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Isolation and Reactions of a Phosphorylated Form of Phosphoryl Transferase from Beef Heart Mitochondria[1]

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Phosphoryl transferase, a mitochondrial protein which increases the phosphorylative capacity of poorly phosphorylating submitochondrial particles and catalyzes an ATP–ADP exchange reaction, is phosphorylated during oxidation either of succinate or pyruvate–malate. Inhibitors of oxidative phosphorylation and electron transfer, as well as uncouplers of oxidative phosphorylation, inhibit the phosphorylation of the transferase when phosphorylation is mediated by electron transfer. The protein is also phosphorylated by ATP, the donor group being specifically the terminal phosphate of ATP. The transphosphorylation reaction is not inhibited by inhibitors of electron transfer and coupled phosphorylation, nor by uncouplers of oxidative phosphorylation. The phosphoryl form of the transferase can phosphorylate ADP in the presence of hexokinase, glucose, and magnesium ion, but the transfer is only 50% complete. During this transfer reaction a portion of the protein-bound phosphate becomes transformed to an acid-stable form. Phosphorus is released from phosphoryl transferase as inorganic orthophosphate at pH 4 and 10 and by heat, but is relatively stable at pH 7.5 at 0°. Hydroxylamine also induces release of protein-bound phosphorus as inorganic phosphate. The possible role of the phosphoryl group of the transferase in oxidative phosphorylation is discussed.

In previous reports from this laboratory the isolation of a protein, phosphoryl transferase, from HBHM[3] and ETPH (Mg^{2+}, Mn^{2+}) has been described. Phosphoryl transferase increased the phosphorylative capacity of poorly phosphorylating submitochondrial particles, ETPH(EDTA-2), and appeared to function in one of the transfer steps in oxidative phosphorylation (1–3). This report describes the phosphoryl transferring reactions catalyzed by the transferase and the isolation of the enzyme in a phosphorylated form. The transfer reaction appears to involve formation of the phosphoryl enzyme as an intermediate. Some

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[3] Abbreviations used: HBHM, heavy beef heart mitochondria; ETPH (Mg^{2+}, Mn^{2+}), phosphorylating submitochondrial particles from sonic extracts of HBHM; ETPH(EDTA-2), poorly phosphorylating submitochondrial particles from sonic extracts of HBHM; Dpm, disintegrations per minute; DEAE, diethylaminoethane; G-6-P, glucose 6-phosphate; EDTA, ethylenediaminetetraacetate; DNP, 2,4-dinitrophenol; F_3CCP, *p*-trifluoromethoxycarbonyl cyanidephenylhydrazone.

of the experiments here reported have been the subject of a preliminary report (4).

METHODS

Submitochondrial particles, HBHM, and phosphoryl transferase were prepared as described previously (3). Carbamyl phosphate (^{32}P-labeled) was synthesized according to a modification of the method of Metzenberg *et al.* (5) and was used as a substrate to synthesize $\gamma(^{32}P)$ATP according to a modification[4] of the method of Hokin and Hokin (6) that depends on carbamyl phosphokinase as the catalyst for transfer of phosphate from carbamylphosphate to ADP. Generous quantities of this enzyme, free of adenylate kinase, were supplied by Dr. Margaret Marshall. $\beta,\gamma(^{32}P)$ATP was obtained by using AMP as phosphate acceptor during oxidative phosphorylation catalyzed by HBHM in the presence of $^{32}P_i$ with pyruvate–malate as substrate. Labeled ATP was purified from the reaction mixture by chromatography on DEAE-substituted cellulose and gradient elution with triethylammonium bicarbonate according to Smith and Khorana (7). Identification and purity of the products was established by paper chromatography in the Pabst system I (8). The amounts of P_i and G-6-P were determined according to the isobutanolbenzene extraction method as described by Lindberg and Ernster (9). Occasionally, G-6-P was also determined directly by the chromatographic method of Bandurski and Axelrod (10), the authentic phosphate ester serving as carrier. Radioactive phosphorus was counted in a thin-window, gas-flow counter or a low-background (less than 2 counts/minute) thin-window, gas-flow counter. Protein was estimated by a biuret procedure (11). All chemicals were of analytical reagent grade or equivalent.

Reagent sources were $\alpha(^{32}P)$ATP, International Chemical and Nuclear Corp.; oligomycin, Wisconsin Alumnae Research Foundation; atractylate, kindly provided by Dr. Renato Santi; and F_3CCP, kindly provided by Dr. Peter Heytler.

RESULTS

Chromatography of phosphorylated mitochondrial protein. Preliminary experiments had indicated that mitochondrial proteins, after solubilization, could be phosphorylated when incubated with particles undergoing oxidative phosphorylation, and that a portion of the phosphorus in the protein fraction was released when the proteins were exposed to 0.1 N acid at 100° for 10 minutes. After separation of the soluble proteins from the particles, the extract was fractionated with ammonium sulfate before chromatography on DEAE-cellulose; the various fractions were examined for radioactivity (Fig. 1). The method of protein fractionation was that employed previously (2, 3) to isolate phosphoryl transferase. Prior to chromatography on DEAE-cellulose, the ammonium sulfate fraction was divided into two equal parts. One part was treated with ADP, Mg^{2+}, hexokinase, and glucose in an effort to determine whether phosphoryl-protein was present and capable of phosphorylating ADP; the other part was not so treated.

