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

EFFECTS OF DIFFERENT ENZYMES AND MODIFYING AGENTS

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Summary

The activation of the coupling factor-latent ATPase enzyme by tryptic proteolysis may resemble the activation of many proenzymes by limited proteolysis. The beta (53 000 dalton) subunit of solubilized coupling factor-latent ATPase from *Mycobacterium phlei* was selectively lost in some trypsin-treated samples. Since a concomitant loss of ATPase activity was not observed, the beta subunit may not be essential for ATPase catalytic activity. Treatment of solublized coupling factor with chymotrypsin rapidly produced an A′-type (61 000 dalton) species from the native alpha (64 000 dalton) subunits with partial activation of the ATPase enzyme. Secondary chymotryptic cleavage yielded an A″-type (58 000 dalton) species and a less-active enzyme. Storage of fresh coupling factor samples at −20°C in the presence of 4 mM $MgCl_2$ with several freeze-thaw cycles resulted in loss of ATPase activity without apparent change in alpha subunit structure. Storage at 4°C in the presence or absence of $MgCl_2$ both decreased ATPase activity and generated A′-type alpha subunit species. Since presence of phenylmethylsulfonyl fluoride prevented these changes, an unknown protease was suspected. The peptide bonds first cleaved by trypsin, chymotrypsin, and the unknown protease are all apparently located within the same small segment of alpha subunit polypeptide chain.

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Abbreviations: A'_{ChT}, A′ species formed by chymotryptic proteolysis; A'_{T}, A′ species formed by tryptic proteolysis; A'_{UP}, A′ species formed by an unknown protease.

Introduction

Limited proteolytic digestion is required for activation of many biologically active proteins. In each case, cleavage of a susceptible peptide bond in an inactive precursor induces subtle conformational changes which lead to expression of catalytic activity. Exposure of new susceptible cleavage sites may accompany these changes, and cleavage at these secondary sites may induce further conformational changes that affect enzyme activity [1—3]. The activation of the latent ATPase (membrane-bound adenosinetriphosphatase, EC 3.6.1.3), enzyme of *Mycobacterium phlei* coupling factor (BCF_1) by tryptic proteolysis may occur in a similar manner.

Both the rate and extent of digestibility of a protein are related to the degree of exposure of susceptible peptide bonds. The relative digestibility will therefore tend to change during subtle conformational shifts in the protein. These conformational rearrangements may be induced by changes in the pH or ionic strength of the medium, temperature variation, or by the binding of ligands [4]. Indeed, binding of a single ligand to an enzyme active site or allosteric regulatory site induces conformational changes that alter the digestibility at remote protein surfaces [5,6]. It was thus of interest to investigate the effects of modifying agents on the tryptic digestibility of the solubilized coupling factor from *M. phlei.*

Degradation of native subunits by limited proteolysis has been reported for several ATPase enzymes. The gamma (32 000 dalton) subunit of the *Escherichia coli* enzyme lost a 3000 dalton peptide during storage [7]. Both alpha (60 000 dalton) and beta (55 000 dalton) subunits of the *Streptococcus faecalis* ATPase spontaneously degraded to a 49 000 dalton stable product [8]. The alpha (60 000 dalton) subunit of the *Micrococcus lysodeikticus* ATPase was converted to a metastable 55 000 dalton intermediate and then to a 50 000 dalton stable product during storage, freezing and thawing, or exposure to pH 10 [9]. The alpha (64 000 dalton) subunit of *M. phlei* solubilized coupling factor underwent a similar spontaneous degradation to a 61 000 dalton species during concentration or handling [10]. Studies were undertaken to determine storage conditions under which this degradation would be retarded and the effect of different storage conditions upon ATPase activity. Since trypsin and chymotrypsin have different proteolytic specificities, it was also of interest to compare the effects of tryptic and chymotryptic proteolysis on the alpha subunit structure and ATPase activation of the *M. phlei* coupling factor.

