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Effects of Proteolytic Digestion by Chymotrypsin on the Structure and Catalytic Properties of Reduced Nicotinamide–Adenine Dinucleotide–Ubiquinone Oxidoreductase from Bovine Heart Mitochondria

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1. Incubation of NADH–ubiquinone oxidoreductase (Complex I) with chymotrypsin caused loss of rotenone-sensitive ubiquinone-1 reduction and an increase in rotenone-insensitive ubiquinone reduction. 2. Within the same time-course, NADH–$K_3Fe(CN)_6$ oxidoreductase activity was unaffected. 3. Mixing of chymotrypsin-treated Complex I with Complex III did not give rise to NADH–cytochrome *c* oxidoreductase activity. 4. Gel electrophoresis in the presence of sodium dodecyl sulphate revealed selective degradation of several constituent polypeptides by chymotrypsin. 5. With higher chymotrypsin concentrations and longer incubation times, a decrease in NADH–$K_3Fe(CN)_6$ oxidoreductase was observed. The kinetics of this decrease correlated with solubilization of the low-molecular-weight type-II NADH dehydrogenase (subunit mol.wts. 53000 and 27000) and with degradation of a polypeptide of mol.wt. 30000. 6. Phospholipid-depleted Complex I was more rapidly degraded by chymotrypsin. Specifically, a subunit of mol.wt. 75000, resistant to chymotrypsin in untreated Complex I, was degraded in phospholipid-depleted Complex I. In addition, the 30000-mol.wt. polypeptide was also more rapidly digested, correlating with an increased rate of transformation to type II NADH dehydrogenase.

Selective proteolytic digestion of enzymes has proven to be a useful technique for the study of the structure and function of enzymes. As part of a general survey of the properties of mitochondrial NADH–ubiquinone oxidoreductase (Complex I of Hatefi *et al.*, 1962) we have been investigating the effects of proteolytic degradation on the activity of this enzyme and on its polypeptide subunits analysed by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (Weber & Osborn, 1969). It has been known for some time that both trypsin and chymotrypsin could transform the soluble high-molecular-weight (type I) NADH dehydrogenase (Ringler *et al.*, 1963) into the low-molecular-weight (type II) NADH dehydrogenase (Cremona *et al.*, 1963). In view of the similarity between Complex I and the soluble type-I NADH dehydrogenase (for a general review of NADH dehydrogenation see Ragan, 1976*c*), we have examined the effects of chymotrypsin on Complex I with a view to determining the underlying structural alterations resulting in modification of enzyme activities and transformation to the type II NADH dehydrogenase. The effects of trypsin have been described (Ragan, 1976*b*). In particular, it was found that nicotinamide–adenine dinucleotide transhydrogenase was very rapidly inactivated by trypsin and that this loss of activity appeared to be correlated with degradation of one of the Complex-I subunits.

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Materials and Methods

Chemicals

α-Chymotrypsin (EC 3.4.21.1) (type II) and phenylmethanesulphonyl fluoride were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Ubiquinone-1 was generously given by Hoffman La Roche, Basle, Switzerland.

Biological materials

Complex I (NADH dehydrogenase, EC 1.6.99.3) was prepared as described by Hatefi *et al.* (1962). Phospholipid depletion of Complex I with cholate and $(NH_4)_2SO_4$ was carried out in the absence of reducing agents as described by Ragan & Racker (1973). Complex III (ubiquinone–cytochrome *c* oxidoreductase) was prepared by the method of Rieske (1967).

Chymotrypsin treatment

Complex I (1 mg of protein/ml) was incubated with chymotrypsin (20 or 100 μg of protein/ml) in 50 mM-sodium phosphate, pH 7.6, at 21°C. At intervals, portions were removed for assay or for preparation of samples for electrophoresis.

