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OCCURRENCE OF PROTEINS IMMUNOREACTIVE WITH ANTI-COUPLING FACTOR B IN PHOSPHORYLATING MEMBRANE PREPARATIONS

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Coupling factor B has been isolated from beef heart mitochondria, apparently in multiple forms which differ in molecular weight and specific activity. Since it has no known intrinsic catalytic activity, detection and quantitation have been based upon the factor B-dependent stimulation of ATP-linked activities in factor B-deficient submitochondrial particles. This communication reports the development of a reliable and more universally applicable enzyme-linked immunosorbent assay (ELISA) for detection and quantitation of factor B in soluble or membranous preparations. The assay requires nanoliter volumes of rabbit antiserum raised against purified factor B and will detect nanogram amounts of the coupling factor. Analysis of beef heart submitochondrial particles using a competitive binding ELISA indicated a factor B content of 0.27 nmol/mg protein, making factor B stoichiometric with F_1 (0.3–0.6 nmol/mg). Furthermore, application of the factor B ELISA has indicated the presence of material cross-reacting with the beef heart factor B-antiserum in phosphorylating membranes from chloroplasts, *Escherichia coli*, *Paracoccus denitrificans* and the thermophilic bacterium, PS3. Negative results were obtained with mitochondria and microsomes from rat liver, purple membranes from *Halobium halobacterium* and sarcoplasmic reticulum from rabbit skeletal muscle.

Introduction

Coupling factor B (F_B) was first isolated from beef heart mitochondria by Lam et al. [1]. This highly purified protein, although lacking in intrinsic catalytic activity, enhanced ATP-linked reactions in F_B-deficient submitochondrial particles (prepared by ammonium/EDTA extraction) [1]. F_B was assayed by its stimulations of the activity of ammonium/EDTA-extracted submitochondrial particles in ATP-driven NAD^+ reduction by succinate. It was also demonstrated that F_B has an essential -SH group and that its coupling activity is sensitive to -SH inhibitors. Its minimum molecular weight was 14 600 by amino acids analysis and 32 000 by sedimentation analysis, indicating a dimeric form. In recent years, several other proteins with similar coupling activity have been isolated [2–6]. All of these showed sensitivity to -SH inhibitors. Some [5,6] also showed immunological cross-activity with the antiserum raised against the original F_B preparation of Lam et al. [1]. However, the various proteins containing F_B activity differed in their molecular weight (11 000–47 000), stability on storage, and most importantly in their specific activity (from 2 to 100 μmol NAD^+ reduced/min per mg) in the coupling factor assay [1]. In view of this and the fact that the native form(s) of F_B in mitochondria is still unidentified, it became necessary to develop another quantitative assay not based on coupling activity. Furthermore, it was recently reported from this laboratory that F_B is an essential component of the energy-transducing ATPase com-

Abbreviation: Ig, immunoglobin.

plex or H^+-ATPase * and is required for P_i-ATP exchange activity [2]. It is not needed for the oligomycin-sensitive ATPase in beef heart mitochondria but is necessary for converting the oligomycin-sensitive ATPase to an H^+-ATPase. Its presence in the H^+-ATPase was further confirmed by the cross-reactivity of the latter to anti-F_B serum [2]. In view of its importance, it has become necessary to explore whether H^+-ATPase from other sources, which have many structural and functional similarities with the beef heart H^+-ATPase, also have an F_B-type protein. If so, it is reasonable to expect that this protein from different species may have some common antigenic determinants with F_B.

In view of the above, an enzyme-linked imunnosorbent assay (ELISA) has been developed to detect and quantitate proteins cross-reacting with anti-F_B serum. The assay is simple, fast, involves no radioisotopes, requires only nanoliter quantities of antiserum and detects nanogram amounts of F_B. It is applicable to soluble as well as membranous proteins. The assay has also been applied to detect anti-F_B cross-reactive material in other phosphorylating membranes.

