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TRYPTIC PROTEOLYSIS OF COUPLING FACTOR-LATENT ATPase FROM *MYCOBACTERIUM PHLEI*

THEORETICAL MODELING OF STRUCTURE-FUNCTION RELATIONSHIPS *

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Summary

Trypsin treatment of solubilized coupling factor-latent ATPase from *Mycobacterium phlei* alters its subunit structure and functional properties. This coupling factor exhibits ATPase activity following trypsin treatment. Concurrently, both the ability of the enzyme to rebind to membranes depleted of coupling factor and its capacity for coupled phosphorylation are lost. The native alpha (64 000 dalton) subunit undergoes limited proteolytic digestion, and the delta (14 000 dalton) subunit is partially lost. During the course of tryptic proteolysis, the coupling factor molecule may exist in one of ten unique structural states (e.g. the native, ATPase-inactive molecule exists in the $\alpha\alpha\alpha$ state). Rigorous analysis of the experimental data by theoretical modeling provided information concerning the intermediate structural states leading to the fully ATPase-activated $\alpha''\alpha''\alpha''$ state under different conditions of trypsin treatment. The theoretical models of structure-function relationships that best-represented the experimental data predicted that the native coupling factor molecule contains three copies of the α (64 000 dalton) form of the alpha subunit, that the α'' (58 000 dalton) alpha subunit species contributes maximally and the α' (61 000 dalton) form about half-maximally to ATPase activity, that

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membrane rebinding ability is proportional to the number of native alpha subunits in the enzyme, and that at least one native α subunit/molecule is required for full expression of coupled phosphorylation. These results indicate an essential role for the alpha subunit in the regulation of ATPase activity and in the ability of the solubilized coupling factor to rebind to depleted membranes.

Introduction

The membrane-bound coupling factor-latent ATPase (membrane-bound adenosinetriphosphatase, EC 3.6.1.3) of *Mycobacterium phlei* plays a key role in the bioenergetic processes of this microorganism [1]. Trypsin treatment of the solubilized enzyme produces a 20—37-fold increase in ATPase activity [1,2], loss of coupled (oxidative) phosphorylation [1], and a diminished ability to rebind to coupling factor-depleted membrane vesicles [3]. The native alpha (64 000 dalton) subunit is simultaneously converted to an α″ (58 000 dalton) stable species via an α′ (61 000 dalton) intermediate. The molecular weight of the treated enzyme is about 20 000 daltons less than that of the native coupling factor [3]. Trypsin treatment of the membrane-bound enzyme elicits ATPase activity but without loss of coupled phosphorylation [1].

This study was undertaken to probe the relationship of the observed changes in the structure of the coupling factor to the changes in function and to account for the effect of trypsin on the membrane-bound form. It also sought to identify the coupling factor subunits that play essential roles in regulating the ATPase activity and in membrane attachment.

Various theoretical models were devised and used to predict the functional properties of the solubilized enzyme from the observed alpha subunit composition (i.e. fractional moles of the A, A′, and A″ subunit forms) of trypsin-treated samples. These predicted properties were then compared to the experimental values for the functional properties. Models giving best representations of the data support the existence of three alpha subunits/molecule, and indicate an essential role for the alpha subunits in regulating ATPase activity and in membrane attachment. The theoretical model for ATPase activity also predicts that one molecule of ATP at a time binds to the ATPase hydrolytic site(s). This was confirmed by the finding that only one molecule of 2′,3′-dialdehyde ATP binds to the enzyme with complete inactivation of ATPase activity [4,5].

Methods

Sucrose-solubilized coupling factor was obtained from electron transport particles [6]. After careful separation of this supernatant from the pellet and recentrifugation, a preparation was obtained that was pure as judged by gel electrophoresis. Solubilized coupling factor samples were treated with trypsin under different conditions as described in the legend to Table I. Those referred to as type I samples were treated with a trypsin : protein ratio of 1 : 1—1 : 4 (w/w) whereas those referred to as type II were similarly treated with a ratio of 1 : 5.4—1 : 12.5 (w/w). Type III samples were treated with immobilized trypsin or contained double-strength buffer, sodium succinate, or NADH.

Aliquots of samples from each type of treatment were taken after different intervals (30 s—90 min) of incubation at 30°C, and assay for subunit structure (10% polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate) [3], coupled phosphorylation [6], ATPase activity [2], and membrane rebinding [3].

The alpha subunit composition of coupling factor aliquots was expressed in terms of fractional mol of the A, A′, A″ species. This composition was estimated from the relative Coomassie blue-stained intensities given by each form on the 10% polyacrylamide-0.1% sodium dodecyl sulfate gels. Based on this composition, each aliquot was assigned a conversion index value. This value reflected the degree of alpha subunit conversion and facilitated correlation of functional property changes with the course of subunit conversion. It also served as a reference for comparing pathways of molecular state conversion for the different types of trypsin-treated coupling factor samples. Aliquots of native coupling factor, in which the fractional mol of A was equal to 1.0, were given an index value of 0. Aliquots in which the alpha subunit had undergone maximal conversion (fractional mol of A″ = 1.0) were assigned an index value of 100. Aliquots for which the fractional mol of A was between 0.99 and 0.23 were given index values between 1 and 51 according to the formula: conversion index = 66.63 (1 — fractional mol of A). Those for which the fractional mol of A″ was between 0.45 and 0.99 were similarly assigned index values between 63 and 99 according to the formula: conversion index = 66.73 (fractional mol of A″) + 33.27. Aliquots with fractional mol of A less than 0.23 and fractional mol of A″ less than 0.45 were given index values between 51 and 63 extrapolation.

