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Reconstitution of Oxidative Phosphorylation in Submitochondrial Particles by a Soluble Protein Phosphoryl Transferase[1]

ROBERT E. BEYER[2]

Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin 53706, and the Laboratory of Chemical Biology, Department of Zoology, The University of Michigan, Ann Arbor, Michigan 48104

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A protein designated *phosphoryl transferase* has been isolated from beef heart mitochondria and has been found to increase the ability of submitochondrial particles, oxidizing either NADH or succinate, to synthesize ATP from ADP and inorganic orthophosphate. Increases in the value of P:O ratios between 0.45 and 1 have been observed with succinate as substrate. Phosphoryl transferase was released from beef heart mitochondria by sonic disruption in the presence of EDTA and was then purified by fractionation with ammonium sulfate, chromatography on DEAE-cellulose, and recycling molecular sieve chromatography on polyacrylamide gel (BioGel P-200). The isolated phosphoryl transferase displayed a single peak in the analytical ultracentrifuge and a single band in strip electrophoresis. Its molecular weight was estimated to be 124,000; its isoelectric point, pH 5.7. The absorption spectrum of the protein showed a maximum at 278–280 mμ, a minimum at 250 mμ, and a shoulder at 290 mμ. At pH 13 two distinct maxima appeared at 282 mμ and 288.5 mμ. Increases in the P:O ratios of submitochondrial particles, induced by purified phosphoryl transferase, were observed only at the site of energy conservation between reduced coenzyme Q and cytochrome *c*. The protein has also been isolated from phosphorylating submitochondrial particles. During recycling gel filtration, a protein was separated from the phosphoryl transferase which inhibited the ATPase activity of submitochondrial particles and counteracted the effect of the phosphoryl transferase in increasing the P:O ratio.

Although the phenomenon of ATP synthesis supported by electron transfer has been known since 1939 (1, 2), knowledge is scant concerning either the mechanism of oxidative phosphorylation or the identity of the catalytic components. One approach to this problem is by way of dissecting the mitochondrial system into component parts and reconstituting oxidative phosphorylation from these relatively purified components. This approach has led to the isolation and study of a class of proteins from various sources generally known as "coupling factors,"[3] which are considered to be

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[3] The term "coupling factor" has been used to describe protein preparations which, when included in the assay of the phosphorylative capacity of submitochondrial particles, enhance the efficiency of oxidative phosphorylation. The term implies that the protein factor is operating on, or influencing directly, the coupling process. Since

component catalytic units required for the terminal step of oxidative phosphorylation (3–25) and of photosynthetic phosphorylation (26–28). In the present communication the isolation and properties of one such protein factor, phosphoryl transferase, are described, together with its effect on beef heart submitochondrial particles of low phosphorylative capacity. Some of these data have been reported in preliminary communications (16, 17).

EXPERIMENTAL PROCEDURES

Preparation of particles. Unless otherwise stated, all steps in the preparation of the assay particle, ETPH(EDTA-2)[4] and of phosphoryl transferase were carried out at 0–4°. Heavy beef heart mitochondria were prepared according to the large scale procedure of Crane *et al.* (30), except that the "light-heavy" split was accomplished at pH 7.8 in a medium which was 0.25 M in sucrose, and 0.01 M in Tris-HCl (sucrose-Tris) according to Hatefi and Lester (31). The procedure employed for the preparation of ETPH(EDTA-2) was essentially that of Linnane and Titchener (7) as described by Beyer (32). The Spinco model L ultracentrifuge was used for all centrifugations. HBHM were suspended at a concentration of 10 mg of protein/ml in a solution which was 0.25 M in sucrose, 0.01 M in Tris-HCl, pH 7.5, and 1 mM in EDTA, and were sedimented at 17,000 rpm for 10 minutes. The pellet was suspended in sucrose-Tris, pH 7.6, at a concentration of 20 mg of protein/ml, stored at −20° for one week, and exposed to at least 5 freeze-thaw cycles. Immediately prior to sonic treatment, the suspension was made 2 mM in EDTA and adjusted to pH 7.5; it was then irradiated with 20 kc sound in a Branson Sonifier (Branson Ultrasonics Corporation, model LS-75) at 7–8 A for 60 seconds. The suspension was irradiated in a jacketed beaker, around which was circulated ethyleneglycol at −10°. The temperature of the suspension did not rise above 6° during sonic treatment. The pH was subsequently adjusted to 7.5 and the preparation was centrifuged at 20,000 rpm for 10 minutes. The supernatant suspension was centrifuged at 50,000 rpm for 30 minutes and the pellet was suspended in sucrose-Tris-EDTA (1 mM) medium, pH 7.5, the final volume being half the original volume before sedimentation. The suspension was then sedimented again at 50,000 rpm for 30 minutes. Suspension and sedimentation were repeated twice in sucrose-Tris, pH 7.5, and the final pellet was suspended in the same medium for immediate use, or in the preserving mixture of Linnane and Titchener (7), in 0.5-ml aliquots for storage at −20°.

Assay procedure. Oxidative phosphorylation was assayed manometrically at 30° as described in detail by Beyer (32). The assay system was 0.25 M in sucrose, 0.033 M in glucose, 6.6 mM in $MgCl_2$, 5.2 mM in P_i, and 1.67 mM in ATP, and contained 0.1 mg of crystalline hexokinase, and 1 mg of ETPH(EDTA-2) in a final volume of 3 ml. When NADH was used as substrate it was generated in a system containing 0.5 μmole of NAD, 0.3 mg of crystalline yeast ethanol dehydrogenase (100 U), 60 μmoles of ethanol, and 90 μmoles of semicarbazide in a final volume of 3 ml. Succinate was used at an initial concentration of 6.67 mM. When reduced cytochrome *c* served as substrate, the system contained 10 μmoles of ascorbate, 3 mμ moles PMS, and 2 μg of antimycin A. The Warburg vessels were kept on shaved ice until they were placed in the water bath. Fractions to be assayed were added last and reactions were initiated, following a 5-minute warm up period, by the addition of oxidizable substrate from a side arm. All assays were terminated after 20 minutes by the addition of 2 ml of 1.5 M perchloric acid. P_i was measured by the isobutanol-benzene extraction method of Martin and Doty (33) as described by Lindberg and Ernster (34), the results being corrected for controls carried out in the absence of substrate and at zero time.