The elution pattern of protein shown in Fig. 1 was typical of this step in the purification of phosphoryl transferase (3). The peak, eluted at approximately 90 ml, which in the routine preparation of the enzyme contains phosphoryl transferase (2, 3), also contained considerable radioactive phosphorus in the control sample. Prior treatment of the crude ammonium sulfate fraction with ADP, Mg^{2+}, hexokinase, and glucose resulted in an 87% reduction of the radioactivity of this peak and the appearance of considerable radioactivity in a peak appearing at 195 ml in part A of Fig. 1. Paper chromatographic analysis of this latter peak indicated that the radioactivity co-chromatographed with authentic G-6-P. This result suggested that this product of the reaction was adsorbed to protein during the final Sephadex treatment. The peak eluted at 200 ml in the control fraction (Fig. 1, part B) did not co-chromatograph with G-6-P. This product has not been identified. It is also of considerable interest that the amount of radioactivity in the wash (eluted between 0 and 20 ml) was greatly diminished as a result of treatment with ADP, Mg^{2+}, glucose, and hexokinase. Also notable was the fact that the protein peak corresponding to phosphoryl transferase and the radioactivity peak (80–95 ml) did not coincide; the radioactive compound was somewhat retarded during elution, a not surprising result if a portion of the protein contained in that peak was in a phospho-

[4] A. Worcel and L. Hokin, unpublished procedure.