Gel electrophoresis

After incubation with chymotrypsin, portions (200 μl) were incubated at 21°C for 30 min with 10 μl of 5 mM-phenylmethanesulphonyl fluoride in ethanol. The samples were then dissociated by adding 20 μl of 10% (w/v) SDS,* 20 μl of 10% (v/v) 2-mercaptoethanol, 2 μl of 0.4% Bromophenol Blue, 20 μl of 1 M-sucrose and boiling for 2 min. Electrophoresis of up to 100 μg of protein was performed on 12 cm × 6 mm (internal diam.) gels containing 12.5% (w/v) acrylamide and 0.34% bisacrylamide at 5.5 mA/gel for 16 h by the procedure of Weber & Osborn (1969). Alternatively, electrophoresis was performed as described by Neville & Glossman (1974) by using a 3% (w/v) acrylamide stacking gel, a running gel as above, and the discontinuous buffer system J4179 of Jovin *et al.* (1971). It was unnecessary to change the ionic composition of the sample buffer from sodium phosphate to the Tris/borate buffer used in the cathodic compartment of the electrophoresis. Electrophoresis was performed at 2 mA/gel for 4 h. Staining and destaining were as described by Weber & Osborn (1969). Destained gels were scanned with a Joyce–Loebl densitometer (Joyce, Loebl and Co., Gateshead, Co. Durham, U.K.). The scanning speed was 2.5 cm/min, and the optical slit-width was approx. 0.3 mm. For duplicate samples electrophoresed in parallel, the peak heights from the densitometer traces agreed within 10–15% of each other. When untreated and chymotrypsin-treated samples were compared, changes in peak height of up to 20% were therefore not considered significant.

Polypeptide molecular weights were determined separately for each electrophoresis system as described by Ragan (1976*a*). For reference, these are listed in Table 1.

Assays

NADH–ubiquinone-1 oxidoreductase and NADH–$K_3Fe(CN)_6$ oxidoreductase were measured at 21°C as described by Ragan (1976*a*). NADH–cytochrome *c* oxidoreductase was assayed at 30°C as described by Hatefi & Rieske (1967). Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin (Sigma, fraction V) as a standard.

* Abbreviation: SDS, sodium dodecyl sulphate.

Table 1. *Subunit composition of Complex I*

The molecular weights of the Complex I subunits were determined separately for both electrophoretic systems as described by Ragan (1976*a*). Subunits that were quantitatively purified into the iron–protein fraction or the type-II NADH dehydrogenase obtained from treatment with chaotropic agents (Hatefi & Stempel, 1967) are also indicated.

Subunit molecular weights determined in the system of:	
Weber & Osborn (1969)	Jovin *et al.* (1971)
75000 (iron–protein)	75000 (iron–protein)
53000	53000* (type II dehydrogenase)
	49000* (iron–protein)
42000	42000
39000	38000
33000†	30000
30000† (iron–protein)	
27000 (type II dehydrogenase)	27000 (type II dehydrogenase)
	26000
25000	25000
23500	23500
22000	22000
20500	20500
18000	18000
15500	16500
	15500
8000	8000
5000	5000

* The subunits of mol.wt. 53000 and 49000 co-migrate in the Weber & Osborn (1969) system.

† The subunits of mol.wt. 33000 and 30000 co-migrate in the Jovin *et al.* (1971) system.

Results

Effect of limited digestion by chymotrypsin on Complex-I activity

Incubation of Complex I (1 mg of protein/ml) with chymotrypsin (20 μg of protein/ml) at 21°C caused progressive loss of rotenone sensitivity of NADH–ubiquinone-1 oxidoreductase, although the overall rate of ubiquinone-1 reduction was only slightly decreased (Fig. 1). Closer examination of this effect revealed that the $V_{max.}$ for ubiquinone-1 was unaffected by chymotrypsin digestion (6.0 μmol/min per mg of protein at 30°C), but the K_m increased from 54 μM to 85 μM after 4 h, by which time the percentage inhibition by rotenone had fallen from 90 to 10%. Within the same time-course, NADH–$K_3Fe(CN)_6$ oxidoreductase was unaffected.