Preparation of phosphoryl transferase (35).

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the coupling between energy-releasing and energy-conserving reactions of the mitochondrion is generally considered to occur at the level of the electron transfer chain, and since evidence has not been provided in favor of "coupling factors" interacting directly with electron transfer components, the term "coupling factor" would appear to be premature and inappropriate in this context. Such proteins, however, do appear to be involved in the sequence of reactions leading to the synthesis of ATP. The term "phosphoryl transferase" describes a known enzymic capability of the factor we have isolated, and as such carries no implication as to exactly how and where it is operative.

[4] Abbreviations used: ETPH(EDTA-2), electron transfer particle prepared from heavy beef heart mitochondria in the presence of EDTA, the numeral 2 referring to the second, and longer, procedure as described in (32); EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)-aminomethane; DEAE, diethylaminoethane; PMS, phenazine methosulfate; 2,4-DNP, 2,4-dinitrophenol; F_3CCP, paratrifluoromethoxycarbonylcyanide phenylhydrazone; CoQ_1, coenzyme Q_1 (ubiquinone-1); HBHM, heavy beef heart mitochondria.

HBHM were suspended at a concentration of 30 mg of protein/ml in a solution which was 0.15 M in KCl, 0.01 M in Tris-HCl, pH 7.5, and 5 mM in EDTA. A volume of 40 ml was treated with 20 kc sound at 7–8 A for 60 seconds. In a typical preparation, 12–15 gm of HBHM protein were used as starting material. The suspension was adjusted to pH 7.5 with KOH and centrifuged at 30,000 rpm for 90 minutes. The supernatant fluid (stage A) was removed carefully, and was then treated with solid ammonium sulfate to 45% saturation at 0° (25.6 gm/100 ml); the mixture was stored at −2° overnight. The suspension was clarified by centrifugation at 15,000 rpm for 10 minutes, and the clear, yellow-red supernatant solution was adjusted to 70% saturation with solid ammonium sulfate (15.6 gm/100 ml). After 30 minutes the precipitate was collected by centrifugation and dissolved in a minimal volume of 0.05 M potassium bicarbonate previously adjusted to pH 7.5 with HCl. This stage in the purification will be referred to as stage B. The stage B preparation was dialyzed for 4–6 hours against 4 liters of 5 mM Tris-sulfate, pH 7.5, with one change of dialysis fluid midway through dialysis. The small flocculent precipitate was removed by centrifugation. DEAE-substituted cellulose was prepared for chromatography by removal of fine particles in distilled water. The cellulose was washed successively with 1 N HCl, water, 1 N NaOH, water, 1 N HCl, water, and 1 M Tris-sulfate until the pH of the suspension reached 7.5. The cellulose, thus treated, was stored in the Tris-sulfate solution until ready for use. As noted by Himmelhoch and Peterson (36), DEAE-substituted celluloses from various manufacturers may differ in their properties. The most consistent resolution was obtained with BioRad Cellex-D (obtained from the California Corporation for Biochemical Research, Los Angeles, California). Columns of DEAE-cellulose (20 × 220 mm) were prepared and washed with approximately 100 ml of 5 mM Tris-sulfate, pH 7.5. Up to 1 gm of the dialyzed and freeze-dried stage B fraction, dissolved in 50 mM Tris-sulfate, pH 7.5, was passed through a column (40 × 450 mm) of Sephadex G-25M, previously equilibrated with 5 mM Tris-sulfate, pH 7.5. The lyophilized product was stable for several months when maintained at −20°. The Sephadex eluate was applied to the DEAE-cellulose column and the column was washed with 5 mM Tris-sulfate, pH 7.5, until the absorbancy of the effluent (1 cm path) at 278 mμ was below 0.1. A linear gradient containing Tris-sulfate at concentrations varying between 0.005 and 0.15 M, pH 7.5, was applied to the column. The mixing chamber and the reservoir each contained 300 ml of solution. Phosphoryl transferase was eluted when the concentration of Tris-sulfate was approximately 80 mM. The fraction at this stage of purification was designated stage C.

Further purification was achieved by multicycle chromatography on a molecular sieve column in the LKB ReCyChrom apparatus according to the techniques described by Porath and Bennich (37). Several lyophilized preparations at stage C were pooled, equilibrated with 50 mM Tris-sulfate, pH 7.5, on a Sephadex G-25M column, and applied to a 40 × 800-mm column of the polyacrylamide gel[5] P-200 previously equilibrated with the same buffer. The column was maintained between 2° and 4° with a cooling jacket. The stage C preparation was applied to the bottom of the column and the developing solvent was pumped in the same direction. Since pressure counteracted the gravity factor, compression was kept to a minimum and a good flow rate was maintained. Three passes of the stage C preparation through the column, corresponding to a total bed height of 2.4 meters, were sufficient to resolve stage C into two discrete fractions. The fraction corresponding to the major, slower moving peak induced increases in the P:O ratio of the test particle and was designated phosphoryl transferase, stage D. This protein fraction was stable for several weeks when stored at −20°, or for a period of several months when stored at −196° in 50 mM Tris-sulfate, pH 7.5.

Electrophoretic mobility on cellulose polyacetate strips (Sepraphore III) was measured in a Gelman strip electrophoresis apparatus. Protein was determined by a biuret method (38), crystalline bovine serum albumin being used as standard. The concentrations of protein in dilute solutions of the phosphoryl transferase were determined spectrophotometrically; when the absorbancy at 279 mμ (1 cm) was multiplied by 1.1 (see Fig. 4) the product had the dimensions of mg protein/ml.

Ethanol dehydrogenase and hexokinase were obtained from Worthington Biochemical Corp., and oligomycin and antimycin A from the Wisconsin Alumni Research Foundation. All other chemicals were of analytical reagent or equivalent grade. Molecular weight measurements were made by Ehrenberg's (39) method of approach to sedimentation equilibrium.