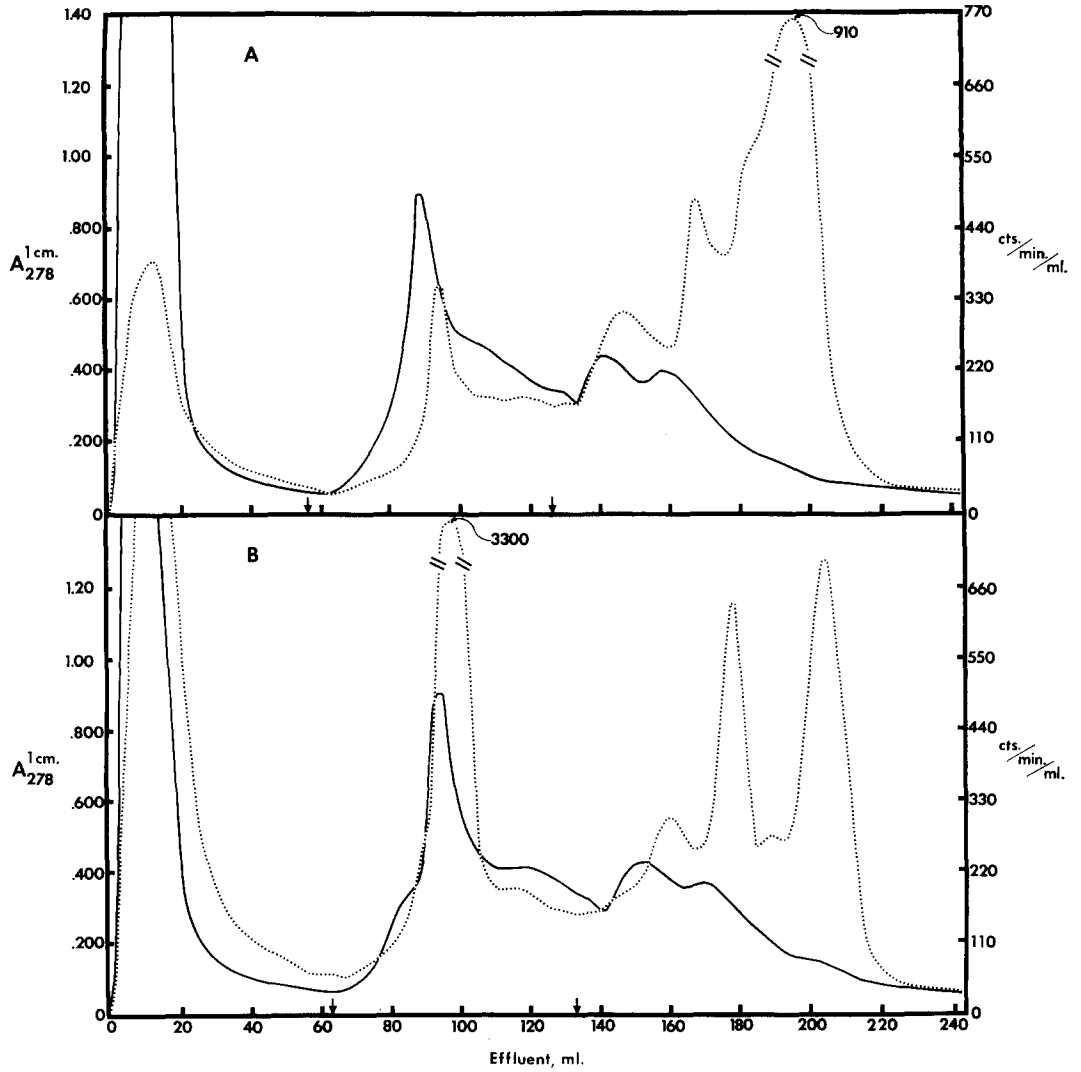

FIG. 1. Chromatography of ^{32}P-labeled mitochondrial proteins. A suspension of HBHM (195 ml containing 30 mg protein/ml) in a solution 0.15 M in KCl and 0.01 M in Tris-acetate, pH 7.5, was treated with 20 kc sound (Branson Sonifier, model S75, 7.5 A output, 60 seconds, sample cooled in a jacketed cell around which was circulated ethylene glycol at $-10°$) in 40-ml portions. After sonic treatment the suspension was made 5 mM with respect to $MgCl_2$ and 10 mM with respect to pyruvate and malate. The suspension was warmed to 30°, and 200 μmoles of $^{32}P_i$ (1 mC/100 μmoles) was added. After 5 minutes of incubation at 30° with the labeled phosphate, the suspension was made 10 mM with respect to EDTA, cooled, and spun at 105,000g for 60 minutes. The supernatant solution was decanted carefully, adjusted to 45% saturation with ammonium sulfate, and maintained at ice temperature for 15 minutes before centrifuging at 35,000g for 5 minutes. The pellet was discarded; the supernatant solution was brought to 70% saturation with ammonium sulfate and allowed to stand at 0° for 15 minutes. The suspension was centrifuged at 35,000g for 5 minutes, the pellet was dissolved in a minimal volume of cold 50 mM Tris-sulfate, pH 7.5, and desalted on a 2 × 20-cm column of Sephadex G-25M previously equilibrated with 50 mM Tris-sulfate, pH 7.5. The yield of product was 286 mg protein at a specific radioactivity of 2630 counts/minute/mg. The resulting solution was divided into two equal parts, and to part A was added 71.5 μmoles ADP, 71.5 μmoles $MgCl_2$, 143 μmoles glucose, and 3.6 mg crystalline hexokinase. A volume of water

form. Indeed, Yankeelov *et al.* (12) have separated two proteins on cellulose, phospho- and dephospho-phosphoglucomutase, that differ by one phosphorus atom per molecule. Experiments similar to that reported in Fig. 1 have been performed (4) in which the transferase was incubated with particles oxidizing succinate in the presence of radioactive P_i and rotenone to prevent reduction of NAD. The results of this experiment were qualitatively the same as in the experiment described above. It is also of interest that ATP is eluted in this type of chromatographic system as a fairly broad fraction with a peak at approximately 160 ml.

Effect of inhibitors on phosphorylation of phosphoryl transferase. A series of experiments was undertaken to study the effects of known inhibitors of electron transfer and oxidative phosphorylation, and uncouplers of oxidative phosphorylation, on the phosphorylation of phosphoryl transferase during the oxidation of succinate. Because of the apparent labile nature of the link between the phosphorus and residues of the phorphorylated protein, the isolation procedure was shortened by using stepwise elution of the DEAE-substituted cellulose columns instead of the more lengthy multiple gradient elution. Uncouplers of oxidative phosphorylation, such as DNP and F_3CCP, severely inhibited the incorporation of radiophosphate into phosphoryl transferase at stage C in the purification procedure (see Ref. 3 for steps in the preparation of the enzyme) (Table I). The transferase was incubated with particles undergoing oxidation in the presence of succinate and in the absence of exogenous ADP as phosphate acceptor. Oligomycin, an inhibitor of oxidative phosphorylation (13), also severely inhibited the incorporation of radiophosphate into phosphoryl transferase, as did antimycin A, an inhibitor of electron transfer. The deletion of substrate (succinate) also resulted in a lower protein-bound phosphate content in the fraction isolated from DEAE-substituted cellulose, but such values obtained in the absence of succinate were considerably higher than those obtained in the presence of inhibitors and uncouplers. The higher values may have been due to exchange acitivity or to the presence of endogenous substrate. Considerably higher blank (no substrate) values were obtained in the absence of rotenone, presumably because of endogenous substrates that are predominantly of the type which reduced NAD in the citric acid cycle (14)

Transfer of protein-bound phosphorus to ADP. It was of interest to determine whether the phosphorus bound to phosphoryl transferase (see above for conditions) could be transferred to ADP to form ATP. This possibility appeared likely since it had been observed that the transferase catalyzed an exchange of phosphate between ADP and ATP (15). Consequently, labeled phosphoryl transferase, stage C, was prepared as described in Table I, but at a higher specific radioactivity. The labeled protein was incubated with an acceptor system consisting of ADP, Mg^{2+}, hexokinase, and glucose in order to ensure that the equilibrium of the reaction would favor the transfer of a phosphoryl group to ADP (Table II). Approximately 60% of the phosphorus bound to phosphoryl transferase was capable of being transferred to glucose. Although the data in Table II represent the result of a typical experiment of this type,

equal to these additions was added to part B. Parts A and B were incubated at 30° for 5 minutes, cooled rapidly, and each passed through a column (1.5 × 20 cm) of Sephadex G-25M previously equilibrated with 5 mM Tris-sulfate, pH 7.5. The two protein-containing eluants were applied to 1 × 10-cm DEAE-substituted cellulose columns and washed with 5 mM Tris-sulfate, pH 7.5. Fractions of 3.5 ml were collected in a fraction collector equipped with drop counters. A linear gradient, applied at the first arrow, was constructed with 150 ml 5 mM Tris-sulfate in the mixing chamber and 150 ml 150 mM Tris-sulfate in the reservoir chamber. At the second arrow both chambers were emptied and the mixing chamber was filled with 150 ml of 150 mM Tris-sulfate and the reservoir chamber with 350 mM Tris-sulfate. The absorbancy at 278 mμ (1-cm path) was measured and the radioactivity of each fraction was determined.