Methods

Sucrose-solubilized coupling factor from *M. Phlei* was prepared and treated with trypsin as described earlier [10—13]. Coupling factor samples were subjected to electrophoresis on 10% polyacrylamide gels in the presence of sodium dodecyl sulfate followed by Coomassie blue staining as indicated [10]. The alpha subunit composition was determined and expressed in terms of fractional mol of the alpha subunit forms also as described [14]. ATPase activity was assayed by the standard method [13], which included pretreatment with trypsin or chymotrypsin. Other procedures are given in figure and table legends.

Results and Discussion

Formation of the A_T''' species (56 000 daltons)

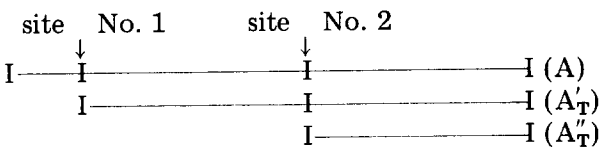
The A_T'' (58 000 dalton) alpha subunit species produced by tryptic proteolysis of solubilized coupling factor was relatively stable under conditions similar to those of the standard ATPase assay, i.e. in samples treated with a trypsin : protein ratio of 1 : 4—1 : 6, w/w, for 10 min or less at 30°C [14]. However, when samples were treated for longer periods of time or when they were treated with higher concentrations of trypsin, a third stable proteolytic product (referred to as A_T''', approx. 56 000 daltons) was formed. A_T''' appeared on the dodecyl sulfate gels between the A_T'' (58 000 dalton) species and the β (53 500 dalton) subunit and was clearly distinguishable. Presence of A_T''' was usually accompanied by a decrease in relative ATPase activity.

Tryptic cleavage pattern

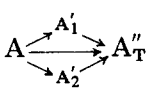
During tryptic proteolysis, the A_T' (61 000 dalton) form of the alpha subunit was formed concurrent with loss of the native A (64 000 dalton) form, and the A_T'' (58 000 dalton) form similarly appeared concurrent with disappearance of the A_T' form [14]. This pattern of alpha subunit conversion indicates that two consecutive, irreversible cleavages occurred in the subunit near the same end of the polypeptide chain [4]. If the two cleavages had occurred independently at opposite ends of the chain, this would have produced A_T' and A_T'' forms simultaneously, not sequentially. These alternate cleavage patterns may be illustrated as follows:

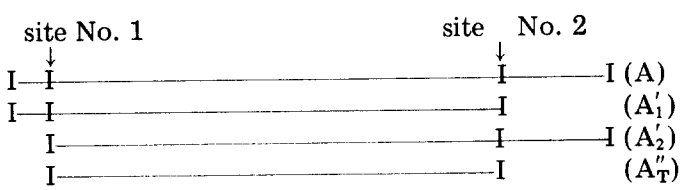
Sequential cleavage:

$A \rightarrow A_T' \rightarrow A_T''$



Independent cleavage:





The observed pattern of tryptic cleavage of the alpha subunit and the production of well-defined, consecutive large fragments (A_T', then A_T'') indicated that (a) a small number of peptide bonds had been broken, that (b) the rate of cleavage at the first site was rapid, that (c) the second site was initially resistant but became susceptible after the peptide bond at the first site had been cleaved, and that (d) a resistant core survived after all the exposed or 'loose' parts had been digested away. Tryptic proteolysis of the coupling factor alpha subunit therefore appears to be a classic case of limited proteolysis [4].

Mechanism of tryptic activation of the M. phlei ATPase

The mechanism of latent ATPase activation by trypsin may be similar to the

limited proteolytic activation of many proenzymes [1]. The first cleavage yielded an enzyme with about half-maximal (45%) activity [14], and the second cleavage produced the fully active enzyme. This contrasts with the activation of certain other proenzymes, where the first cleavage yielded an almost fully active enzyme and the second cleavage produced little further change [4].

Two types of conformational change may be involved in producing catalytic activity during limited proteolytic activation of proenzymes [1]: (a) active site groups may be shifted to create or increase the affinity of a substrate binding site (K_m effects) and/or (b) active site groups may be rearranged to increase the efficiency of the catalytic reaction (V effects). Since the substrate analog 2′,3′-dialdehyde ATP had been found to bind equally well to an ATPase active site on their native (latent) or trypsin-activated *M. phlei* coupling factor [11,12], the second type of conformational changes would be more important for the limited proteolytic activation of this ATPase enzyme by trypsin.