Mixing of Complex I and Complex III leads to reconstitution of NADH–cytochrome *c* oxidoreductase activity (Hatefi, 1963). Table 2 shows that mixing of chymotrypsin-treated Complex I with Complex III gave rise to low rates of NADH–cytochrome *c* oxidoreductase, which were only partially inhibited by rotenone. Therefore the rapid rotenone-insen-

sitive pathway of ubiquinone-1 reduction in the modified Complex I is not related to the physiological pathway of ubiquinone-10 reduction.

Effect of limited digestion by chymotrypsin on the polypeptide composition of Complex I

The polypeptides of Complex I were resolved by polyacrylamide-gel electrophoresis in the presence of SDS (Weber & Osborn, 1969). Fig. 2 shows the progressive changes in polypeptide profile with time when Complex I was treated with chymotrypsin as in Fig. 1. Control experiments established that no proteolytic digestion occurred after treatment with SDS. Polypeptides of mol.wts. 75000, 53000 and 30000 are major components of the iron–protein fragment obtained from Complex I by chaotropic resolution (Hatefi & Stempel, 1967; Ragan, 1976*a*). The low-molecular-weight type II NADH dehydrogenase has subunits of mol.wts. 53000 and 27000 (Ragan, 1976*a*). In the experiment of Fig. 2, the 75000- and 30000-

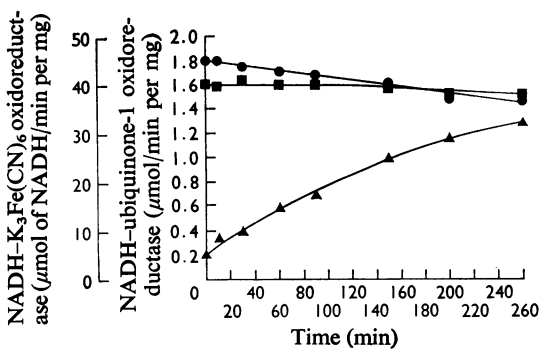

Fig. 1. *Effect of limited digestion by chymotrypsin on Complex-I activities*

Complex I was incubated with chymotrypsin (20μg/ml) and assayed as described in the Materials and Methods section. (●), NADH–ubiquinone-1 oxidoreductase; (▲), NADH-ubiquinone-1 oxidoreductase assayed in the presence of 1μg of rotenone/ml; (■), NADH–$K_3Fe(CN)_6$ oxidoreductase.

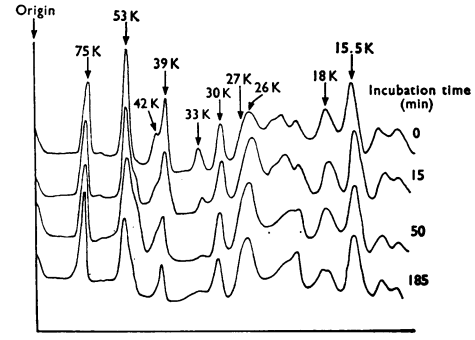

Fig. 2. *Analysis of the effect of limited digestion by chymotrypsin on the polypeptides of Complex I by gel electrophoresis in the Weber & Osborn* (1969) *system*

Complex I was treated with chymotrypsin as in Fig. 1 and, after the indicated times, samples were electrophoresed according to Weber & Osborn (1969) and the gels were scanned as described in the Materials and Methods section. Molecular weights are indicated in thousands by the postscript K.