TABLE I

PHOSPHORYLATION OF STAGE C PHOSPHORYL TRANSFERASE BY MITOCHONDRIA OXIDIZING SUCCINATE IN THE PRESENCE OF P_i

HBHM were treated and incubated as described under Fig. 1 except that rotenone (10^{-6} M) was present during incubation of the particles with substrate (succinate, 10 mM) and inorganic phosphate (1 mM). Inhibitors and uncouplers were added prior to substrate and $^{32}P_i$ at the following final concentrations: DNP, 5×10^{-5} M; F_3CCP, 5×10^{-7} M; oligomycin, 0.5 μg/ml; and antimycin A, 1 μg/mg protein. Treatment after incubation with substrate was the same as described in the legend for Fig. 1 except that DEAE-substituted cellulose columns of 20 × 100 mm were used for chromatography of the incubated protein fraction and gradient elution was not used. After the protein fractions were applied, the column was washed with cold 5 mM Tris-sulfate, pH 7.5, until the A_{278}^{1cm} fell to below 0.05. Phosphoryl transferase stage C was eluted with 0.1 M Tris-sulfate, pH 7.5, and protein and radioactivity were determined. The background of the counter used in this experiment was 21 counts/minute and was subtracted from each radioactive assay. The complete system contained sonic-treated HBHM (20 ml, 30 mg protein/ml), KCl (0.15 M), Tris-acetate, pH 7.5 (0.01 M), $MgCl_2$ (5 mM), succinate (10 mM), $^{32}P_i$ (1 mM, 2.05×10^6 counts/μmole), and rotenone (10^{-6} M).

System	Protein-bound ^{32}P (counts/minute/mg protein)
Complete	7840
+ DNP	426
+ F_3CCP	391
+ Oligomycin	567
+ Antimycin A	1377
− Succinate	1894

values as low as 46% for the transfer of radiophosphate from labeled protein to glucose have been observed. Of further interest in the complete system (Table II) was the distribution of the remainder of the label. Approximately 20–25% of the label originally associated with the protein was recovered in an acid-stable form, while between 5 and 10% was recovered as inorganic orthophosphate. Oligomycin did not affect significantly the extent of transfer. Atractylate and DNP were also without effect. The transfer to glucose required the

TABLE II

TRANSFER OF BOUND ^{32}P FROM PHOSPHORYL TRANSFERASE TO ADP

^{32}P-Labeled phosphoryl transferase (stage C) was prepared as described in Table I except that the specific radioactivity of the P_i was 4×10^6 counts/minute/μmole. Each complete reaction tube contained, in 1 ml, 2.4 mg of phosphoryl transferase protein with a relative specific radioactivity of 12,940 counts/minute/mg; 50 μmoles Tris-sulfate, pH 7.5; 5 μmoles $MgCl_2$; 20 μmoles glucose; 10 μmoles ADP; and 0.25 mg crystalline hexokinase. When present the following compounds were added prior to ADP at the following concentrations: oligomycin, 0.5 μg/ml; potassium atractylate, 5 mμmoles/mg protein; DNP, 5×10^{-5} M; P_i, 5 mM. The reaction was initiated by the addition of ADP and was allowed to proceed for 10 minutes at 30°. The reaction was terminated by the addition of 2 ml of silicotungstic acid (1 N with respect to H_2SO_4) prepared according to (9). The mixture was placed in a boiling water bath for 10 minutes under a condenser to avoid loss of fluid volume, and cooled to room temperature. Three ml of a 1:1 mixture of isobutanol–benzene and 0.5 ml of 10% ammonium molybdate were added, and the mixture was shaken for 30 seconds. The isobutanol–benzene layer was removed and the aqueous layer was reextracted with 3 ml of water-saturated isobutanol–benzene. The isobutanol–benzene layers were combined and both layers were counted. In addition, the protein precipitate was collected from the aqueous phase, washed twice with silicotungstic acid, dissolved in 6 N KOH, and counted. The aqueous layer was checked occasionally for G-6-P by paper chromatography according to Bandurski and Axelrod (10). Radioactivity in the isobutanol–benzene layer was considered to represent inorganic orthophosphate, that in the aqueous layer, G-6-P, and that in the protein precipitate, acid-stable protein-bound phosphate. Within the limits of our technique, no other labeled compounds were observed in the aqueous layer.

System	Counts/minute/2.4 mg protein			Counts recovered (%)
	G-6-P	P_i	Protein	
Complete	18,944	2484	6832	91
+ Oligomycin	18,012	3106	8075	91
+ Atractylate	20,497	1242	6832	90
+ DNP	16,770	3416	6211	85
+ P_i	19,114	2171	6444	89
− ADP	621	25,466	1242	88
− Mg^{2+}	58	26,708	272	87

presence both of ADP and Mg^{2+}. The ADP requirement for formation of G-6-P establishes ATP as an intermediate in the transfer and excludes the possibility that preformed ATP adsorbed to the transferase could account for formation of G-6-P.

