Partial Resolution of Oxidative Phosphorylation in Yeast Mitochondria†

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ABSTRACT: A new coupling factor (YF_6) from yeast was isolated which exhibited properties similar to coupling factor 6 (F_6) from bovine heart. In depleted submitochondrial particles prepared from yeast, YF_6 as well as coupling factor 1 (ATPase) and oligomycin-sensitivity conferral protein (YOSCP) stimulated oxidative phosphorylation. OSCP and F_6 isolated from bovine heart mitochondria were much less effective. Yeast OSCP did not stimulate depleted bovine particles whereas yeast F_6 did. F_6 from either yeast or heart reduced the rate of inactivation of bovine ATPase in the absence of ATP.

Attempts have been made in the past to resolve from yeast mitochondria coupling factors that stimulate oxidative phosphorylation, but they have met with little success. In 1967, Schatz *et al.* successfully purified mitochondrial ATPase from yeast. A requirement for YF_1[1] in oxidative phosphorylation in yeast particles was not observed, although the factor stimulated oxidative phosphorylation in partially depleted A particles from bovine heart. Tzagoloff (1970) succeeded in isolating a protein (OSCP) from yeast required for conferral of rutamycin sensitivity to the ATPase. The protein had properties similar to those of OSCP from bovine heart; however, no data were presented to indicate that yeast OSCP was a coupling factor.

In view of the recent surge of interest in biogenesis of yeast mitochondria (Roodyn and Wilkie, 1968; Schatz, 1970; Linnane and Haslam, 1970), it should be useful to have as much information as possible on the individual components of the inner membrane of these organelles. The mode of interaction between these components is also of primary importance.

This communication presents data on a second protein isolated from yeast that is required for rutamycin sensitivity of the ATPase. It will be shown that both proteins as well as YOSCP function as coupling factors of oxidative phosphorylation in yeast.

Experimental Procedure

Materials. Solutions were prepared as previously described (Racker *et al.*, 1969); Fessenden-Raden *et al.*, 1969). Rutamycin was kindly supplied by Dr. G. E. Mallett of Eli Lilly Co. Phenylmethylsulfonyl fluoride was purchased from Calbiochem. Fleischmann's (bakers) yeast was obtained from a local bakery.

Analytical Methods The $^{32}P_i$–ATP-exchange activity (Racker *et al.*, 1969), oxidative phosphorylation (Fessenden-Raden *et al.*, 1969), and ATPase activity (Horstman and Racker, 1970) were measured according to published procedures. Rutamycin-sensitive ATPase activity was measured as described by Bulos and Racker (1968).

Protein of the bovine heart preparations was determined by the biuret procedure (Jacobs *et al.*, 1956). The method of Lowry *et al.* (1951) was used for all other determinations.

Preparations. Bovine heart mitochondria (Green *et al.*, 1957) with 20 mM EDTA present during the blending of the muscle mince (Fessenden-Raden, 1972), STA particles, pH 9.5, 1% silicotungstate (Racker *et al.*, 1969), or pH 10.4, 2% silicotungstate (J. M. Fessenden-Raden, unpublished data), F_1 (Horstman and Racker, 1970), F_2 (Fessenden-Raden, 1972a), OSCP (MacLennan and Tzagoloff, 1968) from F_4 (Conover *et al.*, 1967), F_3 (Fessenden-Raden, 1972a), F_5 (Fessenden-Raden, *et al.*, 1969), and F_6 (Fessenden-Raden, 1972a) from bovine heart were prepared according to published procedures. F_1 was freed of ATP by passage through a G-50 Sephadex column equilibrated with 0.25 M sucrose, 10 mM Tris-SO_4 (pH 7.5), and 2 mM EDTA. It was immediately frozen and stored in small aliquotes in liquid nitrogen. F_1 from yeast (Tzagoloff, 1969) was the gift of Dr. G. Schatz. F_6 from yeast was prepared according to the procedure for bovine heart (Fessenden-Raden, 1972a) but in the presence of phenylmethylsulfonyl fluoride (16 μg/ml) to inhibit protease activity (Steinman and Jakoby, 1967). OSCP from yeast was prepared as described (Tzagoloff, 1970), through the ammonium sulfate fractionation step except that the acid precipitation was omitted because of loss of activity. Phenylmethylsulfonyl fluoride was included during the entire procedure. Yeast mitochondria were isolated from Fleischmann's (bakers) yeast by an unpublished procedure of D. Wharton[2] except that 20 mM EDTA was present during the initial homogenization. Yeast submitochondrial particles were prepared by sonic oscillation in 0.25 M sucrose–0.01 M Tris-acetate (pH 7.5) for 1 min as described by Tzagoloff (1970) except that

