Coupling factor B involvement in the inhibition of P_i–ATP exchange activity by *N*-ethylmaleimide

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1. INTRODUCTION

Recent results have established coupling factor B (F_B) as a component of the F_o segment of mitochondrial H^+-ATPase and its participation in the P_i–ATP exchange reaction but not in the oligomycin-sensitive ATPase [1]. F_B activity in phosphorylating submitochondrial particles and in the H^+-ATPase is inhibited by membrane-impermeable, polar mercurials [2,3], indicating involvement of an –SH in the hydrophilic, extrinsic milieu. Similarly, it is inhibited by Cd^{2+}, phenylarsine oxide and arsenite in the presence of equimolar 2,3-dimercaptopropanol under conditions specific for inactivation of vicinal dithiol groups [4,5]. This observation is consistent with the presence of two cysteines per F_B monomer as determined by amino acid analysis [6]. The H^+-ATPase has several –SH groups besides that in F_B [7]. Here, we have presented evidence suggesting that inhibition of the P_i–ATP exchange activity of the H^+-ATPase by *N*-ethylmaleimide (NEM) is caused by modification of a thiol group on a protein which co-migrates with F_B on SDS–PAGE. The data have been used to arrive at the stoichiometry of F_B:F_1 in the H^+-ATPase.

Abbreviations: F_B, beef heart mitochondrial coupling factor B; F_o, membrane segment of the H^+-ATPase; NEM, *N*-ethylmaleimide; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; DCCD, *N,N'*-dicyclohexylcarbodiimide; ClCCP, carbonylcyanide *m*-chlorophenylhydrazone

2. MATERIALS AND METHODS

Beef heart mitochondria [8], ETPH [9], F_1 [1], F_B [1], OSCP [10] and F_6 [11] were isolated by published methods. The procedure for the lysolecithin extraction of H^+-ATPase from beef heart mitochondria is modified from [12] and is detailed in [13], as are the methods for the assay of ATPase and P_i–ATP exchange activity [6]. Thiol saturation by [^{3}H]NEM was accomplished by first incubating the H^+-ATPase (20 mg/ml) at 37°C for 10 min in 40 mM Tris–H_2SO_4 (pH 8.0) containing 1 mM EDTA, 1% SDS and 40 mM 2-mercaptoethanol. Aliquots (0.2 ml) were transferred to a small Sephadex column (G-25, 1.5 ml) pre-equilibrated with 10 mM sodium phosphate (pH 7.0) containing 0.1 mM DTT and 1% SDS and eluted by centrifugation. Protein was adjusted to 10 mg/ml and then incubated with 10 mM [^{3}H]NEM (100 μCi/μmol) for 3 h at 20°C. No further binding occurred beyond this time.

Inhibition of P_i–ATP exchange by NEM was determined after incubating H^+-ATPase (2 mg/ml) for 60 min at 20°C in 100 mM Tricine–KOH (pH 8.0) containing 0.25 M sucrose and various [NEM]. Alternatively, 250 μM NEM and variable incubation times were used. The reac-

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tion was terminated by the addition of a 40-fold excess of 2-mercaptoethanol. Samples were diluted with the medium used for the assay of P_i–ATP exchange and the activity determined. Duplicate samples were treated with [^{3}H]NEM and subjected to SDS–PAGE [14].

3. RESULTS

The H^+-ATPase prepared as described has high P_i–ATP exchange activity (400–600 nmol.min^{-1}.mg^{-1}), which is stimulated by OSCP or F_6 and only minimally stimulated (< 10%) by addition of F_1 and/or F_B [13]. Activity is sensitive to energy-transfer inhibitors (2 μg oligomycin, 5 μg rutamycin, 10 μM DCCD) and uncouplers (50 μM ClCCP, 2 mM DNP). The high level of exchange activity plus the lack of any coupling factor requirement have been attributed to a high degree of structural and compositional integrity in this complex.

The Coomassie Blue staining pattern following SDS-electrophoresis of the H^+-ATPase (fig.1) is similar to that obtained with other preparations [15,16]. The peaks representing the 5 subunits of F_1 as well as OSCP, F_6 and the ATPase inhibitor protein have been identified by co-migration with the respective purified proteins. Peak 7 co-migrates with the fluorescamine-labelled monomer of coupling factor B. Components with M_r 60000–100000 are variable and, in agreement with other studies, are considered non-essential for P_i–ATP exchange. Band 4 (M_r 29000) is also a variable component which can be resolved from the complex by sucrose gradient centrifugation without loss of exchange activity as has been observed with other preparations [16].