RESULTS

Purification of phosphoryl transferase. Table I contains data pertaining to the purification of phosphoryl transferase. Relative or absolute units of activity of the enzyme have not been calculated at the various stages of purification because an

[5] BioGel P-200, California Corporation for Biochemical Research, Los Angeles, California.

TABLE I

PURIFICATION OF PHOSPHORYL TRANSFERASE

Stage of purification	Volume (ml)	Protein (mg)	Protein required for saturation[a] (μg/mg)	Δ P:O[b]
HBHM	246	9470		
A	396	1410		
B	19	362	2000	+0.18
C	4[c]	10.2	60	+0.26
D	4[c]	6.4	28	+0.64

[a] The amount of phosphoryl transferase in μg protein required to induce a maximal increase in the P:O ratio of ETPH(EDTA-2).

[b] Difference between the P:O ratio obtained in the presence and absence of phosphoryl transferase in saturating amounts. The P:O ratio was determined in the presence of succinate as substrate; the control P:O ratio was 0.19.

[c] The protein was determined after concentration by lyophilization.

inhibitor appears to be present which is not removed until the final purification step is performed. An indication of degree of purification obtained at each step may be ascertained from the data on the amount of phosphoryl transferase required to obtain maximal stimulation of oxidative phosphorylation (ΔP:O). In the particular preparation represented in Table I, 28 μg of phosphoryl transferase at stage D sufficed to saturate 1 mg of ETPH(EDTA-2), whereas 60 μg of the fraction at the previous stage in the purification (stage C) was required to saturate the same amount of particle. On the basis of saturation data, the purification would be slightly over twofold. This would appear unlikely, however, in view of the fact that over 60% of the protein applied to the P-200 column was recovered. Moreover, some of the protein eluted from the P-200 column was lost during concentration and subsequent solubilization. It is also noteworthy that the amount of phosphoryl transferase at stage C required to saturate 1 mg of particle is more than twice that required for the preparation at stage D. The final step in the purification apparently separates the phosphoryl transferase from an inhibitory substance.

Figure 1 shows a typical pattern of the data obtained by gradient elution when the stage-B fraction was chromatographed on DEAE-substituted cellulose. Every third fraction was assayed for activity in increasing the P:O ratio of ETPH(EDTA-2) under the assay conditions described in EXPERIMENTAL PROCEDURES with succinate as substrate. Each fraction was tested whenever activity was demonstrable in an area of elution. In fractions 90–98 (Fig. 1), eluted by approximately 80 mM Tris-sulfate, activity was demonstrable. Active fractions were pooled and are referred to as stage-C phosphoryl transferase.

Stage C was purified further by chromatography involving ReCyChrom gel filtration (37) as described above and previously (35). Figure 2 illustrates a typical separation pattern of several combined preparations of phosphoryl transferase at stage C. Two distinct peaks were observed and bled from the column. Protein in the larger, slower moving peak increased the P:O ratio of ETPH(EDTA-2). The faster moving, minor component, on the other hand, was found to counteract the increase in P:O ratio induced by phosphoryl transferase. Details of this phenomenon are presented below. In the experiment depicted in Fig. 2 some diffusion of the peaks appears to have occurred as indicated by the spread of the peaks. This was not a consistent observation; the extent appeared to depend upon the packing and previous use of the column. Recovery of protein was usually between 65 and 80% of that applied to the ReCyChrom column. In one experiment, for example, 124 mg of phosphoryl transferase at stage C was applied and 7 mg of the fast-moving and 78 mg of the major peak were recovered. Among other parameters, recovery greatly depended upon where the bleeding valve was opened to remove protein from the column. In these experiments the valve was not opened to remove protein from the ReCyChrom column until the recorder showed a value of 95% transmission. This practice ensured a minimum of cross-contamination of protein tailing from the first into the second peak, but it also lowered the recovery.

Physical properties of phosphoryl transferase. The sedimentation characteristics of phosphoryl transferase at the C and D stages

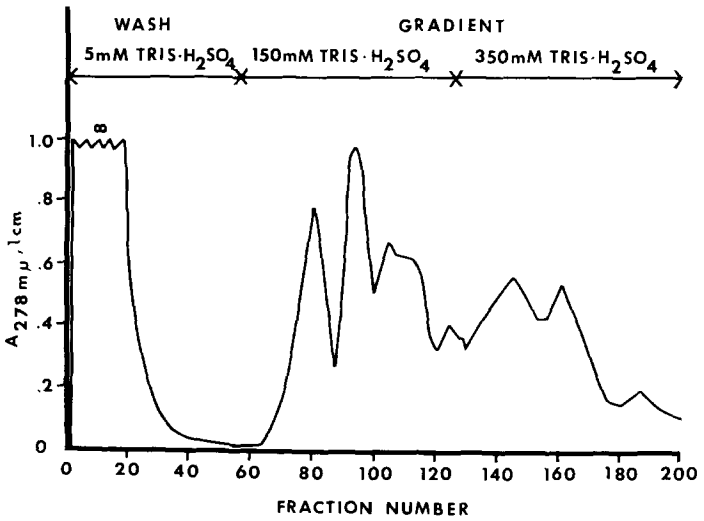

FIG. 1. Gradient elution chromatography of a preparation of phosphoryl transferase at stage B of purification on DEAE-substituted cellulose. A fresh preparation of fraction B (200 mg of protein) was applied to a column of Sephadex G-25M equilibrated with 5 mM Tris-sulfate, pH 7.5. The eluant was applied to a 20 × 220-mm column of DEAE-cellulose which had been equilibrated with 5 mM Tris-sulfate, pH 7.5. The loaded column was washed with approximately 200 ml of 5 mM Tris-sulfate, pH 7.5. Fractions of 3.5 ml each (64 drops) were collected when protein began to be eluted from the column. The column was developed at an approximate rate of 50 ml/hour. A linear gradient system was started (where indicated) by using two 500-ml chambers, the mixer chamber containing 300 ml of 5 mM Tris-sulfate and the reservoir chamber containing an equal volume of 150 mM Tris-sulfate. Where indicated, the mixer chamber was filled to 300 ml with 150 mM Tris-sulfate and a new gradient was started by using 300 ml of 350 mM Tris-sulfate in the reservoir chamber.