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[1] The abbreviations used are: F_1, F_2, F_3, F_4, F_5, and F_6 for bovine heart coupling factors 1 (ATPase), 2, 3, 4, 5, and 6, respectively; YF_1 and YF_6 for yeast coupling factors 1 (ATPase) and 6, respectively; OSCP and YOSCP for oligomycin-sensitivity conferral protein isolated from bovine heart and yeast, respectively; STA, particles for submitochondrial particles prepared from bovine heart mitochondria by sonic oscillation at alkaline pH, then treatment with silicotungstate.

[2] This procedure is available upon request from Dr. D. Wharton, Section of Biochemistry and Molecular Biology, Division of Biological Sciences, Cornell University, Ithaca, N. Y. 14850.

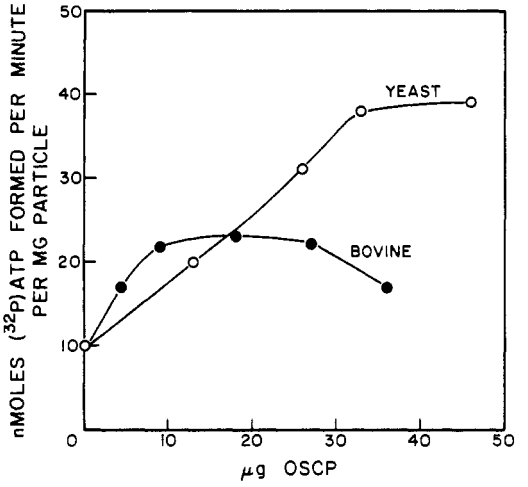

FIGURE 1: Titration of OSCP from yeast and bovine heart in yeast submitochondrial particles. $^{32}P_i$–ATP-exchange activity was measured and incubations were carried out as described in Table I except that 20 μg of F_1 from bovine heart was used in place of Y-F_1.

an initial centrifugation of short duration (12 min) at 58,000*g* was added.

Results and Discussion

Requirement for Coupling Factors. Yeast submitochondrial particles obtained by sonic oscillation catalyze a low rate of oxidative phosphorylation or $^{32}P_i$–ATP exchange as shown in line 1 of Table I. Data presented in this table show that the addition of known coupling factors from yeast, YF_1 (Schatz *et al.*, 1967) and YOSCP (Tzagoloff, 1970), as well as a new coupling factor, YF_6, are required for maximal activity. Titration of all the coupling factors against each other gave saturation curves indicating only minor cross contamination of the preparations. These yeast particles show a 3- to 5-fold stimulation by addition of YOSCP but at most a 2- to 3-fold stimulation by the further addition of YF_1 or YF_6 indicating less depletion of these latter coupling factors. Since the yeast submitochondrial particles still have substantial ATPase activity (2–3 μmoles of P_i liberated per min per mg), one can conclude that, as with bovine heart submitochondrial particles (Racker, 1962), only small portions of the membrane have been denuded of coupling factor 1 during sonication.

Species Specificity. In general, the yeast coupling factors were more efficient in restoring phosphorylating activity in depleted yeast submitochondrial particles than the bovine heart coupling factors, and *vice versa*. Figure 1 shows that an extensively purified preparation of OSCP from bovine heart gave rise to only one-half of the maximal $^{32}P_i$–ATP-exchange activity obtained in the presence of the yeast enzyme. One possible explanation for these results is that the bovine enzyme cannot interact with all the sites in the yeast particles. However, the possible presence of an inhibitor in the bovine OSCP preparation for the yeast system has not been ruled out. These yeast particles did not respond greatly