Theoretical models (sets of assumptions) representing different alpha subunit composition-functional property relationships in the trypsin-treated coupling factor molecule were tested against the experimental data using a Fortran IV computer program and an IBM 370/158 digital computer; a copy of the program is available on request. Input data consisted of values of the parameter(s) defining the assumptions of a given theoretical model and either (a) the relative values of ATPase activity and the alpha subunit composition (in terms of fractional mol of the A, A′, and A″ forms) of 106 aliquots of type I, II, and III coupling factor samples, (b) the relative values of membrane rebinding and the alpha subunit composition of 45 aliquots of type II or III samples, or (c) the relative values of coupled phosphorylation and the alpha subunit composition of 33 aliquots of type I, II, and III samples.

Models incorporating the assumption of three copies of the alpha subunit/molecule were defined by the number of times (K) that each variable (X_1 or X_2) occurs in each of ten possible structural states of the coupling factor molecule. Models based on the assumption of two copies of the alpha subunit were defined by the number of times each variable occurs in each of the six possible structural states. Variable X_1 was given a fixed value of 1.0; variable X_2 was allowed to take on values between 0.0 and 1.0 to optimize the fit of a given model to the data being modeled. For example, a model incorporating the assumptions that the α'' form contributes maximally to a functional property, that the α' form contributes partially and independently, and that there are three alpha subunits

will be defined as follows:

Structural State	Number of times ($K(1)$) ($\alpha'' = X_1$)	Number of times ($K(2)$) ($\alpha' = X_2$)
$(\alpha\alpha\alpha)_1$	0	0
$(\alpha\alpha\alpha')_2$	0	1
$(\alpha\alpha\alpha'')_3$	1	0
$(\alpha\alpha'\alpha')_4$	0	2
$(\alpha\alpha'\alpha'')_5$	1	1
$(\alpha\alpha''\alpha'')_6$	2	0
$(\alpha'\alpha'\alpha')_7$	0	3
$(\alpha'\alpha'\alpha'')_8$	1	2
$(\alpha'\alpha''\alpha'')_9$	2	1
$(\alpha''\alpha''\alpha'')_{10}$	3	0

Modeling of each functional property was performed separately. The following equation was used to predict values of the given functional property from the alpha subunit composition of coupling factor aliquots:

$$\text{Theoretical value } (N) = X_1\left(\sum_{i=1}^{10\ (\text{or } 6)} P(\text{i}) \cdot K(1, i)\right) + X_2\left(\sum_{i=1}^{10\ (\text{or } 6)} P(i) \cdot K(2, i)\right)$$

N is the number of the aliquot, X_1 is variable 1 ($\equiv 1.0$), and X_2 is variable 2. $P(i)$ is the calculated probability of finding coupling factor molecules with the i^{th} structural state in a population of molecules corresponding to the N^{th} aliquot. $K(1, i)$ and $K(2, i)$ indicate the number of times that variable X_1 or X_2, respectively, occurs in the i^{th} structural state.

Goodness of fit of the predicted values to the corresponding experimental values of the given functional property was estimated from the sum of the squares of the errors between the experimental and theoretical values for all coupling factor aliquots considered together. This type of goodness of fit analysis was also used by Cornish-Bowden and Koshland [7] and by Clore and Chance [8] in theoretical modeling of enzyme systems.

The sums of the squares of errors obtained for all the theoretical models tested against the experimental data for a given functional property were then subjected to statistical analysis. The model yielding the smallest value for the sum of squares of errors was designated model Z. The value of the chi square for # degrees of freedom ($\chi^2_{\#}$) was then calculated for all other models (e.g. model Y) that were tested for that property. The value of # is equal to the number of assumptions defining model Z less the number defining model Y. The percentile of the χ^2 distribution for # degrees of freedom ($P_{\chi^2_{\#}}$) indicated the probability that a given model, e.g. model Y, had a goodness of fit to the data equal to the fit obtained for model Z. If the value of $P_{\chi^2_{\#}}$ for a given model was <95%, then its fit to the data was considered statistically equal to the fit given by model Z.

Results

Effects of treatment conditions on the rates of alpha subunit conversion and ATPase activation

The effects of trypsin treatment on the rate of alpha subunit conversion and ATPase activation rate under different conditions are shown in Table I. The rate of complete alpha subunit conversion was most rapid for the type I samples (fractional mol of A'' was equal to fractional mol of A' at 0.53 min) and slowest for the type III samples (fractional mol of A'' equal to fractional mol of A' at 16.6 min). The relative accumulations of the A' species (given by the maximal value for the fractional mol of A' during conversion) indicate that, compared to the rate of A to A' conversion, the rate of A' to A'' conversion is fastest for the type I samples, slower for type II, and slowest for the type III samples.

Maximal ATPase activity was observed after about 5 min when solubilized coupling factor was treated with trypsin under conditions similar to the standard ATPase assay (type II samples) [2]. In contrast, when the chloroplast coupling factor was treated with L-1-(*p*-toluenesulfonyl)amino-2-phenylethyl-chloromethyl ketone (TPCK) trypsin, maximal ATPase activity was attained only after 5 h [9]. Tryptic activation of the *Escherichia coli* or *Salmonella typhinurium* (Ca^{2+}-activated) enzymes produced maximal activity again only after 30 min of treatment [10].