of purity have been observed in the Beckman model E analytical ultracentrifuge. Figure 3 contains pictures of the course of sedimentation of the protein at stage C (Fig. 3A) and at stage D in the purification (Fig. 3B). At stage C, phosphoryl transferase contained two visible components, a minor faster moving component with an $s_{20,w}$ value of 8.9S and a major slower moving component with an $s_{20,w}$ of 6S. After gel filtration on BioGel P-200 (Fig. 3B) the phosphoryl transferase appeared to be homogeneous and sedimented with an $s_{20,w}$ value of 6.1. This value corresponds to that of the major component of the preparation at stage C. From the data for the approach to sedimentation equilibrium at the meniscus for seven periods during the run, a mean molecular weight of 124,000 was computed.

The activity of phosphoryl transferase was destroyed by exposure to 65° for 2 minutes. The protein was nondialyzable and, in addition, was eluted from Sephadex G-25M with an R_F of 1. The absorption spectrum of phosphoryl transferase in the ultraviolet region is typical of many proteins (Fig. 4). Since the shoulder at 290 mμ suggested the presence of ionizable tyrosine groups, the protein was treated with alkali to observe the nature of the 290 mμ contribution. The spectrum after addition of KOH to a final pH > 13 showed a new minimum at 269 mμ, a loss of absorptivity at 278 mμ, and two distinct peaks at 282 and 288.5 mμ, respectively. The difference spectrum between the spectra of the enzyme at pH values 13 and 7.5 revealed a trough at 274 mμ and a peak at 295 mμ.

Figure 5 shows diagrammatically the direction of electrophoretic migration of the enzyme at three pH values. The protein migrated toward the cathode at pH 4.5 (I = 0.05). The protein remained at the point of application when the pH of the medium was 5.7. Only one band was noted on strips to which the enzyme at stage D

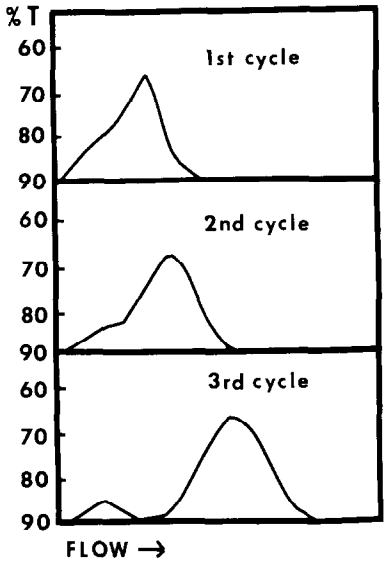

FIG. 2. Chromatography of phosphoryl transferase, stage C, on BioGel in the ReCyChrom apparatus. Six ml of a solution containing 80.4 mg of phosphoryl transferase protein at stage C was applied to a BioGel P-200 column previously equilibrated with degassed 50 mM Tris-sulfate, pH 7.5. The rate of flow was 28 ml/hour. Each cycle required approximately 16–18 hours. Elution (from left to right) indicated that the minor peak traveled faster than the major peak, which contained the phosphoryl transferase.

was applied and electrophoresed at various pH values between 4 and 8. However, when preparations of the transferase at the C stage of purification were examined at pH 4.5, a minor component was found near the origin. The electrophoretic mobility of the minor component has not been studied in detail.

Phosphoryl transferase and oxidative phosphorylation. Table III shows the increment in the value of P:O induced by the purified transferase under various conditions that define the site of energy conservation affected by the protein. It is interesting to note that at no stage in the preparation did the addition of the active fraction increase the phosphorylative capacity of ETPH (EDTA-2) at the third phosphorylation site. The adequacy of the assay system we have used for measuring ATP synthesis at the terminal site of oxidative phosphorylation has been documented previously (32) for submitochondrial particles capable of phosphorylation at the third phosphorylation site. The P:O ratios of the unsupplemented test particle with NADH as substrate ranged from 0.3 to 0.7 depending on the preparation used. Phosphoryl transferase at stage B increased the P:O ratio obtaining with either NADH or succinate as substrate, the increase with NADH being about twice as large as that with succinate. Although this difference between the two substrates was not uniform, it was observed in about 80% of the experiments. The enzyme fraction, after purification on DEAE-substituted cellulose (stage C), increased the P:O ratio in the presence of succinate to a greater extent than did the fraction at stage B. However, no such difference was observed between the two fractions with respect to the increase in P:O induced in the presence of NADH as substrate. After the phosphoryl transferase was further purified on BioGel P-200 the protein induced the same increase in P:O ratios whether succinate or NADH were used as substrate and the increase in P:O per milligram of transferase protein was greater than theory on the basis of protein purification. As stated above, only some 10% of contaminating protein was eliminated by this process. The curves of Fig. 6, showing the saturation of phosphoryl transferase with respect to the test particle, indicate that the enzyme thus purified was three times as active, and the total increase in P:O induced by the preparation at stage-D purification was substantially higher than that induced by the enzyme at the earlier stage of purification.

Esterification of P_i in the presence of the transferase was inhibited by oligomycin, 2,4-DNP, and F_3CCP; the respiration was inhibited by antimycin A. Bovine serum albumin, which has been shown to counteract the effect of uncouplers of oxidative phosphorylation (40), did not increase significantly the P:O ratio of the test submitochondrial particle.