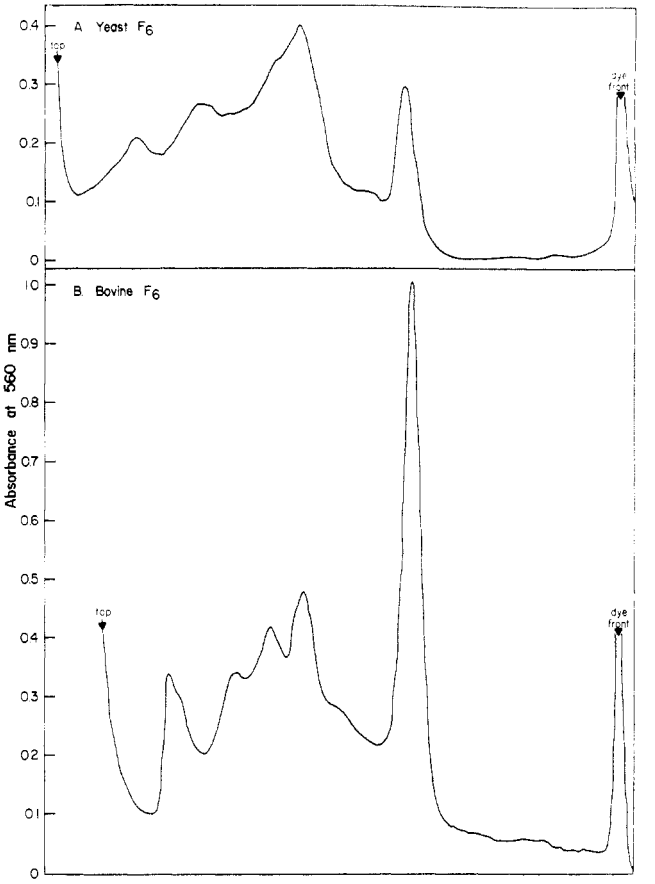

FIGURE 2: Densitometer traces of yeast and bovine F_6. (A) Yeast F_6 (30 μg of protein) and (B) bovine F_6 (30 μg of protein). Electrophoresis (anode at the right) was carried out as described by Davis (1964). The gels were stained with Coomassie Brilliant Blue R in 10% trichloroacetic acid was described by Chrambach *et al.* (1967). Densitometer traces were obtained at 569 nm on a recording Gilford spectrophotometer with a linear transport for gel scanning.

TABLE I: Coupling Factor Requirements in Depleted Yeast Submitochondrial Particles.[a]

Addns to Yeast Particles	$^{32}P_i$–ATP Exchange (nmoles of [^{32}P]ATP/min per mg)	Oxygen Uptake (natoms)	[^{32}P]-Glc-6-P Produced (nmoles)	P:O
None	18	122	17	0.14
YF_1, YOSCP, YF_6	51	109	52	0.48
YOSCP + YF_6	28	93	31	0.33
YOSCP + YF_1	24	141	41	0.29
YF_1 + YF_6	11	99	13	0.13

[a] In a volume of 0.3 ml, 20 μmoles of potassium phosphate (pH 7.8), 20 μmoles of Tris-SO_4 (pH 8.0), 2 μmoles of $MgSO_4$, 2 μmoles of ATP (pH 7.4), 1.2 mg of bovine serum albumin, 165 μg of yeast submitochondrial particles, 40 μg of YF_1 (specific activity 25), 35 μg of YOSCP, and 50 μg of YF_6 as indicated were incubated at 23° for 30 min and $^{32}P_i$–ATP-exchange activity was measured as described (Racker *et al.*, 1969). Oxidative phosphorylation was measured polarographically at 25° as previously described (Fessenden-Raden *et al.*, 1969) with succinate as substrate. Incubations were carried out as above except that 2.4 mg of bovine serum albumin, 295 μg of yeast submitochondria particles, 40 μg of YF_1, 70 μg of YOSCP, and 90 μg of YF_6 were used.

TABLE II: Effect of Yeast Coupling Factors on the Rutamycin-Sensitive ATPase and $^{32}P_i$–ATP Exchange Activities of Bovine Heart STA Particles.[a]

Addns to STA Particles and F_1	ATPase (μmoles of P_i Released/10 min) −Rutamycin	+Rutamycin	Inhibn (%)	Δ (%)	$^{32}P_i$–ATP Exchange (nmoles of [^{32}P]ATP/ min per mg of Particles)	Δ (nmoles/ min per mg)
		Experiment 1				
F_6	0.89	0.70	21		18	
F_6, OSCP	0.88	0.39	56	35	63	45
F_6, YOSCP	0.79	0.51	36	15	30	12
		Experiment 2				
OSCP	1.31	1.13	14		19	
OSCP, F_6	1.35	0.60	56	42	84	65
OSCP, YF_6	*b*				75	56
OSCP, YF_6 (dialyzed)	1.66	0.73	56	42	29	10