Material and Methods

Beef heart mitochondria [8], rat liver mitochondria [9], submitochondrial particles [10], F_1-ATPase [11] and oligomycin sensitivity-conferring protein [12], were prepared according to published methods. F_B, isolated as described by Joshi et al. [2], was used to raise antibodies in female rabbits as described previously [2]. Spinach chloroplasts purified over a Percoll gradient [13] were kindly supplied by Dr. M. Gibbs and Mr. J. Berkowitz, *Paracoccus denitrificans* by Drs. M.F. Henry and P.M. Vignais, sarcoplasmic reticulum from rabbit skeletal muscle by Dr. N. Ikemoto, purple membrane from *Halobacterium halobium* by Dr. H.G. Khorana, and thermophilic bacterium PS3 membrane by Dr. K. Kagawa. Plastic microtiter plates (Immulon) with 'U' bottom wells were obtained from Dynatech Laboratories, Alexandria, VA. Rabbit anti-bovine serum albumin serum was obtained from Miles Biochemicals, Elkhart, IN, and goat anti-rabbit Ig conjugated to horseradish peroxidase from Cappel Laboratories, Cochraneville, PA. *o*-Phenylenediamine was obtained from Eastman Kodak Company, Rochester, NY.

F_B or material cross-reactive with anti-F_B serum was detected by the direct binding assay and quantitative determinations were made using the competitive inhibition assay, both of which are described in detail in the next section.

Results and Discussion

Standardization of the direct binding assay

The procedure is modified from that of Voller et al. [14] as follows: F_B was diluted to various concentrations in coating buffer (50 mM carbonate/bicarbonate buffer, pH 9.6, containing 0.02% NaN_3) and a 200 μl aliquot of each concentration was applied to the microtiter wells. The plate was incubated at room temperature in a humid plastic box to facilitate attachment of F_B to plastic wells. The unbound F_B was removed by aspiration, followed by washing the wells three times with 300 μl of buffer A (50 mM sodium phosphate buffer, pH 7.0, containing 0.8% saline and 0.05% Tween 20). F_B bound to the plates was next allowed to incubate with 200 μl of rabbit anti-F_B serum or rabbit normal serum. Unbound serum was removed by aspiration and washing as described before. The wells were then incubated for 60 min at 37°C with 200 μl of a 0.001% solution of goat anti-rabbit Ig-enzyme conjugate. After removing the unbound conjugate, a 200 μl aliquot of substrate solution (0.01% *o*-phenylenediamine solution in water containing 0.003% H_2O_2) was added to each well and the plate was incubated again at 37°C. The reaction was allowed to proceed until absorbance at 490 nm in the first well approached 0.5–1.5. The reaction in all the wells was then terminated by adding 100-μl aliquots of 2 N H_2SO_4 to each well. The samples were mixed and read at 490 nm in a Stasar II Gilford spectrophotometer equipped with an aspirating microcuvette. Under appropriate conditions, the increase in absorbance at 490 nm is directly proportional to the antigen bound to the microtiter

* We have observed that the ATPase or F_0-F_1 complex made by the lysolecithin extraction procedure [2] is capable of utilizing ATP energy for proton pumping in the same manner as the H^+-translocating ATPase preparation of Serrano et al. [7]. Hence, it is appropriate to call this preparation [2] H^+-ATPase, a term which is being increasingly used in the literature.

wells. The bound antigen determines the amount of rabbit anti-F_B Ig binding, which in turn determines the amount of goat anti-rabbit Ig-enzyme conjugate binding.

The sensitivity of ELISA is dependent upon three parameters. First, the binding of the antigen to the microtiter plate is time dependent as can be seen in Fig.1. In this experiment, other components such as rabbit anti-F_B serum, goat anti-rabbit Ig, or peroxidase substrate levels are in saturating amounts. Hence, it is reasonable to assume that the peroxidase activity, as measured by A_{490}, is in direct proportion to F_B binding, although no direct measurements were made of F_B binding to microtiter wells. The peroxidase activity data indicate that F_B binding increases as a function of the time of its initial incubation in the well. Using a 1 : 100 dilution of rabbit anti-F_B serum, no additional F_B binding occurs after 8–10 h of incubation, while 50% of maximal activity is observable within the first 2 h. Furthermore, the initial incubation of the antigen in the microtiter wells can be extended up to 16 h without loss of activity. Hence, in subsequent experiments, the antigen was routinely incubated overnight (approx. 16 h). The second parameter affecting assay sensitivity involves the interaction of rabbit anti-F_B serum with F_B (which is bound to the microtiter plates) which again is time dependent. The results of incubating wells, pretreated with 250 ng/ml F_B for 16 h, with a 1 : 200 dilution of rabbit anti-F_B serum are presented in Fig. 2. Interaction of antiserum with the bound antigen increases progressively during the first 4 h of incubation. After that time, there is a slight decrease in overall activity. This may be due to inactivation of the bound F_B, since both its coupling activity and immunological activity are temperature sensitive (unpublished observations). The results in Fig. 1 would seem to preclude a time-dependent net release of the antigen from the plate as the cause of this decrease.