Limited chymotryptic proteolysis of solubilized coupling factor

Solubilized coupling factor samples were treated with chymotrypsin as described in Fig. 1, and the alpha subunit composition and relative ATPase activity of sample aliquots were plotted versus time of treatment.

The maximum level of ATPase activity produced by chymotryptic proteolysis of solubilized coupling factor was about 35% of that produced by tryptic proteolysis under similar conditions. The relative rate of ATPase activation by chymotrypsin lagged behind conversion of the native A form of the alpha subunit to an A′-type species, referred to as A'_{ChT} (an A′ species, 61 000 daltons,

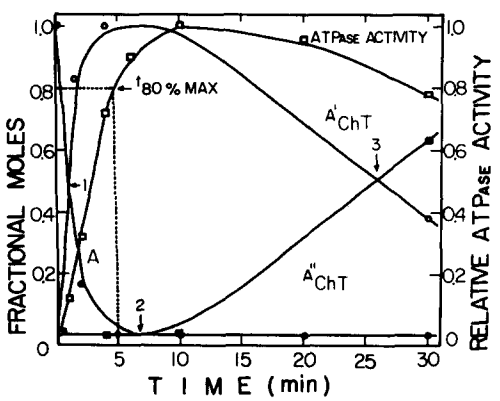


Fig. 1. Time course of alpha subunit conversion and ATPase activation during chymotryptic proteolysis of solubilized coupling factor. A coupling factor sample was treated with bovine pancreatic α-chymotrypsin (chymotrypsin : coupling factor protein = 1 : 20) in standard buffer at 30°C. After 1—30 min, proteolysis was terminated by the addition of 0.05 ml of 10 mg/ml phenylmethylsulfonyl fluoride in 95% ethanol to aliquots of the sample. The alpha subunit composition of each aliquot was determined by dodecyl sulfate gel electrophoresis [14] and ATPase activity was measured by the standard assay [13]. Numeral 1, the point during alpha subunit conversion at which the fractional mol of A was equal to the fractional mol of A'_{ChT}; numeral 2, the point at which the fractional mol of A was equal to the fractional mol of the A''_{ChT} species, and numeral 3, the fractional mol of A'_{ChT} was equal to the fractional mol of A''_{ChT}. This experiment was also performed using carboxymethylcellulose (immobilized)-chymotrypsin with similar results.

formed by chymotryptic proteolysis). Formation of an A''-type (58 000 dalton) species (referred to as A''_{ChT}) was accompanied by a decrease in ATPase activity. Although the A'_{ChT} and A''_{ChT} species had primary structures indistinguishable (after electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate) from their tryptic counterparts (referred to as A'_T, 61 000 daltons, and A''_T, 58 000 daltons) they would not be identical to the latter since trypsin and chymotrypsin have different specificities.

The rate of latent ATPase activation produced by chymotrypsin was similar to the average rate of ATPase activation that had been seen for samples treated with a 1 : 4—1 : 6 ratio (w/w) of trypsin to protein (type II samples) [14]. However, the rate of conversion of A to A''_{ChT} was very slow in the chymotrypsin-treated samples; the point at which the fractional mol of the A'_{ChT} species was equal to the fractional mol of the A''_{ChT} species occurred only after about 26 min of treatment (Fig. 1). The initial rate of conversion, on the other hand, was rapid; the point at which the fractional mol of A was equal to the fractional mol of A'_{ChT} was reached after only 1 min.

These results indicate that, although A'_T and A'_{ChT} species exhibited primary structures indistinguishable on the gels, the conformation at the ATPase active site of these two species must have differed. The rate of conversion of A'_{ChT} to A''_{ChT} was slower than the $A'_T \rightarrow A''_T$ conversion. This would suggest that unfolding of the alpha subunit chain after formation of the A'_{ChT} species was slower than unfolding of the A'_T species during trypsin treatment. Alternatively, the second cleavage site may have been more resistant to chymotrypsin than to trypsin [4].