Trypsin treatment of membrane-bound coupling factor

The maximal degree of alpha subunit conversion observed for membrane-bound coupling factor after tryptic proteolysis (i.e. fractional mol of A = 0.38; fractional mol of A' = 0.62; fractional mol of A'' = 0.0) was far less than the maximal conversion seen for the solubilized enzyme (fractional mol of A'' = 1.0). It is roughly equivalent to one native alpha subunit remaining/molecule. Conversion of all three native alpha subunits was therefore prevented by virtue of attachment of the enzyme to the membrane. This protection is similar to the complete and specific immunity to proteolysis observed for the alpha subunits of the membrane-bound *Streptococcus faecalis* ATPase enzyme [11].

The rate of ATPase activation for the membrane-bound coupling factor was similar to that of the solubilized enzyme. Maximal activation was attained after about 4 min of trypsin treatment; this was accompanied by a slight decrease in coupled phosphorylation (about 20%). This finding is consistent with earlier findings where tryptic activation of the membrane-bound enzyme occurred without loss of coupled phosphorylation [12].

Conversion index plots

The relative ATPase activity values for the trypsin-treated aliquots of solubilized coupling factor were plotted versus the conversion index values of the aliquots. Interdispersion of the data points for type I, II and III is evident in the plot shown in Fig. 1. This indicates that the relationship of alpha subunit composition to ATPase activity is similar for the type I, II, and III samples. This plot does not, however, include data for samples that contained 50 mM NADH or 0.2 mM adenyl-5'-yl imidodiphosphate or lacked 4 mM $MgCl_2$ during trypsin

TABLE I

TRYPSIN TREATMENT CONDITIONS FOR TYPE I, II AND III COUPLING FACTOR SAMPLES, ALPHA SUBUNIT CONVERSION PARAMETERS, AND RATE OF ATPase ACTIVATION

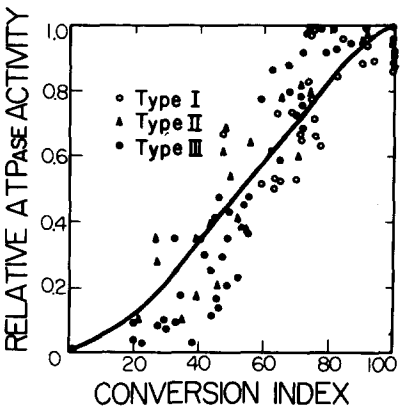
Samples of solubilize coupling factor (0.5 mg protein/ml) were treated with bovine pancreatic trypsin or diphenylcarbamyl chloride-treated trypsin as described previously [3]. Samples contained 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid/KOH (pH 7.5) buffer + 4 mM $MgCl_2$ (standard buffer). (1) After 30 s—60 min incubation at 30°C, soybean trypsin inhibitor was added (trypsin inhibitor : trypsin = 5 : 1, w/w) and aliquots of the treated sample were taken for analysis. (2) Samples were treated with immobilized trypsin (Enzite-ethylene malic anhydride trypsin, Miles-Yeda). After incubation at 30° C for 1—90 min, aliquots were centrifuged, and the supernatant taken for analysis. (3) Samples were treated as in (1) except that they contained 100 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid/KOH (pH 7.5) buffer + 8 mM $MgCl_2$ (double-strength buffer), or (4) standard buffer + 50 mM sodium succinate (pH 7.0), (5) standard buffer + 50 mM NADH, or (6) standard buffer + 0.2 mM adenyl-5′-yl imidodiphosphate. (7) Other samples were treated as in (1) but contained 4 mM $MgCl_2$ only after treatment, and (8) some samples contained standard buffer during trypsin treatment and were made double-strength after treatment. Numerals for treatment conditions indicate type of treatment condition described above. $A = A'$, number of minutes after start of treatment at which the fractional mol of A' was equal to the fractional mol of A. $A = A''$, number of minutes at which the fractional mol of A'' was equal to the fractional mol of A. $A' = A''$, number of minutes at which the fractional mol of A'' was equal to the fractional mol of A'. A'_{max}, the maximal value of the fractional mol of A' during alpha subunit. $t_{80\%\ max}$, number of minutes at which the ATPase activity attained 80% of its maximal observed value.

Sample type	Treatment conditions	[Trypsin/coupling factor protein] (w/w)	Alpha subunit conversion parameters				Rate of ATPase activation ($t_{80\%\ max}$, min)
			$A = A'$ (min)	$A = A''$ (min)	$A' = A''$ (min)	A'_{max}	
I	1,6,7,8	1 : 1—1 : 5	0.56 ± 0.06 *	0.52 ± 0.05	0.53 ± 0.05	0.36 ± 0.01	2.9 ± 0.4
II	1,7	1 : 6—1 : 12.5	0.49 ± 0.09	1.02 ± 0.02	2.31 ± 0.55	0.51 ± 0.03	4.7 ± 1.1
III	2,3,4,5	1 : 2—1 : 6 (or immobilized trypsin, 1 : 2.1—1 : 4.2)	5.03 ± 2.44	9.03 ± 2.62	16.6 ± 4.88	0.70 ± 0.03	12.7 ± 3.4

* Values are expressed as mean ± S.E.

treatment; these gave anomalous values, as discussed later.