Finally, to determine the effect of antiserum concentration, microtiter wells are first treated with aliquots of F_B (125 ng/ml) for 16 h at 25°C. 200-μl samples of various dilutions of rabbit anti-F_B or rabbit normal serum were added and the incubation at 25°C continued for another 4 h. The results (Fig. 3) indicate that a 1 : 300 dilution of rabbit anti-F_B serum provides Ig to saturate bound F_B sites. A 1 : 400 dilution of antiserum gave 80% of the maximal response. Even at 1 : 1 200 dilution, samples with rabbit anti-F_B serum could be readily distinguished from those containing rabbit normal serum.

The sensitivity of the assay was finally tested by

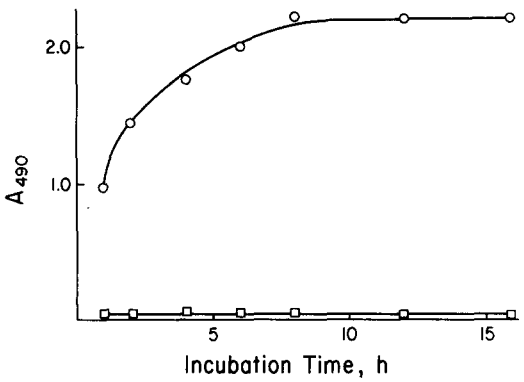

Fig. 1. Time course of incubation of F_B in the direct binding assay. A 200 μl aliquot of F_B (125 ng/ml) was applied to each microtiter well and the plate was incubated for the indicated time intervals. After removing the unbound F_B by washing and aspiration, the wells were incubated sequentially with 200-μl aliquots of (1) a 1 : 100 dilution of rabbit serum for 4 h at room temperature, followed by (2) a 1 : 1 000 dilution of goat anti-rabbit Ig-enzyme conjugate for 1 h at 37°C followed by (3) substrate solution at 37°C. The reaction was terminated by the addition of a 50 μl aliquot of 2 N H_2SO_4. After mixing the samples were read at 490 nm. (o———o) Rabbit anti-F_B serum, (□———□) rabbit control serum.

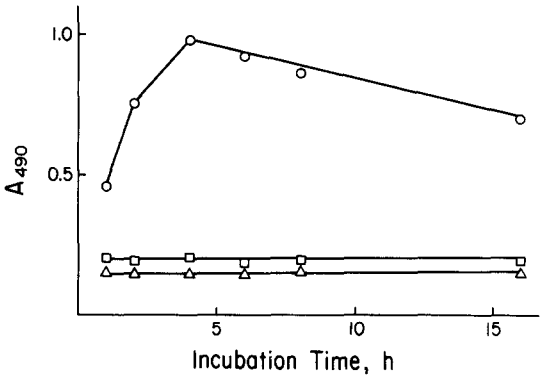

Fig. 2. Time course of incubation of rabbit anti-F_B serum with immobilized F_B in the direct binding assay. The wells were incubated with F_B (250 ng/ml) for 16 h and then with rabbit serum (1 : 200 dilution) for the indicated time intervals. The rest of the assay conditions were as described for Fig. 1. (o———o) Rabbit anti-F_B serum, (□———□) rabbit control serum, (△———△) no serum.