Effects of agents modifying the tryptic digestibility of coupling factor alpha subunits

Solubilized coupling factor samples were treated with trypsin plus soybean trypsin inhibitor in the presence of modifying agents. Table I lists these agents and their effect on the alpha subunit composition, on the relative intensity of the alpha and beta subunit peaks, and on the relative ATPase activity. In this experiment, the effective concentration of trypsin was low. The maximal degree of alpha subunit conversion observed under these conditions was equivalent to 0.60 fractional mol of the A'_T (61 000 dalton) species and 0.40 fractional mol of the native 64 000 dalton alpha subunit.

When either 1 mM KSCN, 1 mM ADP, 12 mM KF, or no agent was present in the sample during treatment (Table I), the fractional mol of A was about 0.45. On the other hand, the fractional mol of A was 0.60—0.69 when either AMP or sodium succinate was present, but only 0.30 when 0.2 mM adenyl-5'-yl imidodiphosphate was present. The proteolytic conversion of the native A form of the alpha subunit to the A'_T species was therefore affected by the presence of certain agents under these conditions of treatment.

The stained intensity of the coupling factor beta (53 500 dalton) subunit peak on polyacrylamide gels in the presence of dodecyl sulfate was diminished in the samples that contained either 12 mM KF or 0.2 mM ADP. However, the sum of the intensities of the A and A'_T forms did not decrease in these samples. This specific loss of the beta subunit was not accompanied by a parallel loss of ATPase activity. These results suggest that the beta subunit of the coupling

TABLE I

EFFECTS OF MODIFYING AGENTS DURING TRYSPIN TREATMENT ON SUBUNIT PROTEOLYSIS AND RELATIVE ATPase ACTIVITY

Each sample contained 0.5 mg of sucrose-solubilized coupling factor, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid/KOH (pH 7.5) buffer + 4 mM $MgCl_2$ (standard buffer), and the specific agents as indicated. Bovine pancreas trypsin + soybean trypsin inhibitor (trypsin coupling factor protein:trypsin inhibitor = 1 : 6 : 5, w/w) was added to each sample, followed by incubation at 30° C for 30 min.

Sample	Modifying agent(s)	Alpha subunit composition (fractional mol)		Intensity of subunit peaks (cm)		Relative ATPase activity (% of control)
				$(A + A'_T)$	β	
		A	A'_T			
1	none	0.40	0.60	40.2	25.1	100
2	50 mM sodium succinate	0.69	0.31	35.0	26.0	73
3	200 mM sodium succinate	0.60	0.40	38.1	22.3	67
4	20 mM AMP	0.61	0.39	26.3	20.8	64
5	10 mM AMP + 50 mM sodium succinate	0.65	0.35	26.8	17.2	58
6	0.2 mM adenyl-5'yl imidodiphosphate	0.30	0.70	20.2	17.3	115
7	1 mM KSCN	0.40	0.60	31.2	21.8	100
8	1 mM ADP	0.44	0.56	21.1	11.4	91
9	12 mM KF	0.44	0.56	22.7	7.1	91
10	0.2 mM ADP	0.49	0.51	24.3	4.1	85

factor may not be essential for ATPase catalytic activity. They are also consistent with the finding that the substrate analog 2',3'-dialdehyde ATP, which was a specific inhibitor for the ATPase active site, bound covalently to the alpha subunit(s) of the coupling factor [11,12].

The $A'_T \rightarrow A''_T$ conversion of the coupling factor alpha subunit by trypsin was retarded by the presence of either double-strength buffer or by 50 mM sodium succinate [14]. The $A \rightarrow A'_T$ conversion was also retarded, but to a lesser degree. These agents may therefore have similar effects on the conformation of the alpha subunits to decrease digestibility through induction of a more compact subunit conformation. Since the $A'_T \rightarrow A''_T$ conversion was retarded more than the $A \rightarrow A'_T$ conversion, the former conversion may have been mediated by a greater degree of conformational expansion.