TABLE III

PHOSPHORYLATION OF PHOSPHORYL TRANSFERASE BY $\gamma(^{32}P)$ATP

Stage D phosphoryl transferase was prepared as described in Refs. 2 and 3, concentrated to 7.7 mg protein/ml with Carbowax 6000, and dialyzed against 50 mM Tris-sulfate, pH 7.5. For phosphoryl transfer from ATP to enzyme the reaction mixture, in 1 ml, contained Tris-sulfate, pH 7.5 (200 μmoles); $MgCl_2$ (5 μmoles), $\gamma(^{32}P)$ATP (0.590 μmole of specific activity 16.2×10^6 counts/minute/μmole), and stage D phosphoryl transferase (5 mg). The reaction was initiated by the addition of enzyme and was allowed to proceed for 5 minutes at 30°. The tube was cooled rapidly and the contents were applied to a Sephadex G-25M column (15 × 200 mm) previously equilibrated with 50 mM Tris-sulfate, pH 7.5. The size of the Sephadex column was adequate for separation of protein from ATP; the separation of the two was checked by the absorption at 260 and 278 mμ in the eluates. The protein fraction was collected. Transfer of radiophosphate from protein to ADP was achieved under the following conditions: To each of two test tubes was added, in the order listed; ^{32}P-labeled phosphoryl transferase (2.4 mg), hexokinase (0.25 mg), glucose (20 μmoles), ADP (10 μmoles) to tube A only, and $MgCl_2$ (10 μmoles), and the mixture was brought to a final volume of 1.95 ml. Both tubes were incubated at 30° for 10 minutes and the reaction was terminated by the addition of 4 ml of silicotungstic acid which was 1 N with respect to H_2SO_4. The tubes were incubated in a boiling water bath for 10 minutes under a condenser to avoid evaporation and cooled, and the contents were centrifuged. G-6-^{32}P, $^{32}P_i$, and ^{32}P-protein were assayed as described in Table II.

Reaction	Counts/minute/mg protein		
$\gamma(^{32}P)$ATP + protein $\xrightarrow{Mg^{2+}}$ ADP + protein-^{32}P	6408		
Transfer reaction	Total counts/minute in		
	G-6-P	Pi	Protein
Protein-^{32}P to glucose	4928	5405	5667
Protein-^{32}P to glucose minus ADP	210	14853	937

(This latter point has been explored in a direct manner and is reported below.) The distribution of radiophosphate after incubation of the transferase in the absence of ADP or Mg^{2+} differed considerably from that in the complete system (Table II). Essentially all of the phosphorus assayed as P_i after exposure of the transferase for 10 minutes at 100° in 1 N acid. Since data derived from control experiments indicated that less than 15% of the phosphorus was released as P_i after exposure of the protein to 30° for 10 minutes (see also Table VII), it would appear that the bulk of the protein-bound phosphorus was stable in the absence of Mg^{2+} or ADP. The presence of 5 mM P_i did not alter the final distribution of the label in the complete system, indicating that the protein-bound phosphorus was not in equilibrium with inorganic phosphate and did not in any way exchange with external inorganic phosphate during the transfer reaction.

Phosphorylation of phosphoryl transferase by ATP. Since phosphoryl transferase catalyses an ATP–ADP exchange reaction (15), the reversibility of the transfer reaction could be anticipated, namely, the phosphorylation of phosphoryl transferase by ATP. Initial experiments were performed with stage C enzyme that has been reported (2, 3) to be approximately 85–90% pure. However, the data in Table III were obtained with stage D enzyme, which was homogenous in the analytical untracentrifuge and which appeared as a single component when examined by molecular gel filtration (3). Forty nmoles of the enzyme [if a molecular weight of 124,000 is assumed for phosphoryl transferase (see Ref. 3)] was incubated with the labeled ATP. If each molecule of the enzyme had contained one phosphoryl group upon reisolation after incubation with labeled ATP, 6.48×10^6 counts should have been recovered in the protein fraction. The data in Table III for the reaction $\gamma(^{32}P)$ATP + protein → ADP + protein-^{32}P show that approximately 0.5% of this amount of radioactivity was recovered bound to the protein, indicating that the equilibrium of the reaction must lie far to the left, i.e., toward the

formation of ATP from the labeled protein, despite the initial high concentration of ATP relative to protein in the reaction mixture. The data in the lower portion of Table III, indicating that the phosphoryl group of the transferase can be transferred to glucose via ADP as intermediate phosphoryl acceptor, thus demonstrate the complete reversibility of the reaction. Of the approximately 16,000 counts/minute/mg enzyme in the assay tube, 30.8% appeared as G-6-^{32}P.

Another question of considerable concern was the form of the phosphorus bound to the enzyme. The experiments in Table II and III indirectly suggested that either the bound form was not ATP, or if it was ATP, it was in a form not accessible to hexokinase. A preliminary experiment comparing the transfer of label from 8-(^{14}C)ATP and γ(^{32}P)ATP to enzyme (1) indicated that the purine portion of ATP was not bound to the enzyme. However, this type of experiment was susceptible to the criticism that the specific activities of the two ATP samples were not similar and the counting methods were not the same. Consequently, two experiments, the results of which appear in Table IV, were performed in which the transfer of radiophosphate from ATP labeled in the γ, the α, and the β and γ positions and of comparable specific radioactivities was measured.