[a] In a volume of 0.75 ml, 25 μmoles of Tris-SO_4 (pH 8.2), 150 μg of STA particles (pH 10.4, 2%), 2 μg of F_1, 2.5 mg of bovine serum albumin, 9 μg of OSCP or 34 μg of YOSCP, 40 μg of F_6 or 60 μg of YF_6, and 5 μg of rutamycin as indicated were incubated for 5 min at 30°, then ATPase activity was measured as described (Bulos and Racker, 1968). $^{32}P_i$–ATP-exchange activity was measured as in Table I and incubations were carried out as above except 40 μg of F_1 and 25 μmoles of potassium phosphate (pH 7.8) were added. Dialysis was carried out as described for bovine F_6 (Fessenden-Raden, 1972a,b). [b] Not measured due to interference by ammonium sulfate.

to either F_1 or YF_1 in $^{32}P_i$–ATP exchange or in oxidative phosphorylation. As noted earlier, these particles exhibited a high ATPase activity which was rutamycin sensitive. The small effects of F_1 noted in the experiments described may have been due to a structural function of F_1 and YF_1 (Schatz *et al.*, 1967). Bovine particles that were completely devoid of F_1 showed an absolute dependency on bovine F_1 for $^{32}P_i$–ATP-exchange activity whereas it had been shown previously that A particles only partially depleted in F_1 could be restored by addition of YF_1 (Schatz *et al.*, 1967). In the latter case, a structural role for F_1 such that it completes or tightens the membrane was suggested. Whereas F_6 from either source appeared to work equally well, YOSCP was less than one-half as effective in bovine particles as OSCP when assayed either by $^{32}P_i$–ATP exchange or by rutamycin sensitivity of the ATPase (Table II).

It should be pointed out, however, that these apparent species differences between F_6 and OSCP may simply reflect incomplete resolution of coupling factors from the different membranes. Thus, the F_1-free STA particles exhibited an absolute specificity for bovine heart F_1; similarly, the realtively high species specificity observed with OSCP may result from the relatively effective depletion of OSCP from the acceptor particles. Tzagoloff (1970) has also observed some species specificity with OSCP. He showed that only YOSCP would bind YF_1 properly to the depleted yeast particle to restore rutamycin sensitivity. Although bovine heart OSCP could also facilitate the binding of YF_1 to the membrane, it did not render the ATPase rutamycin sensitive.

Coupling Factor 6 from Yeast. Employing the procedure for the isolation of F_6 from bovine heart, a protein preparation was extracted from yeast mitochondria that was similar to bovine heart F_6 in that it was stable to 90° for 5 min but labile to trypsin (Table III). Since it replaced F_6 in STA particles (expt 2, Table II) it is termed yeast F_6 (YF_6). As earlier observed with heart muscle, the presence of 20 mM EDTA during preparation of the yeast mitochondria assured more reproducible F_6 preparations. YF_6 showed several bands on polyacrylamide gel electrophoresis (Davis, 1964) as did bovine F_6 (Figure 2). As in the case of bovine F_6 (Fessenden-Raden, 1972b) most of the bands were extractable with isooctane leaving as the major band the most rapidly moving protein. Unlike bovine F_6, however, the yeast protein had lost most of its activity after lyophilization and was inactive following isooctane extraction. It was, therefore, not possible to correlate the remaining band with activity. In contrast to the bovine preparation, YF_6 lost activity after dialysis as

TABLE III: Comparison of Yeast F_6 with Bovine Heart F_6.[a]

Addns to STA Particles and F_1, F_2, F_3, and F_5	$^{32}P_i$–ATP Exchange (nmoles of [^{32}P]ATP/min per mg of Particles)
None	19
F_6 (27 μg)	87
YF_6 (79 μg) unheated	71
YF_6 (60 μg) 90°, 5 min	80
YF_6, trypsin treated	10

[a] Activity was measured and incubations carried out as in Table I except that 160 μg of bovine heart STA particles (pH 9.5, 1% STA), 40 μg of F_1, 120 μg of F_2, 125 μg of F_3, 45 μg of F_5, and F_6 from bovine heart or yeast as indicated were used. Trypsin treatment, dialysis, and lyophilization were carried out as described for F_6 (Fessenden-Raden, 1972a).