Binding of a single ligand to an enzyme active site or to an allosteric regulatory site has been found to induce conformational changes in the entire protein that alters its digestibility at remote surfaces [4—6]. The presence of AMP (Table I) retarded the tryptic digestion of the *M. phlei* coupling factor alpha subunits. Since this agent is an adenine nucleotide, it may have bound to substrate or nucleotide regulatory sites to induce a more compact conformation of the alpha subunits. On the other hand, the adenine nucleotide derivative adenyl-5'-yl imidodiphosphate enhanced the tryptic digestibility of the alpha subunits (Table I). Since this agent was also a non-competitive inhibitor of the coupling factor ATPase [18], it may have bound to a nucleotide regulatory site and induced a more expanded conformation.

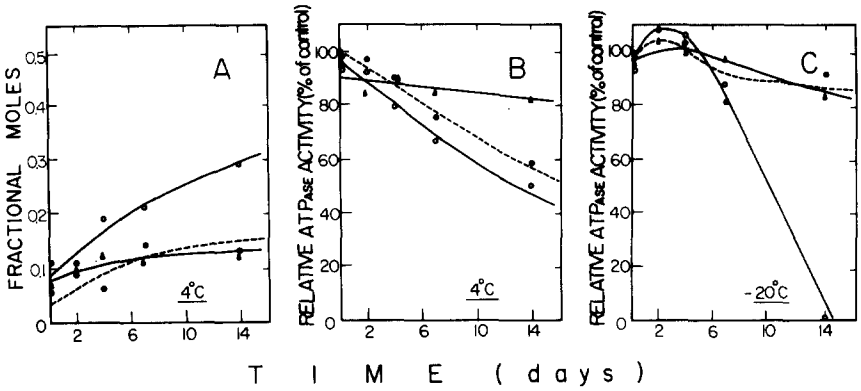


Fig. 2. Effects of storage conditions on the alpha subunit composition and relative ATPase activity of solubilized coupling factor. The fractional mol of the A'_{UP} species for aliquots of fresh coupling factor stored at 4°C is shown in (a) versus days of storage. Samples contained either (a) •- - - - -•, no addition; (b) ○———○, 4 mM $MgCl_2$; or (c) △———△, 2 mM phenylmethylsulfonyl fluoride + 2.5% ethanol. The relative ATPase activity (as percent of control) of aliquots stored at 4°C is shown in (b). Sample compositions were the same as in (a). Relative ATPase activity of coupling factor aliquots stored at −20°C is shown in (c). Samples that were stored for a total of 4 days were thawed and refrozen on day 2; samples stored for 7 days were thawed and refrozen on days 2 and 4; samples stored for 14 days were thawed and refrozen on days 2, 4, and 7. After each thawing on day 2 or 4, fresh phenylmethylsulfonyl fluoride in 95% ethanol was added to samples that contained it initially.

Limited digestion of solubilized M. phlei coupling factor by an unknown protease

Freshly prepared samples of solubilized coupling factor contained the alpha subunit exclusively in the native (64 000 dalton) form. These samples were stored at 4°C or −20°C under different conditions, and activated by trypsin treatment. As seen in Fig. 2, the relative ATPase activity of the samples that contained 4 mM $MgCl_2$ and were stored at either 4°C or −20°C for 14 days with four cycles of freezing and thawing decreased. Untreated samples stored at −20°C retained activity after 14 days, but untreated samples stored at 4°C for 14 days had decreased activity. Samples containing 2 mM phenylmethylsulfonyl fluoride and 2.5% ethanol retained nearly full activity after 14 days either at 4°C or at −20°C with four cycles of freezing and thawing.

The latent coupling factor ATPase enzyme is therefore stable compared to the ATPase enzymes from mitochondria [19], *S. faecalis* [20], *E. coli* [21], and *M. lysodeikticus* [22]. Since the enzyme was stable (in the absence of $MgCl_2$) at −20°C, even with freezing and thawing, as well as at 4°C for more than a day, the BCF_1 ATPase enzyme is not 'cold labile' [23].