The relative rebinding values for treated coupling factor samples is plotted versus index values in Fig. 2a. The data points for the type II and III samples appear to be intermingled, with the type III values falling slightly lower in the distribution than the type II values. The coupling factor aliquots for which rebinding was assayed in standard buffer are distinguished from those for which rebinding was measured in the medium used to measure oxidative phosphorylation in Fig. 2b. A similar distribution of relative rebinding values is apparent for



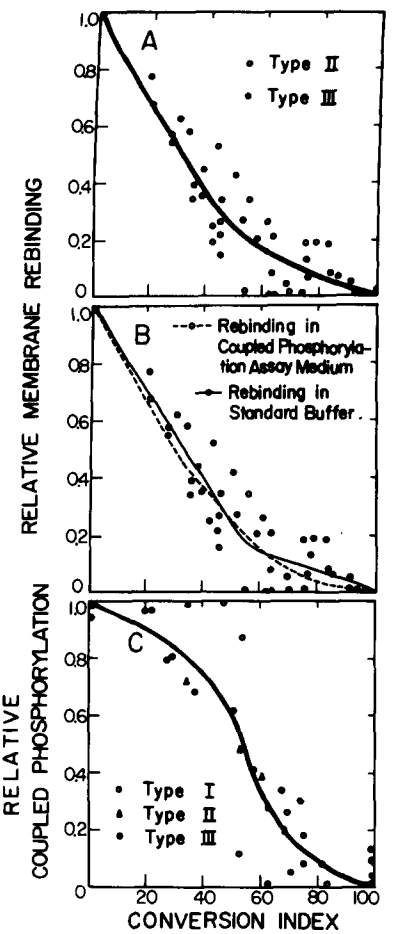


Fig. 1. Conversion index plot of relative ATPase activity for coupling factor treated with trypsin under different conditions. The smoothed curve through activity values for 34 type I aliquots, 23 type II, and 53 type III aliquots is shown.

Fig. 2. Conversion index plots of relative membrane rebinding and coupled phosphorylation. (a) The smoothed curve through rebinding values for 33 aliquots of type II coupling factor samples and 14 type III aliquots. (b) The curve through rebinding values for 31 aliquots measured in standard buffer and the curve through rebinding values of 11 aliquots measured in coupled phosphorylation assay medium. Aliquots were incubated with depleted membranes in either standard buffer or coupled phosphorylation assay medium. After centrifugation at 105 000 × *g* for 60 min, relative rebinding was estimated by the relative amount of ATPase activity in the reconstituted membranes. (c) The smoothed curve through coupled phosphorylation values for 15 type I aliquots, nine type II, and 13 type III aliquots.

these two types of medium. The relative ability of each aliquot to couple phosphorylation to oxidation is similarly plotted versus conversion index value in Fig. 2c. Although the values are fewer in number and more widely scattered that those for the membrane rebinding data, the smoothed curve for coupled phosphorylation clearly declines more slowly than the curve for relative membrane rebinding in Fig. 2a. This indicates that loss of rebinding alone cannot explain the effect of tryptic proteolysis on coupled phosphorylation.

Relative amounts of the coupling factor gamma, delta, and epsilon subunits in aliquots of trypsin-treated enzyme were also plotted versus conversion index. Both gamma (33 000 dalton) and epsilon (8000 dalton) subunits showed small decreases (about 12%) concurrent with complete alpha subunit conversion. The delta (14 000 dalton) subunit, however, diminished by about 75%. In earlier studies [3], the delta subunit was present after partial alpha subunit conversion, which would be equivalent to a conversion index value of 25.

A structural state model of the alpha subunit conversion

In order to carry out theoretical modeling of structure-function relationships in the trypsin-treated coupling factor molecule, it was necessary to derive a probabilistic model representing the alpha subunit conversion in terms of molecular states. The approach was similar to that used by Sears and Beychok [13] for the reduced and partially oxidized immunoglobulin structures. It embodies the following concepts and assumptions:

(1) The symbols A, A′, and A″ represent the macroscopic forms of the coupling factor alpha subunit, i.e. the primary structures observed after electrophoresis of coupling factor samples on polyacrylamide gels in the presence of sodium dodecyl sulfate. The symbols α, α', and α'' signify the microscopic forms of the subunit within the coupling factor molecule.

(2) During tryptic proteolysis, the native 64 000 dalton subunit (α) undergoes limited proteolysis to a 58 000 dalton stable product (α'') via a 61 000 dalton intermediate (α'). Three different microscopic forms are therefore possible for each alpha subunit. This alpha subunit conversion is not accompanied by dissociation of subunits since the decrease in the molecular weight of the fully converted enzyme corresponded (within 1%) to the total observed decrease in the molecular weight of the three alpha subunits [3].

(3) N copies of the alpha subunit are assumed to exist in the coupling factor molecule, where $N = 2$ or 3. These copies are assumed to be geometrically equivalent. There will then be ten possible combinations of the alpha subunit forms if $N = 3$ and six if $N = 2$. Each combination corresponds to a unique structural state of the coupling factor molecule.