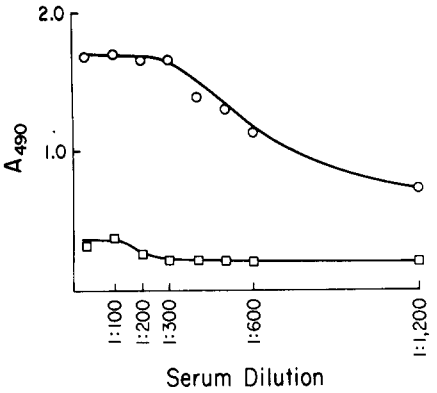

Fig. 3. Titration of rabbit anti-F_B serum with F_B bound to the wells in the direct binding assay. Microtiter wells were incubated with F_B (125 ng/ml) for 16 h and then with the indicated dilutions of rabbit serum for another 4 h. The remaining assay conditions were as described for Fig. 1. (o———o) Rabbit anti-F_B serum, (□———□) rabbit control serum.

applying 200-μl aliquots of various dilutions of F_B in coating buffer. The wells were allowed to incubate for 16 h at room temperature. After removing unbound F_B a 200 μl aliquot of a 1 : 400 dilution of rabbit anti-F_B serum was then added and the assay completed as described previously. As can be seen in Fig. 4, A_{490} increases linearly with increasing levels of F_B present during the initial incubation. In contrast,

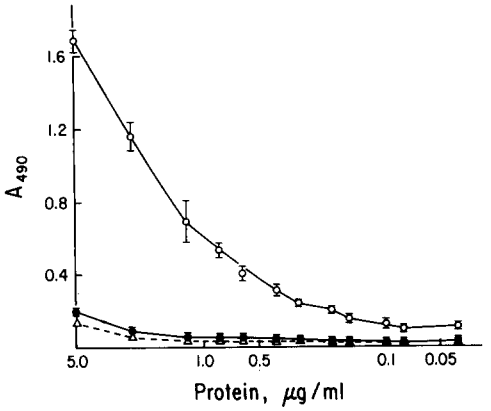

Fig. 4. Antigen titration using F_B as the antigen in the direct binding assay. The microtiter wells were incubated first with the indicated concentrations of F_B for 16 h and then with a 1 : 400 dilution of rabbit serum for 4 h. The remaining assay conditions were as described for Fig. 1. (o———o) Anti-F_B serum, (•———•) normal serum, (△- - - -△) no serum.

when F_B-coated microtiter wells were preincubated with rabbit normal serum or without serum, no increase in A_{490} was detectable, except at the highest antigen concentration (5 μg/ml). Under these conditions, the assay will detect F_B at a concentration of 100 ng/ml in the initial incubation step.

It is evident that under the conditions described above, the increase in peroxidase activity in the wells exposed to rabbit anti-F_B serum as compared to rabbit normal serum is a direct measure of the reactivity of the bound antigen to anti-F_B serum. A unit of cross-reactivity is defined as the change in A_{490} produced when 1 μg/ml F_B is applied to the well and the plate processed as described above.

Cross-reactivity to rabbit anti-F_B serum in other membrane preparations

To verify if other ATP-synthesizing membranes contain components that are immunologically reactive to mitochondrial anti-F_B serum, assays were performed as follows. Sarcoplasmic reticulum from rabbit skeletal muscle, purple membrane from *H. halobium*, rat liver mitochondria and rat liver microsomes were suspended at 10 mg/ml in 10 mM Tris-HCl buffer (pH 7.8) containing 0.25 M sucrose and sonicated for 2 min at 0–4°C, prior to assay. Beef heart mitochondria, *Escherichia coli, P. denitrificans*, thermophilic bacterium PS3 and spinach chloroplast membranes were similarly suspended at 10 mg/ml and sonicated. The sonicated suspensions were subjected to centrifugation at 10 000 × *g* for 20 min to remove larger, unbroken membranes. The supernatant (S_1) was next centrifuged at 100 000 × *g* for 3 h to sediment small membrane particles. The resultant supernatant (S_2) and sediment (P_2) (after suspension in the initial sonication medium) were assayed for immunoreactivity against anti-F_B serum. The test samples were serially diluted in coating buffer and 200-μl aliquots of each dilution were applied to microtiter wells. After a 16 h incubation, unbound material was removed and the assay completed as described previously. Parallel assays with purified F_B were used for comparison of relative cross-reactivity. It is important to realize that the values so derived may not necessarily reflect absolute antigen concentration but only the degree of cross-reactivity towards anti-F_B serum.

It was found that S_1 fractions from beef heart

mitochondria, chloroplasts, *P. denitrificans*, *E. coli* and PS3 were highly immunoreactive. ELISA on samples after centrifugation of S_1 revealed that the cross-reactive material was primarily associated with S_2 or P_2, depending upon the nature of the membrane and the medium in which it was sonicated. The experimental conditions for enrichment of cross-reactive material in P_2 were not identical for each membrane as is illustrated in Table I. This is not surprising, considering that the conditions required for isolation of functionally intact membranes from these sources are also very different.