The apparent loss of the ability to stimulate phosphorylation at the site of energy conservation between NADH and coenzyme Q consequent on the further purification of the stage-B fraction was examined

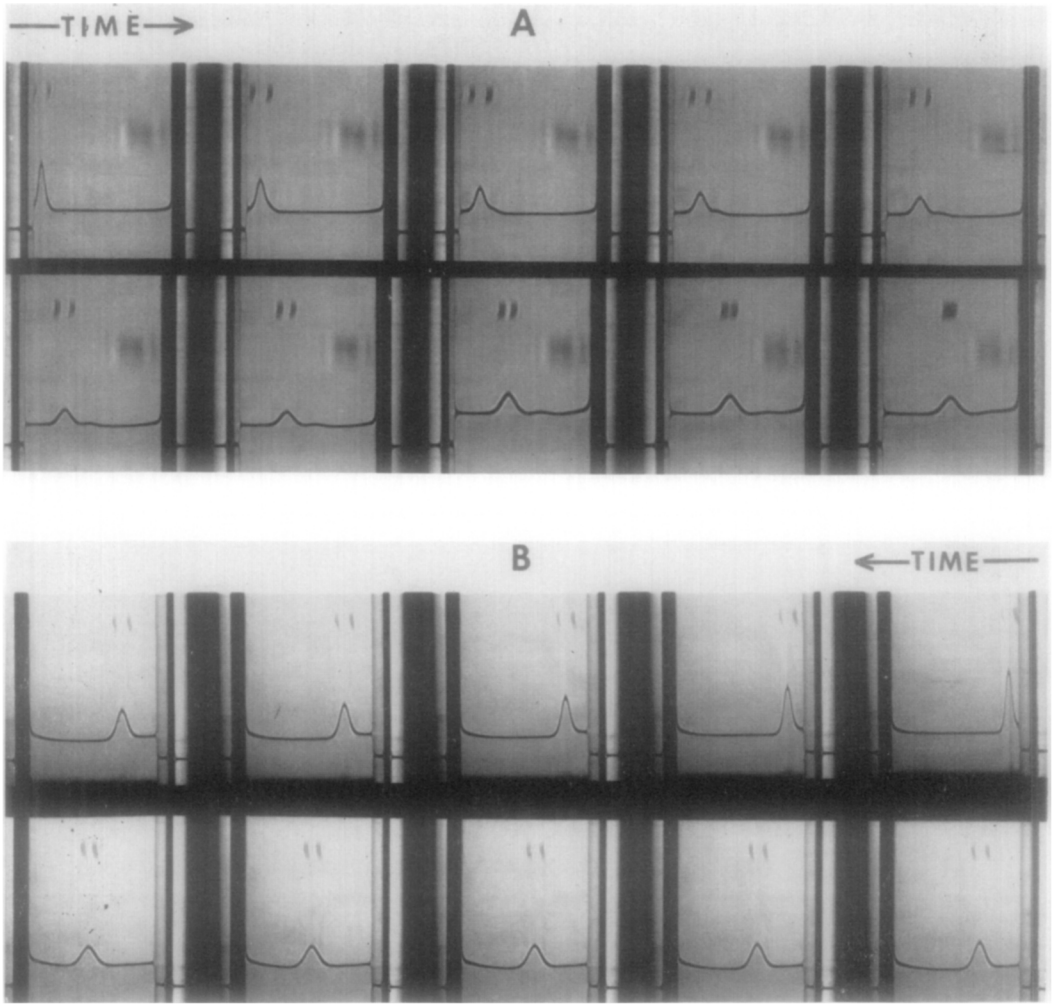

FIG. 3. Analytical ultracentrifugation of phosphoryl transferase. Figure 3A (read from left to right) represents stage C of a preparation containing 5.1 mg of protein/ml. Figure 3B (read from right to left) represents stage D of a preparation containing 6.8 mg of protein/ml. The conditions were as follows: the medium, 0.1 M Tris-sulfate, pH 7.5; temperature, 8.3°; speed, 59,780 rpm; picture interval, 8 minutes.

further in the assay system of Schatz and Racker (41), which is specific for site I. In confirmation of Schatz and Racker, "P:Q" ratios lying between 0.6 and 0.7 have been observed for the oxidation of NADH by coenzyme Q_1 catalyzed by ETPH(Mg^{++}, Mn^{++}) (Table V). The P:O ratio of the same preparation for the oxidation of NADH by molecular oxygen was 2.33. F_3CCP abolished the esterification of P_i coupled to the oxidation of NADH by coenzyme Q_1. The B fraction of the phosphoryl transferase preparation increased the P:Q ratio in the assay system of Schatz and Racker, but the purified enzyme (at the C or D stage of purity) was not active in this respect.

Isolation of phosphoryl transferase from ETPH(Mg^{++}, Mn^{++}). To provide information on the localization of phosphoryl transferase in the mitochondrion, the isolation procedure was applied to the preparation of the transferase from ETPH(Mg^{++}, Mn^{++}). A number of large batches of this type of ETPH were prepared over a period

TABLE II

MOLECULAR WEIGHT OF PHOSPHORYL TRANSFERASE COMPUTED FROM DATA FOR THE APPROACH TO SEDIMENTATION EQUILIBRIUM

Time (minutes)	MW × 10^5
97	1.27
113	1.26
129	1.20
145	1.23
161	1.29
177	1.25
193	1.16
	Mean 1.24

of several weeks and stored at −20°. ETPH in the amount of 14.63 gm was used as the starting material for the preparation. The procedure followed was identical with that for the preparation of the transferase from HBHM. The soluble supernatant fraction obtained after the centrifugation of the sonic-treated suspension (stage A) contained 419 mg of protein, equivalent to 2.86% of the starting material. The peak eluted from DEAE-substituted cellulose contained approximately 23 mg of protein or about 0.16% of the initial ETPH protein. This yield was somewhat higher than the 0.1% average yield of phosphoryl transferase from HBHM.