(4) The fractional mol of each macroscopic subunit form (e.g. fractional mol of A) observed in a coupling factor aliquot is proportional to the fractional mol of each corresponding microscopic form (e.g. fractional mol of α) in the population of molecules comprising the aliquot.

(5) The probability of finding a coupling factor molecule with a given structural state is equal to the product of (a) the fractional mol of each subunit form observed in the aliquot multiplied by the number of times that its corresponding microscopic form occurs in that state and (b) the number of indistinguishable ways in which the given state can be formed.

(6) Specific functional properties are assumed to be associated with each alpha subunit microscopic form. These properties may be modified by the presence of different microscopic forms in the same molecule. The expression of a property of the α form may thus be different when α occurs in the $\alpha\alpha\alpha$ structural state than when it occurs in the $\alpha\alpha''\alpha''$ state.

Based on this structural state model of the solubilized coupling factor during tryptic proteolysis, pathways of conversion between structural states were derived for the solubilized coupling factor under different conditions of proteolysis. These are shown in Fig. 3. The approach for this derivation was similar to the one used by Sears and Beychok [13] for disulfide bond reoxidation pathways in the immunoglobulin molecule. Table II and Fig. 3 give the calculations for type II samples.

Theoretical modeling of structure-function relationships

Computer modeling of alpha subunit composition-functional property rela-

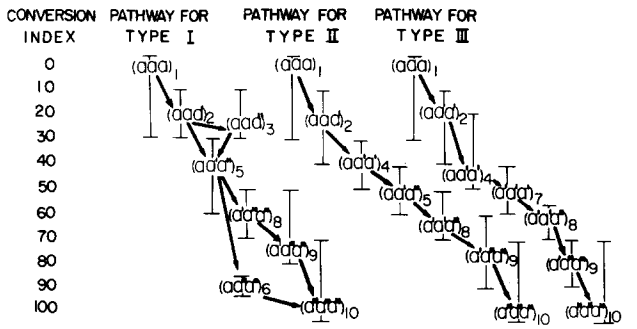
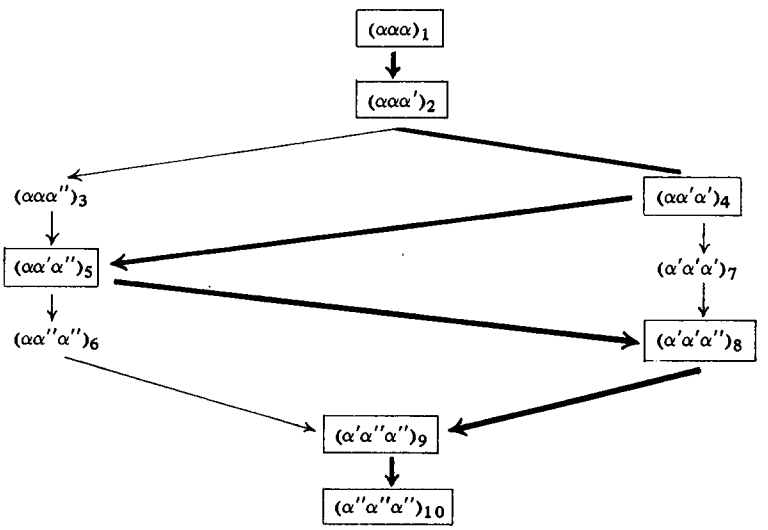


Fig. 3. Pathways of structural state conversion for type I, II, and III coupling factor samples. The pathway for the type II samples was derived by selecting routes between those structural states having a probability of existing ≥ 0.15 in Table II from the diagram of all the possible routes of conversion between the ten structural states:



Pathway derivation was similar for type I and III samples. Bar lengths in Fig. 1 indicate the span of index values over which a given structural state existed with a $P \geq 0.15$.

TABLE II

PROBABILITY DISTRIBUTIONS OF COUPLING FACTOR MOLECULES FOR TYPE II SAMPLES

The average alpha subunit composition at each of ten conversion index values was used to calculated probability distributions of coupling factor molecules for type I, II, and III samples. For example, the type II samples gave average values of fractional mol of A = 0.86, fractional mol of A′ = 0.11, and fractional mol of A″ = 0.04 at index 10. The probability of finding molecules with the $(\alpha\alpha\alpha')_2$ structural state in a corresponding population of molecules is then equal to $P(\alpha\alpha\alpha')_2$ = ((average fractional mol of A)^2 (average fractional mol of A′) × 3) = 0.24. Structural states with a probability of existing ⩾0.15 are underlined.