TABLE IV

TRANSFER OF LABEL FROM α, γ, AND β,γ(^{32}P)ATP TO PHOSPHORYL TRANSFERASE

The three species of labeled ATP were obtained as described in METHODS. After dilution of labeled ATP with carrier ATP, the specific radioactivities of each of the three species of labeled ATP were as follows: γ(^{32}P)ATP, 19.3 × 10^6; α(^{32}P)ATP, 20 × 10^6; and β,γ(^{32}P)ATP, 22.6 × 10^6. The comparisons were made in two separate experiments on different days for technical reasons and are so indicated below. Each incubation tube contained, in 0.7 ml, 35 μmoles Tris-sulfate, pH 7.5; 2 μmoles ATP; 5 μmoles $MgCl_2$; and 1.25 mg of stage D phosphoryl transferase. The reaction was initiated by the addition of enzyme and was maintained at 30° for 5 minutes. Transfer of label from ATP to protein was assayed as described in Table III.

Experiment	ATP	Counts/minute/1.25 mg protein
1	α(^{32}P)	13
	γ(^{32}P)	2078
2	γ(^{32}P)	2214
	β,γ(^{32}P)	1066

TABLE V

PHOSPHORUS CONTENT OF PHOSPHORYL TRANSFERASE

Stage C phosphoryl transferase was extensively dialyzed against 50 mM Tris-sulfate, pH 7.5, and analyzed for phosphorus by the micromethod of Chen *et al.* (16). An equal volume of the fluid external to the dialysis sac was used as blank. Analyses were kindly performed in the laboratory of Dr. Sidney Fleisher. The lower limt of this technique is 0.15 μg phosphorus per assay.

Phosphoryl transferase (mg)	Phosphorus (μg)	Phosphorus (μg)/mg
1	<0.15	<0.07
2	0.16	0.08
4	0.29	0.07
4	0.29	0.07

The data from experiment 1 (Table IV) clearly showed that the α-phosphate of ATP could not serve as a source of protein-bound phosphate. On the presumed basis of one mole of phosphorus bound per mole of enzyme, and a molecular weight of 124,000 for phosphoryl transferase (3), it was estimated that 1% of the enzyme molecules were labeled in this particular preparation. The data from experiment 2 (Table IV) revealed that counts originating from doubly labeled ATP were approximately half of the counts originating from γ(^{32}P)ATP. Since the two samples of ATP in this experiment were of essentially equal specific radioactivities, and the terminal phosphate group of the doubly labeled ATP had one-half the specific activity of the singly labeled ATP the difference in amount of label originating from the doubly labeled ATP clearly indicated that the transfer reaction involved only the γ position of ATP.

Phosphorus content of phosphoryl transferase. The finding that phosphoryl transferase could be converted to a phosphoform

prompted an analysis of the phosphorus content of the protein. Based on a molecular weight of 124,000 for phosphoryl transferase (3), the data in Table V would indicate that the enzyme preparation contained 0.25 mole of phosphorus per mole of enzyme. The enzyme did not appear to be a conventional phosphoprotein in that it did not contain, upon isolation, one or more phosphorus atoms per molecule of enzyme, nor did it appear to contain phospholipid.

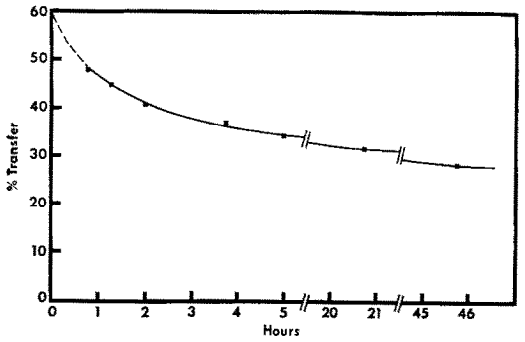

FIG. 2. Loss of phosphoryl transferase activity with time. The phosphorylated form of stage C phosphoryl transferase was prepared as described in Table III and maintained at pH 7.5 and 0°. The product contained 4618 counts/minute/mg. One-ml portions (containing 2 mg protein) were removed at the times indicated in the figure, and the transfer of ^{32}P to ADP was measured as described in Table III. The data are expressed as the percentage of the counts in the original phosphophosphoryl transferase transferred to glucose, via ADP, to form G-6-P. The data were corrected for radio decay of ^{32}P. The broken line represents extrapolation to zero time.

TABLE VI

EFFECT OF pH ON THE STABILITY OF PROTEIN-BOUND ^{32}P

Phosphorylated stage C phosphoryl transferase was prepared as described in the caption of Table III. The specific activity was 1175 counts/minute/mg protein. The solutions, in 50 mM Tris-sulfate, pH 7.5, were divided into three portions of 1 ml, each containing 1 mg protein. The adjustments of pH, monitored with a glass electrode, were made with either 2 N KOH or H_2SO_4. The control sample received approximately equal volumes of a solution of KOH adjusted to pH 7.5 with H_2SO_4. The neutralized samples were passed through 15 × 200-mm Sephadex G-25M columns previously equilibrated with 50 mM Tris-sulfate, pH 7.5; the protein fraction was collected and the content of ^{32}P was analyzed.