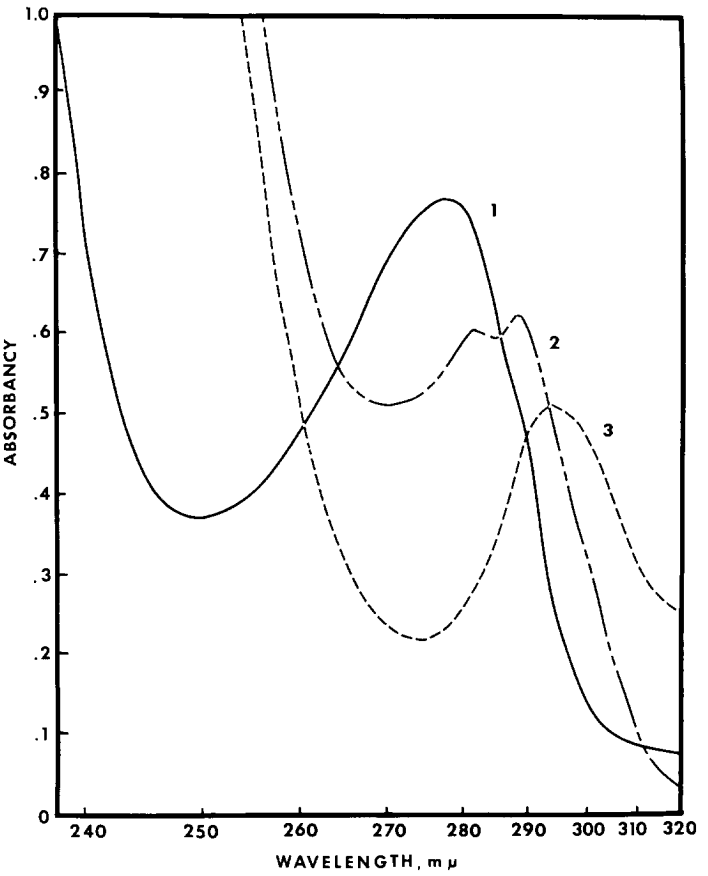

FIG. 4. Spectral shift of phosphoryl transferase (stage D) at high pH. Spectra were recorded in a Beckman DK-2 spectrophotometer, path length, 1 cm. A solution of Tris-sulfate (0.7 ml of 50 mM, pH 7.5), containing 0.71 mg of protein/ml was placed in a 1-ml cuvette, and the direct spectrum was recorded (——). Alkali (0.1 ml of 6 N KOH) was added and the direct spectrum was recorded once more (— — — —). The difference spectrum (alkaline spectrum minus the spectrum at pH 7.5) was normalized to allow for differences in protein concentration (- - -).

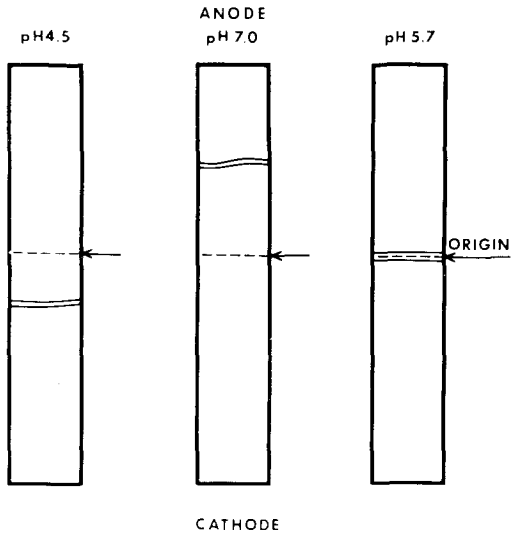

FIG. 5. Electrophoretic mobility of phosphoryl transferase (stage I). Mobility was measured in a Gelman strip electrophoresis apparatus at 2°, 190 V (11.7 V/cm) on Seprephore III cellulose polyacetate strips (2.4 × 16.3 cm) in buffers of ionic strength (I)0.05. Approximately 0.05 ml of a solution containing between 3 and 5 mg of protein/ml was applied to the strip with a Gelman applicator. The time of the runs was either 4 or 8 hours. Caffeine was used to correct for endosmosis. The strips were fixed and stained with 0.5% Ponceau S in 5% trichloroacetic acid and washed in 5% acetic acid. The buffer for the run at pH 4.5 was acetic acid-sodium acetate, and the time was 4 hours. The buffers for the runs at pH 7 and 5.7, respectively, were appropriate mixtures of cacodylic acid and sodium cacodylate. The time of the electrophoresis was 8 hours.

The purified transferase isolated from ETPH(Mg^{++}, Mn^{++}) improved the P:O ratio of ETPH(EDTA-2) with succinate as substrate, despite the fact that the test particles showed a relatively high residual phosphorylating capacity (Table VI). However, the particles from which the transferase had been extracted during its preparation from ETPH(Mg^{++}, Mn^{++}) were not stimulated with respect to coupled phosphorylation by the addition of the transferase. The rates of phosphorylation and of oxidation of succinate were both increased to approximately equal extents by the addition of transferase. Hence, the P:O ratio remained unchanged.

Effect of inhibitor on ATPase and P:O. The phosphoryl transferase after elution from the BioGel P-200 column was more active per milligram of protein than could have been expected on the basis of the proportion of inactive protein removed. Apparently, the faster moving component which was removed by gel filtration counteracted the enhancing effect of the transferase on the P:O ratio. We were led to this interpretation by the demonstration by Pullman and Monroy (42) of a factor in beef heart mitochondria which inhibits the ATPase activity of F_1 [the ATPase isolated from beef heart mitochondria by Pullman *et al.* (8)]. We tested the effect of the faster moving protein fraction on the ATPase activity catalyzed by ETPH(Mg^{++}, Mn^{++}) and found it to be inhibitory (Fig. 7). Inhibition was directly proportional to the concentration of the inhibitor protein up to 10 μg/ml, the maximal inhibition attained being 91% of that of the control activity. A greater extent of inhibition of the particle-catalyzed ATPase activity was not observed in any of the several experiments of this type which were performed.

The inhibitor fraction also interfered with the capacity of the transferase to increase the P:O ratio of ETPH(EDTA-2) (Table VII). Two other observations are of interest. The inhibitor protein saturated the phosphorylating system at lower concentrations than those required for the maximal effect of phosphoryl transferase. Even at saturating levels of the inhibitor some enhancement of the P:O ratio induced by the transferase was demonstrable. This would suggest competition between the inhibitor and the transferase for the same site, and also would argue against the possibility of the inhibitor acting as an uncoupler of oxidative phosphorylation. The fact that the inhibitor protein did not increase the state-4 respiration of intact HBHM with pyruvate as substrate may be interpreted as additional evidence that the inhibitor is not an uncoupler. The inhibitor protein also did not significantly effect the respiratory control ratio of HBHM, which was routinely between 4 and 6. However, the lack of effect of the inhibitor protein on intact beef heart mitochondria may be due to a lack of per-

TABLE III

INCREASE IN P:O INDUCED BY PHOSPHORYL TRANSFERASE

Substrates were placed in the side arm of Warburg flasks, and all other components in the main compartment. The substrate was tipped into the main compartment; after 5 minutes of thermal equilibration oxygen consumption was measured for 30 minutes. All other conditions and concentrations of assay components were those described in the text.