Table 2. *Reconstitution of NADH–cytochrome c oxidoreductase*

Complex I (1 mg of protein in 1 ml) was incubated with or without 20μg of chymotrypsin at 21°C for 5h as described in the Materials and Methods section. Samples were diluted to approx. 10ml with 50mM-sodium phosphate, pH7.6, and centrifuged for 30min at 150000*g* and 4°C in the 10×10ml rotor of an MSE-65 centrifuge. The pellets were suspended by homogenization in the same buffer and re-centrifuged as above. The pellets were finally homogenized in approx. 0.3ml of 0.67M-sucrose/50mM-Tris/HCl, pH8.0, and diluted to a final protein concentration of 2.5mg/ml. Portions (20μl) were incubated at 0°C for 45min with or without 20μl of Complex III (35mg of protein/ml) and 0.67M-sucrose/50mM-Tris/HCl, pH8.0, to a final volume of 50μl. Complex III alone did not catalyse NADH–cytochrome *c* oxidoreduction. Abbreviation: N.D., not determined.

Sample	NADH–ubiquinone-1 oxidoreductase (μmol of NADH/min per mg of Complex-I protein)	NADH–cytochrome *c* oxidoreductase (μmol of cytochrome *c*/min per mg of Complex-I protein)
Complex I	1.89	0.39
+rotenone	0.18	0.00
Complex I+Complex III	N.D.	5.55
+rotenone	N.D.	0.00
Chymotrypsin-treated Complex I	1.35	0.30
+rotenone	1.20	0.11
Chymotrypsin-treated Complex I+Complex III	N.D.	0.99
+rotenone	N.D.	0.33

mol.wt. polypeptides were clearly unaffected, but one of the 53000-mol.wt. polypeptides and others of mol.wts. 42000, 39000 and 33000 were degraded. To distinguish the two 53000-mol.wt. polypeptides and to provide better resolution of the low-molecular-weight polypeptides, electrophoresis was also performed at pH9.5 in the discontinuous buffer system J4179 of Jovin *et al.* (1971). In this system (Fig. 3) the 53000-mol.wt. polypeptide of the iron–protein fraction migrates with an apparent mol.wt. of 49000, and it is clear that it is this polypeptide which is susceptible to proteolytic digestion. The subunits of the type II NADH dehydrogenase (mol.wts. 53000 and 27000) were clearly unaffected. The 33000-mol.wt. polypeptide co-migrates with the 30000-mol.wt. polypeptide in the Jovin *et al.* (1971) system, whereas the low-molecular-weight components of Complex I are more clearly resolved than in the Weber & Osborn (1969) system. Of these, the 26000-, 23500-, 22000-, 18000- and 15500-mol.wt. polypeptides were to a greater or lesser extent degraded, but the 25000-, 20500- and 16500-mol.wt. polypeptides were relatively resistant.

The time-course of degradation of the Complex-I subunits was approximately parallel to the loss of rotenone sensitivity, except for the 53000-mol.wt. polypeptide, which was degraded far more rapidly than the others and more rapidly than the changes in enzyme activity. The effect of chymotrypsin on the 53000-mol.wt. polypeptide of the iron–protein fragment were confirmed by isolating the latter from chymotrypsin-treated Complex I. In Fig. 4, iron–protein isolated from intact and chymotrypsin-treated Complex I was compared with isolated type II dehydrogenase; clearly the iron–protein shows specific degradation of the 53000-mol.wt. polypeptide.

Effect of extensive digestion by chymotrypsin on Complex I activity

Fig. 1 shows that NADH–$K_3Fe(CN)_6$ oxidoreductase activity showed considerable resistance to chymotrypsin. Loss of this activity was only encountered under conditions of more extensive degradation by chymotrypsin, e.g. 100 μg of protein/ml for longer times. Little effect on this activity was detected for approx. 120min, after which the rate declined to a minimum of about 30% of the original after 300min.