Sonicated rat liver mitochondria as well as subfractions therefrom showed negligible reactivity to anti-F_B serum as assayed by ELISA. It is unclear whether this is related to the absence of factor B-type proteins in rat liver mitochondria or to the possibility that the proteins from rat liver and bovine heart share no common antigenic determinations. Similar sensitivity of rat liver and beef heart mitochondrial phosphorylation reactions to cadmium [15] together with recent evidence tentatively identifying F_B as a cadmium-binding protein in beef heart mitochondria [16] would seem to favor the latter explanation. Additional experiments to resolve this point are in progress. Rat liver microsomes, sarcoplasmic reticulum and purple membrane showed negligible reactivity to anti-F_B serum. Treatment of microtiter wells with glutaraldehyde prior to the antigen-binding step to improve adsorption of antigen to the wells [17] did not result in increased activity. We have found that purified H^+-ATPase from *E. coli* shows positive cross-reactivity to anti-F_B serum. Mitochondrial F_1 and oligomycin sensitivity-conferring protein were negative when tested by the direct binding assay. This is consistent with the earlier suggestion [2] that F_B may be part of F_0 of the H^+-ATPase.

The particles (P_2) from rat liver mitochondria, *E. coli* and spinach chloroplasts were also tested for reactivity to anti-F_B serum by the Ouchterlony radial immunodiffusion method. 2–3 mg of crude anti-F_B serum were placed in the central well and 0.1–1.5 mg

TABLE I

DETECTION OF CROSS-REACTIVE MATERIAL IN MEMBRANE PREPARATIONS BY THE DIRECT BINDING ASSAY

Fractions of different membranes were serially diluted in coating buffer, and a 200 μl aliquot of each dilution was applied to microtiter wells. S_2 and P_2 are the high-speed supernatant and pellet fractions, respectively, resulting from centrifugation of S_1. Additional details are provided in the text and Fig. 1. Isolation buffers: (1) 50 mM Tris-acetate, 10 mM Mg^{2+}/acetate, 1 mM ATP (pH 7.4); (2) 50 mM Tris-acetate, 10 mM Mg^{2+}/acetate, 1 mM ATP (pH 7.8); (3) 10 mM Tris-HCl (pH 7.8); (4) 0.25 M sucrose, 10 mM Tris-HCl (pH 7.8).

	Sub-fractions	Cross-reactivity (units/mg)	Total cross-reactivity (units) [a]	Isolation buffer
Bovine heart mitochondria	S_2	23.5	0.45	1
	P_2	59.5	3.75	
E. coli	S_2	155	393	2
	P_2	>2 000	3 571	
P. denitrificans	S_2	60.0	22	3
	P_2	2 000	115	
Spinach chloroplasts	S_2	<5.0	0.35	4
	P_2	6 000	414	
Thermophilic bacterium PS3	S_2	1 280	252	4
	P_2	1 493	89	
Rat liver microsomes		<1.0	–	4
Sarcoplasmic reticulum		<0.5	–	4
Purple membrane from *halobium*		<0.1	–	4

[a] Expressed as total units of cross-reactive material/mg protein of starting material.

of various membrane preparations, with and without prior treatment with 0.1% Triton X-100, were applied to the peripheral wells. The plates were incubated in a humid environment at 2–4°C. Single precipitin lines were visible against 5 μg F_B or 1 mg submitochondrial particles from bovine heart within 48 h but none were obtained against the P_2 fraction of the other membrane preparations even after 2 weeks. This may indicate that the antigen-antibody complexes in the case of phylogenetically distant species are nonprecipitating under the conditions used here. This is not surprising, since antigenic determinants for lattice formation on these membranes may be in insufficient amounts to cause immunoprecipitation, yet sufficient to be detected by ELISA.