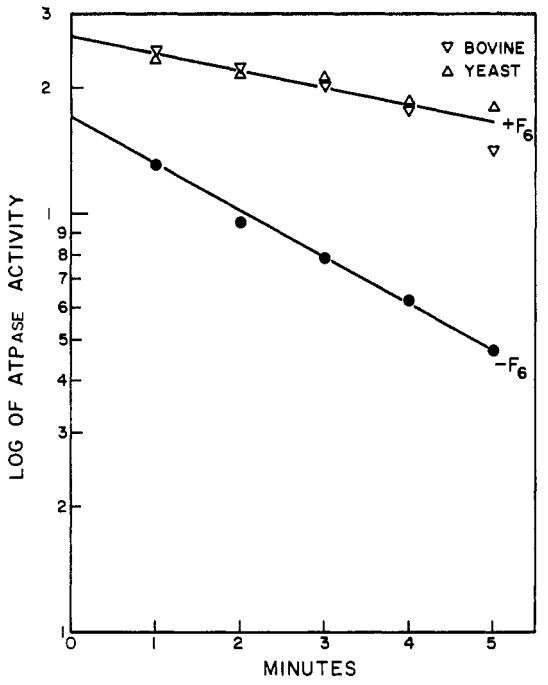

FIGURE 3: Protection of ATPase activity by bovine and yeast F_6. F_1 previously passed through Sephadex to remove ATP was removed from liquid nitrogen storage, thawed as quickly as possible at 30°, and added to three test tubes containing in a final volume of 2.0 ml either 50 μg of F_1, alone or with 25 μg of bovine or yeast F_6 as indicated. Samples (0.1 ml) were removed and assayed at the indicated times. The earliest sample was taken one minute after removal from liquid nitrogen. ATPase activity was assayed as described (Horstman and Racker, 1970).

measured by restoration of the ^{32}P–ATP-exchange activity. Dialysis did not, however, diminish the ability of yeast F_6 to restore rutamycin sensitivity to bovine ATPase (expt 2, Table II). In fact due to the high ammonium sulfate concentration in the YF_6 preparation, dialysis was required to avoid interference with the assay. These results would be consistent with a structural type role for YF_6 in the latter reaction but not in the former one, although the presence of a component in the YF_6 preparation stable to lyophilization and dialysis has not been ruled out.

Interaction of F_6 with F_1. F_1 has been shown to require ATP for stability at room temperature. When F_1 was passed through Sephadex to remove all residual ATP it was inactivated at 23° (Figure 3). This loss of ATPase activity was initially quite rapid and decreased after 5 min. The presence of either bovine heart or yeast F_6 greatly reduced the rate at which ATPase activity was lost as shown in Figure 2. It can be seen that the kinetics of this inactivation were apparent first-order in all cases. F_6 also afforded some protection to F_1 at 0° and at 37° but not at 50°. The protection of ATPase activity at 23° appeared to be specific for F_6 since none of the other coupling factors, or the ATPase inhibitor (Pullman and Monroy, 1963), or bovine serum albumin afforded protection at similar concentrations. The ATPase inhibitor has previously been reported to protect ATPase activity at 0° (Pullman and Monroy, 1963). It should be pointed out that in these latter experiments ATP was present in the F_1 preparations whereas all studies reported here were carried out with F_1 free of ATP and at very low salt concentrations. In fact, the interaction between F_6 and F_1 was prevented by the presence of salt. In the presence of buffer (0.2 M sucrose–5 mM Tris-SO_4–1 mM EDTA, pH 7.5) where F_6 had little effect, the addition of 2×10^{-8} M ATP or ADP to F_1 preserved 80% of the ATPase activity even after 120 min at 23°. The control without any nucleotide had lost 60% of the ATPase activity and without buffer or nucleotide 90% of the ATPase activity was lost after 120 min. D. Hilborn and G. G. Hammes (unpublished experiments) have found that F_1 contains two sites for ADP, one with an estimated dissociation constant of 3×10^{-7} M and a second with an estimated dissociation constant of 4.7×10^{-5} M. It is conceivable that the more tightly bound ADP may be acting to protect the molecule from inactivation and it may be near or at this site that F_6 interacts with F_1. Investigations along these lines will be carried out when homogeneous F_6 can be prepared.

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