Substrate	Transferase added		Oxygen uptake (mμatoms/mg prot.[a]/min)	P_i uptake (mμmoles/mg prot.[a]/min)	P:O	Δ P:O
	Stage	Amount (mg)				
NADH	—	—	453	131	0.29	—
	B	1.72	424	233	0.55	+.26
	C	0.10	433	234	0.54	+.25
	D	0.067	429	399	0.93	+.64
Succinate	—	—	443	93	0.21	—
	B	1.72	447	148	0.33	+.12
	C	0.10	439	197	0.45	+.24
	D	0.067	455	377	0.83	+.62
Reduced cyt. *c*	—	—	139	13	0.09	—
	B	1.72	155	8	0.05	−.04
	C	0.10	146	12	0.08	−.01
	D	0.067	132	12	0.08	−.01

[a] Refers to protein of ETPH(EDTA 2) only.

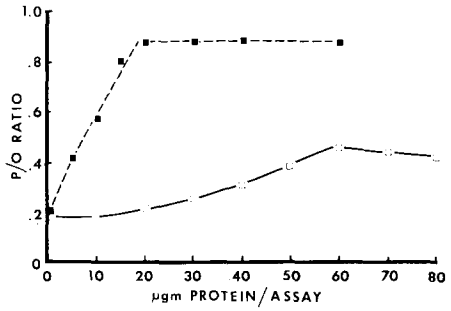

FIG. 6. Saturation of ETPH(EDTA-2) with phosphoryl transferase respectively at stage C (——) and stage D (---). The P:O ratios were determined as described in the text. The units plotted on the abscissa are μg of transferase per assay; 1 mg of ETPH(EDTA-2) protein was present in each assay mixture. The duration of the experiment was 20 minutes. Succinate was used as substrate.

meability of the limiting mitochondrial membrane to the inhibitor protein.

DISCUSSION

A protein (phosphoryl transferase) has been isolated from beef heart mitochondria which appears to play a catalytic role in oxidative phosphorylation. The transferase increases the phosphorylative capacity of submitochondrial particles prepared under specific conditions. The fact that the increase in P:O ratio induced by the transferase is abolished by uncouplers and inhibitors of oxidative phosphorylation and by inhibitors of the electron transfer process

TABLE IV

RESPONSE OF RECONSTITUTED SYSTEM TO UNCOUPLING AGENTS AND INHIBITORS OF ELECTRON TRANSFER

Assays were performed as described in Table III and in the text. The substrate used was succinate. The concentrations used of the following additives were: 2,4-DNP, 5×10^{-5} M; F_3CCP, 5×10^{-7} M; oligomycin, 1 μg/mg of protein; antimycin A, 0.4 μg/mg of protein.

Additions		O_2 uptake (mμatoms/mg prot./min)	P_i uptake (mμmoles/mg prot./min)	P:O
Transferase (μg)	Uncoupler or inhibitor			
—	—	432	82	0.19
40	—	446	343	0.77
40	2,4-DNP	441	4	<0.01
40	F_3CCP	433	3	<0.01
40	Oligomycin	427	8	0.02
40	Antimycin A	<30	<3	<0.01
Bovine serum albumin (2 mg)		441	93	0.21

TABLE V

EFFECT OF PHOSPHORYL TRANSFERASE ON THE P:Q RATIO OF ETPH(EDTA-2)

Assays were performed as described by Schatz and Racker (40) at 30° in a temperature-controlled Beckman DU-2 spectrophotometer in silica cuvettes with a 1-cm light path. The final concentrations of the components in 1 ml were: $MgCl_2$, 2 mM; EDTA, 0.48 mM; Tris-sulfate, pH 7.4, 5 mM; glucose, 32 mM; ATP, 1 mM; hexokinase, 10 units; crystalline bovine serum albumin, 2 mg; $^{32}P_i$, pH 7.4 (3.2×10^5 cpm/μmole), 10 mM; KCN (in 0.1 M Tris-sulfate, pH 7.4), 1.6 mM; CoQ_1, 0.12 mM; sucrose, 0.25 M; ETPH protein, 200 μg. Reactions were initiated by the addition of 0.08 μmole of NADH with rapid stirring. The absorbancy was followed at 340 mμ until the NADH was exhausted. ETPH(Mg^{++}, Mn^{++}) was prepared as previously described (32).

Particle	Additions	G-6-^{32}P formed (mμmoles)	Glu-6-P[a] / NADH
ETPH(Mg^{++}, Mn^{++})[b]	—	53	0.66
ETPH(Mg^{++}, Mn^{++})	F_3CCP, 5×10^{-7} M	3	0.04
ETPH(EDTA-2)	—	14	0.18
	1.5 mg stage-B transf.	27	0.34
	0.10 mg stage-C transf.	12	0.15
	0.06 mg stage-D transf.	15	0.19

[a] The G-6-P:NADH ratio was considered to be equivalent to the P:O or P:Q ratio in the NADH-CoQ span. The value used for the NADH utilized was the amount of NADH added, 80 mμmoles.

[b] The P:O ratio observed for this particulate preparation, with NADH as substrate and oxygen as oxidant, was 2.33.

TABLE VI

ACTIVITY OF PHOSPHORYL TRANSFERASE ISOLATED FROM ETPH(Mg^{++}, Mn^{++})

Assays were performed as described in the text with succinate as substrate. ETPH(Me^{++}, son.) refers to the particles recovered after sonic treatment of ETPH(Mg^{++}, Mn^{++}) in the presence of a medium which was 0.15 M in KCl, 5 mM in EDTA, and 0.01 M in Tris-chloride, pH 7.5. Each flask contained 1 mg of particle protein in a total volume of 3 ml. The experiment was terminated after 30 minutes.