Treatment	Counts/minute/mg protein
pH 7.5	992
pH 4.0	77
pH 10.0	104

TABLE VII

TEMPERATURE STABILITY OF THE PHOSPHATE MOIETY OF ^{32}P-LABELED PHOSPHORYL TRANSFERASE

^{32}P-Phosphoryl transferase was prepared from $\gamma(^{32}P)$ATP as described in the caption of Table III. The activity of the product was 7167 disintegrations/minute/(dpm)/mg. Radioactivity in this experiment was assayed in a Packard three-channel liquid scintillation counter with automatic external standardization. All samples were corrected for quenching. Aqueous samples were counted in the scintillation mixture of Gordon and Wolfe (18), which contains 4% Cab-O-Sil. Each sample contained 0.6 mg ^{32}P-phosphoryl transferase, 4 μmoles $MgCl_2$, and 20 μmoles Tris-sulfate, pH 7.5, in a volume of 0.56 ml. After the experimental period the samples were cooled rapidly in ice and the hexokinase trapping system was added. The transfer reaction to glucose via ADP was accomplished as described in the caption of Table III. The fractions corresponding to $^{32}P_i$ and G-6-^{32}P were counted.

Temperature at which exposed for 10 min (°C)	Total dpm	P_i		G-6-P	
		dpm	%	dpm	%
0	2105	840	39.9	1265	60.1
15	2200	900	40.9	1300	59.1
30	2242	967	43.1	1275	56.9
45	3277	2633	80.3	644	19.7
60	4088	3996	97.8	92	2.3

Stability of the phosphoryl group associated with phosphophosphoryl transferase. The transfer of a phosphoryl group from the transferase to ADP was not affected severely by storage of the enzyme at 0° (Fig. 2). The transfer was still 50% of the original level after almost 2 days of storage in ice. On the other hand, the effect of pH on the retention of labeled phosphorus by the enzyme was profound (Table VI). After the enzyme was exposed to pH 4 for 30 minutes only 6.6% of the original counts was still bound to the protein as compared

with 84.4% in the pH 7.5 sample, and the sample maintained for the same amount of time at pH 10.0 contained 8.9% of its original complement of ^{32}P.

To determine whether incubation of the labeled protein did not by itself result in a disruption of the bond between protein and phosphoryl group, the effect of prior treatment of phosphorylated phosphoryl transferase for 10 minutes at each of five different temperatures on the transfer of protein-bound ^{32}P to glucose via ADP was measured (Table VII). When the phosphorylated protein was maintained for 10 minutes at temperatures between 0° and 30°, the transfer of the phosphoryl group to glucose via ADP was affected to a negligible degree. However, after incubation of the enzyme for the same length of time at 45° and 60° the subsequent transfer reaction was affected profoundly. In addition, a considerable proportion of the phosphorus appeared to have been released as P_i prior to incubation of the protein with the hexokinase trapping system. This release accounts for the increase in the total radioactivity recovered in the latter two samples.

Hydroxylamine and protein-bound ^{32}P. In an attempt to gain information on the type of attachment between the protein and its bound phosphorus, the effect of exposure of the transferase in its phosphorylated form to salt-free hydroxylamine was studied (Table VIII). After the enzyme was incubated for 30 minutes at 30° with hydroxylamine, there was a reduction of 48.2% in the amount of protein-bound ^{32}P transferred to glucose via ADP. Incubation for a period of 60 minutes with hydroxylamine resulted in a reduction of 63% in the amount transferred by comparison with the untreated control sample. The amount of radiophosphate bound to the enzyme in an acid-stable form was not severely altered by treatment with hydroxylamine, whereas the same treatment increased the phosphorus assaying as P_i by 167%.

TABLE VIII

EFFECT OF NH_2OH ON THE RELEASE OF PROTEIN-BOUND ^{32}P

Phosphorylated phosphoryl transferase was prepared as described in the caption of Table III. The relative specific radioactivity of the product was 2070 counts/minute/mg protein. During incubation at 30°, each sample contained 2.33 mg phosphophosphoryl transferase, either 0.25 M NH_2OH (salt-free) or an equivalent volume of water, and 0.1 M Tris-sulfate, pH 7.5. The initial period of incubation with NH_2OH was terminated by the addition of the hexokinase trapping system as described in the caption of Table III. Fractions corresponding to P_i, G-6-P, and protein were collected and counted.

System	Time at 30° (min)	Total counts/minute in		
		Protein	Pi	G-6-P (%)[a]
Control	60	316	1758	2143 (50.9)
0.25 M NH_2OH	30	274	3234	1260 (26.4)
0.25 M NH_2OH	60	298	2942	754 (18.9)

[a] Refers to the percentage of the recovered counts in G-6-P.