Conversion index	Average fractional mol			Probability of finding coupling factor molecules with the given structural state									
	A	A′	A″	$P(\alpha\alpha\alpha)_1$	$P(\alpha\alpha\alpha')_2$	$P(\alpha\alpha\alpha'')_3$	$P(\alpha\alpha'\alpha')_4$	$P(\alpha\alpha'\alpha'')_5$	$P(\alpha\alpha''\alpha'')_6$	$P(\alpha'\alpha'\alpha')_7$	$P(\alpha'\alpha'\alpha'')_8$	$P(\alpha'\alpha''\alpha'')_9$	$P(\alpha''\alpha''\alpha'')_{10}$
0	1.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
10	0.86	0.11	0.04	0.64	0.24	0.09	0.03	0.02	0.00	0.00	0.00	0.00	0.00
20	9.73	0.19	0.09	0.39	0.30	0.14	0.08	0.07	0.02	0.01	0.01	0.00	0.00
30	0.57	0.34	0.09	0.19	0.33	0.09	0.20	0.10	0.10	0.04	0.03	0.01	0.00
40	0.39	0.48	0.14	0.06	0.22	0.06	0.27	0.16	0.02	0.11	0.10	0.03	0.00
50	0.24	0.45	0.32	0.01	0.08	0.06	0.15	0.21	0.07	0.09	0.19	0.14	0.03
60	0.13	0.44	0.43	0.00	0.02	0.02	0.08	0.15	0.07	0.09	0.25	0.24	0.08
70	0.06	0.40	0.55	0.00	0.00	0.01	0.03	0.08	0.05	0.06	0.26	0.36	0.17
80	0.05	0.26	0.70	0.00	0.00	0.01	0.01	0.06	0.07	0.02	0.14	0.38	0.34
90	0.00	0.14	0.86	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.31	0.64
100	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00

tionships in the coupling factor molecule during tryptic proteolysis was carried out. The models tested against the experimental data for each functional property are given in Table III. Theoretical models incorporating the assumption of two copies of the alpha subunit gave significantly poorer fits to the data for each functional property than their three-subunit counterparts. This corroborates the stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$ for the *M. phlei* coupling factor which had been proposed on the basis of sodium dodecyl sulfate gel electrophoresis [3].

Model 9 for ATPase activity (fractional mol of $\alpha'' = 1.0$; fractional mol of $\alpha' = 0.45$, assuming three alpha subunits) is the simpler of the two best-fit models in Table III; it is therefore the best representation of the experimental data. Experimental values for ATPase activity are plotted versus the theoretical values given by model 9 in Fig. 4a. The least-squares regression line is shown,

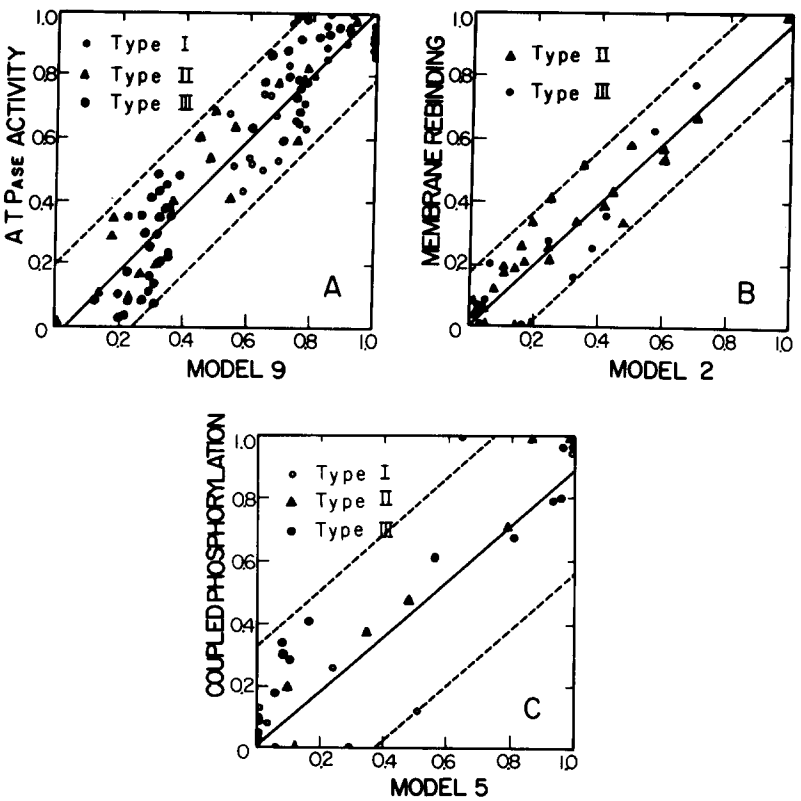


Fig. 4. Experimental values for ATPase activity, membrane rebinding, and coupled phosphorylation versus the theoretical values given by the models best-representing the experimental data. (a) The ATPase activity values for type I, II, and III coupling factor samples versus the corresponding values given by model 9 (fractional mol of $\alpha'' = X_1$, assuming three alpha subunits). The standard error of the least-squares regression line (———) was 0.155, with 95% confidence limits of ±0.226 (- - - - - -). (b) The membrane rebinding values for aliquots of type II and III coupling factor samples versus the corresponding values given by model 2 (fractional mol of $\alpha = X_1$, fractional mol of $\alpha' = X_2$, assuming three alpha subunits). The standard error was 0.088 with 95% confidence limits of ±0.172. (c) The coupled phosphorylation values for aliquots of type I, II, and III samples versus the theoretical values given by model 5 (presence of $\alpha = X_1$, three alpha subunits). The standard error of the line was 0.170 with 95% confidence limits of ±0.333.