Assay particle	Transferase (μg)	P_i uptake (mμmoles/mg prot./min)	P:O	ΔP:O
ETPH(EDTA-2)	0	82	0.50	—
	68	138	1.03	+0.53
ETPH(Me^{++}, son.)	0	51	0.22	—
	68	89	0.28	+0.06

indicates that the effect is referable to the normal coupling mechanism. Phosphoryl transferase has been isolated both from mitochondria and from submitochondrial particles capable of efficient oxidative phosphorylation. Thus it appears to be a component associated with the inner mitochondrial membrane, the membrane which contains the assembly of enzymes required to synthesize ATP (see 43).

The physical measurements of phosphoryl transferase not only provide biochemical data on the nature of the protein but also permit a comparison of this protein with a number of other factors reported to affect increases in phosphorylation. The molecular weight of phosphoryl transferase is approximately 124,000, whereas that of the ATPase (F_1) of Pullman *et al.* (8, 44) is reported to be 284,000. Additional differences between the two proteins have emerged from experiments (with Dr. G. Schatz) which indicate that phosphoryl transferase is not capable of hydrolyzing ATP under conditions in which ATP is hydrolyzed by F_1. In addition, the same experiments demonstrated that conditions which do not affect the ATPase activity of F_1 (65°, 2 minutes) inactivate phosphoryl transferase. The transferase appears to differ in several respects from the factor isolated by Sanadi *et al.* (10, 15, 18), despite the similarity of the procedures by which the two proteins are isolated. Sanadi's factor A, like F_1, shows cold-labile ATPase activity. However, the capability of Sanadi's factor A for improving the P:O ratio of

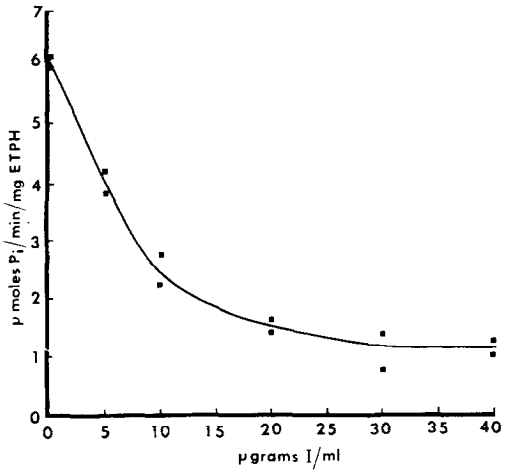

FIG. 7. The effect of inhibitor protein on the ATPase activity of ETPH(Mg^{++}, Mn^{++}). Each assay tube, in a total volume of 1 ml, contained: 5 μmoles of $MgCl_2$, 0.1 μmole of 2,4-DNP, 0.1 mg of particle protein, and 10 μmoles of ATP. The reaction was initiated by the addition of ATP. The tubes were incubated for 10 minutes at 30°. The P_i released from ATP was measured by the colorimetric method described by Lindberg and Ernster (34) and corrected for a control value in the absence of enzyme.

TABLE VII

INHIBITION BY FRACTION I OF THE ENHANCING EFFECT OF PHOSPHORYL TRANSFERASE ON THE P:O RATIO

Succinate was used as substrate; the duration of assay, 20 minutes; the temperature, 30°; the assay particle, ETPH(EDTA-2). Phosphoryl transferase at stage C was chromatographed on BioGel P-200 in the ReCyChrom apparatus, and two distinct fractions were eluted. The faster moving, minor component is referred to as fraction I, and the slower moving, major component as phosphoryl transferase stage D. Other conditions and components were those described in the text.

Additions		P_i uptake (μmoles/assay)	P:O	ΔP:O
Transferase at stage D (μg)	Inhibitor fraction I (μg)			
—	—	1.40	0.17	—
50	—	6.94	0.81	+0.64
50	5	5.61	0.70	+0.53
50	10	3.02	0.38	+0.21
50	20	3.29	0.39	+0.22

treated particles is not cold-labile, and, unlike purified phosphoryl transferase, Sanadi's factor A increases the rate of the ATP-energized reduction of NAD by succinate in extracted submitochondrial particles. Crude phosphoryl transferase, at stage B, stimulates reversed electron transfer catalyzed by ETPH(EDTA-2), but the ability to stimulate this reversal is lost on further purification of the transferase. It is interesting to note that fraction c of Linnane and Titchener (7) also increases the rate of ATP-energized reduction of NAD by succinate, catalyzed by ETPH(EDTA-2). Sone and Hagihara (21) have reported the isolation of a factor which enhances P:O ratios at phorphorylation sites 1 and 2, but not at site 3; it shows no detectable ATPase activity. This latter factor clearly differs from phosphoryl transferase in that its molecular weight is of the order of 200,000 or higher (excluded from Sephadex G-200), and it stimulates the ATP-driven reduction of NAD by succinate. Finally, phosphoryl transferase catalyzes an exchange between ATP and ADP (45) and is phosphorylated during oxidative phosphorylation (46), two properties which clearly distinguish it from the several factors enumerated above. These two properties will be the subject of forthcoming manuscripts.

The appearance of a peak at 2950 Å in proteins at pH 13 is indicative of the emergence of ionized tyrosyl groups and is usually a token of protein denaturation (47, 48). Phosphoryl transferase shares this property with a group of proteins which includes ovalbumin, ribonuclease, lysozyme, insulin, and catalase (cf. 47 and 48).

The data presented herein, as well as those presented previously in preliminary form (45, 46), indicate that phosphoryl transferase is involved either in the transfer within the inner membrane of a phosphoryl group from some phosphoryl donor to ADP, or in the transfer of a phosphoryl group from internal ATP to external ADP. This interpretation is consistent with the findings that phosphoryl transferase catalyzes an exchange between ATP and ADP (45), and that the enzyme forms a phosphorylated intermediate that can phos-

phorylate ADP with formation of ATP (46).

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