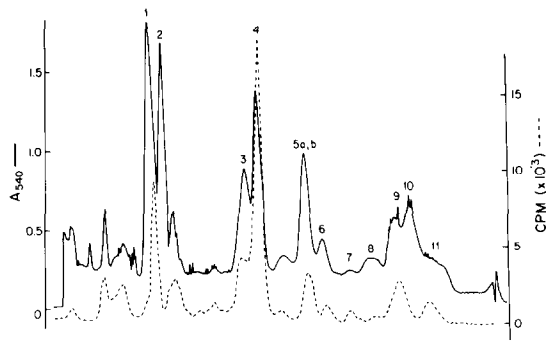

Fig.1. Radioactivity and staining profile of SDS–PAGE of H^+-ATPase. H^+-ATPase was incubated with 10 mM [^{3}H]NEM (10 μCi/μmol) for 3 h at 20°C in the presence of 1% SDS and 0.1 mM DTT as in section 2. Samples (50 μg/gel) were subjected to SDS–PAGE [14] and either stained with Coomassie blue (0.5% in 10% acetic acid–40% methanol, 15 h at 20°C) or sliced and counted.

The total thiol content of the H^+-ATPase was determined following incubation of the complex with [^{3}H]NEM (10 mM) for 3 h in the presence of 1% SDS. Reactive thiols in soluble F_1 are confined to the α, γ and ϵ subunits [17] which is consistent with NEM incorporation into bands 1, 3 and 11 but not 2 (fig.1). Activity associated with peak 8 indicates the presence of a thiol containing component in addition to the thiol free δ subunit of F_1. The best possible stoichiometry for this component is 0.06 nmol/2 nmol F_1 (table 1), suggesting that it cannot represent an essential component. NEM binding to peak 4 is not included since this component is not considered essential for P_i–ATP exchange [13]. Peak 9 and peak 10 probably contain, F_6, proteolipid and possibly the ATPase inhibitor. Both F_6 [18] and the inhibitor protein from beef heart [19] contain no cysteine while the proteolipid does [20]. Consequently, the NEM incorporation into peak 8 may represent binding to the proteolipid molecule. Binding to peak 10 is also substoichiometric with F_1 suggesting that the NEM binding component is a contaminant.

The total thiol content of the F_1 subunits is known [17]. Using these values and by assuming an M_r of 500000 for H^+-ATPase [21] and 360000 for F_1 [22], the amount of [^{3}H]NEM incorporated into the F_1 subunits present in H^+-ATPase (table 1) can be predicted (i.e., 8 nmol NEM incorporated into the α subunit/nmol F_1). The values for the α and ϵ subunits are in good agreement with the experimental values. The γ subunit may not be sufficiently resolved from an adjacent thiol binding component (peak 4, fig.1) which would explain the apparent discrepancy for this subunit (table 1).

The factor B content of beef heart mitochondria has been estimated using an enzyme-linked immunosorbent assay (ELISA) employing a rabbit antibody raised against a homogeneous F_B preparation [23]. The result indicated a F_B stoichiometry of 1:1 with respect to F_1. While the amino acid composition of F_B indicates 2 cysteine residues/monomer [6], the NEM binding to the

Table 1

Maximum incorporation of [^{3}H]NEM into components of H^+-ATPase (nmol/mg)

Band	Predicted	Experimental	
1. (α-F_1)	16.0[a]	16.4	(0.9)
2. (β-F_1)	0[a]	–	–
3. (γ-F_1)	4.0[a]	6.4	(0.4)
4. –	–	–	–
5. a,b, (OSCP,)	–	7.8	(1.1)
7. (F_B (factor B)	–	2.1	(0.2)
8. (δ-F_1)	0[a]	0.06	–
9. (Proteolipid)	12[b]	10.8	(1.1)
10. (ATPase inhibitor)	0[c]	0.1	–
11. (ϵ-F_1)	4.0[a]	3.7	(0.7)
F_1 content	2.0 nmol/mg H^+-ATPase		
F_B content	2.1 nmol/mg H^+-ATPase		

[a] Based on published values [17]
[b] Based on amino acid content [20]
[c] Based on amino acid content [19]

H^+-ATPase (20 mg/ml) was incubated in 40 mM Tris–SO_4 (pH 8.0) containing 1 mM EDTA, 1% SDS and 40 mM 2-mercaptoethanol for 10 min at 37°C and then eluted from Sephadex G-25 with 10 mM sodium phosphate (pH 7.0) containing 0.1 mM DTT, and 1% SDS. Protein was adjusted to 10 mg/ml with phosphate buffer and sufficient NEM added to reach 10 mM final conc. (100 μCi/μmol). Incubation was for 3 h at 20°C. Predicted values are based on the assumption that 1 mg H^+-ATPase (M_r 500000) = 2.0 nmol and a stoichiometry of 1 nmol F_1/nmol H^+-ATPase. Results are means of 3 values, and ( ) = 1 standard derivation

