated with aconitate isomerase the initial velocity falls off rapidly with time; when the enzyme is preincubated, and assays initiated with substrate, the initial velocity is invariant with time but depressed. The addition of 20% glycerol both diminishes the rapid fall off in velocity and gives increased activity with preincubated enzyme. The relationship between enzyme concentration and activity is linear for assays initiated with enzyme in the presence of 20% glycerol, whereas preincubation of aconitate isomerase gives rise to a nonlinear relationship. The K_m of *cis*-aconitate for preincubated enzyme is found to be inversely dependent on enzyme concentration: over a 4-fold range in enzyme concentration, the value of the K_m was 1.90–1.05 mM in the presence of 20% glycerol and 25.0–9.0 mM in the absence of glycerol. The observed relationship between enzyme activity and concentration under the different assay conditions is analyzed and shown to be consistent with a dissociation of enzyme into inactive subunits ($\eta = 2$).

An inhibitor of aconitate hydratase, *trans*-aconitic acid (Anfinsen, 1955), has been found to occur in a number of plant materials (Lippmann, 1879; Beath, 1926; Roberts and Martin, 1954; MacLennon and Beevers, 1964; Burau and Stout, 1965). Rao and Altekar (1961) reported on the presence of an induced enzyme, aconitate isomerase, in a fluorescent pseudomonad grown on a medium containing *trans*-aconitate as the sole carbon source. The presence of considerable amounts of aconitate isomerase in the leaf extracts of sugar cane has also been reported (Altekar *et al.*, 1965). In the case of the bacterial enzyme, aconitate isomerase was prepared free of aconitate hydratase by ammonium sulfate precipitation and shown to catalyze the conversion of *trans*-aconitate into *cis*-aconitate. As originally reported, the assay requirements of aconitate isomerase included an SH and Fe^{2+} requirement.

Aconitate isomerase catalyzes the interconversion of reaction 1. In this paper, the first of a series of three papers, we describe the partial purification of aconitate isomerase and some of its



cis-aconitate *trans*-aconitate (1)

kinetic properties. The second paper considers the mechanism of action of this enzyme. The third paper describes the use of aconitate isomerase to determine the steric course of the electrophilic replacement reactions catalyzed by citrate synthase, ATP citrate lyase, and citrate lyase.

Experimental Section

All chemicals were obtained commercially and were reagent grade unless otherwise noted. Glycerol was spectroquality and was obtained from Matheson Coleman & Bell. Dialysis tubing (Visking) was washed in boiling 0.1 M EDTA (pH 9) and stored in 0.01 M EDTA (pH 7). Bovine serum albumin was obtained from Pentex Biochemical Co. and ribonuclease was obtained from Worthington Biochemicals.

Determinations of pH were carried out on a radiometer (Type TTTlc) equipped with an expanded-scale attachment. Routine spectrophotometric determinations were carried out on Beckman DU spectrophotometers fitted with Gilford light

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