DISCUSSION

The conventional formulation (20) of the sequence of events in oxidative phosphorylation has for some years embodied the explicit assumption of a phosphorylated intermediate in the synthesis of ATP, namely,

$$AH_2 + B + I \rightarrow A \sim I + BH_2$$
$$A \sim I + X \rightarrow A + X \sim I$$
$$X \sim I + P_i \rightarrow X \sim P + I$$
$$X \sim P + ADP \rightarrow X + ATP$$

Sum: $AH_2 + B + P_i + ADP \rightarrow A + BH_2 + ATP$

On the other hand, the elusive character of such intermediates of oxidative phosphorylation has led to a hypothesis which does not require the participation of a stable phosphorylated intermediate in oxidative phosphorylation (21). Several lines of evidence are consistent with the participation of phosphoryl transferase in oxidative phosphorylation: (*a*) the increase in the phosphorylative capacity of poorly phosphorylating submitochondrial particles by the transferase (3), (*b*) its ADP–ATP exchange activity (15), (*c*) the rapid phosphorylation of the protein during oxidative phosphorylation (19), (*d*) inhibition of the phosphorylation of the protein during oxidative phosphorylation by uncouplers and inhibitors of oxidative phosphorylation and by inhibitors of electron transfer (4, 19),

(*e*) the transfer of a phosphoryl group from the phosphorylated protein to ADP, and (*f*) phosphorylation of the enzyme by the terminal phosphoryl group of ATP. The existence of a stable phosphorylated intermediate of oxidative phosphorylation has been postulated by Ter Welle and Slater (22) on the basis that the uncoupling of oxidative phosphorylation by arsenate can be reversed by P_i. These authors have inferred that such evidence affords "perhaps the best evidence that a stable $X \backsim P$ can be formed during respiratory-chain oxidative phosphorylation" (22).

An alternative explanation for the phosphorylation of phosphoryl transferase during oxidative phosphorylation is that this protein would serve as an acceptor of a phosphoryl group originating from ATP formed during oxidative phosphorylation, and, in that case, would not serve as an intermediate in the synthesis of ATP. The fact that oligomycin inhibited the formation of the phosphorylated protein during oxidative phosphorylation does not constitute decisive evidence that the phosphorylated protein preceded the formation of ATP, i.e., does not allow a distinction between

$$P_i \xrightarrow{\text{oligo}} \sim P \rightarrow ATP \quad \text{and}$$
$$P_i \xrightarrow{\text{olig.}} ATP \rightarrow \sim P.$$

Our present evidence does not permit us to distinguish between these alternatives. However, in the event that the latter alternative proves correct, phosphoryl transferase would be engaged in the transfer of a phosphoryl group from endogenous, bound ATP, to exogenous ADP. The finding by Heldt *et al.* (23) that endogenous ADP serves as an early phosphate acceptor during oxidative phosphorylation is consistent with such an interpretation.

The formation of a phosphorylated intermediate is in no way unique to phosphoryl transferase. $Na^+ + K^+ -$ activated ATPase has been shown to form a phosphorylated intermediate (24–28) which appears to have the characteristics of an acyl phosphate (25–27). Phosphoglucomutase has been shown (12) to exist either in a phospho- or dephospho-enzyme form, as has alkaline phosphatase from *Escherichia coli* (29, 30). Succinate thiokinase from mitochondria has been found to form kinetically active intermediates which contain phosphohistidine (31) and enzyme-bound succinyl phosphate (32), respectively. In addition, phosphoproteins are known to be formed rapidly during electron transfer in yeast respiratory particles (33) and in chloroplasts (34). The latter work (34) is of particular interest since the phosphorus bound to chloroplast proteins did not appear to be either protein-bound phosphohistidine or phosphoserine (34). Although we do not pretend that this list represents a complete survey of possible phosphorylated intermediates, it is safe to say that such phenomena are far from being numerically restricted. Of particular relevance to the protein under discussion here, however, is the recent report (35) of the isolation from beef heart mitochondria of a phosphorylated intermediate involved in the ATP–ADP exchange reaction since some of its characteristics appear to be similar to those of phosphoryl transferase in its phosphorylated form. Both proteins catalyze an exchange between ATP and ADP, both are phosphorylated by $\gamma(^{32}P)ATP$, in both bound phosphate does not equilibrate with $^{32}P_i$, and the phosphoryl group in both proteins is unstable outside a limited range of temperature and pH. Also of interest in this regard is the report by Wadkins and Lehninger (36) that an ATP–ADP exchange enzyme isolated from rat liver mitochondria can increase the phosphorylative capacity of mitochondria depleted of this enzyme. The similarities between our preparation and those of Colomb *et al.*, and Wadkins and Lehninger, are quite clear and suggest that the same enzyme is common to all three preparations.

The nature of the binding between phosphorus and protein deserves some mention since considerable difference was found between the stability of the phosphate residue before and after reaction of the ADP. Prior to reaction of the enzyme with ADP the protein-bound phosphorus was easily released by high pH, heat, or acid. After the transfer of phosphate from the protein to ADP, a considerable portion of the phosphorus had been altered, resulting in a stable

relationship such as that found in phosphorylserine. It is not unlikely that reaction of the transferase with ADP results in a conformational change in the enzyme which uncovers a serine side chain capable of forming a stable *O*-phosphoryl derivative. The pH lability of the bond prior to reaction with ADP would appear to argue against the phosphate residue being in the form of phosphorylhistidine, while the effect of hydroxylamine in releasing bound phosphate from the transferase suggests a carboxyl-phosphoryl linkage.

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