band co-migrating in this gel system with fluorescamine-labelled F_B indicates that only 1 nmol NEM is incorporated/nmol F_1. This would suggest that even in the presence of SDS, only one of the two thiols in F_B is reactive towards NEM. The reason is unclear. However, the coupling activity of both soluble and membrane-associated F_B can be inhibited by Cd^{2+} under conditions where Cd^{2+} binding is expected to be specific for dithiols and not monothiols [4]. Consequently, the two thiol components in F_B are sufficiently close to react with a single Cd^{2+} molecule. In addition, when the dithiol components of the H^+-ATPase are labelled with ^{115}Cd and then subjected to SDS–PAGE, the Cd^{2+} remains associated with peak 7 [24]. Therefore, even in the presence of SDS, the two thiols in F_B may remain in such close proximity that the binding of NEM to one may sterically hinder access of NEM to the other.

An alternative explanation for the apparent difference in reactivity of the F_B thiols towards NEM may involve their respective micro environments. One F_B thiol, located in a relatively hydrophobic environment might be expected to react readily with NEM. The second thiol, located in a more hydrophilic environment would perhaps be more accessible to polar and membrane-impermeable mercurials [2,3]. In either case, if the current NEM binding data are interpreted to indicate that only one of the two thiols in F_B is NEM reactive, then the stoichiometry of F_B is 1:1 with F_1, and is therefore consistent with ELISA results.

The stoichiometry of the DCCD binding proteolipid of the H^+-ATPase is unsettled. Values of 3 and 6 copies/H^+ channel (or H^+-ATPase) have been reported [25–27]. Since the proteolipid contains only a single cysteine [20], the maximum NEM incorporated into band 9 suggests a stoichiometry of 10.8 nmol proteolipid/mg H^+-ATPase. Using a value of 2 nmol F_1/mg H^+-ATPase (table 1) the NEM incorporation into band 8 suggests a maximum of 6 proteolipid molecules/F_1.

The lysolecithin-extracted H^+-ATPase also contains an NEM reactive site which is essential for P_i–ATP exchange activity (fig.2). The sensitivity of the exchange activity of the H^+-ATPase to NEM (fig.2, ——) is similar to that of reconstituted F_0 [18], but surprisingly greater than that reported for the extensively characterized complex V [7]. The explanation for this difference is unclear although the relatively high P_i–ATP exchange activity of the lysolecithin H^+-ATPase as compared to complex V may indicate a basically different arrangement of subunits. The identification of the essential NEM-reactive component in the lysolecithin H^+-ATPase was based on the assumption that the extent of NEM binding to an essential component should parallel the loss of exchange activity. Consequently, the incorporation of NEM into each band on SDS gels of H^+-ATPase has been compared with the loss of activity as a function of time of incubation with [^{3}H]NEM. Since incubations were carried out under non-denaturing conditions, the incorporation of [^{3}H]NEM should reflect accessibility of

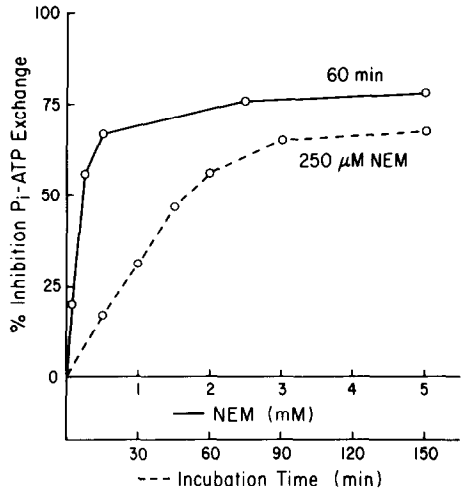

Fig.2. Inactivation of P_i–ATP exchange activity by *N*-ethylmaleimide. H^+-ATPase (2 mg/ml) was incubated at 20°C in 100 mM tricine–KOH (pH 8.0) containing NEM as indicated. P_i–ATP exchange was determined as in [6]. Duplicate samples incubated with [^{3}H]NEM (250 μM) for varying lengths of time and subjected to SDS–PAGE.

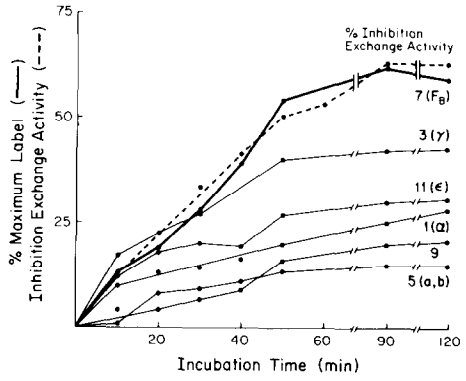

Fig.3. Time course of labeling of the subunits in H^+-ATPase preparation. H^+-ATPase (2 mg/ml) was incubated at 20°C in 100 mM tricine–KOH (pH 8.0) with 250 μM [^{3}H]NEM for varying times. The reaction was terminated by addition of DTT to 10 mM, followed by SDS to 1%. Samples were electrophoresed as in [14] and the incorporation of label into each peak determined. The level of incorporation at each time point was expressed as a percentage of maximum incorporation determined as in table 1. Each point represents the mean of 3 expt.