Effect of extensive digestion by chymotrypsin on the polypeptide composition of Complex I

The polypeptide profile after 120min digestion was similar to that of Fig. 2 and not greatly changed after 300min. However, the decrease in NADH–$K_3Fe(CN)_6$ oxidoreductase activity was paralleled by solubilization of type-II NADH dehydrogenase,

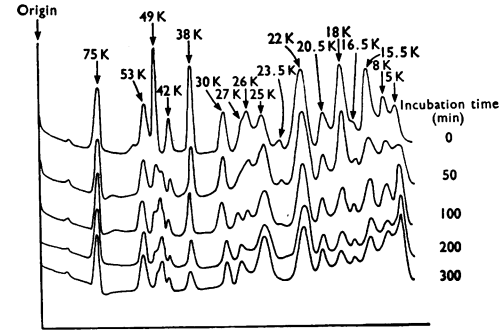

Fig. 3. *Analysis of the effect of limited digestion by chymotrypsin on the polypeptides of Complex I by gel electrophoresis in the Neville & Glossmann* (1974) *system*
Samples from an experiment similar to that of Fig. 2 were analysed by electrophoresis by the method of Neville & Glossmann (1974).

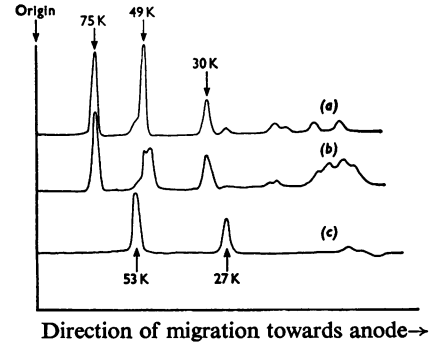

Fig. 4. *Subunit composition of isolated iron–protein and type II dehydrogenase*
Iron–protein (*a*) and type II dehydrogenase (*c*) were purified from Complex I as described by Hatefi & Stempel (1967). Iron–protein from chymotrypsin-treated Complex I (*b*) was isolated as follows. Complex I (10mg of protein in 10ml of 50mM-sodium phosphate, pH7.6) was incubated with chymotrypsin (200 μg of protein) for 100min at 21°C. After cooling on ice, the sample was centrifuged at 100000*g* for 20min at 4°C in the 10×10ml rotor of a MSE 65 centrifuge. The pellet was resuspended by homogenization in 1ml of 0.67M-sucrose/50mM-Tris/HCl, pH8.0 at 0°C, and left for 1h at 0°C after the addition of a 25mM solution of phenylmethanesulphonyl fluoride in ethanol. Then 4M-$NaClO_4$ was added to a final concentration of 0.9M and the mixture incubated at 21°C for 10min. Subsequent steps were exactly as described by Hatefi & Stempel (1967). For electrophoresis, portions containing approx. 30 μg of protein were dissociated, and run by the method of Neville & Glossmann (1974).

shown by the presence of the subunits of mol.wts. 53000 and 27000 and of NADH–$K_3Fe(CN)_6$ oxidoreductase activity in the supernatant after centrifugation at 100000*g* for 30min (results not shown). Small amounts of the 75000-mol.wt. polypeptide and the subunits of chymotrypsin itself were also present in the soluble fraction. The unusual kinetics of release of the type II NADH dehydrogenase suggest that the structure of Complex I is maintained up to a certain extent of degradation, beyond which progressive instability and relatively rapid fragmentation occur. Presumably it is degradation at one particular site which is involved in release of the type II dehydrogenase and of those polypeptides that are clearly resolved on the gels; the 30000-mol.wt. polypeptide of the iron–protein fraction was degraded with time in a manner that quite closely followed the release of the type II dehydrogenase. Thus there was little change in this polypeptide for up to 120min after chymotrypsin addition, but it was subsequently degraded within the next 240min. These results were confirmed by using the discontinuous-electrophoresis system, which also showed little change in the low-molecular-weight polypeptides between 120 and 360min incubation with chymotrypsin. Substantial degradation of the 30000-mol.wt. polypeptide within this time range was also observed, except that there was a decrease in peak height within the first 30min of the incubation owing to degradation of the 33000-mol.wt. polypeptide, which co-migrates with the 30000-mol.wt. polypeptide in this electrophoresis system.