TABLE III

THEORETICAL MODELS OF STRUCTURE-FUNCTION RELATIONSHIPS IN TRYPSIN-TREATED COUPLING FACTOR AND GOODNESS OF FIT ANALYSIS FOR MODELED FUNCTIONAL PROPERTIES

Sum of squares of errors is between experimental and theoretical values for a given functional property. Theoretical modeling procedures and goodness of fit analysis are described in Methods. # is the difference between the number of assumptions defining model Z for a given property and the indicated model. $\chi^2_{\#}$ is

Theoretical models			Goodness of fit analysis for modeled functional				
Model Nr.	Assumptions of the models and defining parameters	Nr. assumptions	Nr. alpha subunits assumed	ATPase activity			
				Sum of squares of errors	#	$\chi^2_{\#}$	$P_{\chi^2_{\#}}$
1.	Fractional mol of $\alpha'' = X_1$	1	3	5.23	3	86.9	>99
2.	Fractional mol of $\alpha = X_1$	1					
3.	Fractional mol of $\alpha + \alpha' = X_1$	1					
4.	Fractional mol of $\alpha' + \alpha'' = X_1$	1	3	7.24	2	>100	>99
5.	Presence of $\alpha = X_1$	1					
6.	Presence of $\alpha'' = X_1$	1	3	4.80	3	74.9	>99
7.	Fractional mol of $\alpha + \alpha' = X_1$; Presence of α needed	2					
8.	Fractional mol of $\alpha = X_1$; Fractional mol $\alpha' = X_2$	2					
9.	Fractional mol of $\alpha'' = X_1$; Fractional mol of $\alpha' = X_2$	2	3 2	1.33 3.21	2 2	3.33 55.6	10—90 >99
10.	Fractional mol of $\alpha = X_1$; Fractional mol of $\alpha' = X_2$; Presence of α needed	3					
11.	Fractional mol of $\alpha'' = X_1$; Fractional mol of $\alpha' = X_2$; Two α kill α' or α''; One α diminishes two α' by half	4	3	1.21 *	**	**	≡0
NMAX (total number of coupling factor aliquots)				106			
NMAX-2 (maximum degrees of freedom)				104			
6^2_Z		1.21/(104 — 4) = 0.012					

* The sum of squares of errors for model Z, i.e. the lowest numerical value for the sum of squares of errors for the given functional property.

** Calculations not meaningful.

and the data points for aliquots of type I, II, and III coupling factor samples are apparently interdispersed about this line. Each regression line (σ = 0.100—0.111) given by individual sample types fell within the standard error limits of the lines for the other types. Model 9 for ATPase activity therefore applies equally well to the experimental data for all three sample types. The alpha subunit composition-ATPase activity relationship under these three types of trypsin treated conditions is also statistically equivalent. Although the relative

the Chi square value of the given model for # degrees of freedom, and $P_{\chi^2_\#}$ is the percentile of the Chi values square distribution for that model.

properties

Nr. alpha subunits assumed	Membrane rebinding: Sum of squares of errors	#	$\chi^2_\#$	$P_{\chi^2_\#}$	Nr. alpha subunits	Coupled phosphorylation: Sum of squares of errors	#	$\chi^2_\#$	$P_{\chi^2_\#}$
[3	0.315	2	0.13	5—10	[3	2.38	1	57.5	>99
2]	0.590	2	17.7	>99	]				
3	7.43	2	>100	>99	3	1.62	1	29.8	>99
[3	3.11	2	>100	>99	[3	0.868	1	2.58	10—90
]					2]	1.13	1	12.2	>99
3	2.15	1	>100	>99	3	0.979	0	>6.3	>99
3	0.314	1	0.13	10—90	3	0.797 *	**	**	≡0
[3	0.313 *	**	**	≡0					
2]	0.590	0	>36	>99					
		45					33		
		43					31		
0.315/(43 — 3) = 0.008					0.797/(31 — 2) = 0.0275				

rates of conversion of one alpha subunit species to another differed under the three types of treatment conditions (Table I), these results indicate that only the rate of formation of the catalytically active α' (61 000 dalton) and α'' (58 000 dalton) species was affected and not the expression of activity by these species.

Data for the coupling factor samples containing 50 mM NADH or 0.2 mM adenyl-5′-yl imidodiphosphate or lacking 4 mM $MgCl_2$ during trypsin treatment

were analyzed separately. The least-squares regression line (σ = 0.207) obtained for the values predicted by model 9 versus the experimental values of ATPase activity for the NADH-containing samples (n = 13) and the regression line (σ = 0.160) obtained for the samples lacking 4 mM $MgCl_2$ (n = 10) both fell within the standard error limits of the lines for the other type I, II, and III samples. The alpha subunit composition-ATPase activity relationship under these two treatment conditions was therefore statistically indistinguishable from the relationship under the treatment conditions of the other samples. Since the scatter of these two data sets was substantial, they were not, however, included in the analysis of ATPase activity. The regression line (σ = 0.299) for the samples (n = 6) containing 0.2 mM adenyl-5′-yl imidodiphosphate fell largely outside the standard error limits of the other type I, II, and III samples. In this case, the quantity and quality of the data was insufficient for meaningful analysis.

Model 2 (fractional mol of α = 1.0, assuming three alpha subunits) was the best representation of the membrane rebinding data (Table III). Two other models giving statistically equivalent best fits had optimized values of X_2 making them equivalent to the assumptions embodied in model 2. Random intermingling of the membrane rebinding experimental versus theoretical data points is evident in Fig. 4. The least-squares regression line (σ = 0.079) for the experimental and theoretical values of the type II samples analyzed separately and the line (σ = 0.108) for the type III samples both fell closely together within each others' standard error limits. Model 2 for rebinding therefore applies equally well to the experimental data for both sample types. The alpha subunit composition-rebinding relationship was also statistically equivalent in both types of samples.