reactive groups to NEM. In each case, [^{3}H]NEM incorporation was compared to values obtained under denaturing conditions where accessibility restraints are removed. Thus, fig.3 represents a time course for the labeling of each individual component by NEM and can be used for comparison with the time course for the development of inhibition by NEM. Fig.3 shows the mean values of 3 expt, presented as percent of total label. Standard deviations did not exceed 15% of the mean and in general were $\leqslant 10\%$.

The NEM binding to peaks 8 and 10 suggests that these components are not present in H^+-ATPase in stoichiometric levels in comparison to F_1 (table 1). The time-dependent binding of [^{3}H]NEM to these components did not parallel the loss of P_i–ATP exchange activity. Consequently, the NEM-binding component in peak 8 and peak 10 cannot be considered essential for the activity of the H^+-ATPase, and the data are not included in fig.3.

The binding of [^{3}H]NEM to band 1 (α-F_1) and band 11 (ϵ-F_1) also did not parallel the loss of exchange activity. This is consistent with the observation that pretreatment of F_1 with NEM (2.2 mM) followed by removal of unreacted NEM, had no effect on subsequent reconstitution of P_i–ATP exchange activity using this NEM-F_1 and untreated F_o (unpublished [28]). In contrast, pretreatment of F_o containing F_B effectively blocked reconstitution of P_i–ATP exchange (see [1]). This suggests that the NEM-reactive group which is essential for exchange activity of the undissociated H^+-ATPase does not reside on F_1.

NEM binding to both band 5a,b and band 9 remained low, even following prolonged incubation. This may indicate that in the undissociated H^+-ATPase, some of the thiols in band 5a,b and 9 either exist as disulfides or are otherwise poorly accessible to NEM. The extent of NEM incorporation into band 5a,b and 9 does not parallel the loss of exchange activity (fig.3). However, because both bands contain multiple thiol groups, the possibility must be considered that only a single thiol in either peak represents an essential component. To completely inhibit exchange activity by binding an essential thiol, 2 nmol NEM would be required/mg H^+-ATPase, based on its F_1 stoichiometry. Since the maximal P_i–ATP exchange inhibition obtained was 65% under the experimental conditions employed, the binding of 1.3 nmol NEM (65% × 2 nmol) would then cor-

relate with the degree of inactivation. The maximum NEM bound to peak 5a,b together was 1.2 nmol which is sufficient by the above criteria to qualify one of them as a candidate for the functional thiol. However, OSCP which is the main component of peak 5a,b is not inactivated by treatment with thiol reagents [29]. The maximum NEM bound to peak 9 (proteolipid) is 2.4 nmol/mg) which is also consistent with the presence of one or even two essential thiols in this component. However, the essential thiol component of F_0 has been shown not to reside on the DCCD binding proteolipid [18]. Consequently, it is considered unlikely that the essential thiol resides in either peak 5a,b or 9.

The time course for NEM binding to band 7 closely parallels the increasing inhibition of P_i–ATP exchange. The actual level of [^{3}H]NEM incorporation into the band 7 at 120 min is 1.0–1.2 nmol/mg of H^+-ATPase. Assuming one NEM binding site/F_B (table 1) 50–60% of the F_B content of the H^+-ATPase is inactivated by NEM following 120 min incubation. This agrees well with the 65% loss of P_i–ATP exchange activity.

4. CONCLUSIONS AND COMMENTS

A previous study suggested that thiol binding to a low-M_r component (8000–10000) and to the α subunit of F_1 resulted in inhibition of ATPase and P_i–ATP exchange activity of complex V [7]. In the present study some binding to components in the same M_r range is observed but it does not appear to correlate with loss of P_i–ATP exchange activity. In contrast, binding of NEM to a component which co-migrates with F_B does correlate with inhibition of P_i–ATP exchange.

The results of this study agree with [1] indicating that pre-treatment of F_B-deficient F_o with high NEM (10 mM) did not influence subsequent reconstitution of P_i–ATP exchange following addition of F_1 and F_B. NEM treatment of F_B prior to reconstitution did prevent restoration of P_i–ATP exchange activity [1].

The failure to observe [^{3}H]NEM incorporation into the peak 7 region in SDS gels of complex V may be due to the loss of this component during detergent extraction of the complex. Such loss of F_B has been reported [5]. Partial loss of F_B would also explain the very low P_i–ATP exchange activity observed in these preparations even after reconstitution with phospholipids and other coupling factors.

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