Effect of digestion by chymotrypsin on phospholipid-depleted Complex I

The transformation by trypsin (EC 3.4.21.4) of Complex I into a type II NADH dehydrogenase was facilitated by depletion of the endogenous phospholipid of Complex I by cholate and $(NH_4)_2SO_4$ treatment (Ragan, 1976*a*). To investigate further the factors involved in solubilization of the type II dehydrogenase by chymotrypsin, the effects of phospholipid depletion were investigated.

Several differences were noted. First, transformation to type II dehydrogenase occurred more readily, e.g. NADH–$K_3Fe(CN)_6$ oxidoreductase began to decrease after treatment with 20μg of chymotrypsin/ml for 60min only, conditions that would not have affected undepleted Complex I. Secondly, there were marked differences in the polypeptide profile, especially in the high-molecular-weight region. As shown in Fig. 5(*a*), after 100min with chymotrypsin there had been more extensive degradation than for the same time in Fig. 3. The major differences were that after 300min the 75000-mol.wt. polypeptide had been considerably digested, whereas the 30000-mol.wt. polypeptide had almost completely disappeared. The 30000-mol.wt. polypeptide was largely degraded between 100 and 240min after the addition of chymotrypsin, following the kinetics of transformation to a type II dehydrogenase. Fig. 5(*b*) shows the effect of 360min incubation with 100μg of chymotrypsin/ml. Only the 53000- and 27000-mol.wt. polypeptides of the type II dehydrogenase remained in the high-molecular-weight region, and a few others together with chymotrypsin subunits, in the low-molecular-weight region.

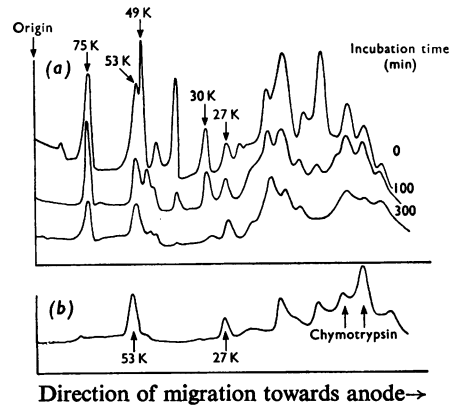

Fig. 5. *Effect of chymotrypsin digestion on the polypeptides of phospholipid-depleted Complex I*

Complex I was depleted of phospholipids as described by Ragan & Racker (1973). Chymotrypsin treatment and electrophoresis (*a*) were exactly as in Fig. 3. In (*b*), the depleted Complex I was treated with 100μg of chymotrypsin/ml for 6h, and electrophoresed by the method of Neville & Glossmann (1974).

Discussion

The conversion by chymotrypsin of rotenone-sensitive into rotenone-insensitive NADH–ubiquinone-1 oxidoreductase is similar to the change induced by trypsin treatment (Ragan, 1976*b*), except that the total ubiquinone-1 reductase activity declined rather more in the latter case. The changes in polypeptide composition accompanying this modification of ubiquinone-1 reduction are also very similar to those caused by trypsin, e.g. the degradation of the 53000-mol.wt. polypeptide of the iron protein, and of the 42000-, 39000-, 33000- and 26000-mol.wt. polypeptides, and the lack of digestion of the 75000-, 53000- (type II dehydrogenase) 30000- and 27000-mol.wt. polypeptides. In the low-molecular-weight region, however, only the 20500-mol.wt. polypeptide was rapidly digested by trypsin, whereas several components were degraded by chymotrypsin under similar conditions. The rapid degradation of the 20500-mol.wt. polypeptide by trypsin followed

the same time-course as loss of nicotinamide–adenine dinucleotide transhydrogenase, and the suggestion was therefore made that this polypeptide was a subunit of this enzyme (Ragan, 1976*b*). Chymotrypsin, however, induced loss of transhydrogenase activity at only 7% of the rate induced by trypsin (results not shown). The resistance of the 20500-mol.wt. polypeptide to degradation by chymotrypsin therefore supports the original proposal that this polypeptide is a transhydrogenase subunit.