It should be noted that the direct binding assay is only semiquantitative. It is limited by the binding characteristics of the antigen to microtiter wells. In the case of antigens with low binding affinity, the results of the direct binding ELISA would underestimate the concentration of the antigen in the test sample. Moreover, the use of crude membrane preparations as test samples may introduce still other undefined components, which could influence the binding of antigen to the wells. Hence, quantitative measurements are best accomplished by the competitive inhibition ELISA. It is more reliable, since it is not dependent upon binding of the antigen to the well. Rather, it is primarily dependent upon the affinity of the antigen for rabbit anti-F_B serum, a property which is not known to be influenced by the presence of other nonimmunoreactive proteins or lipids. Nevertheless, the binding assay is potentially useful for providing rapid preliminary information on relative immunoreactivity. It is also useful in establishing the range of cross-reactive material to be used in the competitive inhibition assay.

F_B quantitation by competitive inhibition with F_B in ELISA

In this assay the antigen (cross-reactive material) is quantitated by its capacity to inhibit the binding of antibody to the antigen adsorbed on the solid phase. To obtain maximal sensitivity in the assay, the amount of antigen used for coating the wells is decreased to the level of detection, and the amount of antibody added should be limiting. To determine the antigen antibody concentrations suitable for this purpose, checkerboard titrations of the antigen (F_B) and of rabbit anti-F_B serum were performed by the direct binding assay as follows. The wells were coated with various concentrations (40 ng–5 μg/ml) of F_B and after the initial binding, each concentration of the antigen was titrated with different dilutions (1 : 100–1 : 3 200) of rabbit anti-F_B serum. The rest of the assay was conducted as described before. The data (not shown) indicated that any concentration of F_B from 315 down to 80 ng/ml gave measurable activity. Similarly, a 1 : 600–1 : 1 600 dilution of rabbit anti-F_B serum was found to be in the limiting range. The competitive inhibition assay was then performed as follows. The test protein sample was diluted serially in 150 μl of buffer A and allowed to equilibrate with an equal volume of a 1 : 600 dilution of rabbit anti-F_B serum or rabbit control serum. During this incubation rabbit anti-F_B Ig reacts with F_B present in the test sample, thus reducing the amount of free rabbit anti-F_B Ig. In the meantime, microtiter wells were coated with 200-μl aliquots of 100 ng/ml F_B just as in the direct binding assay. After removing unadsorbed F_B, 200-μl aliquots of test samples, premixed with anti-F_B serum as described above, were transferred to the wells. The incubation was continued for another 4 h and the remaining assay completed as described for the direct binding assay. Anti-F_B cross-reactive material in the test sample competed with the F_B adsorbed to the wells during the coating step, for the limited number of binding sites on rabbit anti-F_B serum. The amount of rabbit anti-F_B Ig available for binding to F_B adsorbed to the wells was thus inversely related to the amount of F_B present in the test sample. This led to binding of fewer goat anti-rabbit Ig-peroxidase conjugate molecules and ultimately to decreased peroxidase activity. By using known levels of F_B as the test samples, the resultant decrease in peroxidase activity provided a standard curve (Fig. 5). The experiment was then repeated with the unknowns, and the amount of anti-F_B cross-reactive material, if any, was determined by comparison with the standard curve. Inhibition ELISA, like radioimmunoassay, should be applicable to purified as well as partially purified proteins. The validity of this was tested by quantitating bovine serum albumin, in a system containing limited levels of rabbit anti-bovine serum albumin and unknown samples containing (a) bovine serum albumin, (b) *E.*

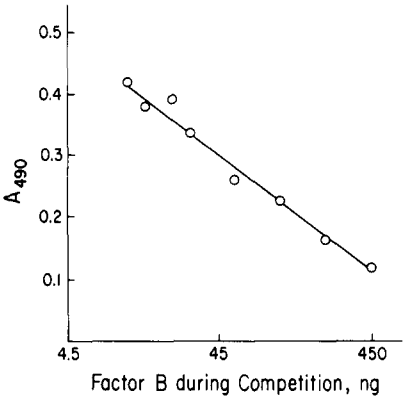

Fig. 5. Estimation of F_B by competitive inhibition ELISA. Microtiter wells were incubated with F_B (100 ng/ml) in coating buffer for 16 h. At the same time, the indicated concentrations of F_B were also indicated with an equal volume of a 1 : 600 dilution of rabbit anti-F_B serum in buffer A. After removing the 'unbound' F_B from the microtiter wells, a 200 μl aliquot of each dilution of mixed F_B anti-F_B serum samples was transferred to the wells and incubation continued for another 4 h. The rest of the assay conditions were as described in Fig. 1.