The regression line (σ = 0.081) for the coupling factor aliquots assayed for rebinding in standard buffer (Fig. 2b) and the line (σ = 0.104) for the aliquots assayed in the medium used to measure coupled phosphorylation each fell within the standard error limits of the other. The alpha subunit composition-rebinding relationship was therefore statistically equivalent for rebinding measured under both conditions.

Model 5 for coupled phosphorylation (presence of α = 1.0, assuming three alpha subunits) was the best model for the coupled phosphorylation data (Table III). The experimental versus theoretical data points in Fig. 4c for the type I, II, and III samples appear randomly scattered. The least-squares regression line (σ = 0.190) for the type I samples analyzed separately and the line (σ = 0.090) for the type II samples each fell closely together within the standar error limits of the other. However, the line (σ = 0.068) for the type III samples deviated from the other lines and fell partly outside the error limits of the type II samples. Model 5 for coupled phosphorylation therefore applies equally well to the type I and type II samples but not as well to the type III sample data. The relationship of the alpha subunit composition of the coupling factor to its capacity for coupled phosphorylation is thus statistically equivalent under the type I and II trypsin treatment conditions but less certain for the type III samples.

Discussion

The modeling result for the ATPase activity of solubilized coupling factor subjected to tryptic proteolysis indicates that relative activity is a simple additive function of the number (or fractional mol) of the α' and α'' species in individual molecules. Each α'' form contributes maximally to activity (relative activity = X_1 = 1.0) and each α' form contributes about half-maximally (relative activity = X_2 = 0.45). Expression of ATPase activity in vitro is therefore accounted for by changes in the primary structure of the alpha subunits. On the other hand, the small (about 12%) loss of the epsilon (8000 dalton) subunit cannot account for expression of activity. This suggests that the native alpha subunit is essential for the in vivo latency of the enzyme and that the epsilon subunit is not likely to be a major regulator. The alpha subunit of the *E. coli* coupling factor ATPase may similarly play an important role in ATP hydrolysis. Thus, the *unc A* mutant, which was incapable of ATPase activity and oxidative phosphorylation, was found to have a defective alpha subunit [14]. Also, 1 mol of the photoaffinity label 8-azido-ATP was found to bind covalently to the alpha subunit of *E. coli* F_1 with more than 90% inhibition of ATPase activity [15]. Tryptic activation of the *Micrococcus lysodeikticus* ATPase enzyme, however, was consistent with a regulatory role for both alpha and epsilon subunits [16,17].

The model for membrane rebinding (fractional mol of α = X_1 = 1.0) predicts that a coupling factor molecule with a $\alpha'\alpha'\alpha'$ structural state will have a rebinding ability of zero and that one with a $\alpha\alpha'\alpha''$ state will have a rebinding ability of one-third. Since the pathways of structural state conversion for type II and III samples were similar except for these states (Fig. 3), the model is consistent with the slightly lower values given by the type III samples in the conversion index plot of membrane rebinding (Fig. 2a). The membrane rebinding model also predicts that a coupling factor molecule can rebind only if it has at least one native α form. This is consistent with the maximal degree of alpha subunit conversion observed for the membrane-bound enzyme, which was equivalent to one native α form remaining/molecule.

The delta subunits of a number of ATPase enzymes have been found to be essential for membrane attachment [11,18,19]. In the *S. faecalis* enzyme, both native delta and alpha subunits were required [18,20]. A similar requirement for the membrane rebinding ability of the *M. phlei* coupling factor is not ruled out, as the effect of the total loss of native alpha subunits may have overshadowed any effects of the partial loss of delta. The modeling results for coupled phosphorylation suggest that solubilized coupling factor molecules with either one, two, or three α forms will have the same observed ability to restore coupled phosphorylation to depleted membranes. On the other hand, the membrane rebinding model predicts that molecules with one or two α forms will have partial rebinding ability. These results, taken together, imply that partially converted molecules containing one or two α forms will have a greater probability of achieving rebinding in a way that permits optimal expression of coupled phosphorylation than the native molecule with three α forms. Observed relative coupled phosphorylation will then be the same for both native and these partially converted molecules. The models for coupled phos-

phorylation and ATPase activity predict that the membrane-bound coupling factor will express both coupled phosphorylation and ATPase activity after maximal alpha subunit conversion (one α form/molecule). This result is consistent with the finding that trypsin activated the membrane-bound ATPase enzyme without loss of coupled phosphorylation [12].

The theoretical modeling results for membrane rebinding ability and coupled phosphorylation are subject to the following sources of error: (1) Some of the molecules with partial rebinding ability may have detached during the washing steps of the rebinding assay. Consistent relative rebinding values were, however, obtained for coupling factor aliquots with the same alpha subunit composition with minimal scatter of the data points (Fig. 4b). (2) Only rebound enzyme molecules were assumed to be capable of phosphorylation. (3) The coupled phosphorylation assay suffered from a lack of reproductibility, and the theoretical model did not apply well to all samples.

Finally, the modeling results are consistent with the simultaneous existence of catalytic sites for both ATP synthesis and hydrolysis in the same coupling factor molecule. Allomorphic states, with corresponding different functional properties, have been described for coupling factors from other sources [21,22]. What is novel in the trypsin-treated coupling factor from *M. phlei* is the existence of different allomorphic states of individual subunits within the same molecule.

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