The native alpha subunit structure was stable in coupling factor samples stored at either 4°C or −20°C in the presence of 2 mM phenylmethylsulfonyl fluoride and 2.5% ethanol. However, the alpha subunit in samples containing 4 mM $MgCl_2$ and stored at 4°C rapidly degraded to an A′-type (61 000 dalton) species. After 14 days at 4°C, the fractional mol of this A′-type species present in the sample was maximally 0.40. This degradation was similar to that which had been seen for some solubilized coupling factor samples after handling or storage [10]. The stability of the native alpha subunit in coupling factor samples stored at −20°C (even in the presence of 4 mM $MgCl_2$ with repeated

freezing and thawing), its degradation in samples stored at 4°C (especially in the presence of $MgCl_2$), and its protection from degradation at 4°C by 2 mM phenylmethylsulfonyl fluoride and 2.5% ethanol suggest that trace amounts of an unknown protease may have produced this A′-type (61 000 dalton) species.

The fractional mol of the A'_{UP} (61 000 dalton, unknown protease) species for each coupling factor sample stored at 4°C (18 samples) was plotted versus relative ATPase activity. An inverse correlation was found between the fractional mol of A'_{UP} and the relative ATPase activity (correlation coefficient = 0.87; standard error = 0.002). After the standard ATPase assay (including trypsin pretreatment), the alpha subunit composition of these samples was indistinguishable from the composition after tryptic activation of fresh coupling factor (i.e. fractional mol of A''_T = 1.0). This negative correlation indicated that formation of the A'_{UP} (61 000 dalton) species was accompanied by inactivation of the ATPase sites associated with this species. The effects of tryptic proteolysis and storage upon the alpha subunit structure and ATPase activity of *M. phlei* coupling factor are similar to the effects of tryptic proteolysis and physical manipulation on the *M. lysodeikticus* ATPase enzyme [9, 24].

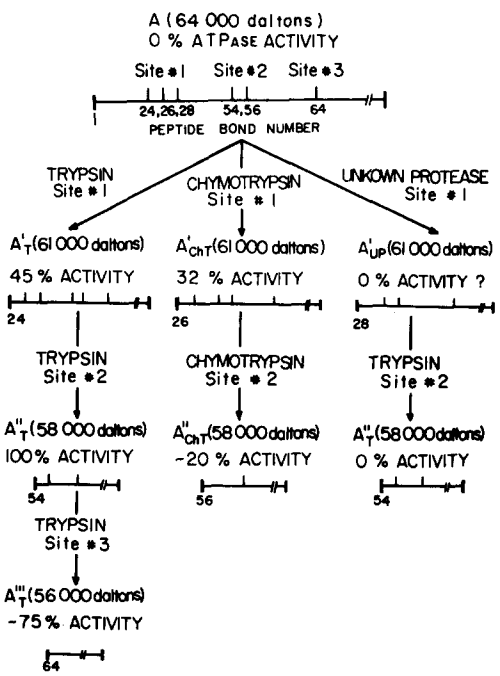


Fig. 3. Comparison of the effects of limited proteolysis by trypsin, chymotrypsin, and the unknown protease on the alpha subunit and relative ATPase activity of coupling factor. Cleavage sites are shown to be near the N-terminal end of the polypeptide chain for sake of illustration. It is not known whether these sites are actually near the C-terminal or N-terminal end of the chain. All sites are, however, located near the same end. The numbering of the peptides is also arbitrary and is given only to shown that each protease cleaves a different bond, corresponding to its particular specificity, at each cleavage site. The relative ATPase activity is also given for each alpha subunit species, with the A''_T species having 100% activity.

Comparison of the limited proteolytic effects of trypsin, chymotrypsin, and the unknown protease

Although trypsin and chymotrypsin have different proteolytic specificities, yet they both produced an A′-type (61 000 dalton) species from the coupling factor alpha subunit. The specific peptide bonds cleaved by these two proteases, as well as the bond cleaved by the unknown protease, were therefore apparently located in the same small segment of polypeptide chain which may have been part of a protruding or flexible loop or similar structure highly vulnerable to proteolytic attack, as suggested by Mihalyi [4]. Limited proteolysis of the *lac* repressor protein by different proteases likewise produced similar proteolytic species [25]. A schematic outline of possible effects of limited proteolysis by trypsin, chymotrypsin, and the unknown protease on the coupling factor alpha subunit structure is given in Fig. 3. Effects on relative ATPase activity are also indicated.

Acknowledgments

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