TABLE II

QUANTITATION OF F_B IN ELECTRON-TRANSPORT PARTICLES PREPARED IN THE PRESENCE OF Mg^{2+} AND Mn^{2+} FROM HEAVY MITOCHONDRIA BY COMPETITIVE INHIBITION ELISA

The microtiter wells were coated with 200-μl aliquots of 100 ng/ml F_B in carbonate/bicarbonate buffer. The particles were suspended in phosphate-buffered saline containing 0.5% Tween 20 at 1 mg/ml and the suspension was centrifuged at 50 000 rev./min for 2 h. The supernatant was serially diluted in buffer A and mixed with an equal volume of a 1 1 : 600 dilution of rabbit anti-F_B serum. After removing the unbound F_B from the microtiter wells, 200-μl aliquots of premixed extracted particle-rabbit anti-F_B serum were transferred to the wells and incubated for an additional 4 h. The wells were next incubated with goat anti-rabbit Ig-enzyme conjugate and substrate as described before.

Particle protein in the assay (μg)	A_{490}	F_B as estimated from the standard curve (ng)
5	0.390	15
10	0.325	32
20	0.260	75
40	0.195	160

coli membranes, and (c) bovine serum albumin and *E. coli* membranes together. It was found that *E. coli* membranes did not compete with 'bound' bovine serum albumin (no immunological cross-reactivity to rabbit anti-bovine serum albumin serum) and caused no interference in the estimation of bovine serum albumin in the test sample c as compared to sample a (data not presented).

The competitive inhibition assay was applied to quantitate F_B in electron-transport particles prepared in the presence of Mg^{2+} and Mn^{2+} from bovine heart mitochondria [10]. These particles had an activity of 250 μmol NAD^+ reduced/min per mg in the ATP-driven NAD^+ reduction by succinate and were not stimulated further by added F_B. The particles were suspended in buffer A at 1 mg/ml and centrifuged at 50 000 rev./min for 2 h to remove any particulate material. The residue was resuspended in coating buffer and tested for the presence of any unextracted F_B by the direct binding assay. There was no measurable activity. The supernatant was serially diluted, mixed with rabbit anti-F_B serum and was applied to the microtiter wells for quantitation of F_B by the competitive inhibition ELISA. Table II shows a distinct decline in A_{490} indicating the presence of cross-reactive material in electron-transfer particles prepared in the presence of Mg^{2+} and Mn^{2+} from heavy mitochondria. The decrease in resultant peroxidase activity is equivalent to the presence of 3.9 μg or 0.267 nmol F_B/mg of these particles. The molecular weight of F_B was taken as 14 600 for these calculations. It should be noted that the calculated F_B content may be an underestimate of the actual F_B content although no F_B activity as measured by the direct binding assay was left in the extracted residue. Coincidentally, this value of 0.267 nmol F_B/mg particle is in good agreement with the published values of 0.3–0.6 nmol F_1-ATPase/mg particle (calculated from published data [18]). From these results, F_B and F_1 bear a stoichiometric ratio of 1 : 1, which is also consistent with the previously published ratio, derived on the basis of *N*-[^{3}H]ethylmaleimide binding to the F_B band of the H^+-ATPase in sodium dodecyl sulfate-polyacrylamide gels [19].

It is evident from the above that ELISA is a useful tool for detection and quantitation of proteins cross-reactive with anti-F_B serum, without having to isolate

and purify cross-reactive material. The assay has the added advantage of detecting antigens that form non-precipitating complexes with antibodies. This is the first report to demonstrate that proteins cross-reactive with anti-coupling F_B serum do occur in phosphorylating membranes of other species, but not in nonphosphorylating membranes. The present data are insufficient to establish the functional role of anti-F_B cross-reactive proteins. However, their presence in phosphorylating membranes and in purified H^+-translocating ATPase preparations of beef heart [2] and of *E. coli* (unpublished observations), and their absence in nonphosphorylating membrane preparations suggest a close functional correlation between mitochondrial proteins in other phosphorylating membrane preparations. Experiments to characterize further these cross-reactive proteins and test whether they would restore energy-linked reactions in F_B-depleted membranes particles are in progress.

Acknowledgments

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