In using any probe to study three-dimensional organization, failure of the probe to react with a particular polypeptide can be due either to inaccessibility of the polypeptide or to the absence of susceptible residues from that polypeptide. In this context, the failure of trypsin and chymotrypsin to degrade the subunits of the type II dehydrogenase and the 75000- and 30000-mol.wt. subunits of the iron–protein cannot be interpreted unambiguously, particularly as the isolated type II dehydrogenase is also resistant to degradation. However, by using lactoperoxidase-catalysed iodination as a structural probe (Ragan, 1975*a*) it was found that the type II dehydrogenase subunits were not labelled in intact Complex I, but were labelled when isolated type II dehydrogenase was used. Moreover, the 75000-, 53000- and 30000-mol.wt. polypeptides of the iron–protein were labelled to only a small extent or not at all in intact Complex I, and heavily labelled in isolation. These result taken together would therefore suggest that in Complex I the type II dehydrogenase and iron–protein subunits are buried in the structure with limited access to the 75000- and 53000-mol.wt. polypeptides of the iron–protein. The remaining clearly resolved polypeptides of mol.wts. 42000, 39000 and 33000 were labelled by lactoperoxidase (Ragan, 1976*a*) and degraded by both trypsin and chymotrypsin, suggesting that they have an exposed configuration in Complex I. The poor resolution of the low-molecular-weight polypeptides and the absence of information as to their possible function makes interpretation of their structural organization unreliable.

The solubilization of type II dehydrogenase by chymotrypsin occurred with a marked lag, during which time most of the proteolytic degradation of the enzyme had already occurred. However, up to the time at which the type II dehydrogenase began to be released, the 30000-mol.wt. polypeptide did not show evidence of degradation, but was almost completely degraded during the release of the type II enzyme. Tentatively, we conclude that the 30000-mol.wt. polypeptide is involved in binding the type II dehydrogenase subunits into the overall structure. In this context, it is noteworthy that papain (EC 3.4.22.2) is capable of degrading the 30000-mol.wt. polypeptide much more readily than chymotrypsin or trypsin (Ragan, 1976*c*) and that this occurs without transformation to type II NADH dehydrogenase, i.e. without loss of NADH–$K_3Fe(CN)_6$ oxidoreductase activity. Obviously, factors other than the 30000-mol.wt. polypeptide are probably involved in the binding of the type II dehydrogenase subunits into the overall structure, but it is also possible that the hydrolysis of the polypeptide by papain does not affect the type II binding site. Preliminary observations suggest that initially this polypeptide is only degraded to a 28000-mol.wt. fragment, and that subsequent cleavage of this on longer treatment with papain does occur with loss of NADH–$K_3Fe(CN)_6$ oxidoreductase.

After incubation with 100 μg of chymotrypsin per ml for 6h, solubilization of the type II dehydrogenase subunits was nearly quantitative, judged from the staining intensity of the 53000-mol.wt. subunit on gels of the soluble fraction obtained after centrifugation of the digested Complex I. By this time, no further losses of NADH–$K_3Fe(CN)_6$ oxidoreductase activity were observed. Apart from some of the 75000-mol.wt. polypeptide, no other subunits of Complex I were solubilized by chymotrypsin and the released enzyme was estimated to be about 75% pure from the Coomassie Blue staining.

The effects of phospholipid removal on the susceptibility of Complex I to chymotrypsin were broadly similar to those seen with trypsin (Ragan, 1976*b*). In general, all susceptible polypeptides were rather more rapidly degraded, but in particular, the 75000- and 30000-mol.wt. polypeptides were now degraded by low concentrations of chymotrypsin. These effects were also observed with trypsin (Ragan, 1976*b*), suggesting increased accessibility of the 75000-mol.wt. polypeptide by phospholipid removal. The more rapid degradation of the 30000-mol.wt. polypeptide by chymotrypsin was probably due to a shortening of the lag period by faster digestion of other polypeptides and was correlated with a more rapid conversion of Complex I into type II NADH dehydrogenase.

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